Gene Therapy (2010) 17, 1333–1340
© 2010 Macmillan Publishers Limited All rights reserved 0969-7128/10
www.nature.com/gt

ORIGINAL ARTICLE

Addition of adenoviral vector targeting of chemotherapy to the MUC-1/ecdCD40L VPPP vector prime protein boost vaccine prolongs survival of mice carrying growing subcutaneous deposits of Lewis lung cancer cells

H Akbulut[1], Y Tang[2], KG Akbulut[3], J Maynard[2] and A Deisseroth[2]
[1]Department of Medical Oncology, Ankara University School of Medicine, Ankara, Turkey; [2]Department of Genetic Therapy, Sidney Kimmel Cancer Center, San Diego, CA, USA and [3]Department of Physiology, Gazi University School of Medicine, Ankara, Turkey

We studied the effect of adding chemotherapy or vector targeted chemotherapy to the administration of an Ad-sig-hMUC-1/ecdCD40L adenoviral vector prime-hMUC-1/ecdCD40L protein boost cancer vaccine (designated hMUC-1/ecdCD40L VPPP vaccine), which were administered to test mice 10 days following subcutaneous (s.c.) inoculation of 500 000 Lewis Lung Carcinoma cells, at a time when the average volume of the s.c. tumors was 50 cu mm. The survival of hMUC-1/ecdCD40L VPPP vaccine-treated mice was twice as long as untreated mice. Addition of vector-targeted chemotherapy (AdCMVCDIRESE1A/5FC) to the hMUC-1/ecdCD40L VPPP vaccine 10 days after tumor inoculation significantly ($P = 0.0062$) prolonged the survival of the test mice over administration of the hMUC-1/ecdCD40L VPPP vaccine alone or the control mice ($P < 0.00001$). Interestingly, the combination of the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy to the hMUC-1/ecdCD40L VPPP vaccine decreased the levels of $CD44^+CD24^-$ cells in s.c. deposits of the human MUC-1-positive Lewis Lung Cancer cell line (LL2/LL1hMUC-1) by 20 fold. These results suggest that the addition of vector-targeted chemotherapy to an adenoviral-based cancer vaccine is a strategy that deserves further testing.
Gene Therapy (2010) **17,** 1333–1340; doi:10.1038/gt.2010.93; published online 1 July 2010

Keywords: *chemotherapy; adenoviral vector; cytosine deaminase; MUC-1; lung cancer cell*

Introduction

The decrease in production of antigen-specific T cells during aging, the lack of appropriate presentation of tumor-associated antigens by the antigen-presenting cells or dendritic cells (DCs), the absence of co-stimulatory cytoadhesion molecules on tumor cells, and the loss of expression among older test subjects of immunostimulatory ligands such as the CD40 ligand (CD40L) on activated CD4 helper T cells make the immune system less responsive to cancer cells among older individuals.[1–4] The decreased ratio of antigen-naive T cells/memory cells and the decreased expression of the OX40 receptor on the activated T cells of the older aged test subjects [3,4] may also have a role.

The strategies aimed at increasing the availability of tumor-associated antigens (TAA) to Class I presentation on DCs either by breaking down the tumor cells with cytotoxic drugs or injecting the vaccines carrying TAA linked to the extracellular domain (ecd) of the CD40L have been reported to increase the tumor-specific immune response. We have previously reported that the linkage of the TAA to the CD40L increased presentation of the TAA fragments on Class I MHC on DCs[5] and induced an increase of the expansion of the TAA-specific CD8 effector T cells.[5–8] In addition to increasing the levels of TAA-specific CD8 effector T cells and antibodies, linking the TAA to the CD40L overcomes anergy in TAA.Tg mice[7] and overcomes the defective immune response in older test subjects such as 18-month-old mice.[7]

Our experiments have also shown that s.c. injection of the TAA/ecdCD40L protein at 7, 14 and 21 days after the injection of the Ad-sig-TAA/ecdCD40L vector (we call this the TAA/ecdCD40L VPPP vaccine), increases the magnitude of TAA-specific immune response over that achievable by two s.c. injections of the Ad-sig-TAA/ecdCD40L vector alone.[5–8] Moreover, the use of chemotherapy (which induces breakdown of cancer cells with attendant release of TAA) concomitantly with vaccination, which increases the amount of TAA available for presentation by DCs might increase the level of TAA for presentation by DCs.[8,9]

However, the use of systemic chemotherapy, delivered at the time of vaccination, could also diminish the

Correspondence: Dr A Deisseroth, US Food and Drug Administration, 10903 New Hampshire Avenue, Building 22, Room 6187, Silver Spring, MD 20903, USA.
E-mail: albert.deisseroth@fda.hhs.gov
Received 5 July 2009; revised 25 February 2010; accepted 15 May 2010; published online 1 July 2010

TAA/ecdCD40L VPP vaccine-induced immune response due to the chemotherapy sensitivity of the TAA-specific CD8 effector T cells, which are proliferating following the vaccination. To spare the proliferating TAA-specific CD8 effector T cells from the effects of chemotherapy, we combined AdCMVCDIRESE1A/5FC vector-targeted chemotherapy (VTCT) with the TAA/ecdCD40 L VPPP vaccination.[8,9]

The $CD44^+CD24^-$ fraction of cancer cells isolated from epithelial cancers has been shown to contain low frequency cells displaying high levels of tumorigenicity, self-renewal and chemotherapy resistance.[10–13] The importance of these cells has been validated by the discovery that cancer-associated gene expression signatures displayed by these cells correlate with therapy outcome and survival in clinical trials in human subjects.[14,15] $CD44^+CD24^-$ cancer cells have been shown to be in the peripheral blood of 30% of patients at diagnosis and over 90% of patients with recurrent disease.[16,17] These cells have also been shown to express stem cell gene expression signatures.[18,19] On the basis of their tumorigenicity, high self-renewal capacity, chemotherapy resistance, expression of stem cell genes and ability to regenerate all of the cells present in the epithelial tissue of origin, at least some of the $CD44^+CD24^-$ cells found in cancer tissue may contain 'cancer stem cells'.

