Neo-Organoid of Marrow Mesenchymal Stromal Cells Secreting Interleukin-12 for Breast Cancer Therapy

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Abstract

Bone marrow–derived mesenchymal stromal cells (MSCs), beneficial for regenerative medicine applications due to their wide differentiation capabilities, also hold promise as cellular vehicles for the delivery of therapeutic plasma-soluble gene products due to their ease of handling, expansion, and genetic engineering. We hypothesized that MSCs, gene enhanced to express interleukin-12 (IL-12) and then embedded in a matrix, may act as an anticancer neo-organoid when delivered s.c. in autologous/syngeneic hosts. We performed such experiments in mice and noted that primary murine MSCs retrovirally engineered to secrete murine IL-12 can significantly interfere with growth of 4T1 breast cancer cells in vivo, with a more substantial anticancer action achieved when these cells are embedded in a matrix. Plasma of mice that received the IL-12 MSC-containing neo-organoids showed increased levels of IL-12 and IFN-γ. Histopathologic analysis revealed less tumor cells in implants of 4T1 cells with IL-12 MSCs, and the presence of necrotic tumor islets and necrotic capillaries, suggesting antiangiogenesis. We also showed that the anticancer effect exerted by the IL-12 MSCs is immune mediated because it is absent in immunodeficient mice, is not due to systemic IL-12 delivery, and also occurs in a B16 melanoma model. This study therefore establishes the feasibility of using gene-enhanced MSCs in a cell-based neo-organoid approach for cancer treatment. [Cancer Res 2008;68(12):4810–8]

Introduction

Bone marrow–derived mesenchymal stromal cells (MSCs) have been increasingly used clinically for regeneration of damaged tissues due to the innate potential of these progenitor cells to turn into a variety of differentiated cell types (1–5). The differentiated progeny of these primary cells includes osteocytes, chondrocytes, astrocytes, adipocytes, neurons, skeletal myoblasts, and cardiomyocytes. Studies have shown the reparative actions of MSCs on bone, cartilage, brain, spinal cord, heart, and other tissues (5–10). For instance, cell therapy with MSCs has aided in the treatment of osteogenesis imperfecta, myocardial infarction, as well as bone marrow recovery when used with hematopoietic stem cells after myeloablation (5, 11–15). Clinical trials involving MSCs are presently ongoing worldwide and comprise phase I/II studies in India for steroid refractory graft-versus-host disease, in Israel for distal tibia bone fractures, in Denmark for severe chronic myocardial ischemia, and in Japan for periodontitis (5).

Besides their favorable implications for regenerative medicine due to their cellular plasticity, MSCs have also been explored following gene enhancement for the secretion of therapeutically beneficial plasma-soluble gene products. For instance, reports have revealed the efficacy of MSCs gene modified to overexpress epidermal growth factor receptor, factor VIII, factor IX, BMP2, BMP4, COL1A1, phenylalanine hydroxylase, and other corrective proteins (16–22). The abundance of MSCs in humans together with the facility with which these cells can be isolated, expanded, and genetically engineered have rendered MSCs a desired autologous cell type for cell and gene therapy purposes.

We have previously described the use of MSCs, gene modified to secrete erythropoietin (Epo), firstly for proof-of-concept studies and subsequently for the treatment of anemia of end-stage renal disease (23–25). More specifically, we noted a pharmacologically relevant effect from Epo gene-engineered MSCs (Epo MSCs), that is, a considerable hematocrit increase, after i.p. or s.c. implantation in normal nonmyeloablated immune-competent mice, with a more significant and durable outcome when these cells were embedded within a matrix before their s.c. administration. The matrices used to form with the MSCs “neo-organoids” comprised the mouse-compatible material Matrigel as well as the human-compatible, Food and Drug Administration (FDA)-approved, bovine collagen–based substance Contigen (23, 24). We thereafter applied our neo-organoid approach in a mouse model of anemia induced by experimental chronic renal failure, and showed that our Epo MSCs embedded in Contigen and implanted s.c. engendered a rise in plasma Epo levels, a cell dose-dependent elevation in hematocrit, and an improvement in exercise capacity which we hypothesized was linked to the cardioprotective action of Epo (25). Moreover, we observed that murine MSCs, although possessing an immunosuppressive ability, were rejected in allogeneic mice and thus not immunoprivileged, signifying that use of MSCs for long-term in vivo action should be limited to autologous settings (26).

