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ORIGINAL ARTICLE

Development of human umbilical cord matrix stem cell-based gene therapy for experimental lung tumors

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Umbilical cord matrix stem (UCMS) cells are unique stem cells derived from Wharton's jelly, which have been shown to express genes characteristic of primitive stem cells. To test the safety of these cells, human UCMS cells were injected both intravenously and subcutaneously in large numbers into severe combined immunodeficiency (SCID) mice and multiple tissues were examined for evidence of tumor formation. UCMS cells did not form gross or histological teratomas up to 50 days posttransplantation. Next, to evaluate whether UCMS cells could selectively engraft in xenotransplanted tumors, MDA 231 cells were intravenously transplanted into SCID mice, followed by intravenous transplantation of UCMS cells 1 and 2 weeks later. UCMS cells were found near or within lung tumors but not in other tissues. Finally, UCMS cells were engineered to express human interferon beta – designated 'UCMS–IFN-β'. UCMS–IFN-β cells were intravenously transplanted at multiple intervals into SCID mice bearing MDA 231 tumors and their effect on tumors was examined. UCMS–IFN-β cells significantly reduced MDA 231 tumor burden in SCID mouse lungs indicated by wet weight. These results clearly indicate safety and usability of UCMS cells in cancer gene therapy. Thus, UCMS cells can potentially be used for targeted delivery of cancer therapeutics.
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Introduction

The umbilical cord contains an inexhaustible, noncontroversial source of stem cells. Worldwide, millions of umbilical cords, each containing millions of stem cells, are routinely discarded after birth. Multipotent stem cells called umbilical cord matrix stem (UCMS) cells are isolated from the mesenchyme-like cushioning material called 'Wharton's jelly' found between the vessels of the umbilical cord.[1] These cells resemble stem cells from several other sources but are also unique in some properties. The properties that promote their potential utility are the large number of cells recoverable from the umbilical cord, the noninvasive harvest following birth, and the abundant supply of umbilical cords.[2,3] Thus, they may offer an immediate avenue for cytotherapy, when time is of the essence for such therapy, for example, in cases of malignant neoplasia. UCMS cells transplanted into rodent neurodegenerative models[4–6] have been extensively characterized[3] and they express many of the genes expressed by primitive stem cells such as embryonic stem cells (ESCs).[7] They can be differentiated into a variety of cell types including muscle,[8] cartilage,[9] bone,[9] and neural[1] cells and have been used for tissue-engineered artificial blood vessels and heart valves.[10]

Previously, several types of stem cells have been shown to migrate selectively into tumors.[11,12] Moreover, they have been engineered to secrete antitumor proteins, such as interferon beta (IFN-β), into the tumor microenvironment with favorable therapeutic effects in rodent animal models.[11–15] In these cases, millions of stem cells have been transplanted systemically. However, similar approaches in humans will likely require an order of magnitude more cells to achieve a comparable effect. Since large numbers of UCMS cells can be obtained in a short time, they potentially could be a solution to this dilemma.

An example of a protein successfully delivered via stem cells for an antitumor effect is IFN-β.[12,15] This cytokine has potent proapoptotic[16,17] and antiproliferative[18,19] effects *in vitro*. Although gene therapy using bone marrow or neural stem cells has been successful in treating cancer mouse models, the preparation of these stem cells usually requires complex methods and a relatively long time.

In the present study, we investigated a key safety issue by transplanting large numbers of UCMS cells into severe combined immunodeficiency (SCID) mice to assess possible teratoma or other tumor formation. Next, we tested UCMS cells for their ability to selectively engraft in lung tumors of SCID mice. Finally, we investigated

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whether UCMS cells, engineered to secrete IFN-β, could reduce lung tumor burden in SCID mice. We show here that UCMS cells themselves do not form tumors; they selectively engraft in lung tumors. In addition, tumor burden in SCID mice bearing lung tumors is significantly reduced following systemic administration of human IFN-β-expressing UCMS cells.

