RESEARCH ARTICLE

Human Embryonic Stem Cells - a Potential Vaccine for Ovarian Cancer

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Abstract

Objective: To investigate the therapeutic potential of human embryonic stem cells (hESCs) as a vaccine to induce an immune response and provide antitumor protection in a rat model. Methods: Cross-reactivity of antigens between hESCs and tumour cells was screened by immunohistochemistry. Fischer 344 rats were divided into 7 groups, with 6 rats in each, immunized with: Group 1, hESC; Group 2, pre-inactivated mitotic NuTu-19; Group 3 PBS; Group 4, hESC; Group 5, pre-inactivated mitotic NuTu-19; Group 6, PBS; Group 7, hESC only. At 1 (Groups 1-3) or 4 weeks (Groups 4-6) after the last vaccination, each rat was challenged intraperitoneally with NuTu-19. Tumor growth and animal survival were closely monitored. Rats immunized with H9 and NuTu-19 were tested by Western blot analysis of rat orbital venous blood for cytokines produced by Th1 and Th2 cells. Results: hESCs presented tumour antigens, markers, and genes related to tumour growth, metastasis, and signal pathway interactions. The vaccine administered to rats in Group 1 led to significant antitumor responses and enhanced tumor rejection in rats with intraperitoneal inoculation of NuTu-19 cells compared to control groups. In contrast, rats in Group 4 did not display any elevation of antitumour responses. Western blot analysis found cross-reactivity among antibodies generated between H9 and NuTu-19. However, the cytokines did not show significant differences, and no side effects were detected. Conclusion: hESC-based vaccination is a promising modality for immunotherapy of ovarian cancer.

Keywords: Ovarian cancer - human embryonic stem cell - antitumor immunity - cancer vaccine

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Introduction

The history of immunizing animals with fetal tissues to generate an antitumor response dates back a century ago (Brewer et al., 2009). Many subsequent reports have affirmed the general idea that immunologic rejection of transplantable tumors, as well as prevention of carcinogenesis, may be affected by vaccination with embryonic/fetal material (LeMevel and Wells, 1973; Coggin et al., 1980). In fact, a significant proportion of the human cancer vaccine trials to date are targeted against embryonic antigens such as carcinoembryonic antigen (Greiner et al., 2002) cancer/testes antigen (Chiriva-Internati, 2011; Mirandola et al., 2011) and α-fetoprotein etc (Toyoda et al., 2011). Unfortunately, targeting one antigen alone is unlikely to generate effective antitumor immune responses to mediate tumor rejection because of rapid appearance of escape mutants and the general inefficiency of monovalent cancer vaccines (Buonaguro et al., 2011; Durrant et al., 2011). Interestingly, it was found that cancer stem cells (CSCs) and embryonic stem cells (ESCs) shared similar cell surface markers and antigens not presented by adult tissues, which played a part in metastasis, angiogenesis and increased chemoradiotherapy resistance in cancer (Field et al., 2010; Kee et al., 2012; Lopez et al., 2012), so immune response against ESCs would cross-react with cancer cells (Li et al., 2009).

Subsequent reports supported the concept that vaccination with embryonic materials could generate cancer-specific immunity and protect animals from transplantable and chemically induced tumors. Li et al. (2009) demonstrated the capacity of human ES cells to effectively immunize against murine colon cancer for the first time. This was further supported by three additional studies that embryonic stem cells had successfully provided activation of antitumor immunity, leading to impressive suppression of proliferation and development of malignant colon tumors and lung cancer (Dong et al., 2010; Mocan and Iancu, 2011; Yaddanapudi et al., 2012).

However, the use of fetal materials or ESCs to induce tumor-specific immunity has always been utilized in mouse models so far, and in colon, lung cancer models and so on. What would happen in rat model? Whether ESCs would be an effective vaccine in ovarian cancer or not? Furthermore, how about the side effects of ESCs if the animals were given repeated vaccinations? In this study, rats were vaccined by hESCs, and then survival time, cellular immunity, humoral immunity, side effects, tumor antigen in hESCs were detected. Both immune responses and clinical responses against ovarian carcinoma were found, and impotantly no obvious side effects were detected.

