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Bone marrow endothelium-targeted therapeutics for metastatic breast cancer

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Abstract

Effective treatment of cancer metastasis to the bone relies on bone marrow drug accumulation. The surface proteins in the bone marrow vascular endothelium provide docking sites for targeted drug delivery. We have developed a thioaptamer that specifically binds to E-selectin that is overexpressed in the vasculature of tumor and inflammatory tissues. In this study, we tested targeted delivery of therapeutic siRNA loaded in the E-selectin thioaptamer-conjugated multistage vector (ESTA-MSV) drug carrier to bone marrow for the treatment of breast cancer bone metastasis. We evaluated tumor type- and tumor growth stage-dependent targeting in mice bearing metastatic breast cancer in the bone, and carried out studies to identify factors that determine targeting efficiency. In a subsequent study, we delivered siRNA to knock down expression of the human STAT3 gene in murine xenograft models of human MDA-MB-231 breast tumor, and assessed therapeutic efficacy. Our studies revealed that the CD31⁺E-selectin⁺ population

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accounted for 20.8%, 26.4% and 29.9% of total endothelial cells respectively inside the femur of mice bearing early, middle and late stage metastatic MDA-MB-231 tumors. In comparison, the double positive cells remained at a basal level in mice with early stage MCF-7 tumors, and jumped to 23.9% and 28.2% when tumor growth progressed to middle and late stages. Accumulation of ESTA-MSV inside the bone marrow correlated with the E-selectin expression pattern. There was up to 5-fold enrichment of the targeted MSV in the bone marrow of mice bearing early or late stage MDA-MB-231 tumors and of mice with late stage, but not early stage, MCF-7 tumors. Targeted delivery of STAT3 siRNA in ESTA-MSV resulted in knockdown of STAT3 expression in 48.7% of cancer cells inside the bone marrow. Weekly systemic administration of ESTA-MSV/STAT3 siRNA significantly extended survival of mice with MDA-MB-231 bone metastasis. In conclusion, targeting the overexpressed E-selectin provides an effective approach for tissue-specific drug delivery to the bone marrow. Tumor growth in the bone can be effectively inhibited by blockage of the STAT3 signaling.

Keywords

breast cancer; bone metastasis; targeted delivery; silicon particle; multistage vector; E-selectin; thioaptamer; siRNA

1. Introduction

Bone is not only host to hematopoietic cancers but also a major organ for metastasis of multiple solid tumors, particularly breast and prostate cancers. In the case of late-stage breast cancer, over 60% of patients carrying estrogen receptor-positive cancer and about 10% of patients with triple negative breast cancer would eventually develop bone metastasis [1,2]. Although breast cancer bone metastasis is usually not the major cause of cancer death, symptoms associated with bone metastasis such as chronic bone pain, pathological fractures, life threatening hypercalcemia, and spinal cord compression pose a severe burden on the quality of life [3–5]. Moreover, there are currently no effective treatments of cancer metastasis to the bone by targeting cancer cells, and for most patients, palliative care is usually the only option. Although several bisphosphonates [6] and a neutralizing antibody targeting RANKL [7,8] have shown promising results on management of tumor progression, benefits on improved survival was only observed in a small population of patients [9,10].

A major challenge in the treatment of metastatic cancer is effective delivery of therapeutics to the tumor lesion. Histological analyses have revealed significant reduced microvessel density in their bone tumor metastases in half number of the breast cancer patients [11], which poses a huge disadvantage on delivering therapeutics to bone metastases than to the primary tumors. In addition, the bone marrow perivascular region not only provides metastatic niches for the cancer cells [12], but also shields the tumor cells from therapeutic agents in the circulation. Furthermore, the perivascular stromal cells and the endothelium support the tumor cells with chemo-attractants and pro-growth factors that facilitate homing of cancer cells. One of the key molecules in enhancing tumor cell homing and promoting tumor growth inside the bone marrow is E-selectin [13], a leukocyte adhesion molecule that is only expressed by the endothelial cells in the organ [14]. It interacts with its ligands on leukocytes at the blood vessel wall to modulate the rolling and subsequent adhesion of these

cells [13]. A recent study demonstrated that E-selectin is a crucial component of the vascular niche in promoting survival of hematopoietic stem cells after mice were treated with chemotherapy agents or radiation [14]. E-selectin was also demonstrated to be a promising target for tumor delivery of chemotherapy drugs [15,16].

