Use of an Extracellular Matrix Material as a Vaccine Carrier and Adjuvant

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Abstract. *Background: The addition of adjuvants frequently enhances the efficacy of vaccine preparations. Interest in the use of vaccines as a means to treat cancer has led to the search for improved adjuvants. Because cancer vaccines based on whole cell preparations might benefit from an adjuvant which enhances expression of antigens expressed during tumor cell growth, we evaluated the utility of an extracellular matrix material, porcine small intestinal submucosa (SIS), as a cancer vaccine adjuvant. Materials and Methods: After tumors were produced in Lobund-Wistar (LW) rats by subcutaneous administration of PAIII prostate adenocarcinoma cells, rats underwent surgical debulking of the tumor mass. Groups of ten rats were then vaccinated directly on the tumor bed with glutaraldehyde-treated tumor (GFT) cells harvested from a PAIII tumor; a 2×2 cm section of glutaraldehyde-treated SIS; or a 2×2 cm section of SIS on which harvested tumor cells were grown for either 3 days (GFT-S3) or 28 days (GFT-S28) and then treated with glutaraldehyde. In addition, a group was left untreated after debulking. Results: When tumors and lungs were harvested 21 days later, there were no significant differences between mean tumor weights of rats vaccinated with GFT cells or SIS and those which were left untreated. In contrast, rats vaccinated with GFT-S3 had a significant ($p<0.01$) reduction of greater than 65% and 58% in mean tumor weight compared to untreated rats and GFT cell-vaccinated rats, respectively. GFT-S28 rats had a significant ($p<0.05$) reduction of 59% and 49% compared to untreated rats and GFT cell-vaccinated rats, respectively. There was no significant difference in mean tumor weight between GFT-S3 and GFT-S28 rats. Furthermore, while most untreated rats had at least one metastatic focus in the lungs, a reduction was seen in rats vaccinated with GFT (7/10 positive), GFT-S3 (2/5 positive) and GFT-S28 (2/5 positive) cells. Conclusion: SIS enhanced the efficacy of a tissue vaccine for prostate cancer, demonstrating the potential utility of extracellular matrices as novel vaccine adjuvants.*

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Cancer of the prostate gland is the most frequently diagnosed malignancy and the second leading cause of death due to non-cutaneous cancer in men in the United States and many Western countries (1, 2). The disease typically begins as an androgen-independent neoplasm and then progresses to an androgen-independent malignancy, which spreads to the lungs and vertebral column (3).

Cancer vaccines have received substantial interest as potential therapeutic modalities. For example, the APC8015 vaccine, composed of autologous dendritic cells which have been pulsed *in vitro* with prostatic acid phosphatase has shown some efficacy against metastatic, androgen-independent prostate cancer (4-6). Likewise, a Phase II clinical trial of a vaccine preparation comprised of two allogeneic prostate cancer cell lines engineered to secrete granulocyte macrophage colony-stimulating factor (GM-CSF) stimulated immunity associated with median survival times of 26.2 and 35.0 months compared with a median survival of 18.9 months in patients receiving the standard of care, docetaxel and prednisolone (7, 8). While such results are encouraging, these approaches have not proven to be curative and substantial room exists for improvement. In this regard, great interest exists in methods to enhance the efficacy of vaccines for cancer, as well as those for infectious pathogens. Adjuvants are substances added to vaccines as nonspecific stimulators of the immune response. In contrast, some vaccines have used cytokines, such as GM-CSF, to stimulate specific aspects of the immune system. At present, the only vaccine adjuvant currently approved for use by the U. S. Food and Drug Administration is alum (9).

Extracellular matrix materials, such as porcine small intestinal submucosa (SIS), have been used for a number of medical applications. For example, SIS has found varied clinical use, including as a hernia repair device, a wound care material, and as an anal fistula plug (10-12). SIS promotes tissue ingrowth and is rapidly incorporated into the native tissue of the host following implantation (13). Because SIS is mildly proinflammatory and supports the