The sum of previous studies, ours and others, supported the notion that a cell and gene therapy strategy with genetically engineered autologous MSCs embedded in a matrix and acting as a neo-organoid for therapeutic protein delivery would not only be feasible but also promising for breast cancer treatment. In our earlier research, we revealed the value of interleukin (IL)-2–engineered MSCs for immunotherapy of melanoma (27). We now here report the effectiveness of IL-12 gene-modified MSCs against breast cancer as well as melanoma. In brief, our results show that murine MSCs can be retrovirally engineered to secrete considerable levels of murine IL-12 (mIL-12), embedded in collagen-based matrices to then generate s.c. neo-organoids delivering IL-12 locally, which can significantly interfere with cancer growth in mice.
Materials and Methods

Generation of retroviral constructs and of retrovirus-producing cells. The eukaryotic expression plasmid pNGVL12 coding for mIL-12 was obtained from National Cancer Institute Laboratories. A fragment comprising the IL-12 p35 and IL-12 p40 genes separated by an internal ribosomal entry site (IRES) was retrieved by restriction enzyme digest of pNGVL12 with Sall and NotI and ligated into our retroviral plasmid construct pEmptyVector (24) cut with XhoI and NotI. The resulting mL-12 retrovector was then cotransfected with the neomycin resistance gene-containing plasmid pEGFP-C1 (Clontech) into GP+E86 retrovirus packaging cells. Stable GP+E86-IL-12 transfectants were used to generate retroparticles for gene transfer. Replication-free control vector retroparticles were likewise generated.

Isolation, culture, and gene modification of marrow MSCs. A female BALB/c mouse (Charles River, L'Arbresle, France) weighing 15 to 20 g was sacrificed, femurs and tibias were isolated, and whole bone marrow was retrieved by flushing these bones with complete medium (Dulbecco's). All of these bone marrow cellswere then cultured for 5 days in bovine serum and 50 units/ml penicillin, 50 µg/ml streptomycin; Wisent Technologies). All of these bone marrow cells were then cultured for 5 days at 37°C with 5% CO2, after which the nonadherent hematopoietic cells were discarded and the adherent MSCs were cultured for four to five passages in complete medium before gene modification.

IL-12 gene-modified BALB/c MSCs were generated by transduction of MSCs twice per day for 2 consecutive days and once more on the third day for each of 2 successive weeks. For each round of transduction, 0.45-µm filtered retroviral supernatant from GP+E86-IL-12 virus producers was placed on 60% to 70% confluent MSCs in the presence of 6 µg/ml Lipofectamine reagent (Invitrogen/Life Technologies). The ensuing polyclonal population of IL-12 gene-modified BALB/c MSCs was plated at limiting dilution for the generation of monoclonal populations of IL-12 MSCs. Control vector retroparticles served to likewise generate control MSCs. Supernatants were collected and ELISA specific for mIL-12 p70 (R&D Systems) showed the in vitro secretion of >60 ng of IL-12 per 10^6 cells per 24 h for the IL-12 MSCs used in this study.

Most experiments were conducted with BALB/c-derived MSCs since testing of the growth of isogenic 4T1 breast cancer cells. To evaluate MSCs from a different mouse strain and on another tumor type, we likewise generated MSCs from a 15 to 20 g C57BL/6 mouse and using our above-described IL-12 retrovectors. As control C57BL/6 MSCs, we used those we had previously produced (23).

In vitro characterization of IL-12 MSCs. IL-12 gene-modified BALB/c MSCs were generated by transduction of C3H-blastin305, CD34-blastinRAM34, CD34-PE1M7, CD45-PE30-F11, CD73-PE2/3Y1, CD80-blastin10A1, CD86-blastin105, CD117-PE2B8, Kd-blastinSf1-1L1, and I-Ad-blastin32.1 (BD Pharmingen) and B7.21H1-blastinMH5 and CD105-blastin17/18 (1:50 dilution; ebioscience) for 1 h at 4°C in the dark and washed with 3% fetal bovine serum (FBS) in PBS. Cells incubated with the biotin-conjugated antibodies were stained with the secondary antibodies (streptavidin-PE, dilution 1:100) for 30 min. Isotypic control analyses were conducted in parallel. Cells were washed with 3% FBS in PBS, fixed with 1% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer with data analysis conducted with CellQuest pro software (BD Immunocytometry Systems).