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Materials and Methods

Cell lines

NuTu-19 cells, an epithelial ovarian carcinoma cell line derived from Fischer 344 rat (Rose et al., 1996) were grown in RPMI 1640 medium. Human ESC line H9 were grown in human ESC medium, and co-cultured with MEFs in a 6 cm dish to maintain their undifferentiated state. NuTu-19 cells were incubated with 10ug/ml mitomycin C for 3 hours in 37°C CO2 incubator and H9 cells were irradiated with 15Gy γ-ray before vaccination. Additionally, H9 cells were processed for paraffin embedding, 3μm sections were prepared for screening tumor antigens and genes by immunohistochemistry and examined with a microscope.

Animal

Specific pathogen-free (SPF) Fischer 344 female rats (100 to 125 g) were obtained from Academy of Military Medical Sciences (Beijing, China) and housed in Peking University People's Hospital with a SPF animal facility. Treatment and care of the animals were in accordance with Institutional Guidelines and the Animal Welfare Assurance Act. The experimental protocol of these animals for these studies was approved by the Institutional Laboratory Animal Care and Use Committee in Peking University People's Hospital (81072141).

Immunization Protocol

Fischer 344 rats were randomly divided into 7 groups with each group containing 6 rats; Group 1 (n = 6) vaccinated pre-irradiated hESCs (1×10^7) in 100μL PBS subcutaneously 3 times at 1-week intervals, and NuTu-19 cells (1×10^6) in 1000μL PBS were inoculated intraperitoneally 1 week after the last vaccination (primary immune response); Group 2 (n = 6) vaccinated pre-inactivated mitotic NuTu-19 (1×10^7) in 100μL PBS subcutaneously 3 times at 1-week intervals, and NuTu-19 cells (1×10^6) in 1000μL PBS inoculated intraperitoneally 1 week after the last vaccination; (this group served as a positive control for group 1); Group 3 (n = 6) vaccinated PBS (100ul) subcutaneously 3 times at 1-week intervals, and NuTu-19 cells (1×10^6) in 1000μL PBS were intraperitoneally inoculated 1 week after the last vaccination (this group served as a negative control for group 1); Group 4 (n = 6) vaccinated pre-irradiated hESCs (1×10^7) in 100μL PBS subcutaneously 3 times at 1-week intervals, and NuTu-19 cells (1×10^6) in 1000μL PBS were inoculated intraperitoneally 4 weeks after the last vaccination (memory immune response); Group 5 (n = 6) vaccinated pre-inactivated mitotic NuTu-19 (1×10^7) in 100μL PBS subcutaneously 3 times at 1-week intervals, and NuTu-19 cells (1×10^6) in 1000μL PBS were inoculated intraperitoneally 4 weeks after the last vaccination (this group served as positive control for group 4); Group 6 (n = 6) vaccinated PBS (100ul) subcutaneously 3 times at 1-week intervals, and NuTu-19 cells (1×10^6) in 1000μL PBS were inoculated intraperitoneally 4 weeks after the last vaccination (this group served as negative control for group 4); Group 7 (n = 6) vaccinated pre-irradiated hESCs (1×10^7) in 100μL PBS subcutaneously 6 times at 1-week intervals (Figure 2A).

Health conditions were monitored daily. Animals were euthanized when they developed large volumes of ascites. Tumorigenesis was recorded by counting the numbers and sizes of tumor foci on each organ.

Blood Analysis and Histology

Rat orbital venipuncture for blood collection was performed after the last vaccination and before NuTu-19 inoculation. The following punctures were performed at the interval of vaccination every month. Both whole blood and serum underwent CBC analysis and assay of metabolic proteins (Peking University Health Science Center, PUHSC). Multiple organs were collected and processed for paraffin embedding. 5μm sections were prepared, H & E stained, and examined with a microscope.

Enzyme-linked ImmunoSorbent Assay, ELISA

As previously described, fresh blood and serum from all groups was obtained at the same time. The serum levels of interleukin-4 (IL-4) and tumor necrosis factor-α (TNF-α) were tested by ELISA kits (NeoBioscience Technology Co., Ltd.) and analyzed on a microplate reader (Tecan Infinite M200, Labsystems Dragon).