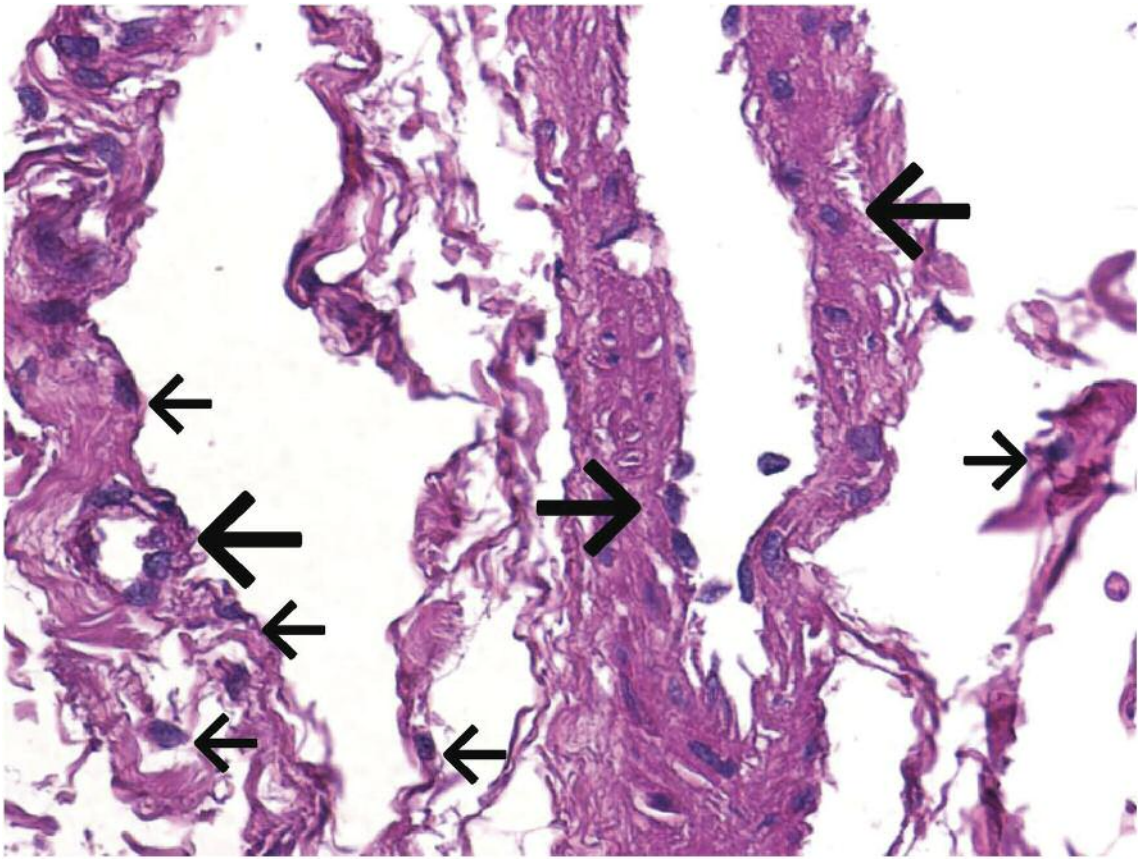

Figure 1. *Photomicrograph of SIS upon which cells from a harvested tumor were cultured. The sample shown represents the material three days after culture was begun. There are numerous cells within the matrix of the SIS (small arrows) and within remnant vascular walls of the SIS (large arrows). Stained with hematoxylin and eosin. Magnification ×400.*

growth of tissue, we investigated the ability of SIS to augment the anticancer response of a tissue vaccine.

Materials and Methods

Animals. All animal studies were approved by the University of Notre Dame Institutional Animal Care and Use Committee. Lobund-Wistar (LW) rats were obtained from a breeding colony maintained at the University of Notre Dame. The LW rat is an established model of prostate cancer which metastasizes to the lungs (14). PAIII cells were originally isolated from an autochthonous, metastatic prostate adenocarcinoma in a LW rat (15). The cells were maintained as tumors by serial passage of tumor samples in LW rats. Typically, these become large subcutaneous tumors, weighing in excess of 10 g and which metastasize to the lungs. Passage of tumors was performed by harvesting a 5-g portion of tumor from a euthanized rat and mincing the tissue in 10 ml of modified Eagle's medium (MEM). Subcutaneous administration of 0.3 ml of this cell suspension consistently resulted in tumor masses which could be palpated as early as 7 days after cell suspension administration.