Since the first description of the malignant stem cell of acute myeloid leukemia by Dick *et al*,[20] many reports have identified cancer stem cells, or tumor-initiating cells in epithelial solid tumor neoplasms,[10–13,21] as well as in central nervous system tumors[22] and in melanoma.[23]

Even though as few as 100 of these highly purified cancer stem cells can generate tumors in NODXSCID mice in 100% of the experiments, many authorities feel that these populations are still genetically or functionally heterogeneous. Nonetheless, therapeutic targeting of the cancer stem cells has become a priority for cancer research due to the possibility that these cells are highly resistant to chemotherapy, and have high self-renewal capacity and tumorigenicity. These cells may be the root cause of recurrence following initial therapy. As these cancer stem cells are highly resistant to DNA-damaging therapy such as chemotherapy, it is logical to develop new directions of therapy of cancer stem cells, based on mechanisms of therapy that are different from those involved in the killing of cells with chemotherapy. One such approach is immunotherapy. As the MUC-1 antigen has been found on the cancer stem cells or tumor-initiating cells as well as on the majority of the more mature cells of epithelial neoplasms,[24–26] we tested if the hMUC-1/ecdCD40 L VPPP vaccine when combined with the AdCMVCDIRESE1A targeted chemotherapy[5–8] could produce a decrease in the level of the $CD44^+CD24^-$ cells in s.c. deposits of the mouse Lewis lung cancer cell line, which was made positive for human MUC-1 by transfection of an expression plasmid for the human MUC-1 gene.

In this study, we tested if the addition of 5-FU systemic chemotherapy or VTCT, which is mediated by the i.t. injection of the AdCMVCDIRESE1A adenoviral vector followed by the i.p. injection of the 5-FU precursor, 5-Fluorocytosine (5-FC), to the hMUC-1/ecdCD40L VPPP vaccination would confer a survival advantage over the hMUC-1/ecdCD40L VPPP vaccination alone. In addition, we tested if the addition of chemotherapy (5-FU) or vector-targeted chemotherapy (AdCMVCDIRESE1A/5FC) to the hMUC-1/ecdCD40L VPPP vaccine would increase the killing of $CD44^+CD24^-$ cancer cells and increase the magnitude of the anti-tumor immune response in mice carrying growing sc nodules of Lewis Lung Cancer.

We tested whether the use of the AdCMVCDIRESE1A/5FC vector to deliver chemotherapy would avoid damaging the hMUC-1-specific CD8 T cells undergoing expansion as a result of the hMUC-1/ecdCD40L VPPP vaccine. It is theoretically possible that the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy would have a greater effect on the cancer cells, specifically the $CD44^+CD24^-$ cancer cells, than does the 5-FU chemotherapy. 5-FU was delivered i.p., whereas the vector targeting of chemotherapy was accomplished by i.t. injection of the AdCMVCDIRESE1A vector followed by i.p. injection of 5-FC (AdCMVCDIRESE1A/5FC). We had previously studied the effect of i.t. injection of the AdCMVCDIRESE1A/5FC in mouse models based on different cancer cells.[9,27,28]

The mice treated with both the hMUC-1/ecdCD40L VPPP vaccine and the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy, Group E, had the smallest tumor volume and survived longer than did mice in the other groups ($P<0.001$). Interestingly, the hMUC-1/ecdCD40L VPPP vaccine strategy targeting MUC-1, significantly decreased the number of $CD44^+CD24^-$ cancer cells by greater than 20 fold in subcutaneous deposits of hMUC-1-positive Lewis lung cancer. In conclusion, the administration of i.t. injections of the AdCMVCDIRESE1A vector followed by i.p. 5-FC concomitant with s.c. injection of the hMUC-1/ecdCD40L VPPP vaccine yielded more potent immune responses than did the hMUC-1/ecdCD40L VPPP vaccine alone.

These results suggest that the TAA/ecdCD40L VPPP vaccine strategy targeting MUC-1 and its combination with AdCMVCDIRESE1A vectortargeted chemotherapy will increase the vaccine-induced immune response against aggressive epithelial cell lines as exemplified by the Lewis lung cancer cell line.

Results

Tests for in vivo *induction of the cellular immune response*

The frequency of IFN gamma and IL-4-secreting T cells from the tumor draining lymph nodes and spleens of vaccinated mice. The frequency of IFN-γ-secreting lymph node and splenic T cells of the experimental treatment groups of mice were assessed by enzyme-linked immunospot (ELISPOT) assay. Lymphocytes isolated from the lymph nodes and splenocytes were stimulated by mitomycin C-treated hMUC-1 LL2/LL1 cells. As shown in Figure 1a, mice injected with the hMUC-1/ecdCD40L VPPP vaccination only (Group no. C) had significantly more IFN-γ-secreting T cells in tumor-draining lymph nodes when compared to the other groups ($P<0.001$). Similarly, VTCT plus the hMUC-1/ecdCD40L VPPP vaccination group (Group E) had more IFN-γ spots than all the other groups except for Group C ($P<0.01$). Splenocytes from the hMUC-1/ecdCD40L VPPP only and VTCT plus hMUC-1/