We have previously developed a porous silicon-based multistage vector (MSV) delivery system [17]. Large payloads of therapeutic agents are packaged into liposomes or micelles and loaded into the nanopores of the porous silicon. Once delivered to the tumor site, the silicon carrier slowly degrades into a non-toxic orthosilicic acid and the drug payload gets sustainably released [18]. The system has been successfully applied to deliver siRNA oligos to primary breast cancer [18] and metastatic ovarian cancers [19,20]. We have also developed a thioaptamer (ESTA) that specifically binds to E-selectin [21]. In the current study, we tested the feasibility of delivering therapeutic siRNA by affinity targeting to E-selectin for the treatment of breast cancer bone metastasis in murine xenograft models of human cancers. We evaluated bone marrow accumulation of ESTA-conjugate MSV (ESTA-MSV) in mice bearing bone metastasis from MDA-MB-231 and MCF-7 tumors. We also explored the underlying mechanism of differential accumulation efficiency between the two tumor lines. Finally, we carried out an efficacy study to demonstrate tumor growth inhibition as a result of effective knockdown of STAT3 expression in metastatic MDA-MB-231 cells.

2. Materials and Methods

2.1 Cell culture

The MCF-7 human breast cancer cell line was purchased from American Type Culture Collection, and the MDA-MB-231-luc-D3H2LN (MDA-MB-231) breast cancer cell line engineered with luciferase expression was from Caliper Life Sciences. MDA-MB-231 cells were cultured in high-glucose Dulbecco's modified Eagle's minimal essential medium with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, 100 µg/mL of streptomycin (complete DMEM) and 0.05 mg/mL zeocin. MCF-7 cells were cultured in complete DMEM. Human microvascular endothelial cells (HMVEC) were from Life Technologies, and were maintained in Medium 131 supplied with 5% microvascular growth supplement (MVGS, Life Technologies). Cells were incubated at 37°C with 5% CO₂.

2.2 Fabrication of porous silicon multistage vector particles

Discoidal porous silicon microparticles were fabricated by electrochemical etching of silicon wafer and surface modified with 3-aminopropyltriethoxysilane (APTES) as previously described [22]. E-selectin thioaptamer was chemically conjugated to the APTES using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as a polylinker. Morphologies of the MSV and ESTA-MSV particles were observed with scan electronic microscope (SEM). To determine grafting density of ESTA on MSV particles, 3.75 billion ESTA-MSV particles were dissolved in 3 mL 1 N NaOH solution overnight. Phosphorus concentration was detected with a Varian 720-ES inductively coupled plasma optical emission spectrometer (ICP, Varian, USA). Yttrium was used as internal control.

To evaluate ESTA stability, the aptamer (4 µg) was incubated in 400 µL murine plasma at 37°C with moderate shaking (500 rpm). Aliquots of samples (20 µL) were collected at

different time points. They were mixed with 1% sodium dodecyl sulfate (SDS), and incubated at 95°C for 5 minutes to eliminate aptamer-protein interaction. The samples were separated by agarose gel (2%) electrophoresis in 1×TAE buffer containing 1×GelRed dye (Biotium, USA) with a current of 100 V. After electrophoresis, the amount of aptamer was visualized under the UV light.

