

expected to be regulated by the same transcriptional regulators. Curiously, miR-23b-27b-189 is localized on the Ch9q22.3, and LOH of this region is strongly correlated with cancer progression and lymph node metastasis [34-36]. In our assay we could not observe any significant differences, however, they could be related to malignancy in different aspects [37,38].

Pathway analysis, which was based on miRNA target prediction, proved that differentially-expressed miRNA cooperatively regulated a large number of signaling pathways, including Wnt, insulin, EGFR1, MAPK and TGF- β receptor, which are relevant to drug resistance as well as tumorigenesis. Concerning the Wnt signaling pathway, it has been reported that activation of the Wnt/ β -catenin pathway plays critical roles in establishment of MLL leukemic stem cells and conferring drug-resistant properties [39]. Additionally, activation of Wnt/ β -catenin signaling in plasma cells induced chemoresistance [40], and RNAi-mediated gene silencing of β -catenin negatively regulated drug-induced apoptosis [41]. Therefore, Wnt/ β -catenin signaling pathway would be a potential therapeutic target to sensitize drug-resistant cancer cell. Furthermore, other pathways were also reported to be associated with drug resistance and apoptosis. Sequential treatment of TGF- β induced MDR1 expression in rat hepatocytes [42]. In contrast, TGF- β also induces apoptotic cell death in hepatocytes and activation of the MAPK/ERK pathway confers resistance to TGF- β -induced cell death [43]. Our findings showed that a lot of pathways were commonly enriched in up- and down-regulated miRNA targets, however, it is hard to decide whether these pathways are positively or negatively regulated in MCF7-ADR, because a large number of target candidates exist in each signaling pathway. Further investigation such as systems biology would be needed to clarify this point.

We believe that the integrative genomic analyses as described here have a huge potential to fundamentally understand transcriptional regulatory networks and identify the novel molecular targets for therapy in the field of cancer biology. By integrating array data and bioinformatics, it could be possible to expeditiously explore the key molecule in the all aspects of pathophysiology. Our studies by means of an integrated genomic analysis not only identified miR-505 as a tumor suppressive miRNA that inhibited cell proliferation by inducing apoptotic cell death but also, more broadly highlighted that various genes and miRNAs orchestrate to temper the drug resistance by intricately controlling genomic status, gene and miRNA expression in cancer cells. Thus, it would be a useful approach to accelerate the understanding of cancer genetics and discover the key targets for diagnosis, prognosis and therapy.

Additional material

Additional file 1: Table S1. Primer list for real-time PCR.

Additional file 2: Figure. S1. Microarray analysis of gene expression and miRNA in MCF7-ADR and MCF7. (A) Scatter plot of gene expression. 41, 000 probes (n = 3). (B) Scatter plot of miRNA expression. 470 probes (n = 2).

Additional file 3: Figure. S2. Validation of accuracy of aCGH in MCF7 and MCF7-ADR. (A) aCGH analysis of MCF7. (B) aCGH analysis of MCF7-ADR. For each sample, the experiment was repeated once, wherein the dye was reversed between the experimental and the reference sample, in order to account for dye-incorporation bias. An aberration filter was set at 2 for the minimum number of probe region and 1 for minimum absolute average log2 ratio for regions in the CGH Analytics to reduce false positives.

Additional file 4: Figure. S3. aCGH analysis of MCF7 and MCF7-ADR as compared with normal human female genome. (A) Blue line shows normal vs. MCF7, and red line shows normal vs. MCF7-ADR (top). Amplified or deleted genome regions (fold change > 2) are highlighted (bottom). (B) The numbers of genes and miRNAs located on the amplified or deleted genome regions (FC > 2).

Additional file 5: Table S2. Up and Down miRNA target gene related pathways.

Additional file 6: Figure. S4. TGF- β signaling pathway. Seventy-four miRNA-targeted genes are plotted in TGF- β signaling pathway map (left), 32 miRNA-targeted genes are plotted (right).

Additional file 7: Table S3. List of down-regulated miRNAs in MCF7-ADR.

Additional file 8: Table S4. List of miRNAs located in genome deletion regions in MCF7-ADR.

Additional file 9: Figure. S5. Polycistronic miRNAs; miR-106-25 cluster. miR-106-25 cluster is located on the deleted genomic region, and the expression is coincidentally downregulated.

Additional file 10: Table S5. List of up-regulated genes in MCF7-ADR judged by T-test in comparison with MCF7.

Additional file 11: Table S6. List of genes associated with apoptosis (KEGG web site).

Additional file 12: Figure. S6. The 3'-UTR assay of Akt3 by miR-505 in MCF7-ADR cells and HEK293 cells. (A) MCF7-ADR cells and (B) HEK293 cells were co-transfected with pre-miR-505 or pre-NC and the psi-Akt3_1 or with psi-Akt3_2. After 48 h, luciferase activities were measured. n.s. represents not significant.

Additional file 13: Figure. S7. Real-time RT-PCR analysis of Akt3 gene by transfection of siRNA in MCF7-ADR cells. Real-time RT-PCR analysis was performed to examine Akt3 from RNA extracted from MCF7-ADR cells transfected with either Akt3 siRNA or negative control siRNA. Akt3 expression levels were normalized to GAPDH expression levels. The mean \pm S. D. of results from triplicate transfections is shown. Results represent the mean \pm S. D. (n = 3). Since Akt3 siRNA-1 was most effectively inhibited the expression of Akt3 genes, it was used for the analysis of cell growth arrest.

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Authors' contributions

YYa, YYo, KM, RT, and FT carried out the experimental work, YYa, KM, TT, RH and YF provided data analysis, YYa, TK, NK and TO designed the study and YYa, NK and TO participated in writing the paper. All authors read and approved the manuscript.

Competing interests

The authors declare that they have no conflict of interest. KM, TT, RH, and YF are Agilent employees.

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ORIGINAL ARTICLE

In vivo delivery of *interferon- α* gene enhances tumor immunity and suppresses immunotolerance in reconstituted lymphopenic hosts

K Narumi^{1,4}, T Udagawa¹, A Kondoh¹, A Kobayashi¹, H Hara¹, Y Ikarashi¹, S Ohnami², F Takeshita³, T Ochiya³, T Okada⁴, M Yamagishi⁴, T Yoshida² and K Aoki¹

T cells recognize tumor-associated antigens under the condition of lymphopenia-induced homeostatic proliferation (HP); however, HP-driven antitumor responses gradually decay in association with tumor growth. Type I interferon (IFN) has important roles in regulating the innate and adaptive immune system. In this study we examined whether a tumor-specific immune response induced by IFN- α could enhance and sustain HP-induced antitumor immunity. An intratumoral IFN- α gene transfer resulted in marked tumor suppression when administered in the early period of syngeneic hematopoietic stem cell transplantation (synHSCT), and was evident even in distant tumors that were not transduced with the IFN- α vector. The intratumoral delivery of the IFN- α gene promoted the maturation of CD11c⁺ cells in the tumors and effectively augmented the antigen-presentation capacity of the cells. An analysis of the cytokine profile showed that the CD11c⁺ cells in the treated tumors secreted a large amount of immune-stimulatory cytokines including interleukin (IL)-6. The CD11c⁺ cells rescued effector T-cell proliferation from regulatory T-cell-mediated suppression, and IL-6 may have a dominant role in this phenomenon. The intratumoral IFN- α gene transfer creates an environment strongly supporting the enhancement of antitumor immunity in reconstituted lymphopenic recipients through the induction of tumor-specific immunity and suppression of immunotolerance. Gene Therapy (2012) 19, 34–48; doi:10.1038/gt.2011.73; published online 26 May 2011

Keywords: IFN- α ; gene transfer; hematopoietic stem cell transplantation; IL-6; regulatory T cells

INTRODUCTION

The development of effective cancer immunotherapy is often difficult because cancer generates an immunotolerant microenvironment against the host immune system.¹ The central objective of cancer immunotherapy is to induce and sustain a tumor-specific immune response; that is, an *in vivo* generation of a large number of highly reactive antitumor lymphocytes that are not restrained by cancer-induced tolerance mechanisms.

It is known that lymphopenia is followed by spontaneous expansion of the remaining T cells in the periphery to restore the original T-cell pool size and maintain homeostasis.² Lymphopenia-induced homeostatic proliferation (HP) of T cells following autologous hematopoietic stem cell transplantation (HSCT) is driven by the recognition of self-antigens, and there is an opportunity to skew the T-cell repertoire during the T-cell recovery by engaging tumor-associated antigens (TAAs), leading to a break in tolerance developed by tumors.² In fact, a variety of animal tumor models showed that lymphopenic conditions are able to create an environment to mount an efficient antitumor immunity through an HP-induced expansion of T cells.^{3–6} However, integration of other immunotherapeutic strategies is necessary to successfully eradicate pre-existing malignant tumors, because HP-driven antitumor responses

decay gradually, as they are vulnerable to a development of tolerance.^{5,6}

The interferon (IFN)- α protein is a pleiotropic cytokine regulating anti-proliferation, induction of cell death, anti-angiogenesis and immunomodulation, and has been used for treatment in a variety of cancers such as chronic myeloid leukemia, melanoma and renal cancer.^{7,8} Although IFN- α was long thought to function mainly by suppressing tumor cell proliferation *in vivo*, more recently it has been established that type I IFNs have important roles in regulating the innate and adaptive arms of the immune system: upregulation of major histocompatibility complex class I gene, promotion of the priming and survival of T cells, enhancement of humoral immunity, increase of the cytotoxic activity of natural killer (NK) cells and CD8⁺ T cells and activation of dendritic cells (DCs).^{9,10} We also reported that in addition to the direct cytotoxicity in the injected site, intratumoral IFN- α gene transfer elicits a systemic tumor-specific immunity in several animal models.^{11,12} Furthermore, our data showed that, because of the effective induction of antitumor immunity and the lower toxicity, an intratumoral route of the IFN vector is superior to an intravenous administration.¹³

In this study, we examined whether HP-induced antitumor activity can be enhanced by IFN- α gene transfer during a physiological

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immune reconstitution, and investigated mechanisms of the enhancement. From the viewpoint of *IFN- α* immune therapy also, an autologous HSCT following a preconditioning is expected to introduce a fresh immune system, in which tolerance to tumor cells is not yet induced, and may present a unique opportunity for *IFN- α* to augment efficacy of the immune therapy.

RESULTS

Adenovirus-mediated *IFN- α* gene transfer induces significant antitumor effect with synHSCT

To examine whether HP of T cells could induce antitumor immunity in lymphopenic hosts, BALB/c mice were injected subcutaneously with CT26 colon cancer cells shortly after lethal (9 Gy) irradiation, and then bone marrow and T cells were infused into the mice. Tumor growth was significantly suppressed in the syngeneic HSCT (synHSCT) recipients (Figure 1a) as previously reported.⁶ HSCT with immunodeficient mice did not show the tumor growth suppression as compared with non-transplanted mice (data not shown), indicating that the antitumor effect is not mediated by a nonspecific

effect of irradiation or lymphocyte infusion. Then, to examine whether a combination of intratumoral *IFN- α* gene transfer enhances the antitumor effect of synHSCT, 5×10^6 PFU (plaque forming unit) of Ad-mIFN was injected once into the tumor at 5 days after the CT26 inoculation. To detect a synergistic effect, we used a low dose (5×10^6 PFU) of Ad-mIFN in this experiment, although the antitumor effect of intratumoral Ad-mIFN injection is dose-dependent, and a strong antitumor effect is induced by a high dose (5 – 10×10^7 PFU) of Ad-mIFN alone.^{12,13} Although the low dose of Ad-mIFN alone suppressed tumor growth only slightly in naïve mice as expected, a significant growth suppression was observed in the synHSCT recipients compared with the injection of a control Ad-AP (Figure 1b).