Extracellular matrix. Small intestinal submucosa (SIS; Surgisis®, Cook Biotech, Inc., West Lafayette, IN USA) was provided as a sterile, lyophilized sheet of extracellular matrix. The SIS was of porcine origin and derived by removal of all mesenteric tissues, serosa and tunica muscularis from segments of jejunum. Prior to culture with tumor cells and implanation into animals, SIS was cut into 2 cm × 2 cm sections.

Preparation of vaccines. Two vaccine preparations were evaluated: glutaraldehyde-fixed tumor (GFT) cells harvested directly from a subcutaneous PAIII tumor and GFT cells which were grown and fixed with glutaraldehyde on SIS (GFT-S vaccine). The GFT vaccine was prepared by harvesting 3 g of a subcutaneous tumor and mechanically dissociating it by fine mincing followed by passage through a 80-mesh screen to create a cell suspension in MEM (16). The suspension was incubated in 2.5% glutaraldehyde (v/v) at 37˚C for 60 min and then washed thoroughly with medium to produce the final vaccine preparation. The GFT-S vaccine was produced by incubating 1×10^6 harvested tumor cells, obtained as for the GFT vaccine, in MEM at 37˚C under 5% CO_2. Following 3 days or 28 days of growth, SIS with attached cells then underwent glutaraldehyde fixation and washing as for the GFT vaccine. Additional samples were fixed in 10% neutral buffered formalin and saved for histological preparation and examination.

Surgical resection of tumors. Rats underwent surgical excision of subcutaneous tumors fourteen days after administration of PAIII cells.