2.3 Preparation of siRNA polyplexes and loading of siRNA into MSV

The polyplexes were prepared with various ratios between nitrogen in cationic polymer and phosphorus in siRNA oligo (N/P ratio). siRNA oligos were first mixed with PEG(5k)2-PEI(10k) (PEG-PEI), and then incubated at 20°C for 15 minutes to form the polyplexes. Hydrodynamic diameter and zeta potential of the PEG-PEI/siRNA polyplexes were characterized with a Zetasizer Nano ZS (Malvern Instruments, UK) in 20 mM phosphate buffer (pH 7.4) at 25°C with a laser wavelength of 633 nm and a scattering angle of 90 degree. siRNA packaging capacity of PEG-PEI was measured by agarose gel (2%) electrophoresis in 1×TAE buffer containing 1×SYBRsafe (Invitrogen, USA).

To prepare siRNA nanoparticles, scramble (Scr) siRNA (Sigma), STAT3 siRNA (Sigma), or Alexa555-siRNA (Qiagen) was mixed with PEG-PEI polymer at N/P ratio of 15:1. The PEG-PEI/siRNA polyplexes were then loaded into the ESTA-MSV particles by sonication in a water bath for 3 minutes. ESTA-MSV particles were then spun down at 12,000 rpm for 5 minutes, and the supernatant containing excess PEG-PEI/siRNA was removed. PEG-PEI/Alexa555-siRNA loaded ESTA-MSV was prepared to calculate polyplexes loading capacity, loading efficiency and release pattern. Loading capacity was determined by the difference of fluorescent intensity before and after loading. To measure siRNA release from ESTA-MSV, Alexa555-siRNA loaded ESTA-MSV was incubated in FBS at 37°C under moderate shaking (500 rpm). Aliquots of samples were taken at different time points, silicon particles were spun down by centrifugation, and fluorescent intensity in the supernatant was measured with a Bio Tek microplate reader at excitation/emission wavelengths 550/570 nm.

2.4 Murine models of human breast cancer bone metastasis

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of The Methodist Hospital Research Institute in Houston, Texas. Female athymic nude mice (6 weeks old) were purchased from the Charles River Laboratories. They were housed in a pathogen-free facility under a 24-hour light-dark cycle, and fed with a pathogen-free diet and water *ad libitum*. To establish murine models of bone metastasis, nude mice were inoculated with 1×10^5 MDA-MB-231 or 5×10^5 MCF-7 cells by intracardiac inoculation. Tumor growth was monitored with a Xenogen IVIS 200 imaging system.

2.5 Histological analysis

Mice with tumor bone metastasis were anesthetized and euthanized at different time points. Femur and spine samples were collected, fixed in 10% formalin, decalcified with 14% ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin. Four-micrometer sections were processed with haematoxylin and eosin (H&E) staining for morphology observation, or with rabbit anti-mouse E-selectin antibody (1:100 diluted, Abcam) or rat

anti-mouse CD31 antibody (1:100 diluted, BD Biosciences) for immunofluorescence. Images were captured with a Nikon Eclipse 80i microscope.

2.6 E-selectin expression analysis

To determine E-selectin-positive endothelial cells in the bone marrow, femur and spine tissues were cut into small pieces, digested with 200 units/mL collagenase type III at 37°C for 2 hours, and single cells were isolated by centrifugation. Cells were stained with anti-mouse CD31 and E-selectin antibodies (both 1:50 diluted in PBS containing 2% FBS) for 30 minutes on ice. Samples were separated with a BD LSR Fortessa analyzer and analyzed with the Flowjo software (Tree Star, USA).