Injection of *IFN- α* -expressing plasmid suppresses tumor growth in synHSCT recipients

We observed a significant growth suppression of colon cancer in a lymphopenic host by an injection of a low dose of Ad-mIFN (Figure 1b). Although the *in vivo* gene transduction efficiency of the plasmid vector is lower than that of the virus vector, the lipofection/

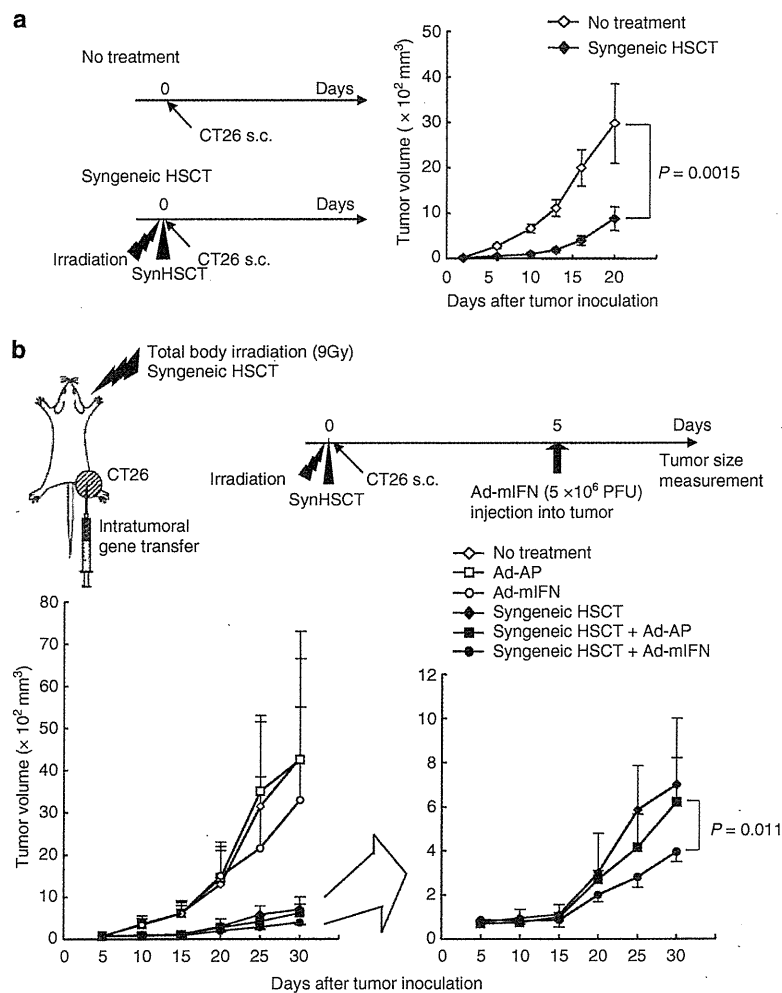


Figure 1 Adenovirus-mediated *IFN- α* gene transfer enhances antitumor effect in synHSCT recipients. (a) Growth suppression of subcutaneous tumors in the synHSCT mice. The mice received a lethal dose (9 Gy) of irradiation, followed by a transfusion of bone marrow and splenic T cells, and then CT26 cells were inoculated into right legs. As a control, CT26 cells were inoculated in non-irradiated mice ($n=7$). (b) A combination of syngeneic HSCT and *IFN- α* adenovirus injection. When CT26 subcutaneous tumors were established, 5×10^6 PFU of Ad-mIFN or control vector (Ad-AP) were injected once into the tumors ($n=6$ – 8). The experiments were repeated two times.

polyfection of the plasmid vector has an excellent safety profile.¹⁴ Therefore, we examined whether tumor growth suppression would be observed by an injection of an *IFN- α* -expressing plasmid (p*IFN- α*). First, we confirmed that CT26 and Renca cells transfected with the p*IFN- α* produced significant amounts of *IFN- α* protein in the culture medium (Figure 2a), and showed growth suppression *in vitro* (Figure 2b). In CT26 tumor-bearing BALB/c mice, the injection of p*IFN- α* complexed with cationic liposome (DMRIE-DOPE) expressed *IFN- α* in the tumors in a dose-dependent manner, and an *IFN- α* concentration by the injections of p*IFN- α* (30 μ g, three times) was comparable with that of Ad-m*IFN* (5×10^6 PFU, once) (Figure 2c). The plasmid-mediated *IFN- α* expression continued for more than 10 days after gene transfer and returned to a control level at 14 days after gene transfer (Figure 2d).

Then, to examine the *in vivo* antitumor effect of a plasmid-mediated *IFN- α* gene transfer, a p*IFN- α* /liposome complex was injected into the CT26 subcutaneous tumors at 7 days after the transplantation. The injection of p*IFN- α* slightly suppressed tumor growth compared with the non-injected control group, whereas the tumor-suppressive effect by the injection of p*IFN- α* was significantly enhanced in the lymphopenic mice that received synHSCT (Figure 2e). The results suggested that an *in vivo* injection of p*IFN- α* induced antitumor immunity in lymphopenic hosts as effectively as the Ad-m*IFN*. Regarding the timing of intratumoral *IFN- α* gene transfer, the injection of p*IFN- α* at 8 weeks after transplantation did not enhance the antitumor immunity, whereas a substantial antitumor effect was observed by *IFN- α* gene transfer in the earlier period (2–6 weeks) after the transplantation (Figure 2f), suggesting that intratumoral *IFN- α* gene transfer during immune reconstitution can induce a synergistic antitumor effect. In the mice treated with *IFN- α* gene transfer at 6 weeks after HSCT, tumor growth suppression was still recognized ($P=0.016$) at day 42 and the survival of the treated mice was significantly prolonged as compared with the injection of the control plasmid (Figure 2g). All treated mice looked healthy during the course of the experiments, and the blood chemistry (albumin, alanine transaminase, total bilirubin, alkaline phosphatase, blood urea nitrogen, creatinine) showed no abnormal values in the treated mice at 5 weeks after the HSCT.

Intratumoral *IFN- α* gene transfer increases tumor-infiltrating lymphocytes after synHSCT

It is known that a large number of tumor-infiltrating lymphocytes (TILs) results in a better prognosis for cancer patients.¹⁵ To examine whether an increase of TILs in treated tumors is related to tumor growth suppression, immunohistochemical staining of CD4 and CD8-

positive cells was performed at 1–4 weeks after synHSCT. In this experiment, CT26 cells were inoculated on legs at 1 week before synHSCT, and the number of CD4⁺ and CD8⁺ T cells infiltrated into the tumors was examined at the day of synHSCT to evaluate how the number of immune cells increases in the tumors by the combination therapy as compared with the pre-treatment status. In this established tumor model also, a significant antitumor effect was recognized in the synHSCT mice with the *IFN- α* gene transfer (Figure 3a).

The numbers of CD4⁺ and CD8⁺ cells gradually diminished in the non-treated tumors, whereas the TILs increased in the tumors of synHSCT mice. Intratumoral *IFN- α* gene transfer further increased the numbers of TILs significantly in synHSCT recipients at 4 weeks (Figure 3b), suggesting that intratumoral *IFN- α* gene transfer enhances and prolongs HP-induced antitumor immunity after synHSCT.

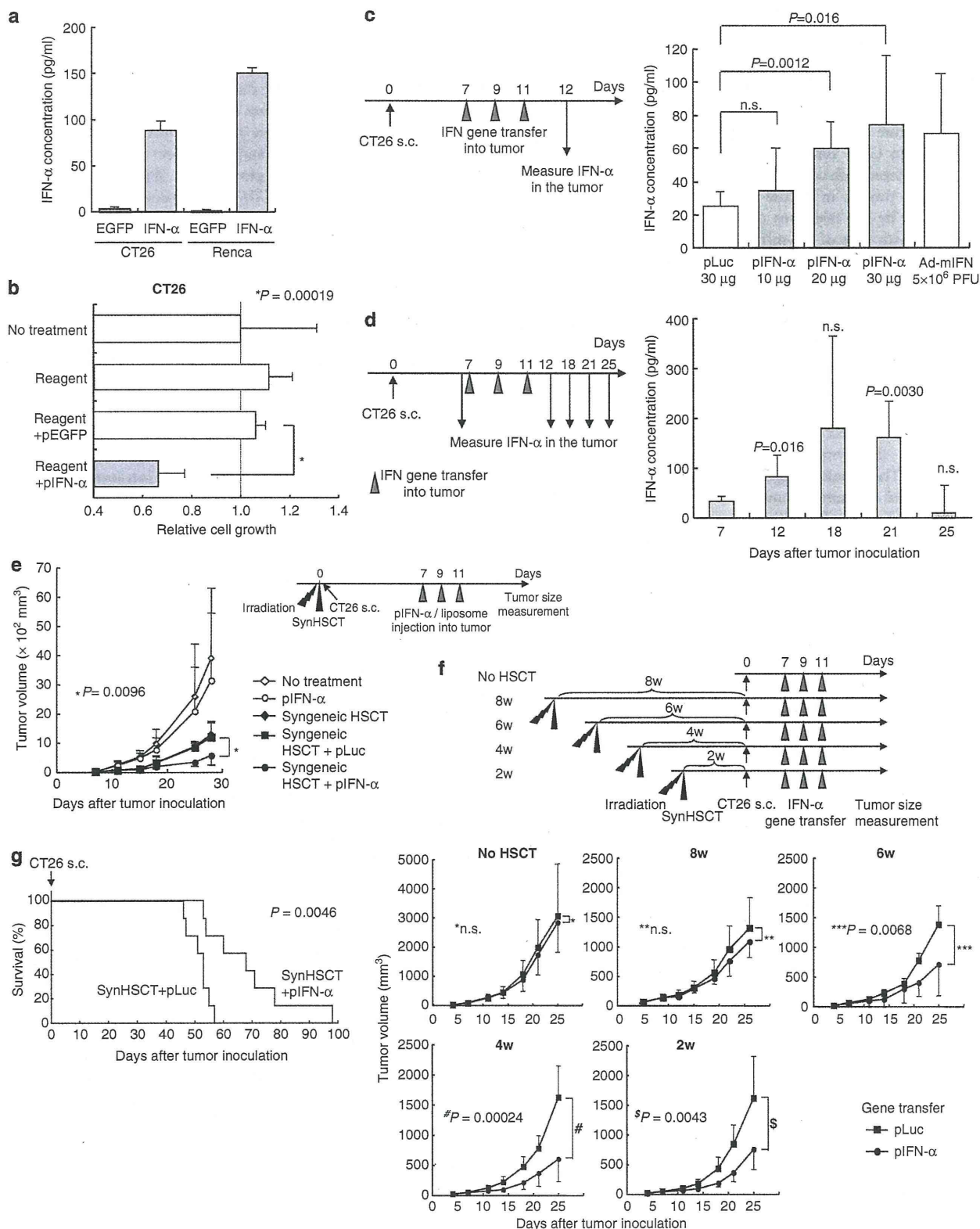
Then, to examine the cytotoxic activity of TILs, CD8⁺ T cells were isolated from tumors in the treated mice. The flow cytometry showed that the expression of perforin on CD8⁺ TILs was significantly enhanced in the synHSCT alone group and in the combination therapy-treated group, suggesting that synHSCT creates an environment to enhance the killing activity of TILs in the tumors (Figure 3c).