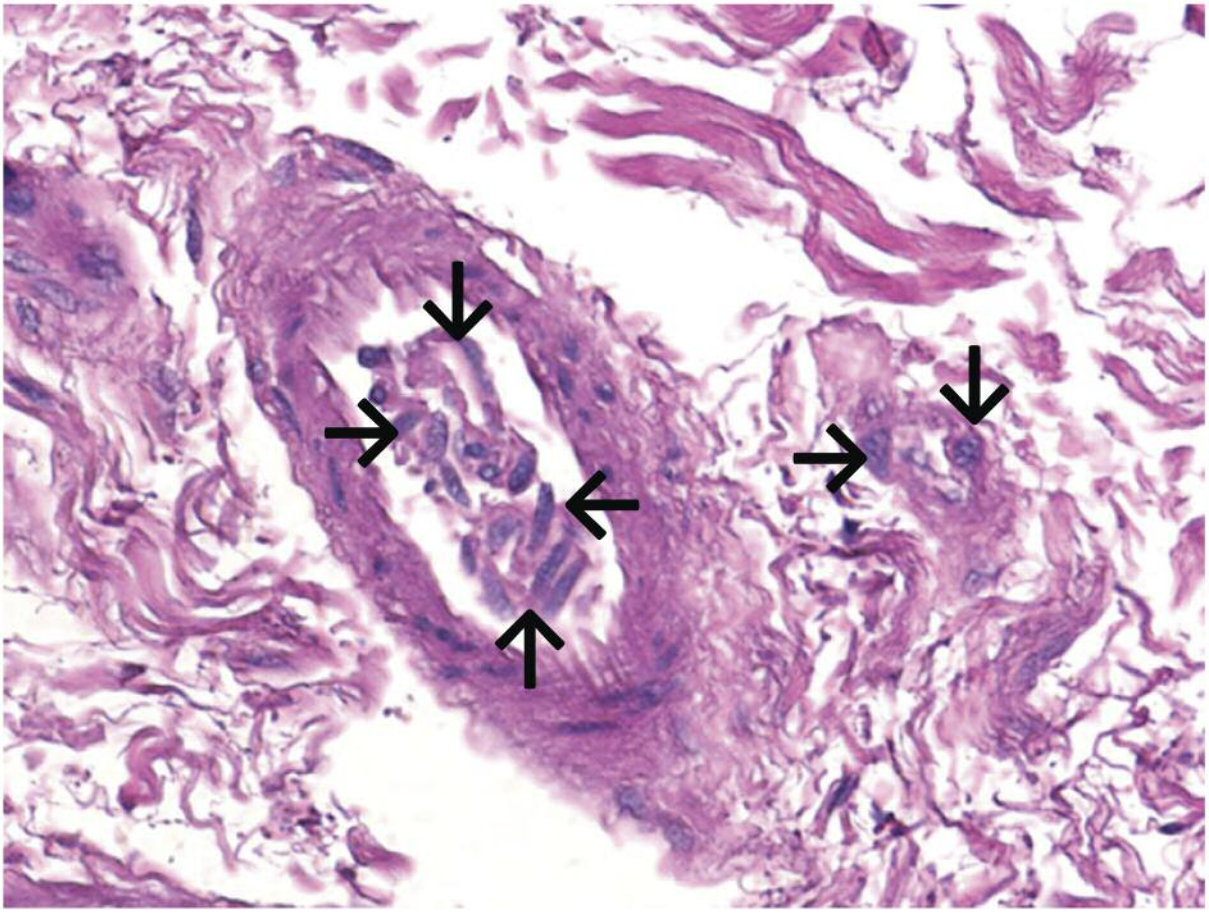

Figure 2. *Photomicrograph of SIS three days after culture with harvested tumor cells. Numerous small vascular structures were present (arrows), including some forming within the lumen of a vascular remnant of the SIS. Stained with hematoxylin and eosin. Magnification ×400.*

Following induction of surgical anesthesia with an intraperitoneal dose of ketamine (90 mg/kg) and xylazine (10 mg/kg), the hair overlying the tumor was clipped and the skin scrubbed with an iodophore. Using an aseptic technique, tumors were surgically excised and the skin closed with surgical staples. Rats were administered a subcutaneous dose of butorphanol (2 mg/kg) for post-surgical analgesia. With this technique, a small residual tumor bed remains and tumors typically regrow within 10-14 days.

Histological examination of GFT-S samples and tumor samples. Samples of GFT-S and tumors of rats harvested at the time of euthanasia were fixed in 10% neutral buffered formalin. Samples were washed in 70% ethanol and embedded in paraffin. Following sectioning at 4-5 μm and stained with hematoxylin and eosin for histological examination.

Study design. To generate subcutaneous PAIII tumors, 50 male LW rats, 3-4 months old, were administered 1×10^6 freshly harvested tumor cells subcutaneously in a volume of 0.3 ml of MEM. Fourteen days later, all rats had palpable subcutaneous tumors and underwent surgical resection of the tumors. Rats were then randomly assigned to groups of ten which were either left untreated or treated by placement upon the tumor bed of glutaradehyde-fixed SIS, GFT cells, or GFT-S vaccine which was produced following growth of cells on SIS for either 3 days (GFT-S3) or 28 days (GFT-S28) in culture. Twenty-one days later, rats were euthanized by carbon dioxide narcosis and the tumors weighed. At the same time, the lungs were harvested and the presence or absence of subpleural metastatic foci noted. Samples of tumors were processed for histological evaluations. The differences in the mean tumor weights were evaluated for significance between groups using one-way analysis of variance with significance reached when $p\leq0.05$.

Results

Growth of cells on SIS. To determine the ability of SIS to support prostate tumor cell growth, cells harvested directly from tumor tissue were grown on SIS for either 3 days or 28 days. Histological examination of samples from each time point demonstrated abundant growth of cells within the SIS matrix. Cells appeared to grow along the collagen matrix of the SIS as well as within remnant vascular walls of the material (Figure 1). Cells appeared to form numerous small vessels, suggesting angiogenesis (Figure 2). Histological examination

Table I. *Mean tumor weights and number of rats with metastatic foci in the lungs.*

Treatment group	Mean tumor weight (g)	Number with pulmonary metastases/ rats in group
Resection only (untreated)	14.9±2.12	10/10
SIS	15.6±1.82	10/10
GFT Cells	11.80±1.46	7/10
GFT-S3	4.77±1.17	4/10
GFT-S28	5.00±2.61	4/10