To measure E-selectin overexpression in HMVEC, cells were either treated with the positive control TNF- α (25 ng/mL) or with 50% conditioned medium from MDA-MB-231 or MCF-7 cell culture for 8 hours. They were then stained with 1:10 diluted mouse anti-human E-selectin antibody (BD Biosciences), and analyzed with a BD LSR Fortessa analyzer. To examine intra-cellular E-selectin recycle and receptor mediated endocytosis, HMVEC cells were cultured in 50% MDA-MB-231 conditioned medium for 5 hours, and untargeted MSV or ESTA-MSV particles were then added to the cell culture at a ratio of 1/100 (cell/particle). Cells were harvested at different time points, and the amount of cell surface E-selectin was measured by flow cytometry. To identify factors that were involved in activation of E-selectin expression, HMVEC cells were treated with TNF- α or 50% conditioned medium from MDA-MB-231 or MCF-7 cell culture for 30 minutes. Cells were then immediately washed with cold PBS, and lysed with cold lysis buffer with protease and phosphatase inhibitors (Pierce). Western blot analysis was then applied to detect phosphorylation of the NF- κ B p65 subunit (p65), stress-activated protein kinase/Jun-amino-terminal kinase (JNK), and p38 MAP kinase (p38) with respective antibodies. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the positive control. All antibodies for Western blot analysis were from Cell Signaling Technology.

2.7 Knockdown of STAT3 expression *in vitro* and *in vivo*

To test knockdown of STAT3 expression in cell culture, MDA-MB-231 cells were seeded in 6-well plate at 1×10^5 cells/well. ESTA-MSV/Scr or ESTA-MSV/STAT3 particles were added into the culture 24 hours later. Cells were harvested at the indicated time points, and STAT3 expression was measured by Western blot analysis using the rabbit anti-human STAT3 α antibody (1:1,000, Cell Signaling Technologies).

To measure mammosphere formation efficiency, MDA-MB-231 cells were seeded in a 96-well ultralow attachment plate (Corning) at a seeding density of 1,000 cells/well and cultured in 0.1 mL complete human MammoCult medium (STEMCELL Technologies). ESTA-MSV/Scr or ESTA-MSV/STAT3 particles were added into the cell culture (5 wells per group). Number of mammospheres was counted under microscope 7 days later.

To examine knockdown of STAT3 expression in metastatic tumor nodules inside the bone marrow, mice with MDA-MB-231 tumors were divided into 4 groups (3 mice/group), and treated with MSV/Scr (20 μ g siRNA), MSV/STAT3 (20 μ g siRNA), ESTA-MSV/Scr (20 μ g siRNA), or ESTA-MSV/STAT3 (20 μ g siRNA). They were sacrificed 3 days later, and cells

were isolated from the femur and spine. They were then stained with mouse anti-HLA-A,B,C antibody (1:10, BD Biosciences) and mouse anti-human STAT3 antibody (1:10, BD Biosciences) followed by flow cytometry.

2.8 Tissue biodistribution of MSV and ESTA-MSV

Mice bearing early stage MDA-MB-231 bone metastasis were used to measure tissue biodistribution of MSV and ESTA-MSV. Each mouse was administrated with 5 billion particles by *i.v.* injection (3 mice per group). Mice were sacrificed 4 hours later, and major organs (heart, liver, spleen, lung, kidney, femur, thyroid) and blood samples were collected. Silicon content in each sample was measure by ICP [23]. Briefly, tissue samples were weighted and homogenized in 20% ethanol containing 1 N sodium hydroxide. They were kept in a shaker at 20°C for 48 hours. Samples were spun down at 4,200 rpm for 25 min, and 0.5 mL supernatant was collected from each sample, mixed with 2.5 mL de-ionized water, and used to measure silicon content by ICP. To measure silicon content in the femur, samples were first decalcified in 10% hydrochloride prior to the homogenization and digestion procedure.

2.9 Evaluation of therapeutic efficacy

Mice bearing MDA-MB-231 tumor in the bone were randomly divided into 3 groups (8 – 9 mice/group) 7 days after tumor inoculation, and treated weekly with 1) PBS, 2) ESTA-MSV/Scr (20 µg siRNA), or 3) ESTA-MSV/STAT3 (20 µg siRNA) by tail vein injection. The animals were sacrificed at signs of paralysis or low body condition score.