Tumor-specific lymphocytes are activated by intratumoral *IFN- α* gene transfer in synHSCT recipients

To examine the immune reaction to intratumoral *IFN- α* gene transfer in synHSCT recipients, splenocytes were extracted from the treated mice and cultured with CT26 cells. An enzyme-linked immunosorbent spot assay showed that the average number of *IFN- γ* -producing splenocytes in response to CT26 cells was slightly increased in the synHSCT alone group, whereas a combination of synHSCT and intratumoral *IFN- α* gene transfer further increased the *IFN- γ* -positive spots (Figure 4a, left panel). The numbers of the spots in splenocytes co-cultured with syngeneic lymphocytes were not changed in the treated groups (Figure 4a, right panel). To analyze the subset of activated lymphocytes, the frequency of tumor-reactive immune cells was determined by intracellular cytokine staining and flow cytometry. The percentage of CD4⁺ and CD8⁺ T cells stimulated to produce *IFN- γ* in response to CT26 cells increased significantly in the mice treated by a combination of synHSCT and intratumoral *IFN- α* gene transfer, and there was also an increase in the percentage of *IFN- γ* -positive NK cells (Figure 4b). The results indicated that the numbers of tumor-reactive lymphocytes were increased synergistically by a combination therapy.

An *in vitro* cytotoxic assay showed that the splenocytes derived from the synHSCT mice recognized and lysed CT26 cells, and *IFN- α* gene transfer enhanced the cytolysis to CT26 cells (Figure 4c, left panel). To

Figure 2 Plasmid vector-mediated *IFN- α* gene transfer induces a significant antitumor effect in synHSCT recipients. (a) *In vitro* *IFN- α* concentration in the medium of cancer cells transfected with p*IFN- α* . CT26 or Renca cells were transfected with p*IFN- α* , and 48 h later, *IFN- α* concentration in the culture medium was measured by enzyme-linked immunosorbent assay (ELISA; Immunotech, Marseille Cedex, France). pEGFP was used as a control. (b) *In vitro* cytotoxicity of p*IFN- α* transfection. The cell growth was determined by an *in vitro* cell proliferation assay at 5 days after transfection. The data are expressed as relative cell growth (OD₄₅₀ of indicated cells/OD₄₅₀ of untreated cells). Reagent; Lipofectamine 2000. (c) *In vivo* *IFN- α* concentration in the tumors transfected with p*IFN- α* . Various amounts (10–30 μ g) of p*IFN- α* complexed with liposome were injected three times into the CT26 tumors at 7, 9 and 11 days after the tumor inoculation. Ad-m*IFN* (5×10^6 PFU) was injected once into the tumors at day 9. Tumors were collected at day 12, and the *IFN- α* concentration was measured by ELISA ($n=4$). (d) Time course of *IFN- α* expression in the tumors. The *IFN- α* levels were measured in the subcutaneous tumors during 14 days after intratumoral injection of p*IFN- α* (30 μ g)/liposome complex at days 7, 9 and 11 ($n=4-5$). The statistical difference between *IFN- α* concentration in the indicated days and at day 7 is presented. (e) A combination of syngeneic HSCT and intratumoral *IFN- α* gene transfer caused marked tumor growth suppression. The p*IFN- α* (30 μ g)/liposome complex was injected into the CT26 tumors at 7, 9 and 11 days after the tumor inoculation in the synHSCT mice ($n=7$). The experiments were repeated two times. (f) Intratumoral *IFN- α* gene transfer during an early phase of immune reconstitution enhances the antitumor effects. Syngeneic HSCTs to the BALB/c mice were staggered at intervals of 2 weeks, followed by a subcutaneous injection of CT26 cells and intratumoral *IFN- α* gene transfer at days 7, 9 and 11 ($n=6-8$). (g) Intratumoral *IFN- α* gene transfer significantly extends the survival of synHSCT mice ($n=7$). The p*IFN- α* /liposome complex was injected into the CT26 tumors at 6 weeks after the HSCT, and survival of the mice was compared with the mice injected with a control plasmid ($n=7$).



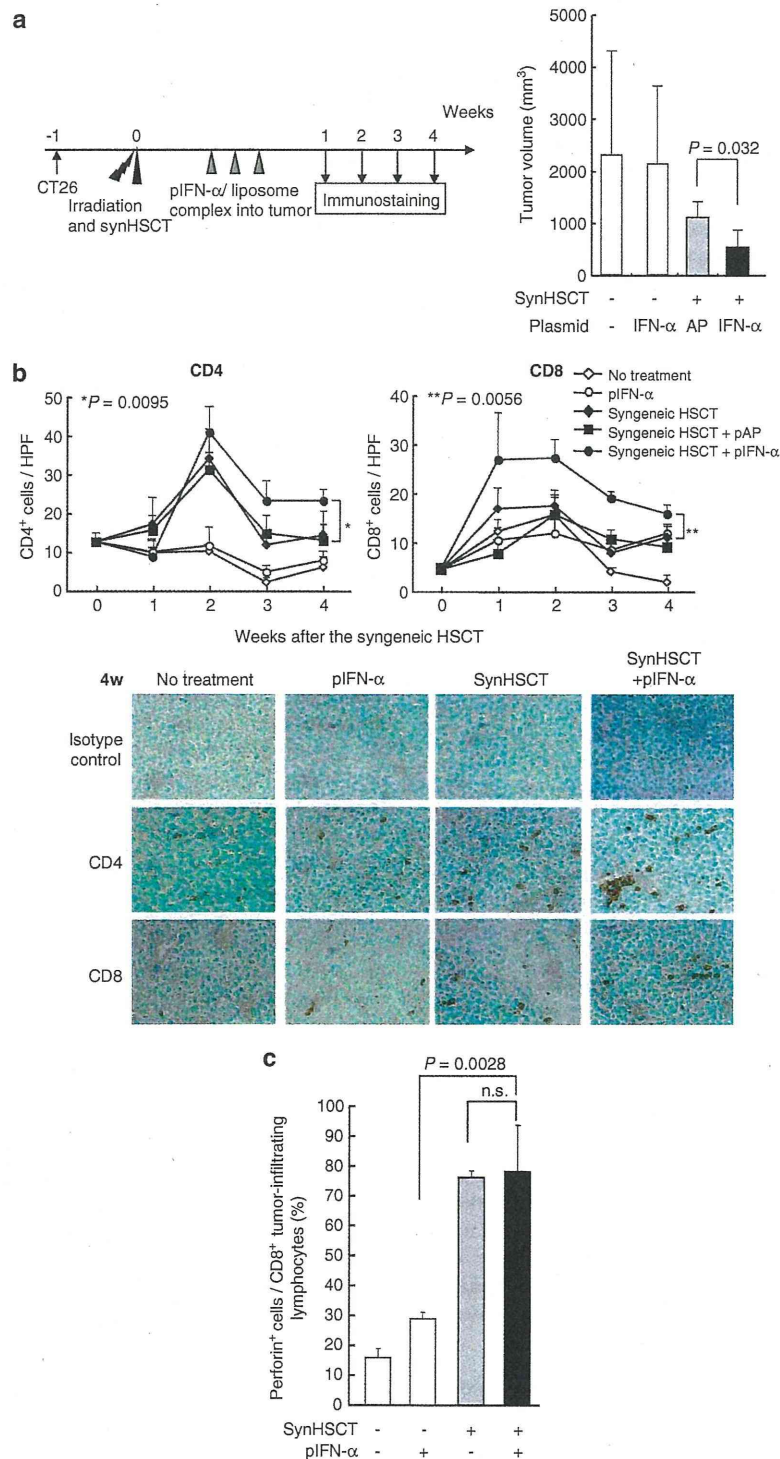


Figure 3 Immunostaining of treated tumors. (a) Antitumor effect of *IFN- α* gene transfer for established tumors in the synHSCT recipient mice. CT26 cells were inoculated on the right legs of BALB/c mice at 1 week before the synHSCT, and the pIFN- α /liposome complex was injected into CT26 tumors for three times. The tumor volumes at 3 weeks after the *IFN- α* gene transfer are presented. The subcutaneous tumors were resected at the indicated days ($n=3$), and stained with CD4 and CD8 antibodies. The CD4⁺ (upper left) and CD8⁺ cells (upper right) were counted by microscopy in five high-power fields ($\times 400$). Representative photographs of stained cells at 4 weeks after the gene transfer are presented in the lower panel ($\times 400$). (c) Expression of perforin on CD8⁺ tumor-infiltrating lymphocytes. The CD8⁺ T cells were isolated from the tumors using mouse CD8 MicroBeads and AutoMACS magnetic sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The flow cytometry of perforin (eBioOMAK-D; eBioscience, San Diego, CA, USA) was performed on the CD8⁺ TILs ($n=3$). The frequency of perforin⁺ cells per CD8⁺ cells is presented.

show the major histocompatibility complex class I-restriction of cytotoxicity, lymphocytes were pre-incubated with the anti-CD4 or anti-CD8 antibodies before the cytolysis for CT26 cells. The addition of anti-CD8 antibody markedly inhibited the cell lysis (Figure 4c, right panel).

To explore what kind of lymphocytes contribute to antitumor immunity *in vivo*, the mice were treated with anti-CD4, anti-CD8 and anti-asialo GM1 antibodies intraperitoneally to deplete CD4⁺ T cells, CD8⁺ T cells and NK cells, respectively. Depletion of all of the three populations canceled antitumor effect almost completely. Depletion of each CD4⁺ T-cell, CD8⁺ T-cell or NK cell showed some growth advantages but still resulted in significant tumor growth inhibition and, in particular, CD8⁺ T cells appeared to contribute more strongly than CD4⁺ T and NK cells (Figure 4d).

Intratumoral *IFN- α* gene transfer causes growth suppression in not only treated but also distant tumors

To evaluate the therapeutic efficacy of *IFN- α* gene transfer against tumors at distant sites in synHSCT recipients, the mice were inoculated with CT26 cells on both legs and Renca cells on the back, and an *IFN- α* plasmid/liposome complex was injected into the CT26 tumor on the right leg. The *IFN- α* gene transfer significantly suppressed the growth of not only the right leg CT26 tumors but also the left CT26 tumors that were not transfected with the *IFN- α* gene, whereas the growth of Renca tumors on the back was not influenced by the *IFN- α* gene transfer into the CT26 tumors (Figure 5a). When we exchanged CT26 and Renca cells, the antitumor effect was *vice versa* (Figure 5b). The results indicated that intratumoral *IFN- α* gene transfer enhances a vector-injected-tumor specific immunity systemically in the synHSCT recipients.

Liver metastasis is one of the most frequent causes of mortality in patients with gastrointestinal cancer such as colorectal cancer. As another model of distant metastasis, CT26-Luc cells were injected beneath the splenic capsule to generate liver metastasis, and CT26 cells were inoculated into the right leg. Intratumoral *IFN- α* gene transfer suppressed tumor growth of subcutaneous tumors on the legs (Figure 5c, upper left panel) as observed in Figure 2e, and the growth of abdominal tumors was also markedly suppressed, which were evaluated by photon counts on the IVIS imaging system (IVIS; Xenogen, Alameda, CA, USA), in the synHSCT mice (Figure 5c, upper right and lower panels). Twenty-one days after tumor inoculation, the mice were killed and the livers were examined by the IVIS imaging. Livers from non-treated mice showed many photon-positive spots, whereas livers from the mice treated by a combination therapy revealed a fewer number of photon-positive spots (Figure 5d, upper panel), which was confirmed by photon counts in those livers (Figure 5d, lower panel). These results indicated that a combination therapy is effective for preventing and regressing liver metastases.