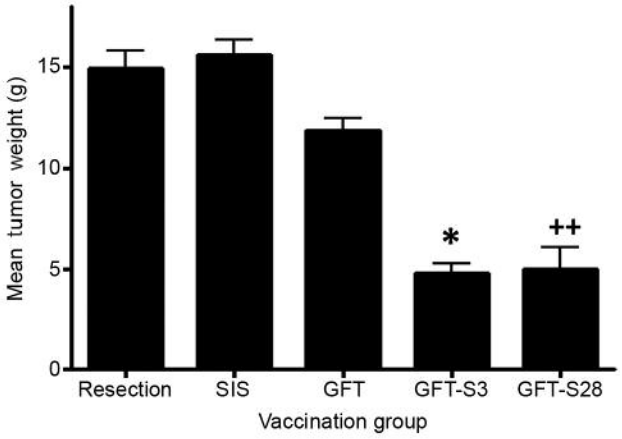

Figure 3. *Mean tumor weight at the time of sacrifice. Rats had undergone resection of subcutaneous tumors followed by either no treatment (Resection) or treatment with glutaraldehyde-fixed SIS (SIS); glutaraldehyde-fixed tumor cells (GFT), SIS upon which harvested tumor cells were grown for 3 days and then fixed with glutaradehyde (GFT-S3), or SIS upon which harvested tumor cells were grown for 28 days and then fixed with glutaraldehyde (GFT-S28). Twenty-one days later, there was no benefit of treatment with SIS or GFT compared to resection-only control rats; however, treatment with GFT-S3 or GFT-S28 resulted in significant decreases in mean tumor weight (*$p \leq 0.01$; $^{++}p \leq 0.05$).*

did not reveal any notable difference in the types or distribution of cells growing after 3 days *versus* 28 days of growth.

Inhibition of tumor regrowth and metastasis. Following surgical excision of subcutaneous PAIII prostate tumors, rats were administered a vaccine preparation and then evaluated three weeks later for tumor regrowth and metastasis. As shown in Table I, there were no significant differences between mean tumor weights of rats vaccinated with GFT cells and control rats which were treated with glutaraldehyde-fixed SIS having no added cells, or control rats which had received no further treatment and tumor excision. In contrast, rats vaccinated with the GFT-S3 vaccine had a significant ($p \leq 0.01$) reduction of greater than 65% in mean tumor weight compared to control animals and a mean tumor weight reduction of approximately 58% compared to rats vaccinated with GFT cells. Furthermore, rats vaccinated with GFT-S28 demonstrated a significant ($p \leq 0.05$) reduction of greater than 59% in mean tumor weight compared to control animals and a reduction of 49% compared to rats vaccinated with GFT cells. There was no significant difference between mean tumor weights of rats vaccinated with GFT-S3 and those vaccinated with GFT-S28.

The incidence of pulmonary metastasis was reduced in rats that were vaccinated with either GFT cells, or the GFT-S3 or GFT-S28 vaccines (Table I). While all untreated and SIS-treated control rats had at least one grossly visible metastatic focus on the subpleural surface of the lungs, a reduction was seen in rats vaccinated with GFT cells (7/10 positive) and GFT-S3 (4/10 positive) and GFT-S28 (4/10 positive).

Discussion

Vaccination as an approach to cancer offers great potential due to the general lack of untoward complications, such as those associated with chemotherapy. It stands to reason that vaccines which include the broadest spectrum of antigens have the greatest chance of stimulating a protective immune response. In this regard, tissue vaccines include a tremendous antigenic repertoire, one that adds antigens relevant to the connective tissue matrix which supports tumor growth and progression, as well as antigens that are uniquely expressed *in vivo versus in vitro* (17). In earlier work, the GFT cell tissue vaccine was shown to reduce the incidence of prostate cancer in LW rats by 90% (16). Moreover, incubation of human PC346C prostate cancer cells with splenocytes from mice vaccinated with the GFT cell vaccine eliminated the ability of tumor formation when those PC346C cells were subsequently transplanted into immunodeficient nude mice (18).

As with vaccines for infectious disease, cancer vaccines benefit from strategies to enhance the resulting immune response. Approaches to vaccine enhancement may include addition of nonspecific immunostimulating adjuvants (9, 19, 20), specific immunostimulatory cytokines (9, 21), or special vaccine delivery systems (22-24). For tissue vaccines, a method which would allow presentation of vaccine antigens in a way that might simulate the architecture of tumor tissue in a mildly proinflammatory milieu would have obvious benefit for optimizing vaccine immunogenicity.

In the present study, we evaluated the ability of an extracellular matrix, SIS, to enhance the anticancer response of a tissue vaccine. We found that the GFT-S3 and GFT-S28 vaccines significantly enhanced the efficacy of the GFT cell vaccine. Moreover, the GFT-S3 and GFT-S28 vaccines were equally effective at both inhibiting regrowth of a prostate tumor following resection and at preventing pulmonary metastasis from the tumor. From this data it appears that relevant antigenic factors are expressed and presented as a result of growth on the SIS relatively quickly, as the GFT-S3 vaccine was as equally

effective as the GFT-S28 vaccine. This suggests that factors expressed during the early growth of a tumor may represent particularly important antigens for vaccine targeting.