2.10 Statistical analysis

For statistical comparisons, a Student's *t* test was performed (two-tailed distribution, two-sample equal variance) except for the efficacy evaluation. A value of $P < 0.05$ was considered statistically significant. For the therapeutic efficacy study, significance was calculated with the Gehan-Breslow-Wilcoxon test. Data were presented as mean $\pm$ SD.

3. Result

3.1 Characterization of MSV and ESTA-MSV particles

The discoidal porous silicon microparticles were fabricated by electrochemical etching of silicon wafer, and surface modified with 3-aminopropyltriethoxysilane (APTES). They were 1 µm in diameter, and 400 nm in height. The particles were about 80% in porosity with nanopores ranging from 45 to 80 nm. Surface chemical modification with APTES and conjugation with the ESTA targeting moiety did not significantly change particle size (Fig. 1A). The thioaptamer was stable in murine plasma for up to 7 hours, and gradually degraded in 48 hours (Supplementary Fig. 1A). ICP was applied to measure grafting density of ESTA on MSV particles. Since the phosphorus element comes exclusively from the 73-mer aptamer, the amount of phosphorus mass reflects the grafting efficiency of the aptamer. There were on average 1.68×10^5 ESTA molecules per MSV particle in ESTA-MSV.

3.2 Formation of PEG-PEI/siRNA polyplexes and MSV loading

siRNA packaging in PEG-PEI polymer, in function of N/P ratio, was investigated. siRNA oligos could be fully incorporated into positively charged nano-polyplexes with 30–40 nm in diameter when the N/P ratio was above 5 (Fig. 1B). Agarose gel electrophoresis was applied to study siRNA-PEG-PEI binding capacity. Strong electrostatic interaction between siRNA and PEG-PEI prohibited separation of siRNA from the complex when N/P ratio was above 4.0 (Fig. 1C). N/P ratio of 15 was chosen for all follow-up studies.

Loading of siRNA polyplexes into ESTA-MSV was measured by difference in fluorescent intensity in solution. After sonication, 64% of the total feeding siRNA polyplexes were encapsulated, and the calculated loading content was 34.8 µg siRNA per 1 billion ESTA-MSV particles (Fig. 1D). Fluorescent microscopic analysis revealed strong red fluorescent signal from the PEG-PEI/Alexa555-siRNA loaded MSV particles (Fig. 1E). *In vitro* siRNA release was performed in FBS at 37°C. Within the first hour, 12.9% of total siRNA polyplexes got released. These siRNA polyplexes most likely were either on the surface of MSV or in the shallow area of the nanopores. Sustained release was achieved after the initial burst release, and up to 70% of the total siRNA polyplexes was released in the next 7 days (Fig. 1F).

3.3 Murine models of human breast cancer bone metastasis

Murine models of human breast cancer bone metastasis were generated by intracardiac inoculation of human MDA-MB-231 or MCF-7 cells into *nu/nu* athymic nude mice. Since the tumor cells were engineered with luciferase expression, tumor growth *in vivo* was monitored by bioluminescent intensity. Major tumor nodules could be identified both in the femur and the spine (Fig. 2A). Tumor growth was arbitrarily divided into early, middle, and late stages based on bioluminescence. Histological analysis confirmed tumor growth in the bone at different stages, as indicated by localized small tumor nodules by day 20, moderate size nodules by day 30, and massive tumor growth by day 40 (Fig. 2B and 2C). While MDA-MB-231 tumors could be found across the whole area of femur, MCF-7 tumors were limited to the epiphysis region (data not shown). Most tumor-bearing mice developed paralysis or severe body weight loss within 30 – 45 days, and had to be euthanized. Bone fractures caused by tumor invasion and osteoclastic bone resorption could be identified in late-stage tumor samples (Fig. 2C).