To ascertain their in vitro differentiation ability, IL-12 BALB/c MSCs were cultured, when ~70% confluent, in medium inductive of osteogenic or adipogenic differentiation for 1 mo and stained with Alizarin Red S or Oil Red O solutions, respectively, as previously described (25, 28, 29).

In vivo experiments assessing IL-12 MSCs on 4T1 breast cancer cell growth. 4T1 murine breast cancer cells purchased from the American Type Culture Collection and cultured in complete medium were first implanted s.c. in the right flank of isogenic BALB/c mice at 2.5 x 10^4 per mouse with or without IL-12 or control MSCs. Mouse were sacrificed, and tumors were measured for over 1 mo.

In vivo testing of IL-12 MSCs contralateral to 4T1 breast cancer cell implantation. BALB/c mice were implanted s.c. in the right flank with 4T1 cells at 2.5 x 10^4 per mouse in RPMI 1640. The following day, two of the three groups of 4T1-implanted mice received s.c. injection in the opposite side (i.e., left flank) IL-12 or control BALB/c MSCs, at 1 x 10^6 per mouse, mixed in Matrigel. Tumor volumes were measured for over 1 mo.

Results

In vitro characterization of IL-12 gene-modified MSCs. To genetically engineer MSCs to render them capable of secreting significant levels of IL-12, we first generated the mL-12 retrovector, a construct containing the two subunits of mL-12, specifically p35 and p40, separated by an IRES, which we then used to produce replication-free IL-12–containing retroparticles. We likewise generated the control retrovector (i.e., not expressing IL-12) and then replication-free control retroparticles. Replication-free retroparticles were used for the gene modification of MSCs. Using MSCs isolated from BALB/c and C57BL/6 mice, we created polyclonal and monoclonal populations of IL-12 gene-modified murine MSCs secreting >60 ng of mL-12 per 10^6 cells per 24 h in vivo by ELISA on cell supernatants. More precisely, mL-12 secretion by the IL-12 MSCs mainly used was 75 ± 3.7 ng of mL-12 per 10^6 cells per
24 h \((n = 5, \text{mean } \pm \text{SE})\). Control MSCs were found not to secrete detectable levels of mIL-12 (<2.5 pg/mL).

To determine if the IL-12 secreted in vivo by IL-12 gene-modified BALB/c MSCs can engender a beneficial effect against 4T1 cells in vivo, we implanted isogenic BALB/c mice with 4T1 cells s.c. in the presence or absence of IL-12 MSCs or control MSCs. We observed a

**Figure 1.** Characterization of IL-12 MSCs. A, the BALB/c mouse-derived MSCs, gene modified to secrete mIL-12 and used in this study, were analyzed by flow cytometry analysis for the expression of cell surface antigens CD31, CD34, CD44, CD45, CD73, CD80, CD86, CD105, CD117, Kd (MHC class I), I-Ad (MHC class II), and B7H1. B, for analysis of their mesenchymal differentiation ability, IL-12–secreting MSCs, undifferentiated and confluent (left), were cultured, when ~70% confluent, in conditions inductive of osteogenic or adipogenic differentiation, respectively. Middle, following osteogenic differentiation, calcium in the mineralized extracellular matrix was shown by Alizarin Red S staining; right, following adipogenic differentiation, lipid droplets were evident by light microscopy as well as by their staining with Oil Red O. Photographs were taken under light microscopy using a Contax167MT camera (Kyocera) with a 400 ISO film attached to an Axiovert25 Zeiss microscope (Carl Zeiss).
significant effect against breast cancer progression in recipients of IL-12 MSCs compared with the other groups of mice (P < 0.01, log-rank test; Fig. 2A). At 3 weeks after implantation, there were 43% of mice tumor-free among those that had received IL-12 MSCs, in contrast to 0% of control mice that had been implanted with 4T1 cells alone or with control MSCs. By 5 weeks after implantation, there were still 14% of tumor-free mice in the IL-12 group (Fig. 2A).