A combination therapy enhances maturation of CD11c⁺ cells and their antigen presentation

To verify whether the intratumoral *IFN- α* gene expression promotes the maturation of DCs in the tumor, we isolated CD11c⁺ cells from the regional lymph nodes of treated tumors. Flow cytometry showed that expressions of CD40, CD80, CD83 and CD86 were clearly upregulated by the *IFN- α* gene transfer (data not shown and see Narumi *et al.*¹³). Then, the expression of CD83 was examined in the CD11c⁺ cells isolated from tumors. The frequency of CD83⁺ cells was increased in IFN/HSCT-CD11c⁺ cells compared with those in IFN-CD11c⁺ and HSCT-CD11c⁺ cells (Figure 6a, left panel), suggesting

that *IFN- α* expression results in the maturation of CD11c⁺ cells. Then, to examine the antigen-presentation capacity of the CD11c⁺ cells in the tumors, the lymphocytes isolated from naïve mice were co-cultured with the CD11c⁺ cells and mitomycin C-treated CT26 cells for 3 days. An enzyme-linked immunosorbent spot assay showed that the IFN/HSCT-CD11c⁺ cells increased the number of IFN- γ -positive lymphocytes in response to CT26 cells (Figure 6a, middle panel). The production of IFN- γ from the CD11c⁺ cells *per se* was minimal. Lymphocytes under.HP are considered to be primed to TAAs. When lymphocytes isolated from tumor-bearing synHSCT mice were co-cultured with the CD11c⁺ cells and mitomycin C-treated CT26 cells, the stimulation by IFN/HSCT-CD11c⁺ cells resulted in a higher number of IFN- γ -secreting lymphocytes than those of IFN-CD11c⁺ and HSCT-CD11c⁺ cells (Figure 6a, right panel). The results indicated that the antigen-presentation by CD11c⁺ cells was enhanced by the *IFN- α* expression in the tumors in synHSCT mice.

CD11c⁺ cells treated by a combination therapy suppresses the activity of regulatory T cells

To analyze the cytokine expression profile of the CD11c⁺ cells, we collected CD11c⁺ cells from the treated tumor at 2 weeks after *IFN- α* gene transfer, cultured the cells *in vitro* for 48 h and measured the expression of various cytokines in the medium. The IFN/HSCT-CD11c⁺ cells produced a large amount of immune-stimulatory cytokines such as interleukin (IL)-1 β , IL-6 and IL-12 (Figure 6b), which may enhance the proliferation and activation of lymphocytes in the treated mice. Among the immunosuppressive cytokines (IL-4, IL-10 and transforming growth factor- β), IL-10 production is increased in IFN/HSCT-CD11c⁺ cells. The enhanced IL-12 production might promote the production of IL-10 in some population of the CD11c⁺ cells as a negative feedback mechanism.¹⁶ However, the large amount of immune-stimulatory cytokines may overcome the suppressive effect of IL-10 in the tumors treated with the combination therapy.

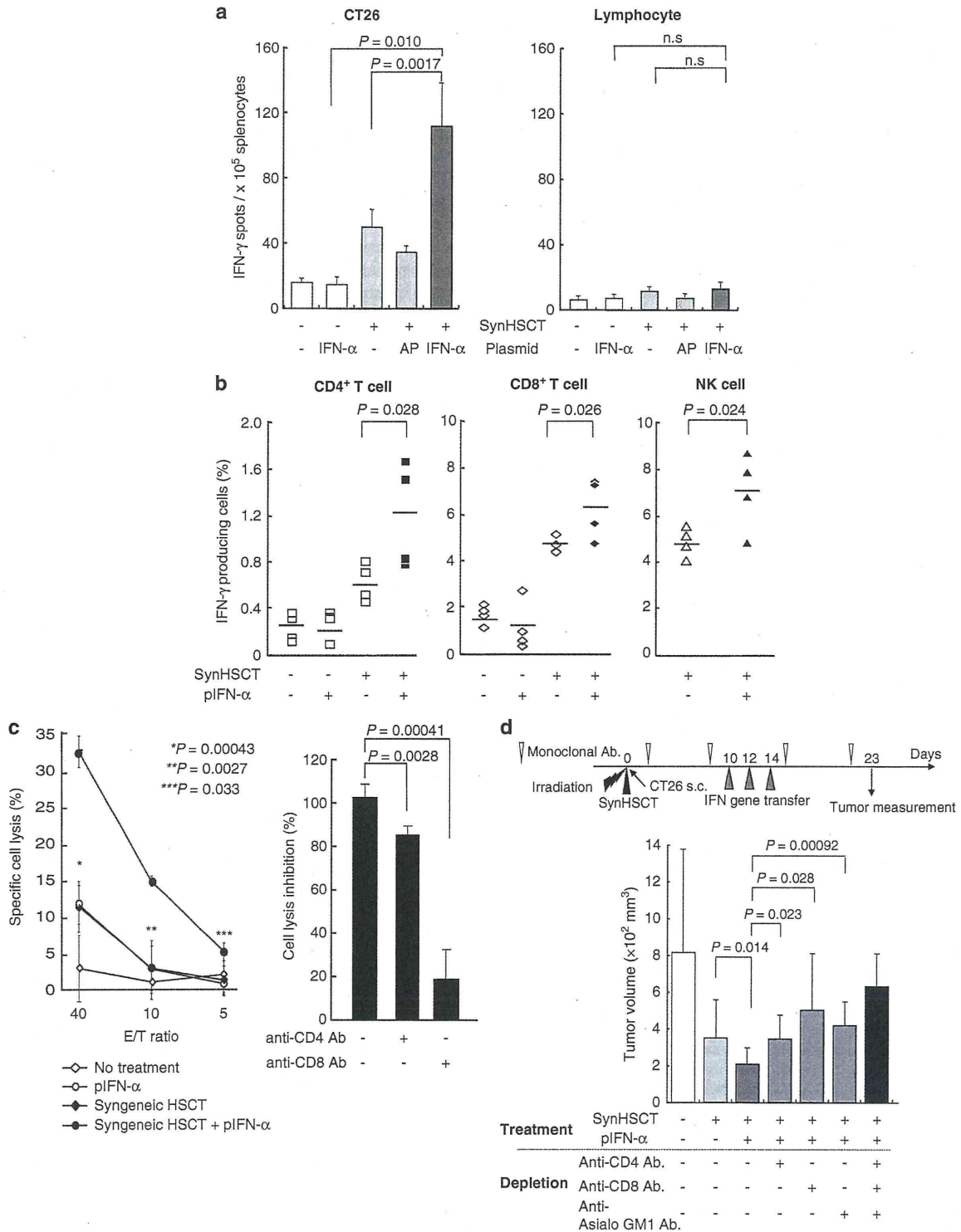
IL-6 is of particular interest, because, when released from DCs, it is critical for overcoming Tregs-mediated immune suppression.¹⁷ When the CD11c⁺ cells isolated from the spleen of synHSCT and non-HSCT mice were cultured in the medium containing *IFN- α* protein, the cells produced IL-6 in a dose-response manner (Figure 7a). To examine whether the CD11c⁺ cells inhibit the suppressive activity of Tregs, CD11c⁺ cells were isolated from regional lymph nodes and co-cultured with CD4⁺CD25⁻ T cells (target) and CD4⁺CD25⁺ Tregs in mouse anti-CD3 T-cell activation plates. When Tregs were co-cultured with the control CD11c⁺ cells isolated from non-treated mice, Tregs effectively suppressed the proliferation of target T cells. The co-culture with IFN/HSCT-CD11c⁺ cells canceled the suppressive activity of Tregs for effector T cells (Figure 7b). The supernatant of IFN/HSCT-CD11c⁺ cells also inhibited the suppressive activity of Tregs, and the addition of anti-IL-6 antibody in the medium restored the activity of Tregs, indicating that the critical factor of CD11c⁺ cell-mediated inhibition of Tregs is IL-6, produced from activated CD11c⁺ cells (Figure 7c).

Then, to examine the *in vivo* effect of intratumoral *IFN- α* expression on Tregs, we collected lymphocytes from treated tumors in synHSCT mice. Flow cytometry showed that intratumoral *IFN- α* gene transfer decreased the ratio of Foxp3⁺ cells per CD4⁺ T cells in the tumors of synHSCT mice, whereas the ratio of Foxp3⁺ cells in the spleen was same as that in the non-treated mice (Figure 7d). The results suggested that IL-6 produced from CD11c⁺ cells decreases the ratio of Tregs in the treated tumors. *IFN- α* expression may result in the extensive infiltration of T cells into the tumors of synHSCT mice (Figure 3b), and the number of Foxp3⁺ cells also might increase in the

tumors. However, as the antitumor immune response is based on the balance between the effector and regulatory sides of immune cells; the decrease of Treg ratio (Figure 7d) and inhibition of Treg activity (Figure 7b) may lead to a strong antitumor immunity.

DISCUSSION

In this study, we showed that an intratumoral *IFN-α* gene transfer significantly enhances a systemic tumor-specific immunity in the synHSCT recipients. The precise mechanism for the enhancement is



not completely understood, but it should include an effective stimulation of DCs by the expression of *IFN- α* in the tumors in synHSCT recipients, because (1) intratumoral expression of *IFN- α* effectively induces cell death of cancer cells and exposes TAAs in large quantity to DCs (CD11c⁺ cells);¹² (2) *IFN- α* promotes maturation of CD11c⁺ cells, which facilitates the presentation of TAAs on CD11c⁺ cells (Figure 6a); (3) CD11c⁺ cells in the tumors transduced with the *IFN- α* gene produce a large quantity of immune-stimulatory cytokines such as IL-12 (Figure 6b); (4) the CD11c⁺ cells in the treated tumors suppress the inhibitory activity of Tregs (Figure 7b). The combination of immune-stimulatory effects by *IFN- α* and the reconstitution of a fresh immune system following HSCT could create an environment strongly supporting the activation of an antitumor response. We propose a model showing the integrated mechanisms of inducing a strong antitumor immunity by a combination therapy (Figure 8).

Although the conditioning of HSCT with irradiation and/or immunosuppressive reagents can destroy the immunotolerance deployed by the tumor, the tumors restore a tolerant microenvironment by induction of Tregs and the production of immune-inhibitory cytokines,¹⁸ which may be one of the main reasons for the failure to fully sustain HP-mediated antitumor immunity. An analysis of the cytokine profile unexpectedly showed that the *IFN/HSCT-CD11c⁺* cells produce a large amount of IL-6 as well as other immune-stimulatory cytokines (Figure 6b). It has been reported that IL-6 increases methylation of upstream Foxp3 enhancer and represses the Foxp3 transcription in natural Tregs.¹⁹ Our findings demonstrated that CD11c⁺ cells isolated from the tumors transduced by *IFN- α* gene significantly suppress the activity of Tregs (Figure 7b), which may inhibit or delay the reconstitution of an immunotolerant microenvironment in the tumor. CD11c⁺ cells seem to have a capacity to produce IL-6 in response to *IFN- α* in a dose-dependent manner (Figure 7a). The IL-6 production was increased in the *HSCT-CD11c⁺* cells also (Figures 6b and 7b). The intratumoral *IFN- α* expression and the immune-stimulatory condition by elevated cytokine levels after synHSCT may synergistically influence CD11c⁺ cells to produce IL-6. It is also reported that IL-6 promotes carcinogenesis through multiple signal pathways.²⁰ The role of IL-6 in tumors is probably based on the balance between the positive and negative effect of IL-6 on the tumor growth. Although it is known that DCs secrete proinflammatory cytokines such as IL-6 by toll-like receptor stimulation,¹⁷ further research is needed to clarify the critical factor/pathway for the production of IL-6 from DCs in synHSCT recipients.

As HP leads to a break in tolerance against self-antigens, the expression of *IFN- α* could theoretically promote T-cell response not only against tumor cells but also against host normal cells, which may

cause an autoimmune reaction. However, no overt toxicity was observed for the treated mice, including their blood chemistry. The immunogenic DCs at the *IFN- α* vector-injected tumor site were able to capture both TAAs and normal self-antigens shared by tumor and normal cells, and promote tumor-specific immunity and a local autoimmune reaction, whereas resting host DCs away from the tumor site present only normal self-antigens and may induce tolerance or exhaustion of host-reactive T cells. Alternatively, we recently found that the percentage of Foxp3⁺ cells per CD4⁺ T cells in the spleen was clearly elevated at an early phase after syngeneic HSCT (data not shown), which differed from the low frequency of Foxp3⁺ T cells in the tumor (Figure 7d). The finding suggests that among CD4⁺ T cells, Tregs rapidly proliferate during HP in the body, which might protect patients against autoimmunity after autologous HSCT.