3.4 Targeted delivery of ESTA-conjugated MSV to bone marrow

To test bone marrow accumulation of E-selectin-targeted MSV particles, we conjugated MSV with the free Cy5 dye or Cy5-labeled ESTA, and performed intravenous injection (*i.v.*) into mice bearing bone metastatic MDA-MB-231 tumors. Mice were sacrificed 4 hours later, and the femur and spine were collected and processed for histological analysis. In mice dosed with Cy5-MSV, the red fluorescent particles inside the bone marrow were sparse, and did not colocalize with the E-selectin-positive endothelial cells that were stained in green with an anti-E-selectin antibody (Fig. 3A). In comparison, more particles could be found inside the bone marrow when mice were treated with the ESTA-MSV particles, and most of the red fluorescent particles co-localized with the E-selectin-positive endothelial cells (Fig. 3B), indicating effective targeted delivery of the thioaptamer-conjugated particles. The

majority of the ESTA-MSV particles had exited bone marrow sinusoidal vessels and entered the perivascular region following affinity binding to E-selectin inside the medullary cavity (Fig. 3C). This distribution pattern was in contrast to particles outside the bone marrow. All ESTA-MSV particles in the periosteum were inside the blood vessel, as indicated by their colocalization with the endothelium.

3.5 Tumor stage-dependent enrichment of ESTA-conjugated MSV to bone marrow

As an initial step to develop a targeted delivery strategy, we systematically investigated bone marrow accumulation of targeted and untargeted MSV particles in mice bearing MDA-MB-231 or MCF-7 metastatic tumors in the bone at different tumor growth stages. The femur and spine tissues were then processed, and the tissue blocks were stained with DAPI to identify the bone marrow cells in a dark field under the fluorescent microscope. The number of red fluorescent MSV particles in bone marrow was counted in each microscopic field. A total of 20 views per slide were randomly selected, and the average number of particles per view was calculated. In mice bearing MDA-MB-231 tumors, an average of 4-fold increase of ESTA-MSV in the femur and up to 6-fold increase in the spine were observed compared to the untargeted MSV (Fig. 4A). This distribution pattern was largely independent of tumor growth stage. Silicon content analysis confirmed enrichment of ESTA-MSV in the bone in mice bearing early stage MDA-MB-231 metastatic tumors (Supplementary Fig. 2). Although most particles still got trapped in the filtering organs including the liver, lung, and spleen, there was a 4-fold increase of silicon particles inside the femur in mice treated with ESTA-MSV comparing to those dosed with untargeted MSV. In mice bearing MCF-7 tumors, however, the benefit of ESTA-MSV accumulation was only observed in the middle and late stage tumors (Fig. 4B). There was no significant enrichment of ESTA-MSV in femur or spine in mice bearing early stage metastatic MCF-7 tumors compared to the untargeted MSV.

To understand the disparity of particle distribution between mice with MDA-MB-231 and MCF-7 tumors, we isolated cells from bone marrow in the femur of tumor mice, and examined E-selectin expression by flow cytometry (Fig. 4C). As expected, E-selectin expression was restricted to endothelial cells with CD31 expression. About 5% of CD31⁺ cells in a tumor-free mouse were also E-selectin⁺ indicating low E-selectin co-expression in these cells (data not shown). The CD31⁺ E-selectin⁺ cells increased to 20–30% in mice bearing early stage, middle stage and late stage MDA-MB-231 tumors. In comparison, this population of cells was at the basal level in mice with early stage MCF-7 tumors, but increased to over 20% in mice with middle and late stage tumors (Fig. 4C). This result correlates with the tumor stage-dependent ESTA-MSV accumulation data in mice with MCF-7 tumors (Fig. 4B).