Effect of Matrigel-embedded IL-12 MSCs on 4T1 breast cancer cell growth. To reveal the potential of a clinically translatable anticancer approach where genetically engineered MSCs would be implanted embedded in a matrix as a neo-organoid at the tumor site, we mixed the IL-12 BALB/c MSCs in Matrigel and injected these at the site where 4T1 tumor cells had been implanted on their own, and without any matrix, 1 day earlier. In mice implanted s.c. with 4T1 cells and the following day with Matrigel-embedded IL-12 MSCs in the same area, 100% were tumor-free at 20 days after implantation versus 0% of mice implanted with 4T1 cells only or with next day Matrigel-embedded control MSCs (P < 0.001, log-rank test). Furthermore, 67% of IL-12 MSC-implanted mice remained tumor-free for >55 days (Fig. 2B).

To determine IL-12 plasma levels, mL-12 p70–specific ELISA was conducted on plasma samples collected 1 week after implantation and revealed concentrations of 362 ± 61.3 pg/mL in IL-12 MSC recipients versus 27.3 ± 26.0 pg/mL in control MSC-implanted mice (P < 0.05, Student’s t test; Fig. 2C, left). To establish if increased IL-12 plasma levels led to elevated IFN-γ plasma concentrations, mIFN-γ-specific ELISA was performed and revealed IFN-γ levels of 452 ± 113 pg/mL in these IL-12 mice, in contrast to no detectable levels (i.e., <2 pg/mL) in control MSC recipients (P < 0.05, Student’s t test; Fig. 2C, right). However, plasma concentrations of both IL-12 and IFN-γ declined over time following implantation of IL-12 MSCs, as we determined a drop of ~35% in IL-12 and ~95% in IFN-γ at 3 weeks after implantation, more precisely to 233 ± 46.1 pg/mL and 24 pg/mL, respectively (data not shown).

Histologic analysis of implants of 4T1 cells with/without IL-12 or control MSCs. To analyze and compare the histology of implants of tumor cells in the presence or absence of gene-modified MSCs, implants were retrieved 2 weeks after implantation. Matrigel implants from mice implanted with 4T1 cells alone and IL-12 MSCs contained much less tumor cells (Fig. 3A) than Matrigel implants from mice implanted with 4T1 cells alone (Fig. 3A) or 4T1 cells with control MSCs (Fig. 3B). Tumors were subdivided in large ill-defined lobules by thin delicate fibrovascular bands, and within lobules, tumor cells formed a solid sheet where few capillaries were interspersed. Lobules contained small and few areas of lytic necrosis where cell debris were mixed with few neutrophils. Tumor cells were poorly differentiated: they were closely and disorderly packed (they did not form glandular acini), and their nuclei were large, vesicular, and highly pleomorphic and often contained multiple amphophilic nucleoli. The chromatin was coarsely clumped. The cytoplasm was scarce and cellular outline was indistinct. We noted that there were no striking quantitative or qualitative changes between the IL-12 group and both control groups regarding inflammation. A quantitative analysis of neutrophils revealed the presence of 3.3 ± 2.2, 1.2 ± 0.5, and 0.7 ± 0.3 neutrophils (mean ± SE) per high-power field in implants retrieved from mice 2 weeks after implantation with 4T1 cells in Matrigel, 4T1 cells and control MSCs in Matrigel, and 4T1 cells and IL-12 MSCs in Matrigel, respectively. There were no significant differences between the three different groups. Furthermore, lymphocytes were noted to be even scarcer than neutrophils.

However, an antiangiogenic effect was determined based on the following observations. Besides the smaller implant size and apparent lower number of tumor cells in implants from IL-12 MSC recipient mice, the striking feature of this group was the presence of “ghost” necrotic capillaries in the acellular central part of the implants (Fig. 3C). Quantitative analysis revealed counts of 13 ± 2.4 necrotic capillaries (mean ± SE) within the acellular central core of implants retrieved from recipient mice 2 weeks following implantation with 4T1 cells and IL-12 MSCs in Matrigel. In the other two groups, necrotic capillaries were very rarely seen (less than 2) because these implants, in contrast to those with IL-12 MSCs, were almost entirely populated by tumor cells.
In addition, between these necrotic capillaries, rare islets of tumor cells, also necrotic (ghost like), were present. This type of necrosis, coagulation necrosis, strongly suggests ischemia as a causal factor.