There have been several animal studies showing the potential efficacy of gene- and cell-based immunotherapy in syngeneic HSCT mice. The vaccination with syngeneic tumor cells expressing granulocyte-macrophage colony stimulating factor showed a strong antitumor effect in the transplanted mice.⁵ An immunization with DCs pulsed with whole tumor cell lysates led to efficient antitumor responses in a mouse breast tumor model.²¹ Adoptive transfer of tumor-specific T cells has also shown enhanced antitumor immune responses after HSCT in lymphopenic mice,² and recently, Morgan *et al.*²² reported efficacy of a strategy composed of immunodepletion and adaptive cell transfer for patients with metastatic melanoma. As tumor-reactive T cells are mostly polyclonal, and heterogeneous expressions of various TAAs coexist even in a tumor mass, the *in vivo* stimulation of multiple tumor-reactive lymphocytes might be critical in the clinical application. Moreover, compared with the previous approaches, another major advantage of an *in vivo* *IFN- α* gene transfer is that it does not involve a manipulation and culture of the immune and tumor cells *ex vivo*, making this strategy more feasible for many patients with solid cancers. We previously reported that an allogeneic MHC gene transfer also could enhance an effective antitumor immunity in HSCT recipients.⁶ The major difference between the allogeneic MHC and *IFN- α* gene therapies is in their local effects on tumor sites transduced with the therapeutic genes: *IFN- α* gene transfer significantly induces cell death and growth inhibition (Figure 2b and see Hara *et al.*¹²). In addition, *IFN- α* seems to have direct effects upon DCs such as the maturation of the cells and production of immune-stimulatory cytokines and enhancement of inhibitory activity against Tregs. Therefore, a local *IFN- α* gene therapy is a promising therapeutic strategy, especially in a case in which cancer conditions need strong local tumor control and systemic antitumor activity.

Some of the experiments in this study showed important points to consider in the clinical feasibility of the combination therapy.

Figure 4 A large number of *IFN- α* -producing cells are induced by intratumoral *IFN- α* gene transfer in synHSCT recipient mice. (a) Enzyme-linked immunosorbent spot assay of *IFN- γ* -producing cells in response to stimulation of CT26 cells. At 2 weeks after plasmid-mediated *IFN- α* gene transfer, mouse splenocytes were isolated from treated mice and co-cultured with CT26 cells or control lymphocytes ($n=3$). The experiments were repeated three times. (b) Intracellular cytokine staining of *IFN- γ* -producing cells in response to stimulation of CT26 cells. The splenocytes (1×10^6) from treated mice were incubated with CT26 (1×10^5) and stained by allophycocyanin-anti-mouse *IFN- γ* . The activated cell fractions were analyzed by staining with fluorescein isothiocyanate-anti-mouse CD4, CD8 or CD49b (labeling NK cell) antibody ($n=3-4$). The experiments were repeated twice. (c) *In vitro* cytotoxic assay of splenocytes. Splenocytes were isolated from the treated mice, and their cytotoxicity was evaluated in a standard 4 h ⁵¹Cr release assay against CT26 cells ($n=3$). The statistical difference between cell lysis in the synHSCT mice with *IFN- α* gene transfer and synHSCT alone mice is presented (left panel). Splenocytes isolated from synHSCT mice with *IFN- α* gene transfer were pre-incubated with the anti-CD4 or anti-CD8 antibodies for 1 h before cytotoxicity for CT26 cells (right panel). The data are expressed as cell lysis inhibition (%) at E/T ratio=40 (cell lysis with co-incubation of antibody/that with no antibody). The experiments were repeated twice. (d) Antitumor effect of *IFN- α* gene transfer after *in vivo* depletion of CD4⁺ T cells, CD8⁺ T cells and NK cells. A group of transplanted mice were treated with anti-CD4, anti-CD8 or anti-asialo GM1 antibodies (targeting NK cells) to deplete these cell populations, and the CT26 tumors were injected with p*IFN- α* vector ($n=7-9$). Tumor volumes at 10 days after *IFN- α* gene transfer are presented.

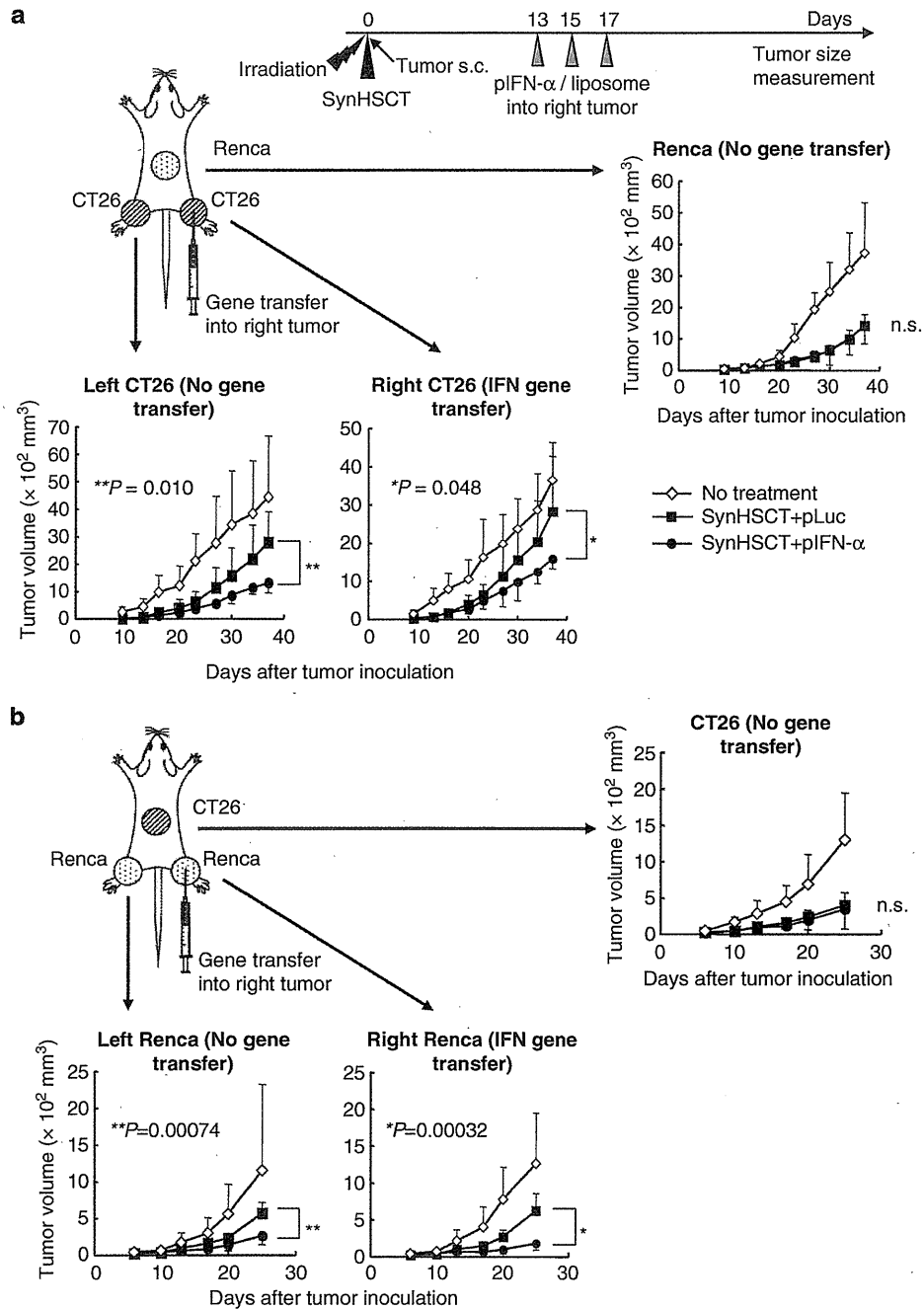


Figure 5 Suppression of tumors at distant site by *IFN-α* gene transfer during immune reconstitution. The experiments were repeated two times. (a) Intratumoral *IFN-α* gene transfer into CT26 tumors. CT26 cells were inoculated on both legs and Renca cells were inoculated on the back in synHSCT mice. The pIFN-α/liposome complex was injected into CT26 subcutaneous tumors on the right legs at days 13, 15 and 17 ($n=4-8$). (b) Intratumoral *IFN-α* gene transfer into Renca tumors. Renca cells were inoculated on both legs and CT26 cells on the back in synHSCT mice. The pIFN-α/liposome complex was injected into Renca subcutaneous tumors on the right legs at days 13, 15 and 17 ($n=4-8$). (c) Suppression of liver metastasis. CT26-Luc cells were injected beneath the splenic capsule to generate liver metastasis, and CT26 cells were inoculated on the right leg. After the *IFN-α* gene transfer, the size of subcutaneous tumors on the legs was macroscopically measured, and photon count of abdominal tumors was evaluated by the IVIS imaging system. (d) *Ex vivo* imaging of the liver. The livers were resected from treated mice at 21 days after the synHSCT, and photon spots and counts of the livers were evaluated by the imaging system. Arrowheads: liver tumors.

First, a liposome-mediated *IFN-α* gene transfer effectively suppressed tumor growth in synHSCT recipients. Although the peak level of *IFN-α* expression was not very high, the expression continued for more

than 10 days after gene transfer (Figure 2d), suggesting that a high concentration of *IFN-α* in the tumors is not necessary and that the continuous expression is important to induce an effective antitumor

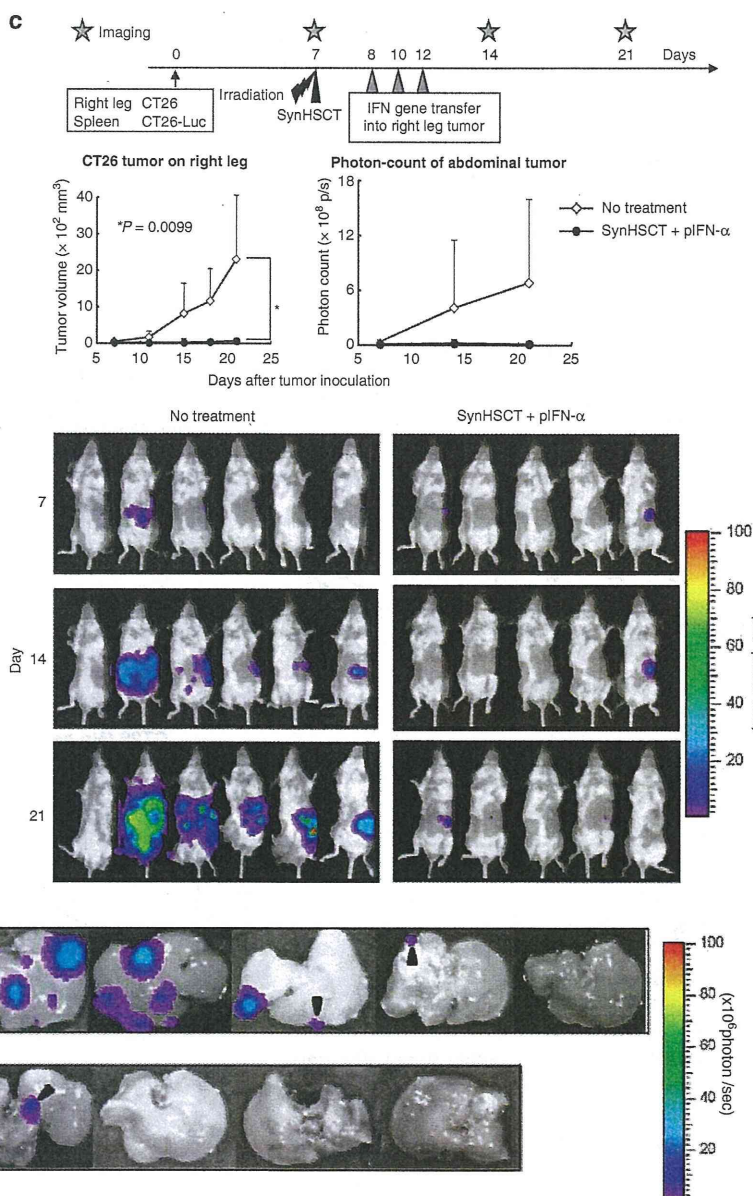


Figure 5 Continued.

immunity. This facilitates a clinical application, because *in vivo* lipofection of an *IFN-α*-expressing plasmid is much safer than virus vectors, although the latter has a high gene transduction efficacy.¹⁴ Second, the intratumoral *IFN-α* gene transfer at 6 weeks after synHSCT significantly suppressed tumor growth and prolonged the survival of synHSCT mice (Figures 2e and f). At 6 weeks after synHSCT, neutrophil count returned to a normal level (data not shown), which avoids the risk of bacterial infection that accompanies a needle injection for intratumoral gene transfer. Third, a combination therapy was effective in suppressing not only the vector-injected tumors but also the vector-uninjected distant tumors in the liver metastasis model, which resembles a clinical setting. The results indicated that this treatment strategy might be feasible for many

patients with solid cancers. The next step in research may include a further elucidation of the main mechanism of *IFN-α* in inducing tumor immunity and the synergism between *IFN-α* and synHSCT, including identification of potential key factors other than IL-6 and DC, development of methods to further sustain and control tumor-specific immunity and to predict and monitor HP followed by an individualization of the combination therapy.