Materials and methods

Tissue culture of human UCMS cells and MDA 231 cells

Human UCMS cells were harvested following deliveries at the time of birth with the mother's consent. The methods to isolate and culture human UCMS cells were previously described.[3]

MDA 231, a human breast carcinoma cell line that engrafts in the lung of nude mice[20] was maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), 1X Pen/Strep (Invitrogen, CA) and 10% fetal bovine serum (FBS) (Mediatech, Herndon, VA) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Adenoviral vectors and transduction of human UCMS cells

Since UCMS cells do not express the adenoviral receptor, fiber-modified viruses were used to allow efficient transduction.[12,15] Recombinant adenoviral particles carrying human IFN-β were used.[12,15] For transduction, the human UCMS cells were plated in T-75 flasks and infected when the cells reached 80–90% confluency. The cells were washed twice with DMEM without serum and incubated with DMEM containing adenovirus at 12 500 viral particles (VP) per cell (for adenovirus-IFN-β) for 4 h at 37°C. The cells were cultured in UCMS media (a mixture of 56% low-glucose DMEM (Invitrogen), 37% MCBD 201 (Sigma, St Louis, MO) and 2% FBS (Mediatech, MTT 35–010-CV) containing 1 × insulin-transferrin selenium-X (ITS-X, Invitrogen, CA), 1X AlbuMAX I (Invitrogen, CA), 1X Pen/Strep (Invitrogen, CA), 10 nM dexamethasone (Sigma), 100 μM ascorbic acid 2-phosphate (Sigma), 10 ng/ml epidermal growth factor (EGF, R&D systems, Minneapolis, MN) and 10 ng/ml platelet-derived growth factor-BB (PDGF-BB, R&D systems) for 24 h and then used for transplantation studies.

IFN-β ELISA

The amount of IFN-β secreted by UCMS–IFN-β cells into the media was quantified by a human IFN-β enzyme-linked immunosorbent assay (ELISA) kit (PBL biomedical laboratories, NJ). UCMS cells were plated at 50 000 cells per well in a 12-well plate. UCMS cells were transduced with adenovirus IFN-β at various amounts (12 500, 6400 and 3200) of adenoviral particles per cell as described above. One day later, the IFN-β level in the medium was determined according to manufacturer's protocols using recombinant IFN-β as a standard.

In vitro *effect of conditioned media from IFN-β-expressing UCMS cells on MDA 231 cells*

To condition the media, fresh media was added to subconfluent (15 000 cells/cm^2) UCMS cells or UCMS–IFN-β cells for 24 h before being removed and centrifuged, and its bioactivity was examined. MDA 231 cells were plated in six-well plates at a density of 160 000 cells per well with MDA 231 growth media (DMEM and 10% FBS). After 24 h, the media was removed from all the wells and the MDA 231 media was replaced with either conditioned media from UCMS cells alone, from UCMS–IFN-β cells or unconditioned UCMS media as a control. After 3 days, the cells were trypsinized and counted on a hemocytometer. The percentage of dead cells was estimated using trypan blue exclusion. Results were also expressed as the percentage of cell growth, calculated with the following formula:[15] (number of MDA 231 cells cultured with conditioned media on day 3−the number of MDA 231 cells on day 0)/(number of MDA 231 cells cultured in UCMS media on day 3−number of MDA 231 cells on day 0) × 100. Three different isolates of UCMS cells were used in separate trials and each experiment was done in triplicate.

Immunohistochemical staining

For immunofluorescence staining, tissue sections were washed with phosphate-buffered saline 0.2% containing Triton X-100 (PBS TX) and fixed with 70% ethanol and acetone (1 : 1). This was followed by washing with three changes of PBS TX. Tissue sections were blocked with 5% normal goat serum in PBS TX for 30 min, and followed by incubation with primary antibody, anti-human mitochondrial antibody (1 : 1000, Chemicon, Temecula, CA), in PBS TX overnight. The tissues were then washed three times with PBS TX and incubated with Alexa Fluor 488-conjugated secondary antibody (1 : 1000, Molecular Probes, Carlsbad, CA) for 3 h. The tissues were incubated for 30 min in Hoechst 33342 (1 : 100, Sigma) as a counterstain to label the nuclei followed by a triple rinse with PBS TX. The antigens were localized using epifluorescence microscopy (Nikon Eclipse, Boyce Scientific Inc., MO) and images were captured using a Roper Cool Snap ES camera and Metamorph 7.