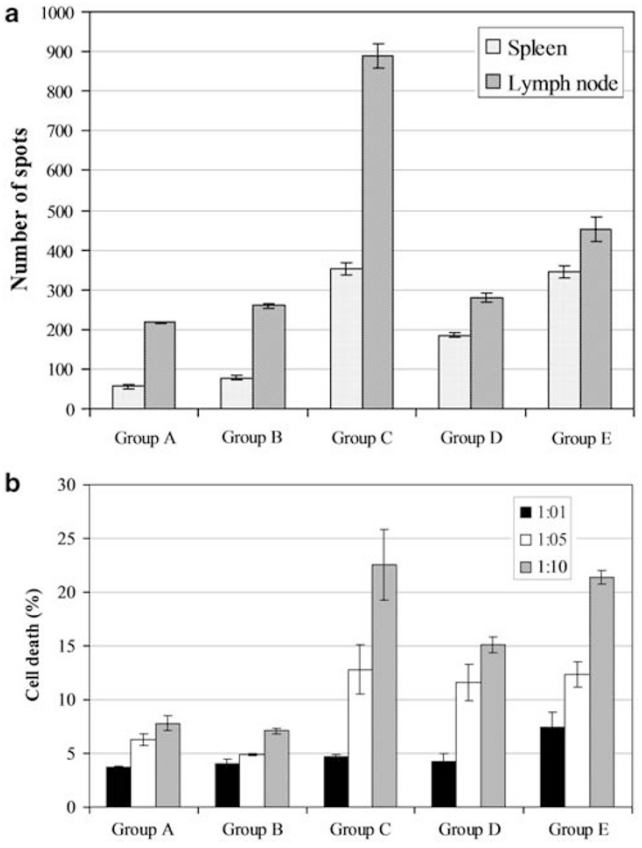


Figure 1 Induction of specific immunity by VPPP vaccination. (**a**) Results of ELISPOT analysis of splenic and tumor-draining lymph node cells of C57Bl/6 mice. Either splenic or tumor-draining lymph node cells of three mice from each group were pooled for ELISPOT analysis and activated by mitomycin-treated LL2/LL1 cells. The immune cells from the hMUC-1/ecdCD40 L VPPP vaccine alone and hMCU-1/ecdCD40L VPPP vaccine plus VTCT (Groups no. C and no. E) had significantly more IFN-γ spots than the other groups (Group no. A: Control (PBS); Group no. B: systemic 5-FU; Group no. D: hMUC-1/ecdCD40 L VPPP vaccine plus systemic 5-FU). (**b**) Cell-mediated cytotoxicity of tumor-draining lymph node CD8(+)T-cells from test mice. Following the activation by mitomycin C-treated LL2/LL1 cells, splenic CD8(+) T cells from three mice of each group of test mice were used to test their cytotoxicty against LL2/LL1 cells. The T-cells from the hMUC-1/ecdCD40L VPPP only and hMUC-1/ecdCD40L VPPP plus VTCT-treated groups (Groups no. C, and no. E) had significantly more cytotoxicity than the other groups (Group no. A: Control (PBS); Group no. B: systemic 5-FU; and Group no. D: VPPP plus systemic 5-FU). Groups no. C and E mice showed significantly higher cell-mediated cytotoxicity than seen with T cells from other groups.

ecdCD40L VPPP vaccine groups had more spots than the other groups ($P<0.05$). 5-FU significantly decreased the number of specific T-cells when administered in combination with hMUC-1/ecdCD40L VPPP vaccine (Group D).

Cell-mediated cytotoxicity assay of splenic T cells and lymphocytes of tumor draining nodes from the vaccinated mice. Cell-mediated cytotoxicity was assayed through the use of an antibody to apoptosis-associated caspase-3 by flow cytometry. Seven days after the injection of the last protein boost, the spleens and the tumor-draining lymph nodes of the mice were removed and CD8 (+) cells were isolated. The lymphocytes from lymph nodes of the mice injected with the hMUC-1/ecdCD40L VPPP either alone or in combination with systemic 5-FC chemotherapy or AdCMVCDIRESE1A vector-targeted chemotherapy had significantly greater cytotoxicity than did the control and systemic 5-FU chemotherapy alone groups (Group A and Group B) as shown in Figure 1b. However, systemic 5-FU chemotherapy significantly decreased the magnitude of cell-mediated cytotoxicity induced by the hMUC-1/ecdCD40L VPPP vaccination (see Group C and Group D of Figure 1b). On the other hand, i.t. administration of AdCMVCDIRESE1A vector followed by i.p. administration of 5-FC, had no significant effect on the response of T-cell cytotoxicity when combined with the hMUC-1/ecdCD40L VPPP vaccination (see Groups E and Group C of Figure 1b).

Effect of MUC1 vaccination on the In vivo *growth of hMUC-1 positive LL2/LL1 Lewis lung cancer cells in C57BL/6 mice*

Test mice were separated into five groups: (A) control (untreated); (B) 5-Fluorouracil (5-FU) chemotherapy only; (C) hMUC-1/ecdCD40 L VPPP vaccine only; (D) 5-FU chemotherapy plus hMUC-1/ecdCD40 L VPPP vaccine; and (E) AdCMVCDIRESE1A/5FC VTCT plus hMUC-1/ecdCD40 L VPPP vaccine. These mice were initially inoculated s.c. with 100 000 of the hMUC-1-positive LL2/LL1 cancer cells. The mice in the vaccine only (Group C), 5-FU chemotherapy plus VPPP vaccine (Group D) and VTCT plus vaccine (Group E) were immunized with a single s.c. injection of the Ad-sig-hMUC-1/ecdCD40L vector 10 days after the s.c. inoculation of the Lewis lung cancer cells (at which the average tumor volume was 50 mm^3) followed in 1, 2 and 3 weeks by an s.c. injection of the hMUC-1/CD40 L protein boost recombinant protein. Chemotherapy only (Group B) and 5-FU chemotherapy plus hMUC-1/ecdCD40L VPPP vaccination (Group D) were given 5-FU i.p. starting on the 10th day following tumor inoculation and continued weekly for a total of 7 weekly treatments. The AdCMVCDIRESE1A/5FC vector (VTCT) in Group E was given i.t. with the hMUC-1/ecdCD40L VPPP vaccine starting on the 10th day following tumor inoculation. All the mice in Group E were given 5-FC i.p. for 10 days in the period between the 10th to 20th day following tumor inoculation.

Effect of hMUC-1/ecdCD40L VPPP vaccination and either systemic 5-FU or VTCT on the volume of s.c. tumor nodules of hMUC-1-positive LL2/LL1 explants in C57BL/6 mice. The effect of the addition of VTCT and 5-FU systemic chemotherapy to the hMUC-1/ecdCD40L VPPP vaccination was tested in C57BL/6 mice bearing hMUC-1-positive LL2/LL1 tumor explants. We started injecting the hMUC-1/ecdCD40L VPPP vaccine on the 10th day following inoculation of the tumor cells at which time the volume of the s.c. tumor nodules was approximately 50 mm^3 (Groups C, D, E).

The mice in all groups were followed until death or killing because of large tumor volume (1000 mm^3). In the mice treated with combined hMUC-1/ecdCD40L VPPP vaccine and VTCT (Group E), the tumor nodules disappeared after the second week of vaccine injection in one mouse and after 3 weeks in the other two test mice. The complete remission (CR) rate was 30% in

Group E mice. There was only one CR in the hMUC-1/ecdCD40L VPPP plus systemic 5-FU combination therapy (Group D). No CRs were seen in the control, 5-FU alone or vaccine alone (Groups A, B or C, respectively) treatment groups. However, the hMUC-1/ecdCD40L VPPP vaccination significantly decreased the volume of the s.c. tumor nodules in test Groups C, D and E (see Figure 2a). The AdCMVCDIRESE1A/5FC vector-targeted chemotherapy group (those receiving the AdCMVCDIRESE1A plus i.p. 5-FC) with hMUC-1/ecdCD40L VPPP vaccination (Group E) had the best tumor response pattern.