SIS is a naturally occurring, bioactive extracellular matrix that has proven successful as a tissue graft material in a variety of clinical applications related to tissue repair (10-12). The material serves as a bioscaffold for in-growth of, and subsequent incorporation into, normal, repaired tissue. Characterized as a collagen-glycosaminoglycan (GAG) material, SIS has been shown to have intrinsic bioactive growth factors that contribute to its clinical usefulness. These growth factors include transforming growth factor-beta (TGF-β), which is important in wound healing, and the highly angiogenic growth factor, basic fibroblast growth factor (FGF-2) (25, 26).

In view of the ability of SIS to support growth of tissue *in vivo*, it is not surprising that SIS also served as an effective substrate for *in vitro* growth of harvested tumor cells. Badylak *et al*. (27) showed that SIS supported the growth of fibroblasts, keratinocytes, vascular endothelial cells and a rat osteosarcoma cell line. Coculture of fibroblasts with keratinocytes resulted in a distinctive spatial orientation of the two cell types, indicating that SIS provided a 3-dimensional scaffold that allowed for cell migration and spatial organization (27). The cell and its extracellular matrix co-exist in a state of "dynamic reciprocity" (28), with the latter not only providing a mechanical framework for tissue architecture but also playing an active role in regulating the signaling process (29). In this way, cells communicate with other cells within the three-dimensional architecture of the extracellular matrix to collectively give rise to differentiated form and function.

Growth of cells on extracellular matrix material, such as SIS, may confer "phenotypic stability" to cells cultured on it, as reported by others (27, 30, 31), and this study supports that concept because the vaccines with either 3 days or 28 days of culture had the same effects *in vivo*. This is in contrast to the well-known phenomenon that cancer cells cultured for many days on plastic dramatically change their phenotypic expression and morphological characteristics (32-34).

In the present study, a tissue vaccine was produced by culturing harvested tumor tissue on SIS. This GFT-S vaccine stimulated a significantly stronger anticancer response than a vaccine produced from noncultured tumor tissue, the GFT vaccine. Although growth of high-grade metastatic bladder cancer cells on SIS resulted in less aggressive behavior and a more organized growth pattern (35), relevant antigens of PAIII prostate cancer cells must have been expressed and preserved in the GFT-S vaccine to an extent sufficient to generate an effective anticancer response. Possibly, the SIS provided a scaffold for harvested tumor cells to organize in a way to more closely simulate *de novo* tumor architecture. While an earlier study demonstrated that SIS suppressed *in vitro* Th1 cell expansion in a TGF-β dependent manner (36), others have shown *in vivo* that SIS interferes with neither the humoral nor cell-mediated immune responses (37). In any case, the sum effect of intrinsic cytokines and the unique antigens that might be expressed by allowing growth of tumor tissue on an extracellular matrix facilitated a dramatic antitumor response in our model.

The substance of the SIS appeared to be well-populated with a variety of cells, some of which formed presumptive vascular structures. Components intrinsic to the SIS, such as FGF-2, are strongly angiogenic and may have promoted the angiogenic potential of some harvested tumor cells. That factors within the SIS might encourage additional growth *versus* simple colonization of tumor and tumor connective tissue cells within the three-dimensional matrix suggests that the SIS promotes growth of tumor tissue and processes which could result in expression of additional antigenic factors. Interestingly, SIS did not promote the growth of PAIII tumors *in vivo* following surgical tumor resection (38). Though SIS is rich in hyaluronic acid, which has been demonstrated to inhibit adhesion of ovarian cancer cells *in vitro* (39), our results did not indicate a significant anticancer effect associated with SIS alone.

In summary, an extracellular matrix material provided adjuvancy for a prostate cancer vaccine. Adjuvancy may have resulted from a number of factors, including the mildly proinflammatory nature of SIS, the simulation of tumor architecture, and expression and sequestration within the SIS of factors associated with a growing tumor. Future studies will be needed to more precisely define the mechanisms involved in vaccine enhancement. Extracellular matrix adjuvants represent a very novel approach to vaccine enhancements and may also be extended to vaccines for other cancer types.

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