Fluorescent labeling of human UCMS cells

For transplanted human UCMS cell identification, the red fluorescent dye SP-DiI (Molecular Probes) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml. SP-DiI dye was added to culture medium to a final concentration of 10 μg/ml and human UCMS cells were labeled by adding 10 ml of medium with SP-DiI in a T-75 flask for 24 h. Then, cells were washed with PBS, incubated with dye-free medium for 4 h and used for transplantations.

Evaluation of tumor formation by human UCMS cells

For the following three *in vivo* studies, 5-week-old female CB-17 SCID mice were obtained from Harlan laboratories (Indianapolis, IN). Mice were held for 1 week after arrival to allow them to acclimatize. They were housed in

a barrier room and individual cages containing high-efficiency particulate air filters. Both subcutaneous and systemic transplantation of UCMS cells suspended in 200 μl PBS were carried out under sterile conditions in a laminar flow hood in the barrier room. Animals were randomly assigned to experimental groups. The first set of mice was transplanted with 2×10^6 human UCMS cells ($n = 5$) or 1×10^7 human UCMS cells ($n = 4$) subcutaneously at the flank region. The remaining mice was transplanted with 2×10^6 human UCMS cells ($n = 5$), 3×10^6 human UCMS cells ($n = 2$) or 6.5×10^6 human UCMS cells ($n = 1$) intravenously via the lateral tail vein. All the mice were killed 50 days after transplantation by cervical dislocation. Lung, kidney and liver were collected, snap frozen in liquid nitrogen-chilled isopentane and used for immunohistochemistry studies. All *in vivo* experiments were carried out with proper IACUC and IBC institutional approval.

Evaluation of selective engraftment of human UCMS cells

MDA 231 cells and UCMS cells were transplanted without anesthesia into the lateral tail vein using sterile conditions in a laminar flow hood in the barrier room. Animals were randomly assigned to experimental groups. For tumor induction, one set of mice was transplanted with 1×10^6 MDA 231 cells ($n = 8$). This was followed by two injections of 1×10^6 fluorescently labeled UCMS cells on day 17 and day 24 after injection of MDA 231 cells. A second set of mice was transplanted with 2×10^6 MDA 231 cells ($n = 8$). This was followed by two injections of 1×10^6 fluorescently labeled UCMS cells on day 11 and day 18. All the mice were killed 8 days after the last UCMS cell transplant. Other groups included mice injected with fluorescently labeled UCMS cells alone ($n = 4$), and mice injected with sterile PBS only ($n = 4$) as controls. Lung weights of control and tumor-bearing animals were measured to estimate tumor burden. Lungs and other organs including spleen, liver, kidney and bone marrow were also harvested as described above for immunohistochemical analysis.

Evaluation of the effect of IFN-β-expressing human UCMS cells on the growth of MDA 231 xenografts

MDA 231 cells and UCMS cells were transplanted without anesthesia as described above. Animals were randomly assigned to experimental groups. For tumor induction, 2×10^6 MDA 231 cells were given via the lateral tail vein ($n = 22$). Fluorescently labeled UCMS–IFN-β cells (0.5×10^6, $n = 9$) or fluorescently labeled UCMS cells (0.5×10^6, $n = 5$) were transplanted on day 8 after tumor inoculation. The UCMS cell transplant was repeated twice at 1-week intervals subsequent to the first transplant. Experimental groups also included a negative control group that received neither MDA 231 cells nor UCMS cells ($n = 6$; PBS injections only), and a group with three weekly injections of fluorescently labeled UCMS–IFN-β cells (0.5×10^6, $n = 6$) into nontumor-bearing mice. All the mice were killed after 30 days by cervical dislocation. Lung weights were measured to estimate tumor burden. Lung, spleen, liver, kidney and bone marrow were harvested and subjected to histological analysis.