Effect of hMUC-1/ecdCD40L VPPP vaccination and either systemic 5-FU or VTCT on the survival of C57BL/6 test mice. We also studied the survival of the assigned treatment groups of C57BL/6 mice following the injection of assigned treatment modalities. All the treatment groups had significantly better survival than the control group (Group A) as shown in Figure 2b ($P = 0.0001$). As shown in Figure 2b, the VTCT plus hMUC-1/ecdCD40L VPPP vaccine group had a remarkably extended survival curve, which was 3-fold longer than that of the control untreated mice ($P < 0.00001$). The survival of mice treated with a combination of the AdCMVCDIRESE1A/5FC (VTCT) with the hMUC-1/ecdCD40L VPPP vaccine (Group E) was longer than the survival of mice in Group C, which were treated with vaccine alone ($P = 0.0062$), and was significantly longer than Group A mice that were the control mice (2 fold longer). This shows that the hMUC-1/ecdCD40L VPPP vaccine itself is active, and that the addition of the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy

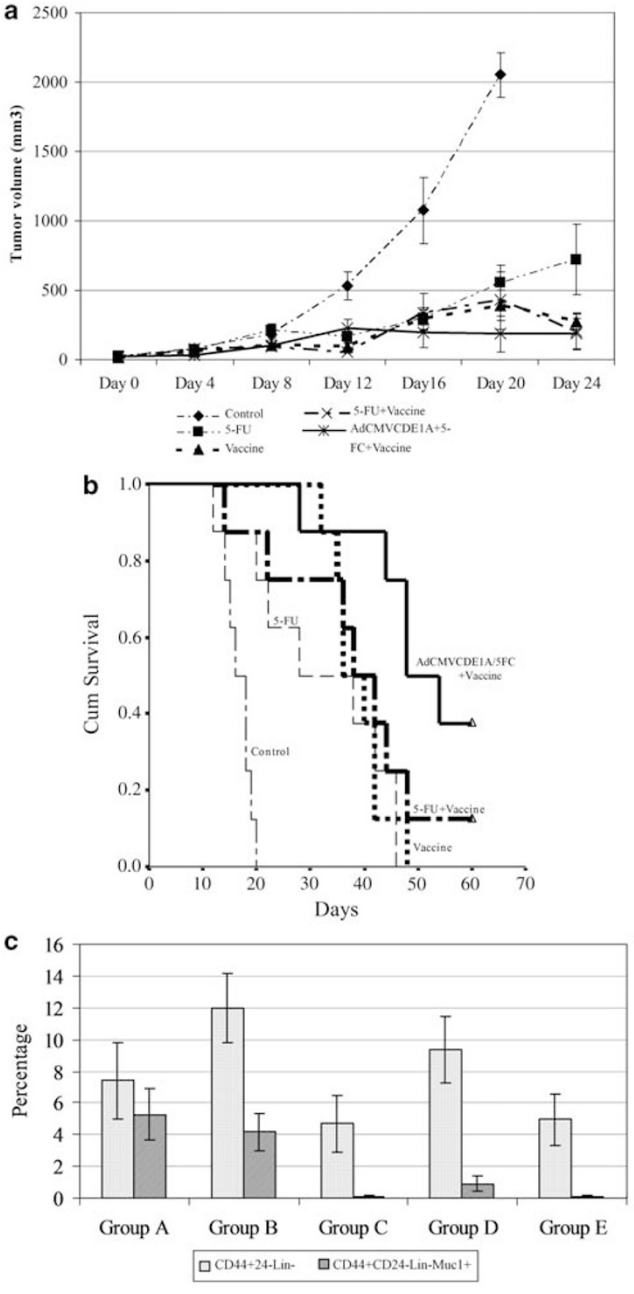


Figure 2 *In Vivo* efficacy of the combination of the hMUC-1/ecdCD40L VPPP vaccination, systemic 5-FU chemotherapy and VTCT. The following are the treatment groups: Group no. A: control (PBS only); Group no. B: intraperitoneal (i.p.) 5-FU systemic chemotherapy; Group no. C: Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (hMUC-1/ecdCD40L VPPP vaccine); Group no. D: 5-FU plus hMUC-1/ecdCD40L VPPP vaccination; Group no. E: intratumoral (i.t.) AdCMVCDIRESE1A vector followed by 10 days of i.p. 5-FC plus hMUC-1/ecdCD40L VPPP vaccination. (**a**) Effect of VPPP vaccine on s.c. tumor volume in C57BL/6 mice-injected s.c. with LL2/LL1 cells. The response of the s.c. nodules of Lewis Lung Cancer to the administration of the Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost and the AdCMVCDIRESE1A vector followed by i.p. 5-FC. The treatment groups listed above are represented by the following symbols in (**a**) Group 1: control (solid diamonds); Group 2: intraperitoneal 5-FU (solid squares); Group 3: The Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (solid triangles); Group 4: intraperitoneal 5-FU and the Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (dashes alternating with X's); Group 5: the AdCMVCDIRESE1A followed by i.p. 5-FC and the Ad-sig-hMUC-1/ecdCD40 L vector prime-hMUC-1/ecdCD40L protein boost vaccine (solid bold line with asterisks). After the second week of the treatment, the tumor nodules of three mice treated with the VPPP vaccine combined with i.t. injection of the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy Group no. E (CR percentage: 42%), 1 mouse from Group no. B (CR percentage:14%) and 1 mouse from Group no. D (CR percentage: 14%) have disappeared. (**b**) Effect of VPPP vaccine on survival of C56BL/6 mice. The treatment groups listed above are represented by the following symbols in (**b**): Group 1: control (long broken line alternating with dashes); Group 2: i.p. 5-FU (dashes); Group 3: The Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (faint short dashes and faint broken lines); Group 4: i.p. 5-FU and the Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (bold faint short dashes and faint broken lines); Group 5: the i.t. administration of the AdCMVCDIRESE1A vector followed by i.p. 5-FC and the Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (solid bold line). (**c**) Effect of VPPP vaccine on the percentage of $CD44^+CD24^-$ cells of the total number of $CD44^+CD24^-$ cancer cells in subcutaneous tumor nodules derived from LL2/LL1 cells in C56BL/6 mice. The following are the treatment groups: Group no. A: control (PBS only); Group no. B: i.p. 5-FU systemic chemotherapy; Group no. C: Ad-sig-hMUC-1/ecdCD40 L vector prime-hMUC-1/ecdCD40L protein boost vaccine (hMUC-1/ecdCD40L VPPP vaccine); Group no. D: 5-FU plus hMUC-1/ecdCD40L VPPP vaccination; Group no. E: i.t. AdCMVCDIRESE1A vector followed by 10 days of i.p. 5-FC plus hMUC-1/ecdCD40L VPPP vaccination. The light grey histograms are $CD44^+CD24^+$ cells from subcutaneous nodules derived from Lewis Lung Cancer cells; the dark grey histograms are from $CD44^+CD24^-MUC\text{-}1^+$ cells from subcutaneous nodules-derived from Lewis Lung Cancer cells.