Western Blot

H9 and NuTu-19 cells were rinsed with PBS and lysed in 100μL Laemmli sample buffer. The samples were separated by electrophoresis on a 10% denaturing and reducing SDS-polyacrylamide gel, then transferred onto an Immobilon-polyvinylidene fluoride membrane (Millipore). Membranes were blocked in 5% skim milk for 1 h and then incubated with the indicated primary antibodies overnight. Then incubated with appropriate secondary antibodies for 1h 30min. Secondary antibodies were incubated at a 1:50 dilution of sera from either naive or different cell-immunized rats. Specific proteins were detected using Enhanced Chemiluminescence.

Teratoma formation

H9 cells grown to approximately 70% confluence in a 6 well plate were injected into the rear leg muscles of severe combined immunodeficient (SCID) male mice aged 4 weeks. Ten to twelve weeks after injection, the resulting teratomas were histologically examined.

Statistical analysis

Survival time was estimated by Kaplan-Meier analysis. Statistical significance of differences in tumor growth rates was determined by ANOVA test analysis of variance using SPSS 20.0. Data were presented as mean ± SD, and a p value <0.05 was considered statistically significant.

Results

Human ESCs present tumor antigens

Immunohistochemical methods were used to screen tumor markers in H9 cell line, we found that several oncogenes, tumor suppressor genes, and metastasis-related genes had high expression in hESCs. Such markers included nm23 (+++), p53 (++), C-myc (++), HER-2 (+).

Table 1. Summary of Metastasis of Rat i.p. Injected with NuTu-19 Cells in Each Vaccine Group

Group	Group1,4 (H9)	Group2,5 (MMC-NuTu-19)	Group3,6 (PBS)
Vaccine route	S.C	S.C	S.C
Vaccine interval	Per week, 3 times	Per week, 3 times	Per week, 3 times
Tumor cells injection route	i.p	i.p	i.p
NO. rat	12	12	12
Metastatic organs	Diaphragm, peritoneal wall, intestine, mesentery, and omentum	Diaphragm, peritoneal wall, intestine, mesentery, omentum, kidney, liver surface and parenchyma, lung	Diaphragm, peritoneal wall, intestine, mesentery, omentum, kidney, liver surface and parenchyma, lung
Liver parenchyma metastasis (%)	0% (0/12)	16.7% (2/12)	33.3% (4/12)
Lung metastasis (%)	0% (0/12)	33.3% (4/12)	50.0% (6/12)
Metastatic tumor size >0.5mm3 (%)	25.0% (3/12)	75.0% (9/12)	83.3%(10/12)

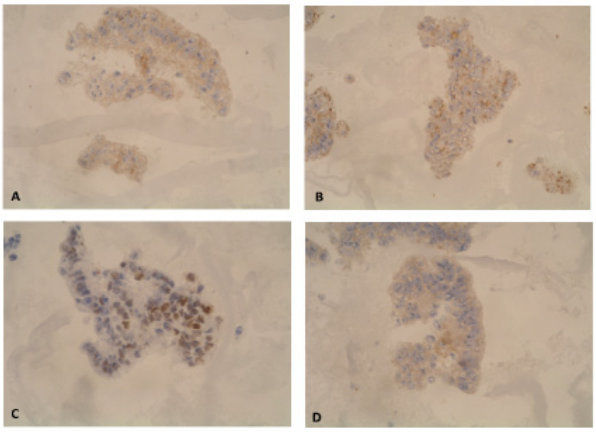

Figure 1. Immunohistochemical Staining of tissue Showing Representative Expression of Each Protein. A. nm23 (+++), Cell membrane and Cytoplasmic staining (×400); B. C-myc (++), Cytoplasmic staining (×400); C. P53 (++), nuclear staining (×400); D. PTEN (+), Cytoplasmic staining (×400)

We also identified some tumor markers, such as PTEN (+), CK (++), (Figure 1). Human embryonic stem cells express a broad spectrum of tumor antigens, markers, and genes related to tumor growth and metastasis. Results were interpreted by two pathologists independently, and a mean percentage of positive cells was determined in at least 5 areas at×400 and assigned to 1 of 4 categories: (-), <5%; (+), 5% to 25%; (++) 25% to 50%; and (+++), >50%.