In conclusion, a combination of intratumoral *IFN-α* gene transfer with synHSCT is a promising immunotherapy for solid cancers, because of the activation of tumor-specific immunity, suppression of the immunotolerant environment and excellent safety features. This therapeutic strategy deserves an evaluation in future clinical trial for solid cancers.

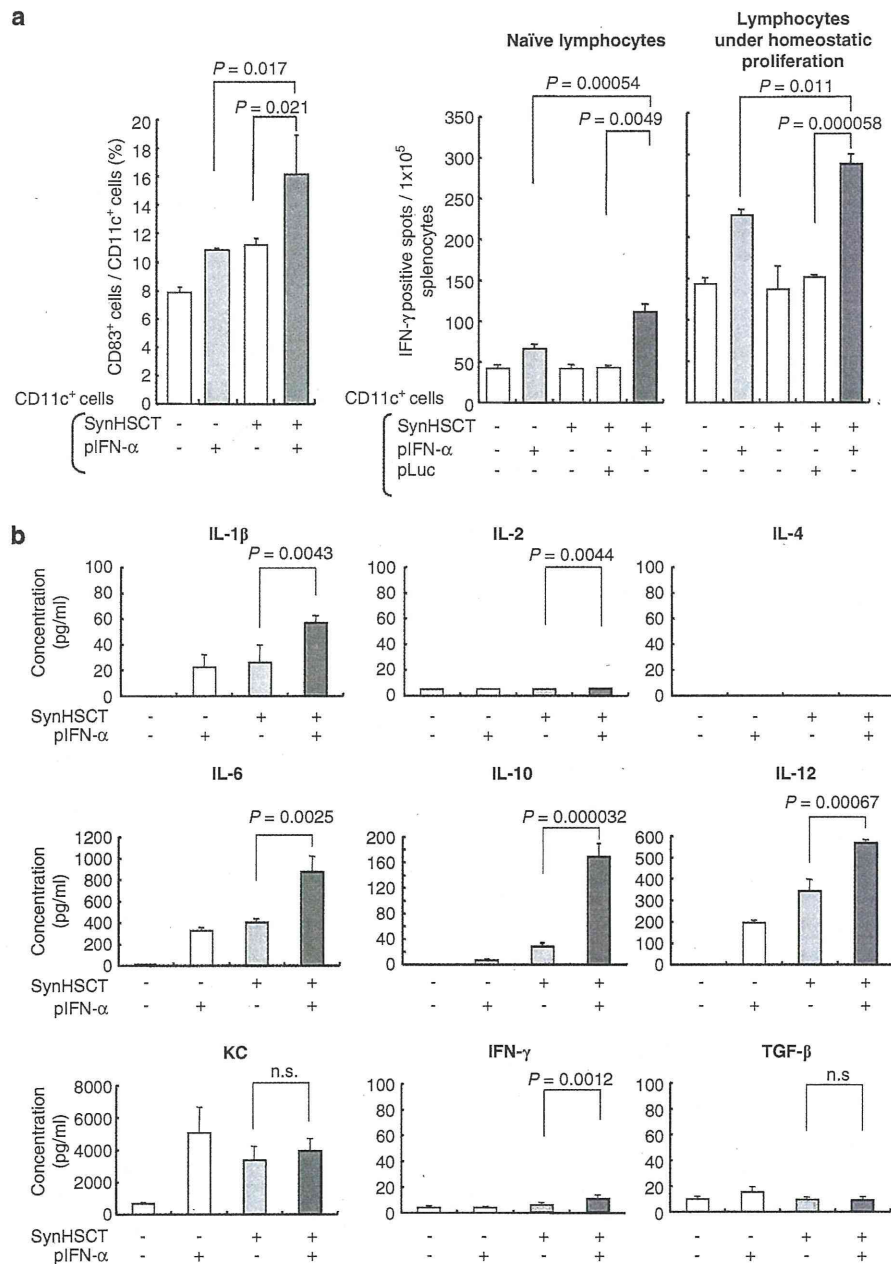


Figure 6 IFN- α expression promotes the maturation of CD11c⁺ cells in the tumor. (a) Number of IFN- γ -positive cells by enzyme-linked immunosorbent spot assay. Flow cytometry of CD83⁺ cells (Michel-19; BD Pharmingen) was performed in the CD11c⁺ cells isolated from tumors ($n=3$). The frequency of CD83⁺ cells per CD11c⁺ cells is presented (left panel). CD11c⁺ cells from treated tumors were co-cultured with lymphocytes isolated from naïve BALB/c mice (middle panel) or synHSCT mice (right panel), and lymphocyte activation was measured by IFN- γ -enzyme-linked immunosorbent spot assay ($n=3$). The experiments were repeated twice. (b) Cytokine production of CD11c⁺ cells. CD11c⁺ cells were isolated from treated tumors ($n=3$), and were seeded in a 48-well plate (1×10^5 cells per well). After the incubation for 48 h, cytokines in the medium were measured by a cytokine array (Procarta Cytokine Assay Kit; Panomics, Inc., Fremont, CA, USA). IL-10 level was measured by enzyme-linked immunosorbent assay (Quantikine; R&D Systems, Minneapolis, MN, USA). The experiments were repeated three times.

MATERIALS AND METHODS

Animals and hematopoietic stem cell transplantation

Seven-to-nine-week-old female BALB/c (H-2^d Ly-1.2) mice were purchased from Charles River Japan, Inc., (Kanagawa, Japan). Animal studies were carried out according to the *Guideline for Animal Experiments of the National Cancer Center*

Research Institute and approved by the Institutional Committee for Ethics in Animal Experimentation. Nine-to-ten-week-old BALB/c mice received a lethal (9 Gy) irradiation on the day of transplantation. The irradiated BALB/c mice were injected intravenously with 5×10^6 of bone marrow cells and 2×10^6 splenic T cells from donor BALB/c mice. Bone marrow cells were isolated from donors by

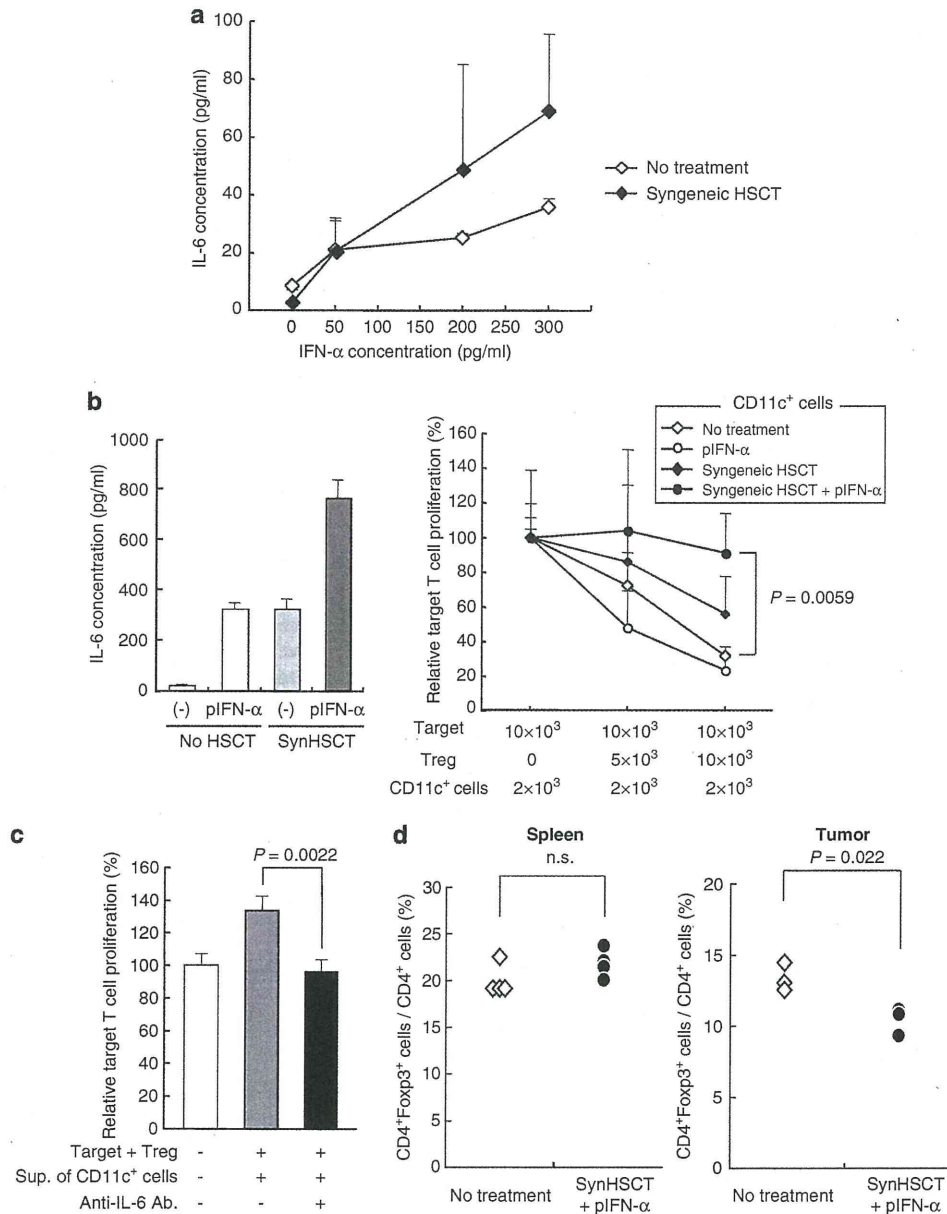


Figure 7 CD11c⁺ cells in the tumor inhibit the immunosuppressive activity of Tregs. (a) CD11c⁺ cells produce IL-6 in response to IFN- α . CD11c⁺ cells isolated from the spleens of non-HSCT and synHSCT tumor-bearing BALB/c mice were seeded in a 96-well plate (1.5×10^5 cells per well) and cultured in the medium containing IFN- α protein at indicated concentration for 2 h. After the change of medium, the cells were incubated for 48 h, and IL-6 in the medium was measured by ELISA (Quantikine; R&D Systems). The experiments were repeated twice. (b) The inhibition of activity of Tregs by CD11c⁺ cells. The CD11c⁺ cells were isolated from treated tumors and seeded in a 48-well plate (1×10^5 cells per well), and after incubation for 24 h ($n=4$) (left panel), IL-6 production from the CD11c⁺ cells was measured by ELISA. Target cells (CD4⁺CD25⁻ T cell) and Treg (CD4⁺CD25⁺ T cell) were isolated from the spleen of naïve BALB/c mice, and were co-cultured with the designated CD11c⁺ cells in a CD3-coated 96-well plate, and the proliferation of target cells was examined by ³H-thymidine uptake assay ($n=3$) (right panel). The experiments were repeated three times. (c) IL-6-mediated suppression of Treg activity. Target cells and Tregs were cultured in a CD3-coated 96-well plate with the supernatant of CD11c⁺ cells from treated tumors, and the proliferation of target cells was evaluated by ³H-thymidine uptake assay ($n=3$). The addition of anti-IL-6 antibody (R&D systems) was used to neutralize mouse IL-6 in the medium. The experiments were repeated twice. (d) The frequency of Tregs in the spleen and treated tumors. The lymphocytes were collected from the spleens ($n=4$, left panel) and treated tumor ($n=3$, right panel), and Foxp3⁺ and CD4⁺ cells were analyzed by flow cytometry.