Assessing if effect of IL-12 MSCs on 4T1 tumor growth is local or systemic. To establish if the slowing of 4T1 tumor progression observed in mice that also received IL-12–secreting gene-modified MSCs is due to a local or systemic action of the IL-12, we conducted experiments where Matrigel-embedded MSCs were injected s.c. in the flank contralaterally to where 4T1 cells were injected the previous day. As revealed in Fig. 4, there was no slowing of tumor growth when IL-12 MSCs were not administered in the same flank and location as 4T1 cancer cells. Tumor growth in mice implanted with 4T1 cells alone or with contralateral injection of control MSCs or IL-12 MSCs in Matrigel was similar (P > 0.1, Student’s t test). At day 29 after implantation, tumor volumes in these groups of mice were 639 ± 125, 739 ± 236, and 894 ± 301 µL, respectively (Fig. 4).

Effect of IL-12 MSCs on 4T1 cancer growth in immunodeficient mice. Aiming to determine the importance of the immune response in the anticancer effect exerted by the IL-12 MSCs, we similarly tested these cells on 4T1 tumor cell growth in immunodeficient mice. We therefore implanted NOD-SCID mice with 4T1 cells and the next day with Matrigel-embedded gene-modified BALB/c MSCs at the same injection site. We noted that no beneficial effect arose from the IL-12 MSCs because 4T1 tumor growth was not significantly different than that in the other two groups of mice (P > 0.05, Student’s t test; Fig. 5).

Effect of IL-12 MSCs on 4T1 tumor growth when embedded in a human-compatible matrix. As the matrix material for embedding MSCs, we first used Matrigel, a mouse-compatible basement membrane derived from EHS mouse sarcoma. We then...
implantation (Fig. 6). One hundred percent of mice implanted with IL-12 MSCs were tumor-free at day 13 versus 0% of mice that received B16 cells alone or with next day control MSCs. Fifty-seven percent of IL-12 were tumor-free at day 13 versus 0% of mice that received B16 cells alone or with next day control MSCs.

Discussion

IL-12, produced mostly by antigen-presenting cells, is a multifunctional, heterodimeric Th1 cytokine shown to induce a cellular immune response, cancer cell apoptosis, as well as antiangiogenesis (31–33). Many studies have evaluated the anticancer effectiveness of IL-12 (34, 35). However, significant toxicity from recombinant human IL-12 administration has been noted in clinical trials in advanced cancers, limiting its clinical use (36–38). Therefore, sustained delivery of IL-12, avoiding toxic peaks seen with intermittent i.v. infusions, would be desirable. One means of achieving this and thus providing sustained and sufficient amounts of IL-12 at the tumor site is through a cell and gene therapy approach. In such a strategy tested in mice, investigators observed IL-12–mediated antiangiogenesis in SCID mice bearing human or murine tumors that were coinoculated with IL-12–secreting gene-modified 3T3 fibroblasts (39). In a phase I dose-escalation study, autologous human fibroblasts retrovirally transduced to secrete human IL-12 and injected in the peritumoral surroundings showed promise for the treatment of disseminated cancer (40). In another more recent study, autologous dendritic cells were transfected with an adenovirus encoding for human IL-12 and injected in the tumors of 17 patients with metastatic gastrointestinal tumors (41). These and other reports support the feasibility of a cell therapy platform for IL-12 delivery to overcome its dose-limiting toxicity.

Based on our previous work with MSCs (24, 25, 27), we here proposed using MSCs as part of a neo-organoid to obtain optimal amounts of IL-12 for cancer treatment. We assessed our IL-12 gene-enhanced cell therapy “neo-organoid” approach mainly against breast cancer cell growth.

We established that IL-12 gene-modified MSCs maintained their progenitor cell properties and that IL-12 did not seem to alter their phenotype, which is consistent with the absence of IL-12 receptor expression by MSCs. We then initially ascertained that IL-12 MSCs can engender an anticancer effect on their own without any matrix, as we noted a slowing of tumor progression in mice implanted with IL-12–secreting BALB/c MSCs mixed with 4T1 breast cancer cells compared with control mice (Fig. 2A).