Human ESCs prolong the survival time of tumor bearing rats

We used a well-established Fischer 344 rat epithelial ovarian cancer model (Rose et al., 1996). After administration of NuTu-19, kinetics of tumor growth and survival time were monitored. The survival time in group 1, 2, 3 was 54.3±2.5 days, 50.7±1.5 days and 48.5±2.4 days ($p<0.05$) separately. While the survival time in group 4, 5, 6 was 48.8±3.7 days, 48.2±4.7 days, 46.7±2.7 days ($p>0.05$), separately (Figure 2B). Rats in the hESC vaccine group 1 obtained stronger antitumor responses and longer survival, and H9 vaccination protected the rats from malignant cancer progression more effectively compared to rats in the control group ($p<0.05$). However, survival of rats in group 4 and its control groups did not show obvious benefits, and no significant differences were detected ($p>0.05$). The results suggested that anti-tumor effects of different vaccination protocols of H9 were different, primary immune response was stronger than memory immune response.

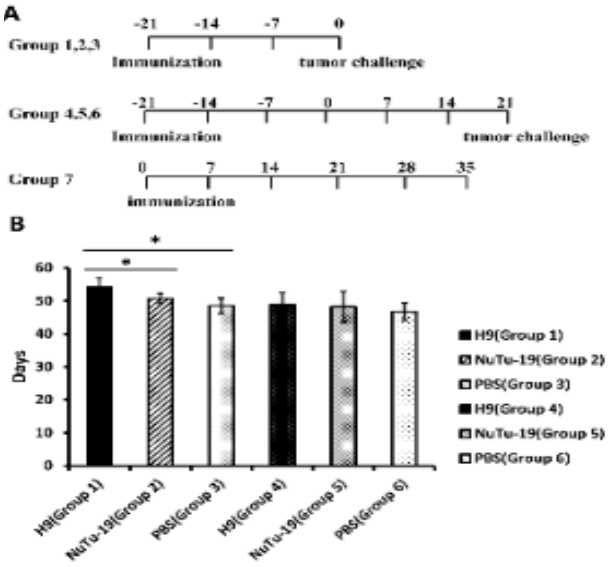

Figure 2. A. Scheme of hESCs Immunization and Tumor Inoculation. B. Human ESCs Vaccination Prolonged Survival in Rat Model. The survival time in group 1, 2, 3 was 54.3±2.5 days, 50.7±1.5 days and 48.5±2.4 days (*$p<0.05$) separately. The survival time in group 4, 5, 6 was 48.8±3.7 days, 48.2±4.7 days, 46.7±2.7 days ($p>0.05$), separately. H9 vaccination resulted in a dramatic longer survival time compared with the control groups

Human ESCs inhibit tumor distant metastasis

We vaccinated rats subcutaneously in groups 1 and 4 with hESCs 1 week and 4 weeks before the inoculation of NuTu-19 respectively. The tumor formation time was closely monitored. Upon gross visual inspection of the peritoneum, numerous tumors were observed in control rats, whereas there were significantly fewer tumors in the hESC-vaccinated rats. In groups 1 and 4 (H9 vaccined groups), metastatic lesion were found in diaphragm, peritoneal wall, intestine, mesentery and omentum, moreover, the metastatic tumor size≥0.5 mm3 were only found in 3 rats; however, in groups 2 and 5 (NuTu-19 vaccinated groups), the metastasis transfered to the kidney, liver surface parenchyma and lung, the metastatic tumor size≥0.5 mm3 were found in 9 rats, and in groups 3 and 6 (PBS vaccinated groups), such tumor size were found in 10 rats. Furthermore, the liver parenchyma metastasis and lung metastasis were found in 6 rats and 10 rats respectively in group 2, 3, 5, 6. However, there was no case developed such distant metastasis in group 1 and group 2. The results indicated obvious antitumor immunity and rejected tumor masses from proliferation and development