Statistical analysis

To evaluate the significance of overall differences in lung weights between all *in vivo* groups, statistical analysis was performed by analysis of variance (ANOVA). A *P*-value less than 0.05 was considered as significant. Following significant ANOVA, *post hoc* analysis using Fisher's protected least significance difference was used for multiple comparisons. Significance for *post hoc* testing was set at $P < 0.05$. All the lung weight data were represented as mean $\pm$ s.e. on graphs. Statistical analyses were performed by Stat View software, version 5.0.1. (Cary, NC).

Results

Secretion of IFN-β by UCMS–IFN-β cells

The amount of IFN-β in international units secreted by the UCMS–IFN-β cells into the media was measured by IFN-β ELISA kit. IFN-β was secreted by the UCMS–IFN-β cells in significant amounts, and there was a trend for more IFN-β release into the media when UCMS cells were transduced with a greater number of VP per cell (Table 1). IFN-β was not detected in the conditioned media with UCMS cells (data not shown).

In vitro *effect of IFN-β secreted by human UCMS–IFN-β cells on MDA 231 cells*

To investigate whether human UCMS–IFN-β cells have an effect on growth and cell viability on MDA 231 cells, conditioned media from human UCMS–IFN-β cells was added to MDA 231 cells and its effect was analyzed. MDA 231 cell proliferation was markedly inhibited when cultured with conditioned media from UCMS–IFN-β cells in comparison with MDA 231 cells cultured in unconditioned UCMS media ($P < 0.001$, Figure 1b). The percentage of dead MDA 231 cells in cultures with conditioned media from UCMS–IFN-β cells was significantly higher than in MDA 231 cells cultured in unconditioned UCMS media ($P < 0.001$, Figure 1a).

Table 1 IFN-β secreted by 1×10^6 human UCMS–IFN-β cells when transduced with 12 500, 6400 and 3200 IFN-β adenoviral particles/cell

Transduction number of viral particles/cell	*Total amount IFN-β secreted by 10^6 UCMS–IFN-β cells in picograms*	*Total amount secreted by 10^6 UCMS–IFN-β cells in international units*
12 500	929 364.7	37 175 $\pm$ 7548
6400	692 657.2	27 706 $\pm$ 1022
3200	509 227.3	20 369 $\pm$ 3958

Abbreviations: IFN-β, interferon beta; UCMS, umbilical cord matrix stem.

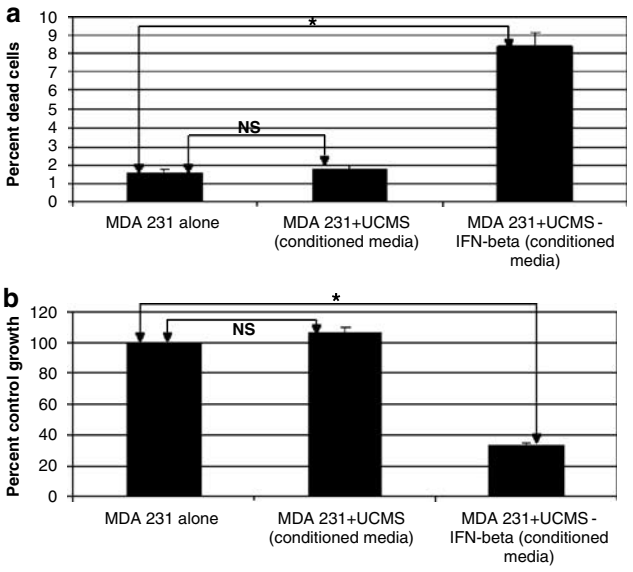

Figure 1 *In vitro* effect of human umbilical cord matrix stem (UCMS) cells and human UCMS–IFN-β cells conditioned media on MDA 231 cells. (**a**) Percentage dead cells and (**b**) percentage control cell growth of MDA 231 cells when treated with UCMS cells and UCMS–IFN-β cells-conditioned media. *Statistically significant in comparison with mice with MDA 231 only (*P*-value <0.001).