to the hMUC-1/ecdCD40 L VPPP vaccine is of value. The durable complete remissions seen in Groups D (10%) and Group E (30%) were translated into the extended survival times beyond 3 months.

Effect of hMUC-1/ecdCD40 L VPPP vaccination with the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy on the number of CD44⁺CD24⁻ cells in the SC nodules of hMUC-1 positive Lewis lung syngeneic mouse cancer cells (LL2/LL1hMUC-1) in C57BL/6 mice. We studied the effect of the hMUC-1/ecdCD40 L VPPP vaccination on the percentage of $CD44^+CD24^-$ cancer cells in the s.c. tumor nodules of the assigned treatment groups. The tumor nodules derived from the hMUC-1 positive LL2/LL1hMUC-1 Lewis lung cancer cells were excised and the $CD44^+CD24^-$ cancer cells expressing hMUC-1 were sorted. As shown by the light grey histograms in Figure 2c, the hMUC-1/ecdCD40L VPPP vaccine only and the hMUC-1/ecdCD40L VPPP vaccine plus VTCT groups (Groups C and E) had the lowest percentage of $CD44^+CD24^-$ cells to total number of cancer cells ($P<0.001$). Interestingly, although systemic 5-FU treatment given by itself (Group B) has decreased the tumor growth, it increased the percentage that the $CD44^+CD24^-$ cells constituted in the total number of cancer cells in the s.c. tumor nodules when compared with the control group (see Figure 2c, Groups A and B) ($P<0.05$). On the other hand, the addition of the vector-targeted chemotherapy to the hMUC-1/ecdCD40L VPPP vaccine in Group D significantly decreased the percentage of $CD44^+CD24^-$ cells in the tumor nodules over that seen in Group B (Group B vs D, ($P<0.001$). The percentage of MUC1-expressing $CD44^+CD24^-$ cells in the total number of cancer cells (dark grey histograms) in the hMUC-1/ecdCD40L VPPP vaccination groups (Groups C, D and E) was significantly lower than that of the control (Group A) and 5-FU only (Group no. B) at the $P<0.001$ level (see Figure 2c).

It is apparent that the decreases seen in Figure 2c in the levels of the $CD44^+CD24^-Lin^-MUC\text{-}1^-$ cells among mice in Groups C, D and E when compared with Groups A and B, are accompanied by increases in the survival of treatment Groups C, D and E when compared with treatment Groups A and B (see Figure 2b). This data suggests that the combination of vector-targeted chemotherapy to the hMUC-1/ecdCD40L VPPP vaccine may be of advantage over the addition of chemotherapy (5-FU) to the hMUC-1/ecdCD40L VPPP cancer vaccine.

Discussion

Animals previously treated with the AdCMVCDIRESE1A/5-FC vector-targeted chemotherapy or tumor cells transfected with the *cytosine deaminase* (*CD)* gene and then treated with 5-FC, have been reported to be resistant to a subsequent challenge with wild-type tumor cells (not containing the *CD* gene). These results have raised the possibility that a systemic immune response can be developed against the TAA with therapeutic vaccination involving viral vectors.

It has been known that the apoptosis seen in tumor cells induced by conventional chemotherapy was associated often with the induction of a tumor-specific immune response.[8,9] Although the exact mechanism of the effect of dying tumor cells on the induction of an anti-tumor immune response is not completely understood, the immune cells attracted to the region of tumor cell death by the released tumor antigens might have a role. We have therefore tested whether it is possible to increase the level of the anti-tumor immune response induced by s.c. injection of the Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost VPPP vaccine, by concomitant administration of systemic chemotherapy (5-FU) or by AdCMVCDIRESE1A/5FC vector-targeted chemotherapy.

Systemic chemotherapy is the treatment of choice for most of the tumors in advanced stage. However, the detrimental effects of systemic chemotherapy on immune response to the cancer cells are usually disregarded. In this report we have shown that, although systemic chemotherapy with 5-FU alone decreased the tumor size and prolonged survival of tumor mice, its effect on survival was less than that of vector-targeted chemotherapy combined with the hMUC-1/ecdCD40L VPPP vaccination ($P=0022$). In addition, 5-FU chemotherapy increased the percentage of $CD44^+CD24^-$ cancer cells within the tumor nodules; whereas the combination of vector-targeted chemotherapy with the hMUC-1/ecdCD40L VPPP vaccination decreased the percentage of cancer cells, which carried the $CD44^+CD24^-$ immunophenotype. This is an important finding in that it may help us understand the reasons for the failure of systemic chemotherapy in advanced tumors.

To overcome this weakness, we substituted systemic chemotherapy (5-FU) with vector-targeted chemotherapy and combined it with hMUC-1/ecdCD40L VPPP vaccination. The results of our experiments showed the survival of mice carrying s.c. deposits of Lewis Lung Cancer was longer when they were treated 10 days after inoculation of the lung cancer cells with VTCT and hMUC-1/ecdCD40L VPPP vaccine than control mice ($P<0.00001$), or in mice treated with 5-FU chemotherapy ($P=0.0022$), or in mice treated with the combination of 5-Fu chemotherapy with the hMUC-1/ecdCD40L VPPP cancer vaccine.