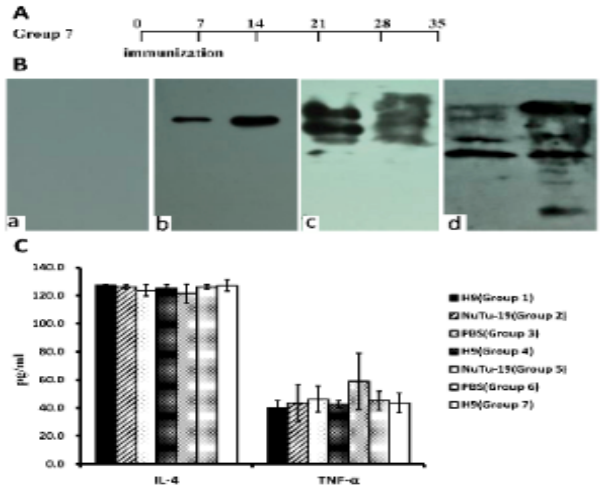

Figure 3. Immunization with H9 Generates a Cross-Reactive Antibody Against NuTu-19 Ovarian Cancer. A. Protocols of hESCs vaccination in group 7. B. Western blot analysis.. Numbers indicate the molecular weight marker (kDa).a. Sera from non-immunized naive rat western blot with H9 and NuTu-19 cell lysate; b. GAPDH as internal reference; c. Sera from immunized hESCs rat western blot with H9 and NuTu-19 cell lysate; d. Sera from NuTu-19-immunized rat western blot with H9 and NuTu-19 cell lysate. C. IL-4 and TNF-α showed no significant statistical differences between hESC vaccine group and control group

compared to the control group (Table 1), with considerably fewer tumors in the vaccinated rats.

Comparison of the immunogenicity between undifferentiated hESCs and pre-inactivated mitotic NuTu-19 Cells

We compared H9 and pre-inactivated mitotic NuTu-19 cells in the same ovarian cancer protection model. The primary antitumor responses of H9 and pre-inactivated mitotic NuTu-19 were evaluated according to the same immunization procedure as used for H9. We demonstrated that immunization with undifferentiated H9 cells only, and not immunization with pre-inactivated mitotic NuTu-19 cells, could inhibit tumor growth. These results suggest that the immunogenicity of hESCs differs from that of pre-inactivated mitotic NuTu-19 cells.

Vaccination with hESCs induced antibody response against ovarian cancer

To address whether a cross-reactive antibody was generated between H9 and NuTu-19, we tested the immunoreactivity of sera against both H9 and NuTu-19 cell lysates from rats that were immunized with H9 and NuTu-19, respectively. Sera from non-immunized naive rats did not react with either H9 or NuTu-19. However, we demonstrated that sera from NuTu-19-immunized rats were able to recognize multiple proteins in NuTu-19 as well as H9 lysates by Western blot analysis. Moreover, prominent 34kDa, 42kDa, 52kDa, and 80kDa molecules were recognized by sera from H9-immunized rats and detected in NuTu-19 ovarian cancer cells (Figure 3B). This suggested that an antitumor antibody response was produced after H9 immunization with shared antigens between NuTu-19 and undifferentiated H9 cells.

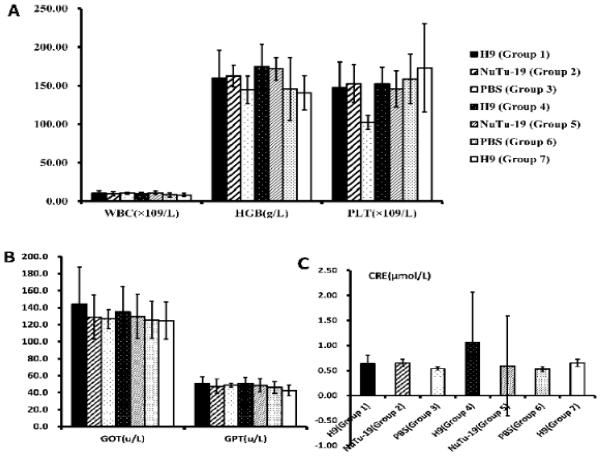

Figure 4. Immunization with Human Embryonic Stem Cells does not Result in a Significant Hematology Toxicity and Side Effect. A. Complete blood count (CBC) tests showed no significant differences in each group in rats. B and C. Blood serum enzymes in liver and kidney showed no significant differences in each group in rats