Human UCMS cells do not form tumors in SCID mice

All the mice were killed 50 days after UCMS cells injection. Mice appeared healthy and no clinical symptoms were found throughout the experimental period. No gross visible tumors were found around the transplantation sites or in any other regions of mice. Immunohistochemical analysis using serial sections stained by antihuman mitochondrial antibodies also failed to detect evidence of tumors in tissues such as kidney, liver and lungs (Figures 2a, b and c). These results suggest that human UCMS cells are nontumorigenic in SCID mice and thus are potentially usable for transplantation studies.

Engraftment of human UCMS cells in MDA 231 xenografts in lungs of SCID mice

Red fluorescent human UCMS cells were found selectively near or within MDA 231 xenografts in lungs of SCID mice after intravenous transplantation (Figures 2e and f). In contrast, in mice injected with MDA 231 cells alone, no red fluorescent cells were found near or within the tumors (data not shown). Similarly, in mice injected with UCMS cells alone, and mice with PBS alone, no fluorescent cells were found in the lungs. These results suggest that UCMS cells are capable of tumor-targeted migration and survival in the tumor microenvironment. Lung weights from nontumor-bearing mice that received fluorescently labeled UCMS cells alone were not different than mice injected with PBS. As reported, there was significant difference ($P<0.05$) in lung weights in mice that received MDA 231 cells than the mice that did not receive any MDA 231 cells (Figure 3). Lungs from tumor-bearing mice, that were not given stem cells transplanted, were not different from tumor-bearing mice transplanted with fluorescently labeled UCMS cells, indicating there was no significant effect of UCMS cells on tumor burden (Figure 3). In the lungs of mice injected with MDA 231 cells and fluorescently labeled UCMS cells, a large number of fluorescently labeled UCMS cells were found near or within tumors (Figures 2e and f) but were not found in kidney, liver or spleen.

Effect of UCMS–IFN-β cells on MDA 231 lung tumor burden in SCID mice

To test the hypothesis that UCMS–IFN-β cells would reduce the tumor burden in lungs of mice injected with MDA 231 cells, lung weights were used to estimate of tumor burden and lungs from various groups were photographed (Figure 4). Tumor burden was significantly reduced in MDA 231 inoculated mice transplanted with UCMS–IFN-β cells compared to mice inoculated with MDA 231 cells alone ($P<0.01$). In fact, the burden was reduced by about 50% (Figure 5). Lungs weights from mice that received only UCMS–IFN-β cells but not MDA tumor cells were not different than sham-treated mice (not significant (NS), $P>0.05$). Mice injected with UCMS–IFN-β cells alone did not show any adverse effects. Histological analysis revealed that engraftment of UCMS cells was detected in close proximity or within lung tumor tissues of tumor-bearing mice that either received fluorescently labeled UCMS–IFN-β cells or fluorescently labeled unengineered UCMS cells (Figure 2d). No fluorescently labeled UCMS cells were found in other tissues such as liver, spleen and kidney.

Discussion

Here, we provide evidence that unique, primitive human stem cells that can be harvested rapidly in large numbers from umbilical cord Wharton's jelly do not themselves form tumors, and thus they are safe, practical delivery vehicles for specific genes. For the first time, we show that UCMS cells selectively engraft in tumors, can be engineered to secrete a therapeutic protein, IFN-β, and significantly reduce lung-metastasized breast carcinoma tumor burden in an animal model.