flushing each femur and tibia with RPMI-1640 medium (RPMI) supplemented with 5% heat-inactivated fetal bovine serum (ICN Biomedicals, Inc., Irvine, CA, USA), and splenic cells were prepared by macerating the spleens. After lysis of the

erythrocytes, splenic cells were incubated with anti-Thy-1.2 immunomagnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at 4 °C for 15 min, followed by selection of T cells by AutoMACS (Miltenyi Biotec).

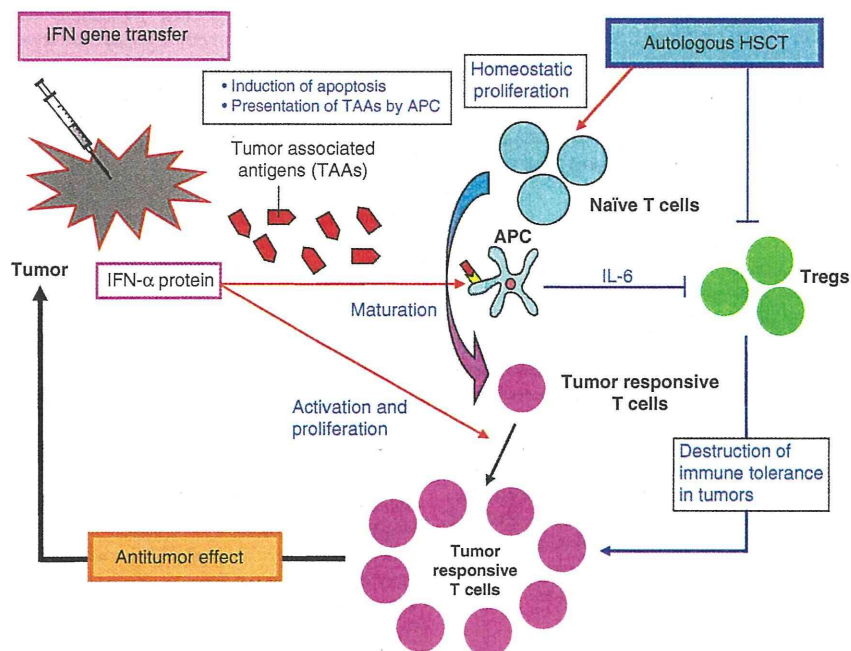


Figure 8 Model showing integrated mechanisms of inducing strong tumor immunity by a combination therapy. In the 'homeostatic proliferation' condition after synHSCT, T cells effectively recognize the low-affinity self-antigen including TAAs, leading to an induction of antitumor immunity. The conditioning of HSCT with irradiation and/or immunosuppressive reagents can destroy the immunotolerance mechanisms developed by the tumor. Furthermore, *IFN- α* expression in the tumors exposes TAAs in large quantity to DCs, and *IFN- α* promotes maturation and enhances the antigen-presenting capacity of DCs. In addition, DCs produced a significant amount of IL-6 in response to *IFN- α* , which suppress the proliferation and activity of Tregs. The integrated mechanisms are capable of inducing a strong antitumor immunity against solid cancers.

Tumor cell lines, recombinant adenovirus vectors and plasmid vectors

CT26 and Renca (American Type Culture Collection, Rockville, MD, USA) are weakly immunogenic BALB/c-derived colon and renal cancer cell lines, respectively. Cells were maintained in RPMI containing 10% fetal bovine serum, 2 mM L-glutamine and 0.15% sodium bicarbonate (complete RPMI). A CT26 cell line that stably expresses the firefly luciferase gene was generated by retrovirus vector-mediated transduction and designated as CT26-Luc. The recombinant adenovirus vectors expressing mouse interferon- α (Ad-mIFN) and alkaline phosphatase cDNA (Ad-AP) were prepared as described.^{23,24} The recombinant adenoviruses are based on serotype 5 with deletions of the entire E1 and a part of the E3 regions, and have the CAG promoter, which is a hybrid of the cytomegalovirus immediate early enhancer sequence and the chicken β -actin/rabbit β -globin promoter. A cesium chloride-purified virus was desalted using a sterile Bio-Gel P-6 DG chromatography column (Econopac DG 10; Bio-Rad, Hercules, CA, USA) and diluted for storage in a 13% glycerol/phosphate-buffered saline solution. All viral preparations were confirmed by PCR assay to be free of E1⁺ adenovirus. A plasmid DNA (pIFN- α) expressing the *IFN- α* gene under the control of the CAG promoter was also used for intratumoral gene transfer. The plasmids that express an alkaline phosphatase (pAP) or luciferase gene (pLuc) were used as a negative control.

In vitro cell proliferation assay

Cultured cells were seeded at 2×10^3 per well in 96-well plates and plasmid DNA-liposome (Lipofectamine2000; Invitrogen, Carlsbad, CA, USA) complex was added according to the manufacturer's protocol. The cell numbers were assessed by a colorimetric cell viability assay using a water-soluble tetrazolium salt (Tetrazolium One; Seikagaku Corp., Tokyo, Japan) at 5 days after the transfection. Absorbance was determined by spectrophotometry using a wavelength of 450 nm with 595 nm as a reference. The assays (carried out in four wells) were repeated three times.

In vivo tumor inoculation and *IFN- α* gene transfer

CT26 cells (1×10^6) or Renca cells (5×10^6) were injected subcutaneously into the leg of BALB/c mice. When the subcutaneous tumor was established (~ 0.6 cm in diameter), it was injected once with 50 μ l of Ad-mIFN or control vector (Ad-AP). Plasmid DNA-liposome complex was prepared by the addition of 30 μ g plasmid DNA into a total of 75 μ l phosphate-buffered saline per mouse, followed by the addition of 75 μ l of 0.15 mmol l⁻¹ DMRIE-DOPE ((+/-)-N-(2-hydroxyethyl)-N, N-dimethyl -2,3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoylphosphatidylethanolamine), which was provided from Vical, Inc., (San Diego, CA, USA). The mixture solution was incubated at room temperature for 15 min, and then injected directly into the tumor three times every other day. The shortest (*r*) and longest (*l*) tumor diameters were measured at indicated days and the tumor volume was determined as $r^2 l/2$. Data are presented as mean \pm s.d. The experiments were repeated two times.

Enzyme-linked immunosorbent spot assays

IFN- γ ELISpot kit (BD Bioscience, San Jose, CA, USA) was used according to the manufacturer's instructions. Briefly, splenocytes (1×10^5) and mitomycin C-treated tumor cells (1×10^4) were co-cultured in 96-well plates pre-coated with mouse IFN- γ (BD Bioscience) for 20 h at 37 °C in complete RPMI medium in triplicate. After washing the wells, biotinylated anti-mouse IFN- γ antibody (2 μ g ml⁻¹) was added and incubated for 2 h at room temperature. Then, a streptavidin-horseradish peroxidase solution was added and incubated for 1 h at room temperature. After the addition of an aminoethyl carbazole substrate solution, spots were counted under a stereomicroscope.

Flow cytometry of cell surface marker and intracellular cytokine staining

Allo-phycocyanin-conjugated monoclonal antibody (mAb) to identify mouse IFN- γ and fluorescein isothiocyanate-conjugated mAb to detect CD4, CD8 and CD49b were purchased from BD Pharmingen (San Jose, CA, USA). Splenocytes (1×10^6) were incubated with medium alone (control) or CT26 (1×10^5) cells

for 2 days; brefeldin-A ($10 \mu\text{g ml}^{-1}$) was then added for 2 h of incubation. After washing, cells were incubated with the CD4, CD8 or CD49b mAbs in a total volume of $100 \mu\text{l}$ phosphate-buffered saline with 5% fetal bovine serum for 30 min at 4°C , and then fixed and permeabilized with a permeabilization buffer (BD Biosciences). Cells were finally stained with antibody to IFN- γ for 15 min at room temperature, washed again and analyzed by FACSCalibur (BD Biosciences). Irrelevant immunoglobulin G mAbs were used as a negative control. Ten thousand live events were acquired for analysis.

Cytotoxic assays

An *in vitro* cytotoxic assay was performed as previously described.¹² Briefly, splenocytes were cultured for 4 days with mitomycin C-treated CT26 stimulators, and then the responder cells were collected and used as effector cells. CT26 target cells were labeled with ^{51}Cr (Perkin-Elmer Japan Co., Kanagawa, Japan). For a 4 h chromium release assay, 4×10^5 , 1×10^5 and 5×10^4 effector cells were mixed with 1×10^4 target cells in a 96-well round-bottom plate (Corning Incorporated, New York, NY, USA). To evaluate the relative contributions of CD4⁺ and CD8⁺ T cells for the tumor cell lysis, effector cells were incubated with mAbs against mouse CD4 (L3L4; BD Pharmingen) or CD8 (Ly-2; BD Pharmingen) for 1 h at 37°C before mixing with target cells. Supernatants were harvested and counted in a gamma counter (Packard Bioscience Company, Meriden, CT, USA). The percentage of cytotoxicity was calculated as ((experimental c.p.m.-spontaneous c.p.m.)/(maximum c.p.m.-spontaneous c.p.m.)) $\times 100$. Each assay was carried out in triplicate.

Immunohistochemistry

Immunostaining was performed using streptavidin-biotin-peroxidase complex techniques (Nichirei, Tokyo, Japan). Consecutive cryostat tissue sections ($6 \mu\text{m}$) were mounted on glass slides and fixed in 99.5% ethanol for 20 min. After blocking with normal rat serum, the sections were stained with rat anti-mouse CD4 and CD8 antibodies (BD Pharmingen). Parallel negative controls with antibodies of the same isotype were examined in all cases. The sections were counter-stained with methyl green.

In vivo depletion of T and NK cells

To deplete the subsets of immune effector cells before and during the treatment with *IFN- α* gene transfer, the synHSCT mice received intraperitoneal injections of 0.3 mg. Monoclonal antibody from the anti-CD4⁺ hybridoma (clone GK1.5, rat IgG2b) or 1.5 mg mAb from the anti-CD8⁺ hybridoma (clone Lyt-2.1, mouse IgG2b; see Nakayama and Uenaka²⁵) or 0.5 mg of anti-asialo GM1 antibody (targeting NK cells: Wako Pure Chemical Industries, Ltd, Tokyo, Japan). Administration of antibodies started at 2 days after the inoculation of CT26 cells, and the injection was repeated every 5–6 days, throughout the entire experimental period. Flow cytometry showed that $\sim 80\%$ of CD4⁺, $\sim 60\%$ of CD8⁺ T cells and $\sim 80\%$ of NK cells were depleted in the Ab-treated mice.