Cytokines

We next examined whether hESC immunization confers a memory antitumor immune response against NuTu-19. We observed both humoral and cell-based immunity, represented by production of NuTu-19-specific antibodies. However, significant differences in interleukin-4 (IL-4) and tumor necrosis factor-α (TNF-α) were not detected. IL-4 in group 1, 2, 3 was 127.4±0.7, 126.1±1.4, 123.7±4.1 pg/ml ($p>0.05$) separately. IL-4 in group 4, 5, 6 was 125.1±2.8, 121.4±6.8, 126.2±1.5 pg/ml ($p>0.05$) separately. And IL-4 in group 7 was 127.2±4.0 pg/ml. TNF-α in group 1, 2, 3 was 39.8±5.6, 43.4±13.4, 46.2±9.2 pg/ml ($p>0.05$) separately. TNF-α in group 4, 5, 6 was 42.6±2.6, 58.9±20.1, 45.2±6.8 pg/ml ($p>0.05$). IL-4 in group 7 was 43.7±7.0 pg/ml (Figure 3C).

Immunization with human embryonic stem cells does not result in a significant autoimmune response

One important consideration for stem cell-based vaccines is the possibility of breaking immune tolerance against self-antigens, such as cross-reactive antibodies against the hematologic system and side effects in liver and kidney. This question also has bearings on the application of stem cells for regenerative medicine in an immunocompetent host. As an index for inhibition in the hematologic system and side effects in important organs, we performed dynamic CBC assays, and the levels of several blood serum enzymes in the sera of rats that were immunized with PBS, H9, or NuTu-19 cells were determined. We used group 7 as positive control, since this group underwent repeated as many as 6 times inoculation with H9 cells only. CBC assays showed no differences among rats immunized with PBS, H9, or NuTu-19 cells. Creatinine and serum liver enzyme levels were normal in control and H9 cell-immunized rats (Figure 4).

Discussion

The recent surge in interest in pluripotent stem cells arose from promising results in the area of regenerative

medicine, indicating that stem cell-derived adult cells may offer treatment options for a variety of degenerative diseases, such as Parkinson's disease, type I diabetes mellitus, and Alzheimer's disease (Park et al., 2008) The first in vitro published observation of the potential of embryonic materials as a vaccine to prevent the development of tumor xenografts in animal models stimulated significant interest and research, leading to rapid development in this field. Preliminary data support the role of ESCs as effective cellular agents that reverse the immune dysfunction that is responsible for causing cancer. Moreover, ESCs induce antitumor immunity in tumor loading mice of different types and stages, such as colon and lung carcinoma (Li et al., 2009; Dong et al., 2010; Mocan and Iancu, 2011; Yaddanapudi et al., 2012). It has been a long time since embryonic materials have been used as vaccines to prevent the formation and development of tumors in animal experiments. Interestingly, this treatment helps support the hypothesis that tumor-embryonic antigens (oncofetal antigens) are expressed in cancer cells and in embryonic material. Thus, anti-embryonic antigens play a role in the anti-tumor immune response through cross-immune reactivity (Brewer et al., 2009).

We first screened tumor-embryonic antigens and several genes related to tumors by immunohistochemical methods. We found several genes or markers related to tumorigenesis, tumor growth, and metastasis. Many of these were involved in critical tumor signal transduction pathways. For example, nm23 and HER-2 were negatively correlated with tumor metastasis and prognosis. Interestingly, HER-2 has been exploited as a promising candidate for peptide-based cancer vaccines (Kedrin et al., 2009; Lekka et al., 2010; Niitsu et al., 2011). PTEN, p53, and c-myc, which are all well known to play important roles in carcinogenesis, have also been shown to be associated with prognosis (Schade et al., 2009; Chen et al., 2011; Huang et al., 2011). These results showed that human embryonic stem cells expressed a broad spectrum of tumor markers, many of which were also shared by ovarian cancer. This provided us with a basis for examining tumor markers for tumor immunotherapy.