The combination of vector-targeted chemotherapy with the hMUC-1/ecdCD40L VPPP vaccine was also better at decreasing the level of the $CD44^+CD24^-$ cancer cells in the tumor than in the mice in the control group, or in the chemotherapy alone group (Figure 2c). The results also showed that the level of the hMUC-1-positive $CD44^+CD24^-$ cancer cells in the s.c. nodules of the Lewis Lung Cancer cells decreased by >20 fold following the administration of the vector-targeted chemotherapy with the hMUC-1/ecdCD40L VPPP vaccine. This result is expected on the basis of many reports that the hMUC-1 marker is expressed on the cancer stem cells as well as the more mature epithelial neoplastic cells.[24–26] Clinical trials involving i.p. injection of therapy sensitization adenoviral vectors have been carried out successfully. In addition, the use of adenoviral vectors for induction of an adaptive immune response to cancer cells has been carried out. Thus, it will be possible for us to study in phase I trials, the use of the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy alone, the hMUC-1/ecdCD40L vector alone and the combination, to assess the potential value for this combination of vector therapies for cancer treatment.

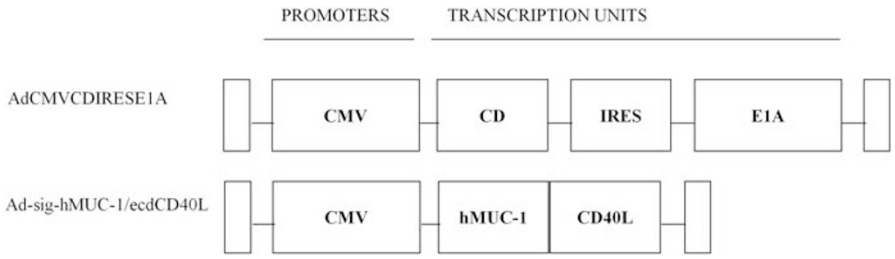


Figure 3 Vectors used in the study. The Ad-sig-hMUC1/ecdCD40L vector was used to activate an immune response against the hMUC1-expressing LL2/LL1 Lewis Lung Cancer cells. The AdCMVCDIRESE1A/5FC vector-targeted chemotherapy was abbreviated as VTCT. CMV = cytomegalovirus transcriptional promoter; CD = cytosine deaminase; IRES = intracistronic re-entry sequence; E1A = adenoviral gene; hMUC-1 = human Mucin-1; CD40L = the extracellular domain of the CD40 ligand.

Materials and methods

Cell lines and mice

The HEK293 human kidney cancer cell line, and the HTB-38 and CRL 2638 human and mouse colon cancer cell lines, the MCF-7 human breast cancer cell line and the LL2/LL1 cells were purchased from American Type Culture Collection (ATCC, Manasas, VA, USA). Six to eight-week-old C57Bl/6 mice were purchased from Harlan (Indianapolis, IN, USA). Rat Her-2-Neu transgenic mice (rH2N.Tg mice) were obtained from DuPont Inc. (Johnston, IA, USA) and then bred on site.

Construction of recombinant adenoviruses

Wild-type adenovirus type 5 was obtained from the American Type Culture Collection (ATCC). The Ad-CMVCDIRESE1A, a replication competent bicistronic adenoviral vector carrying the CMV promoter driven *CD* and *E1A* genes in a single continuous bi-cistronic transcription unit linked by an IRES element, Ad-GFP, Ad-CD, Ad-E1A, Ad-sig-ecdCD40L, Ad-sig-TAA/ecdCD40L vectors were previously engineered in our laboratory,[9,27–29] using the AdEasy vector system[30] and titrated as described previously.[28,29] The Ad-sig-ecdCD40L vector was constructed by linking the aminoterminal end of the ectodomain of CD40L to a secretory signal sequence as described in our previous publications.[5–8] Similarly, the *hMUC1/ecdCD40L* fusion gene was constructed by ligating the cDNA encoding the amino terminal end of the ecd of CD40L to an octapeptide linker (NDAQAPKS), which was linked in turn to a cDNA encoding the carboxyl terminal end of a 40 aminoacid segment of extracellular subunit of human MUC-1. This cDNA was linked to a cDNA encoding a secretory signal sequence. This transcription unit was used to engineer the Ad-sig-hMUC-1/ecdCD40L vector.[5–8] The diagrams of the vectors constructed and used in this study are given in Figure 3.

Production of the hMUC1/ecdCD40L protein

We have previously described a method for purifying the TAA/ecdCD40L protein from bacterial host strains.[7] The hMUC1/ecdCD40L cDNA was amplified and inserted into the pTriEx-2 hygro Vectors (Novagen, EMD Chemicals Inc., Gibbstown, NJ, USA). The resultant bacterial host strain was lysed following incubation in supplemented medium (EMD Chemicals Inc., Gibbstown, NJ, USA) for 4 hours. The cell lysate was prepared by the CellyticB Plus Kit (Sigma, St Louis, MO, USA). The hMUC1/ecdCD40L protein was purified from the soluble fraction by HIS-select Nickel Affinity Gel (Sigma). Then, the protein was concentrated and desalted by centrifugation through an Ultrafree-15 Biomax-50 filter (Millipore, Billerica, MA, USA) and eluted with phosphate-buffered saline (PBS).

Isolation of $CD44^+CD24^-$ cancer cells

The tumor nodules were minced into 2 mm pieces and then digested with collagenase I (1 mg/ml) and DNAse I (1 U/ml) in RPMI 1640 medium for 2 h at 37°C. Following the digestion, the red blood cells were lysed by ammonium chloride and the lineage-positive cells were positively depleted by using microbeads bound with antibodies against anti-mouse CD45, CD14, CD19, CD31 or CD15. Following the depletion of lineage-positive hematopoietic cells, the remaining cells were stained with a DNA-binding dye (Hoecsht 33342) to select the side population including the tumor stem cells and then positively selected for the following immunophenotypic markers: CD133, MUC-1 and ESA using monoclonal antibodies.[31–33]

Tests for the cellular immune response: isolation of T-cells from mice

To test the induction of cellular immunity, we measured the cytokine release and cell-mediated cytoxicity of the splenic T cells.[5–9] The T cells from the spleen and tumor-draining lymph nodes of the mice from each experimental test group were collected 7 days following injection of last treatment. Cytotoxic T cells (CD8 positive) were purified by magnetic separation using MACS CD8a-positive T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Purified T cells were resuspended in RPMI-1640 medium containing Gentamycin, Penicillin, Streptomycin, Mercapthoethanol, 10% fetal bovine serum and T-cell supplement.