When undifferentiated ESCs are transplanted they sometimes form tumors;[21–24] therefore, we tested whether UCMS cells might have that tendency. UCMS cells are karyotypically stable over passages and do not lose anchorage dependency, contact inhibition, or serum dependence,[2,3] which are characteristics of cancer cells. Our *in vivo* findings support the *in vitro* published data, since they do not become tumorigenic and hence are safe from that standpoint. This is an important consideration, since these cells express some of the same primitive genes expressed by ESCs.[7]

IFN-β induces apoptosis in cancer cells mainly by disrupting mitochondria and activation of the caspase cascade.[25] It also is a potent inhibitor of proliferation for

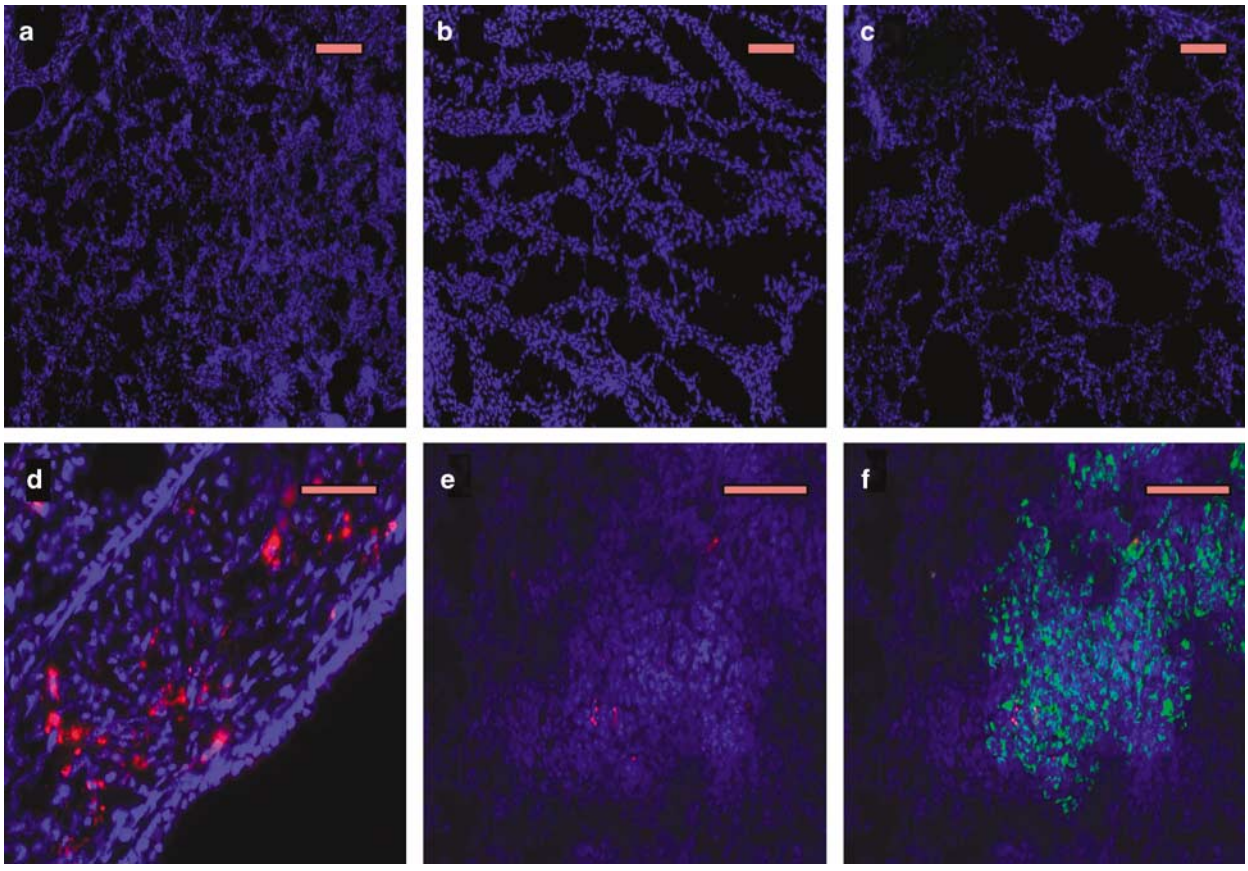

Figure 2 Absence of tumor formation in SCID mice injected with human UCMS cells. (**a**) Kidney section negative for UCMS cells after immunostaining with antihuman mitochondrial antibody and Hoechst 33342 nuclear stain (blue) (**b**) Cross section of liver and (**c**) lung stained with same antibody (scale bar = 100 μm). (**d**–**f**) Selective engraftment of fluorescently labeled human UCMS cells in MDA 231 lung tumors counterstained with Hoechst 33342 nuclear stain. (**d** and **e**) Fluorescent micrograph showing red labeled human UCMS cells (SP-DiI) counterstained with Hoechst 33342 nuclear stain (blue) and Figure 2 (**f**) is the same section as (**e**) but with tumor cells shown as green fluorescing cells after detection with antihuman mitochondrial antibody (scale bar = 100 μm).

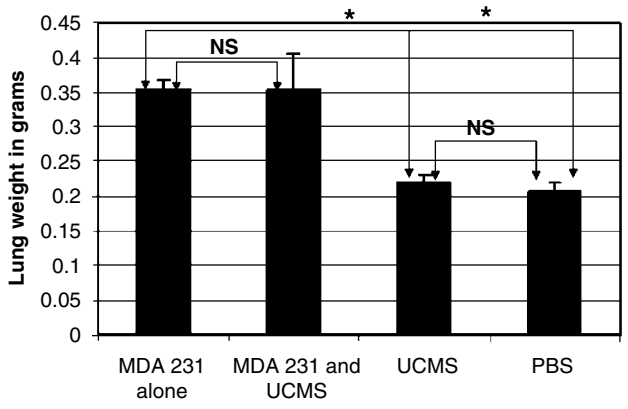