All mice, however, in this experiment did develop tumors by day 36 (Fig. 2A). Hence, seeing as we had earlier published that the effect of a secreted transgene product can be augmented and prolonged by embedding the gene-modified MSCs in a matrix before their s.c. delivery (23, 24), we sought out to determine whether a greater and more persistent antitumor effect can be
realized with matrix-embedded IL-12 MSCs. This indeed did occur. The antitumor effect exerted by the IL-12 MSCs was very markedly superior and longer lasting when these cells were delivered mixed with the matrix material Matrigel (Fig. 2B). This finding is of great interest as it indicates that a neo-organoid containing IL-12 MSCs implanted in the vicinity of already present breast cancer cells can lead to a significant slowing of cancer growth and to increased survival with the possibility of complete tumor regression. We also showed that these IL-12–secreting neo-organoids had engendered increased systemic levels of mIL-12, as concentrations were over 10-fold higher than in the control mice (Fig. 2C, left). Voest and colleagues (33) have shown that IL-12 has antiangiogenic effects that are indirect and that can inhibit tumor growth, and proposed that this antiangiogenic action may be due to IL-12–induced IFN-γ. Consequently, due to the fact that IL-12 has been reported to lead to a significant slowing of cancer growth and to increased mouse survival also occurred when these IFN-γ MSCs were tested by intratumoral injection in B16F10 melanoma-bearing C57BL/6 mice and found to slow tumor growth and prolong survival (44). However, the authors suggested that the immune rejection of these xenogeneic MSCs impaired their effectiveness (44). Other than for IL-12 delivery, studies by us and others have revealed the preclinical efficacy of genetically engineered MSCs in cancer treatment. For instance, human MSCs retrovirally engineered to express rat IL-12 were tested by intratumoral injection in B16F10 melanoma-bearing C57BL/6 mice and found to slow tumor growth and prolong survival (44). However, the authors suggested that the immune rejection of these xenogeneic MSCs impaired their effectiveness (44). Other than for IL-12 delivery, studies by us and others have revealed the preclinical efficacy of genetically engineered MSCs in cancer treatment. For instance, human MSCs retroviroically transduced to express human IFN-β were noted to suppress, after i.v. administration, the growth of A375SM human melanoma in immunodeficient mice (45). These cells were found to have a tropism for the tumor site where local production of the IFN-β then led to the antitumor effect. A slowing of tumor growth and increase in mouse survival also occurred when these IFN-β MSCs were mixed with the melanoma cells and administered s.c. (45). No beneficial effects were observed when the IFN-β MSCs were administered distally (i.e., on the side contralateral to the tumor).
tumors; ref. 45), an observation we also make in our present study (Fig. 4). We here established that the antitumor effect seen with our IL-12 MSCs was due to local, and not systemic, IL-12 delivery because we did not observe this result when the IL-12 MSCs were implanted in the flank contralateral to the 4T1 breast cancer cells. Tumor progression was similar in all groups of mice (Fig. 4), therefore indicating the importance of the proximity of the IL-12 MSCs to the cancer cells for an antitumor effect to arise from the secreted IL-12. In a more recent investigation by Studeny and colleagues (46), IFN-β–gene-modified human MSCs were noted following i.v. administration to reduce the pulmonary metastasis from established human A375SM melanoma or MDA-231 breast cancer cells, an effect the authors suggest is due to tumor site production of IFN-β. In another investigation, these IFN-β–enhanced human MSCs tested in mice with human tumors were also found to lead to increased survival following intravascular delivery or via intratumoral injection (47). This antitumor effect again was only obtained through local delivery of the IFN-β MSCs. In these reports, such as in ours, a high concentration of the transgene at the tumor site plays an important role.

Other than IL-12 and IFN-β, research using rat MSCs genetically engineered to produce human IL-2 showed decreased tumor growth and significantly increased survival time following intratumoral injection in an established rat glioma model (48). We have previously reported that mIL-2–engineered mouse MSCs can generate an anticancer immune response engendering a significant slowing of B16 melanoma tumor growth in recipient mice following s.c. delivery (27). We have also found that MSCs can act as antigen-presenting cells and proposed that this feature can be exploited for augmenting an immune response against a tumor (49, 50).

In conclusion, our investigation shows the potential of using MSCs gene enhanced for delivery of therapeutically relevant levels of a plasma-soluble gene product with an antineoplastic effect, in this case IL-12, as part of a cell-based neo-organoid strategy for cancer therapy. The ease of implantation and removal makes this approach safe and clinically desirable.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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