In vivo imaging of the tumors in a liver-metastasis model

CT26-Luc cells were injected beneath the splenic capsule to generate liver metastasis. The BALB/c mice with CT26-Luc tumors were administered with D-luciferin (150 mg kg^{-1}) (Wako Pure Chemical Industries) by intraperitoneal injection. At 10 min later, photons from animal whole bodies were counted using an *in vivo* imaging system.

Isolation of CD11c⁺ cells and T-cell proliferation assay

Dendritic cells were isolated using mouse CD11c MicroBeads and AutoMACS magnetic sorter (Miltenyi Biotec) from tumors of non-HSCT mice treated by intratumoral *IFN- α* gene transfer, tumors of synHSCT mice injected with control plasmid and tumors of synHSCT mice treated by intratumoral *IFN- α* gene transfer, and designated as IFN-CD11c⁺, HSCT-CD11c⁺ and IFN/HSCT-CD11c⁺, respectively. The flow cytometry showed that $\sim 90\%$ of isolated cells express CD11c, and that $\sim 80\%$ of the isolated CD11c⁺ cells are negative for CD14 (macrophage marker), suggesting that a major population of isolated CD11c⁺ cells is DCs. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were isolated from the spleen of naïve BALB/c mice using mouse CD4 pre-enrichment kit, mouse CD25 selection kit and RoboSep magnetic sorter (StemCell Technologies,

Vancouver, BC, Canada). These populations were stained with anti-Foxp3 antibody, and flow cytometry revealed that about 80% of CD4⁺CD25⁺ cells expressed Foxp3. CD4⁺CD25⁻ T cells were incubated in a 96-well plate (1×10^4 per well) with 2×10^3 of CD11c⁺ cells, $0.5 \mu\text{g ml}^{-1}$ of anti-CD3 antibody and the indicated number of CD4⁺CD25⁺ T cells for 48 h. T-cell proliferation was determined as ^3H -thymidine incorporation during the last 12 h of culture.

Statistical analysis

Comparative analyses of the data were performed by the Student's *t*-test, using SPSS statistical software (SPSS Japan Inc., Tokyo, Japan). $P < 0.05$ was considered as a significant difference.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Competitive Interactions of Cancer Cells and Normal Cells via Secretory MicroRNAs^{*[5]}

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Background: Homeostatic cell competitive system between cancerous cells and non-cancerous cells is considered as the reason for tumor initiation.

Results: Exosomal tumor-suppressive microRNAs secreted by non-cancerous cells inhibit the proliferation of cancerous cells.

Conclusion: Exosomal tumor-suppressive microRNAs act as an inhibitory signal for cancer cells in a cell-competitive process.

Significance: This provides a novel insight into a tumor initiation mechanism.

Normal epithelial cells regulate the secretion of autocrine and paracrine factors that prevent aberrant growth of neighboring cells, leading to healthy development and normal metabolism. One reason for tumor initiation is considered to be a failure of this homeostatic cell competitive system. Here we identify tumor-suppressive microRNAs (miRNAs) secreted by normal cells as anti-proliferative signal entities. Culture supernatant of normal epithelial prostate PNT-2 cells attenuated proliferation of PC-3M-luc cells, prostate cancer cells. Global analysis of miRNA expression signature revealed that a variety of tumor-suppressive miRNAs are released from PNT-2 cells. Of these miRNAs, secretory miR-143 could induce growth inhibition exclusively in cancer cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process. This study provides a novel insight into a tumor initiation mechanism.

Competitive interactions among cells are the basis of many homeostatic processes in biology. In *Drosophila*, normal epithelial cells compete with transformed ones for individual survival, which is a process called cell competition (1, 2). If a given group of cells was exposed to some stress, it would be separated into subpopulations of cells with different levels of damage. In noncompetitive conditions, cells with severe damage die in a

short time, whereas moderately damaged cells survive to the next generation, indicative of the transduction of a negative phenotype. On the other hand, in competitive conditions even slightly damaged cells are eliminated from the cell group because healthy cells, the “winners,” convey death signals to damaged cells, the “losers,” and the losers reciprocally confer growth signals to the winners. This feed-forward regulation enables the cell population to eradicate abnormal cells and maintain the same number of normal cells in a limited niche.

Oncogenesis is characterized by genetic and metabolic changes reprogramming living cells to undergo uncontrolled proliferation (3). This suggests that the abnormal cells that are originally destined for elimination can survive and expand against the cell competitive regulation, leading to the formation of a tumor mass. Consistently with this concept, Bondar and Medzhitov (4) showed that the cell competition process involves p53, a tumor-suppressive gene, between the hematopoietic stem cells and progenitor cells, suggesting that gene modifications of p53 could disturb the homeostatic mechanism and give rise to tumor initiation. It is conceivable that p53 target genes could be associated with intercellular communication between winners and losers; however, this literature has not answered the question of whether this regulatory system is mediated by contact-dependent or contact-independent manner. More than 10 years ago a pioneer study suggested that non-cancerous cells co-cultured with cancer cells inhibit the growth of cancer cells *in vitro* (5). This result indicated that humoral factors could be involved in cell competition as intercellular communicators (6).

As recently as a few years ago it was believed that RNAs could not behave as extracellular signal molecules because of their vulnerability to the attack of ribonucleases largely existing in body fluid. Evidence is presently increasing to show that miRNAs⁴ contained in exosomes are released from mammalian

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⁴ The abbreviations used are: miRNA, microRNA; CM, conditioned medium; luc, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QRT-PCR, quantitative real time PCR.

Secretory miR-143 as an Anti-cancer Signal

cells and act as a signal transducer (7). It is important that many different tumor-suppressive miRNAs, such as miR-16 and miR-143, are down-regulated in cancer cells, resulting in tumorigenesis, tumor progression, and metastasis (8–11). Taken together, these findings suggest that secretory miRNAs may have favorable aspects for anti-proliferative signals mediating cell competition.

In this report we show that miR-143 expression in normal prostate cells, PNT-2 cells, is higher than that in prostate cancer cells, PC-3M-luc cells, and that miR-143 released from non-cancerous cells transfers growth-inhibitory signals to cancerous cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs might be a death signal from winners to losers in the context of cell competition. Secretory miRNAs can be conducive to the maintenance of normal growth and development.

EXPERIMENTAL PROCEDURES

Reagents—Mouse monoclonal anti-KRAS (F234) (sc-30) was purchased from Santa Cruz. Rabbit polyclonal anti-ERK5 (#3372) was purchased from Cell Signaling. Mouse monoclonal anti-actin, clone C4 (MAB1501), was obtained from Millipore. Mouse monoclonal anti-human-CD63 antibody (556019) was purchased from BD Pharmingen. Peroxidase-labeled anti-mouse and anti-rabbit antibodies were included in the Amersham Biosciences ECL PLUS Western blotting Reagents Pack (RPN2124) (GE Healthcare). Synthetic *Caenorhabditis elegans* miRNA cel-miR-39 was synthesized by Qiagen (Valencia, CA). Synthetic hsa-miR-143 (pre-miR-143), the negative control 1 (NC1), has-miR-143 inhibitor molecule (anti-miR-143), and the negative control inhibitor molecule (anti-NC) were purchased from Ambion (Austin, TX). GW4869 was purchased from Calbiochem. Geneticin was purchased from Invitrogen.

Cell Culture—PNT-2 cells, immortalized normal adult prostatic epithelial cell line, were purchased from the DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HEK293 cells, a human embryonic kidney cell line (CRL-1573), were obtained from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO₂. PNT-2 and the prostate cancer cell line, PC-3M-luc cells, continuously expressing firefly luciferase (Xenogen, Alameda, CA), were cultured in RPMI containing 10% heat-inactivated FBS and an antibiotic-antimycotic at 37 °C in 5% CO₂.

Preparation of Conditioned Medium and Exosomes—Before the collection of culture medium, cells were washed 3 times with Advanced RPMI containing an antibiotic-antimycotic and 2 mM L-glutamine (medium A), and the medium was switched to fresh medium A. After incubation for 3 days, medium A was collected and centrifuged at 2000 × *g* for 10 min at room temperature. To thoroughly remove cellular debris, the supernatant was centrifuged again at 12,000 × *g* for 30 min at room temperature or filtered through a 0.22-μm filter (Millipore). The conditioned medium (CM) was then used for miRNA extraction and functional assays as well as exosome isolation.

For exosome preparation the CM was ultracentrifuged at 110,000 × *g* for 70 min at 4 °C. The pellets were washed with 11

ml of PBS, and after ultracentrifugation they were resuspended in PBS. The exosome fraction was measured for its protein content using the Micro BCA Protein Assay kit (Thermo Scientific, Wilmington, DE).

Isolation of MicroRNAs—Isolation of extracellular and cellular miRNAs was performed using the miRNeasy Mini Kit (Qiagen). Two hundred microliters of conditioned medium or cell lysate was diluted with 1 ml of Qiazol Solution. After 5 min of incubation, 10 μl of 0.1 nM cel-miR-39 was added to each aliquot followed by vortexing for 30 s. Subsequent extraction and filter cartridge work were carried out according to the manufacturer's protocol.

Quantitative Real Time PCR (QRT-PCR)—The method for QRT-PCR has been previously described (7). PCR was carried out in 96-well plates using the 7300 Real Time PCR System (Applied Biosystems). All reactions were done in triplicate. All TaqMan MicroRNA Assays were purchased from Applied Biosystems. Cel-miR-39 and RNU6 were used as an invariant control for the CM and cells, respectively.

Immunoblot Analysis—SDS-PAGE gels, SuperSep Ace 5–20% (194–15021) (Wako), were calibrated with Precision Plus Protein Standards (161–0375) (Bio-Rad), and anti-KRAS (1:100), anti-ERK5 (1:1000), anti-CD63 (1:200), and anti-actin (1:1000) were used as primary antibodies. The dilution ratio of each antibody is indicated in parentheses. Two secondary antibodies (peroxidase-labeled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:10,000. Bound antibodies were visualized by chemiluminescence using the ECL PLUS Western blotting detection System (RPN2132) (GE Healthcare), and luminescent images were analyzed by a LuminoImager (LAS-3000; Fuji Film, Inc.). Only gels for CD63 (BD Biosciences) detection were run under non-reducing conditions.

Plasmids—The primary-miR-143 expression vector was purchased from TaKaRa BIO. For luciferase-based reporter gene assays, pLucNeo was constructed by inserting a firefly luciferase gene derived from the pGL3-control (Promega) into the pEYFP-1 vector (Clontech) at BglII and AflIII sites. The sensor vector for miR-143 was constructed by introducing tandem binding sites with perfect complementarity to miR-143 separated by a four-nucleotide spacer into the NotI site of psiCHECK2 (Promega). The sequences of the binding site are as follows: 5'-AAACCTAGAGCGGCCGCGAGCTACAGTGTCTCATCTCAAAGAATTCTTGAGCTACAGTGCTTCA-TCTCAGCGGCCGCTGGCCGCAA-3' (sense) and 5'-TTGCGGCCAGCGGCCGCTGAGATGAAGCACTGTAGCTC-AAGAATTCTTTGAGATGAAGCACTGTAGCTCGCGGCCGCTCTAGGTTT-3' (antisense). The "seed" sequence of miR-143 is indicated by bold italics. In a mutated miR-143 sensor vector, the seed sequence, TCATCTC, was displaced with GACGAGA. All the plasmids were verified by DNA sequencing.

Transient Transfection Assays—Transfections of 10 nM miR-143 mimic and 3 nM anti-miR-143 were accomplished with the DharmaFECT Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. The total amounts of miRNAs for each transfection were equally adjusted by the addition of NC1 and anti-NC, respectively.