Tests for the cellular immune response: ELISPOT assay

The presence of TAA tumor cell-specific effector T cells was assessed by carrying out an ELISPOT assay (BD ELISPOT Assay, San Diego, CA, USA) as previously described.[5–9] Briefly, 1×10^6 purified spleen T-cells were added to the MMC-treated (5 μg/ml) LL2/LL1 cells grown in T-25 flask and incubated for 5 days at 37°C for cell-mediated cytotoxicity tests. Following 2 days of

incubation of T-cells with MMC-treated tumor cells, the supernatants were kept at −80 °C for cytokine assay. Mononuclear cells were used for the IL-4 and IFN-γ ELISPOT tests. The area of the spots of the IFN-γ and IL-4-secreting T cells from the spleens of mice were measured. This method is adapted from the one previously published.[5–9]

Tests for the cellular immune response: cell-mediated cytotoxicity

Cell-mediated cytotoxicity was assayed by measuring the active caspase-3 antibody apoptosis by flow cytometry (BDTM FITC-conjugated monoclonal active caspase-3 antibody apoptosis kit, BD Pharmingen, San Diego, CA, USA). Briefly, the splenic mononuclear cells were incubated with hMUC-1-positive LL2/LL1 cells labeled with red-fluorescent dye for 4 h at 37 °C with varying effector-target ratios (10:1, 5:1 and 1:1) at 37 °C, in culture media containing 5% fetal bovine serum. Following the incubation, the percentage of apoptotic tumor cells was measured by flow cytometric analysis of FITC-conjugated active caspase-3 antibody. This method is adapted from the one previously published.[5–9]

Tests for the cellular immune response: cells from the tumor draining lymph nodes and spleens of test mice

The percentage of cells from the lymph nodes draining the tumor nodules or the vaccination injection site, which was positive for a given cell surface marker was measured quantitatively by flow cytometric analysis. Labeled anti-mouse antibodies for each antigen were purchased from e-biosciences (San Diego, CA, USA). The percentage of lymphocytes positive for each antigen was assayed using the FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA) in the Cell Sorting Core Lab of SKCC. This method was adapted from one previously published.[5–9]

Animal studies

To test the *in vivo* efficacy of the combination of VTCT and hMUC-/ecdCD40L VPPP vaccination on cancer stem cells, we used a hMUC-1-positive LL2/LL1 tumor model. A total of 50 mice were separated into five groups:

Group no. A: control (PBS only);

Group no. B: i.p. 5-FU systemic chemotherapy;

Group no. C: Ad-sig-hMUC-1/ecdCD40L vector prime-hMUC-1/ecdCD40L protein boost vaccine (hMUC-1/ecdCD40L VPPP vaccine);

Group no. D: 5-FU plus hMUC-1/ecdCD40 L VPPP vaccination;

Group no. E: i.t. AdCMVCDIRESE1A vector followed by 10 days of i.p. 5-FC (AdCMVCDIRESE1A/5FC) plus hMUC-1/ecdCD40 L VPPP vaccination.

These mice were inoculated s.c. with h-MUC-1-positive LL2/LL1 tumor explants (5×10^5 cells/mouse). The mice in the hMUC-1/ecdCD40L VPPP vaccine only (Group C) and 5-FU systemic chemotherapy plus hMUC-1/ecdCD40L VPP vaccine (Group D) and VTCT plus hMUC-1/ecdCD40L VPPP vaccination (Group E) were immunized with s.c. injections of the Ad-sig-hMUC1/ecdCD40L vector at the dose of 1×10^8 p.f.u. on the 10th day following tumor inoculation (average tumor volume was 50 mm^3). These mice were also injected s.c. with the hMUC1/CD40L protein boost recombinant protein (10 μg per mouse) weekly for 3 consecutive weeks starting 7 days after the s.c. injection of the Ad-sig-hMUC-1/ecdCD40L vector prime. 5-FU chemotherapy only and 5-FU chemotherapy plus hMUC-1/ecdCD40L vaccine groups (Groups B and D) were given 5-FU intraperitonally at a dose of 100 mg/kg weekly for 7 weeks starting on the 10th day following tumor inoculation. The mice in Group E were given the hMUC-1/ecdCD40L VPPP vaccine as described above plus a single i.t. injection of the AdCMVCDIRESE1A/5FC vector-targeted chemotherapy at a dose of 1×10^8 p.f.u. on the 10th day following tumor inoculation (average tumor volume was 50 mm^3). The mice in Group E group were given i.p. 5-FC at a dose of 500 mg/kg/d for 10 days in the period between the 10th and 20th days following tumor inoculation. Control mice were injected with equal amounts of PBS. Seven mice from each group were used in tumor response and survival experiments.

Statistical analysis

One-way Analysis of variance (with LSD *post hoc* comparisons) and Mann–Whitney tests were used for the comparison of immunological responses, CSC numbers and tumor volumes. Tumor growth rates were evaluated by regression analysis. Survival analyses were performed according to the Kaplan–Meier method and the log-rank test was used for survival comparisons.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We acknowledge support from the support of the SKCC by Sidney Kimmel and the following grants to Albert Deisseroth: DOD (Grants 17999457 and BC022063), the California Breast Cancer Research Program (CBCRP 12IB-0159), the NIH Grant PPG (CA 104898), a grant from the Kaye and Richard Woltman Foundation, and a grant from the Breast Cancer Research Foundation. We acknowledge support to Hakan Akbulut from the Turkish Academy of Sciences (TUBA-GEBIP-2001-1), the National Cancer Institute of United States of America, The Lechner/Haag Foundation and UICC-Yamagiwa-Yoshida international study grants. The views expressed are the result of independent work and do not represent the views or findings of the US FDA or the US government.

References

1 Deng Y, Jing Y, Campbell AE, Gravenstein S. Age-related impaired type 1T cell responses to influenza: reduced activation *ex vivo*, decreased expansion in CTL culture *in vitro*, and blunted response to influenza vaccination *in vivo* in the elderly. *J Immunol* 2004; **172**: 3437–3446.