Figure 3 Effect of human UCMS cells (not expressing IFN-β) on tumor burden. Lung weights of mice injected with only MDA 231 cells, MDA 231 and UCMS cells, UCMS cells alone (sham MDA 231 transplant) and PBS alone (sham tumor, sham transplant) are shown. *Statistically significant in comparison with mice with MDA 231 only (*P*-value <0.05).

many cancer cells *in vitro*.[18,19] However, it cannot be used effectively as cancer therapy because the maximum tolerated dose is not high enough to attain these effects when given systemically[26–28] and it has a short half-life. Although it has been shown that serum levels of interferon delivered by engineered stem cells are insignificant in contrast to interferon given systemically,[15] the local production of IFN-β appears to be the key for tumor growth attenuation. The stem cell-based gene therapy can also avoid potential systemic adverse effects of IFN-β. Since we found no evidence of long-term persistence of UCMS cells in the evaluation of teratoma formation by stem cells (Figures 2a–c), it is conceivable that the engrafted stem cells will not remain for a long period of time after attenuation of the tumor. In more recent work, we have engineered the UCMS cells with a thymidine kinase suicide gene as an additional safety feature (unpublished data). Taken together, our UCMS cell-based tumor-targeted IFN-β gene delivery appears to be a safe, effective and practical cancer gene therapy.

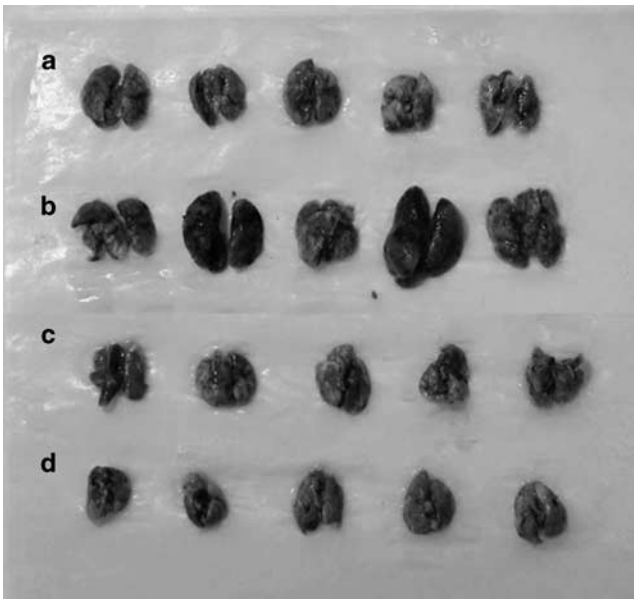

Figure 4 Representative lungs of experimental groups. Group A, MDA 231 + UCMS–IFN-β; group B, MDA 231 alone; group C, UCMS–IFN-β cells, no tumor cells and group D, PBS alone.