Embryonic stem cells (ESCs) are associated with a high degree of plasticity, which allows them to self-renew and differentiate into every somatic cell. During differentiation, ESCs follow a hierarchically organized pattern towards tissue specificity, which ultimately results in permanent cell cycle arrest and a loss of cellular plasticity. In contrast to their normal somatic counterparts, cancer cells retain elevated levels of plasticity that include switches between epithelial and mesenchymal phenotypes. Transitions between these cell stages have lately been linked to the reacquisition of stem cell features during cellular reprogramming and dedifferentiation in normal and neoplastic cells (Strauss et al., 2012). All of these properties make ESC vaccines against cancer feasible and reasonable.

In this system, as few as 1×10^5 NuTu-19 tumor cells, incubated intraperitoneally, could lead to progressive local growth of the tumor and eventual death due, sometimes, to distal lung metastasis in rats. We found that immunization with H9 cells, in the absence of any exogenous adjuvant therapy, could lead to significant protection against live tumor challenge with high number of cells (1×10^6). In our study, we discovered yet another novel application of ESCs. Specifically, the administration of ESCs in rat could generate effective antitumor effects and protect rats from tumor proliferation and/or further development. More importantly, we found that the tumor distal metastasis in the H9 vaccine group were much less than those occurring in the positive control group (pre-inactivated mitotic NuTu-19) and negative group (PBS group). We speculated that hESCs could suppress tumor distal metastasis.

Currently, it is unclear whether tumor cells are able to immunize against cancer. We demonstrated that the immunogenicity of H9 dramatically rivals that of pre-inactivated mitotic NuTu-19 cells. The exact antigens shared by hESCs and NuTu-19 ovarian carcinoma cells remain to be identified. Most likely, the antigens that were reactive with the H9 immune sera were oncofetal antigens present in both NuTu-19 and the hESC. Stem cell immunization might trigger an immune response against these gene products that are also expressed by tumor cells. Additionally, the immune response against H9 could lead to antigenic spread to induce protective immunity against NuTu-19 unique tumor antigens, a concept akin to what was proposed to explain the efficiency of xenogeneic antigen immunization.(Huebener et al., 2009) We have found cross-reactive proteins between H9 and NuTu-19 by Western-blot. The ability to separate and purify these proteins in order to find the exact antigen and to explore the antitumor mechanism of embryonic stem cells are both interesting questions worthy of further exploration.

Additionally, we have examined the potential mechanism of tumor rejection by stem cell-immunized rats. Unfortunately, we didn't find significant differences in IL-4 and TNF-α. However, it is difficult to attribute responsibility for tumor rejection to a single mechanism, as the effector arm of tumor rejection is known to be a complex one, and the field of tumor immunology is still in desperate need of a suitable immunological surrogate marker to predict the clinical effectiveness of cancer vaccines (Zaritskaya et al., 2010). It is thus not surprising that not all H9 cells induced significant numbers of IL-4-producing splenocytes and TNF-α against NuTu-19. However, protective antitumor immunity appeared to wane over time (Byrne et al., 2011). So it may need for regular strengthen immunity. Furthermore, immunity is made of a multifaceted set of integrated responses involving a dynamic interaction of thousands of molecules. It may be the case that cell-mediated immunity might be so complicated that there might be other mechanisms and factors involved (Nolz et al., 2011).

More importantly, we have not observed any significant side effects in the hESC-immunized rats, the animal's weight, hair, joint swelling and neuromuscular tension were normal. Immunized rats were generally healthy without clinical evidence of autoimmune diseases. Rat's blood CBC, kidney and liver function were normal.

In a broad context, our study has raised a number of intriguing questions that deserve further research. For example, with further optimization, could a hESC-based vaccination strategy be effective against pre-established

cancer? What is the exact mechanism of antitumor effects of ESCs? However, additional follow-up studies are needed before hESC-based cancer vaccines move into clinical testing, as human with hereditary, chronological or environmental predispositions to neoplastic disease, which are essentially different from animal models.

In conclusion, we demonstrate the capacity of human ES cells to effectively immunize against rat ovarian cancer. This suggests the presence of shared embryonic antigens between hES cells and tumor cells. ES cells may present as a prophylactic vaccine for various types of cancers.

Acknowledgements

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