2 Weinberg AD, Thalhofer C, Morris N, Walker JM, Seiss D, Wong S *et al*. Anti-OX40 (CD134) administration to nonhuman primates: immunostimulatory effects and toxicokinetic study. *J Immunother* 2006; **29**: 575–585.

3 Eaton SM, Burns EM, Kusser K, Randall TK, Haynes L. Age-related defects in CD4T cells cognate helper function

lead to reductions in humoral responses. *J Exp Med* 2004; **200**: 1613–1622.
4 Dong L, Mori I, Hossain J, Liu B, Kimura Y. An immunostimulatory oligodeoxynucleotide containing a cytidine-guanosine motif protects senescence-accelerated mice from lethal influenza virus by augmenting the T helper type 1 response. *J Gen Virol* 2003; **84**: 1623–1628.
5 Zhang L, Tang YC, Akbulut H, Zelterman D, Linton P-J, Deisseroth A. An adenoviral vector cancer vaccine. *Proc Natl Acad Sci USA* 2003; **100**: 15202–15206.
6 Tang Y, Zhang L, Yuan J, Akbulut H, Maynard J, Linton PJ *et al.* Loading of APC by CD40 ligand/tumor antigen secretory protein generates protection from cancer cell lines. *Blood* 2004; **104**: 2704–2713.
7 Tang YC, Maynard J, Akbulut H, Petersen L, Fang X, Zhang WW *et al.* Vector prime/protein boost vaccine which overcomes defects acquired during aging and cancer. *J Immunol* 2006; **177**: 5697–5707.
8 Akbulut H, Tang Y, Maynard J, Zhang L, Pizzorno G, Deisseroth A. Vector-mediated delivery of 5FU. *Clin Cancer Res* 2004; **10**: 7738–7746.
9 Akbulut H, Tang YC, Akbulut KG, Maynard J, Zhang L, Deisseroth A. Antitumor immune response induced by i.t. injection of vector activated dendritic cells and chemotherapy suppresses metastatic breast cancer. *Mol Cancer Ther* 2006; **5**: 1975–1985.
10 Liu S, Dontu G, Mantle ID, Patel S, Ahn NS, Jackson KW *et al.* Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res* 2006; **66**: 6063–6071.
11 LaPointe J, Li C, Higgins JP, van de Rijn M, Bair E, Montgomery K *et al.* Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc Natl Acad Sci USA* 2004; **101**: 811–816.
12 Feng H, Ghazizadeh M, Konishi H, Araki T. Expression of MUC1 and MUC2 Mucin Gene Products in Human Ovarian Carcinomas. *Japanese J Clin Oncol* 2002; **32**: 525–529.
13 Tamada Y, Takeuchi H, Suzuki N, Susumu N, Aoki D, Irimura T. Biological and therapeutic significance of MUC1 with sialoglycans in clear cell adenocarcinoma of the ovary. *Cancer Sci* 2007; **98**: 1586–1591.
14 Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death from cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest* 2005; **115**: 1503–1521.
15 Liu R, Wang XH, Chen GY, Dalerba P, Gurney A, Hoey T *et al.* The prognostic role of a gene signature from tumorigenic breast cancer cells. *New England J Med* 2007; **356**: 217–226.
16 Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L *et al.* Isolation of rara circulating tumour cells in cancer patients by microchip technology. *Nature* 2007; **450**: 1235–1241.
17 Terstappen LWMM. Monitoring and characterization of circulating tumor cells in cancer patients. In: *American Association for Cancer Research 99th Annual Meetiung Education Book; 2008 Aor 12-16*. AACR: San Diego, CA, USA, 2008, pp 617–624.
18 Berezovska OP, Glinskii AB, Yang Z, Li XM, Hoffman RM, Glinsky GV. Essential role for activation of the Polycomb group (PcG) protein chromatin silencing pathway in metastatic prostate cancer. *Cell Cycle* 2006; **5**: 1886–1901.
19 Balic M, Lin H, Young L, Hawes D, Giuliano A, McNamara G *et al.* Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res* 2006; **12**: 5615–5621.
20 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierachy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730–737.
21 O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106–110.
22 Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T *et al.* Identification of human brain tumour initiating cells. *Nature* 2004; **432**: 396–401.
23 Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M *et al.* Identification of cells initiating human melanomas. *Nature* 2008; **451**: 345–349.
24 Engelmann K, Shen HM, Finn OJ. CDF7 side population cells with characteristics of stem/progenitor cells express the tumor antigen MUC-1. *Cancer Res* 2008; **68**: 2419–2426.
25 Mahanta SJ, Fessler SP, Park JH, Bamdad C. A minimal fragment of MUC1 mediates growth of cancer cells. *PLoS ONE* 2008; **3**: e2054.
26 Hikita ST, Kosik KS, Clegg DO, Bamdad C. MUC1 mediates the growth of human pluripotent stem cells. *PLoS ONE* 2008; **3**: e3312.
27 Tang YC, Akbulut H, Maynard J, Zhang L, Petersen L, Deisseroth A. Vaccine strategies for cancer and infectious diseases in the elderly. In: Chiai T, Shimada H, Tagawa M (eds). *Gene Therapy* 2007: Japanese Ministry of Science and Education: Chiba, Japan, 2007, pp 78–85.
28 Akbulut H, Zhang L, Tang Y, Deisseroth AB. The efficiency of replication-competent adenoviral vectors carrying L-plastin promoted cytosine deaminase gene in colon cancer. *Cancer Gene Ther* 2003; **10**: 388–395.
29 Zhang L, Akbulut H, Peng XY, Pizzorno G, Deisseroth AB. Adenoviral vectors with E1A Tumor specific promoters are selectively cytolytic for breast cancer and on melanoma. *Mol Ther* 2002; **6**: 386–393.
30 He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* 1998; **95**: 2509–2514.
31 Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cdell lines and tumors is enriched with stem-like cancer cells. *Cancer Res* 2007; **67**: 4727–4733.
32 Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U *et al.* A distinct 'side population' of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci USA* 2004; **101**: 14228–14233.
33 Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D *et al.* Purification and unique properties of mammary epithelial stem cells. *Nature* 2006; **439**: 993–997.