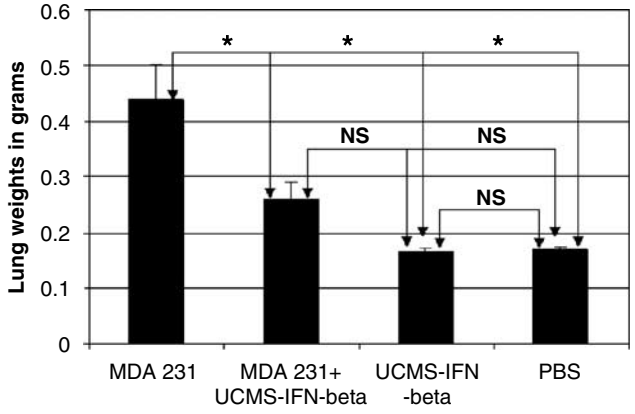

Figure 5 Lung weights of mice injected with both MDA 231 and UCMS–IFN-β cells compared with mice injected with MDA 231 alone, UCMS–IFN-β cells alone and sham-transplanted mice with no tumors. *Statistically significant in comparison with mice with MDA 231 only (P-value <0.01).

The adenoviral system used to engineer the UCMS cells was fiber modified to facilitate viral entry into the cells, since some cells may not express the coxsackie virus and adenovirus receptor necessary for cell entry.[29] Our *in vitro* testing of this fiber-modified vector established that it was considerably more efficient than transduction with unmodified adenoviral vectors (data not shown); this is consistent with other reports.[29–31]

The finding that UCMS cells administered via the tail vein exhibited a selective engraftment to the MDA 231 lung tumors of SCID mice suggests that they may respond to chemotactic signals emanating from the tumor cells. Others have reported that tumors secrete factors that recruit stromal, vascular, bone marrow and other stem cells to the tumor; theoretically the recruited cells provide a scaffolding and source of nutrients.[32,33] Several chemokines are known to be secreted by tumors that may mediate the observed tropism, including vascular endothelial growth factor, transforming growth factor-β (TGF) family members, fibroblast growth factor (FGF) family members, platelet-derived growth factor family members, MCP-1, epidermal growth factor and interleukin-8.[34] Cells related to UCMS cells, bone marrow stromal cells, have been shown to exhibit a tropism for damaged or rapidly growing tissues as well as tumors.[12,14,15,35,36] The human UCMS cells have been characterized by gene array and cytokine array previously,[3] and express possible chemokine receptors such as SDFR1, TGFBR3 and FGFR2.[3]

In summary, human UCMS cells are unique stem cells derived from the Wharton's jelly of the umbilical cord and do not form tumors in SCID mice. Second, they selectively engraft in MDA 231 lung tumors. Third, UCMS cells that were engineered to express IFN-β can deliver sufficient IFN-β to kill MDA 231 cells *in vitro*. Finally, UCMS–IFN-β cell transplantation into lung tumor-bearing SCID mice produces a significant reduction of the tumor burden. Thus, these results suggest that UCMS cells may be a platform for targeted delivery of therapeutic proteins to human breast cancer and other types of cancer.

Abbreviations

DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; ESCs, embryonic stem cells; FBS, fetal bovine serum; HEPA, high-efficiency particulate air; IACUC, institutional animal care and use committees; IBC, institutional biosafety committee; IFN-β, interferon beta; PBS, phosphate-buffered saline; SCID, severe combined immunodeficiency; SP-DiI, sulfonated derivatives of dialkyl indol dye; UCMS cells, umbilical cord matrix stem cells; UCMS–IFN-β cells, interferon-beta-expressing UCMS cells; MDA 231 cells, MD Anderson 231 human breast carcinoma cells.

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