Immunohistochemical (IHC) staining confirmed tumor stage-dependent E-selectin expression in mice bearing metastatic MCF-7 tumors. In the femur from a mouse carrying early stage tumors, high level of E-selectin expression was limited to the tissue surrounding the tumor nodule (Fig. 4D). In comparison, high E-selectin expression could be observed across the whole bone marrow in a femur with late-stage MCF-7 tumors (Fig. 4E). These results indicate that certain potent stimulators secreted by the tumor cells might be

responsible for tumor cell type-dependent and tumor growth stage-dependent E-selectin expression. Expression levels of such factors should vary significantly between MDA-MB-231 and MCF-7 cells, so that it takes only a limited number of MDA-MB-231 tumor cells inside the bone marrow to spread the effect across the whole femur, while a critical mass of MCF-7 cells is needed to achieve a similar result.

3.6 Modulation of E-selectin expression

We collected conditioned media from MDA-MB-231 and MCF-7 cell culture and used them to treat HMVEC cells. Tumor necrosis factor- α (TNF- α) served as the positive control, as previous studies have shown that E-selectin expression could be stimulated by this cytokine [24]. Flow cytometry was applied to analyze E-selectin-positive cells. As expected, the positive control TNF- α triggered robust E-selectin expression. The growth medium from MDA-MB-231 cells, but not from MCF-7 cells, also stimulated E-selectin expression (Fig. 5A). This result confirms that the MDA-MB-231 growth medium contains stimulation factor(s) responsible for E-selectin expression.

It has been reported that TNF- α stimulates E-selectin expression by activating the NF- κ B pathway, the SAPK1/JNK pathway, and the SAPK2/p38MAP kinase pathway [25]. We carried out Western blot analysis to check the phosphorylation status of the key factors in these pathways (Fig. 5B). As with the TNF- α positive control, treatment with MDA-MB-231 growth medium triggered phosphorylation of the NF- κ B p65 subunit, JNK, and p38 MAP kinase. On the other hand, the MCF-7 growth medium did not stimulate phosphorylation of any of these factors.

3.7 Targeted delivery of gene-silencing agent to bone marrow

Although targeted delivery improved accumulation of MSV particles inside the bone marrow in tumor-bearing mice, careful analysis of tissue histology revealed uneven distribution of these particles between tumor nodules and the adjacent bone marrow. Clear boundaries between tumor nodule and bone marrow could be identified by the unique dense structure of the tumor by H&E staining and the dramatically reduced DAPI staining of tumor cells under the fluorescent microscope (Fig. 4D–4E and 6A). In general, more E-selectin-positive cells could be identified in the area adjacent to tumor nodules and in the bone marrow than inside the tumor (Fig. 4D–4E), indicating hypovascularity inside the tumor nodules. Consequently, more depots of ESTA-MSV particles could be found on the boundary between bone marrow and the tumor nodule (Fig. 6A and 6B), and in the region close to the endosteum than inside the tumor (Fig. 6A).

In order to determine whether ESTA-MSV could be used to effectively deliver therapeutics to tumor tissues, we packaged Alexa555-labeled siRNA oligos into Cy5-ESTA-MSV and performed *i.v.* injection into tumor-bearing mice. Histological analysis of the femur tissues collected 8 hours after particle injection confirmed MSV deposition in the perivascular space of the tumor nodule. There were more particles at the tumor/bone marrow boundary than inside the cluster of tumor cells (Supplementary Fig. 3). Most cells were Alexa555 positive in the same region with MSV deposit (Supplementary Fig. 4), and pockets of intense fluorescence could be detected inside the tumor nodule (Fig. 6C). Release of siRNA was time dependent.

The Alexa555-siRNA was exclusively inside the Cy5-ESTA-MSV one hour after injection, and some of them had escaped the silicon particles and penetrated to the nearby cells by 4 hour. By 8 hours, most cells in the region with MSV deposit had received siRNA (Supplementary Fig. 4). These observations indicate effective penetration of the released fluorescent siRNA into the tumor tissue.

Since E-selectin played a pivotal role in targeted MSV delivery, we carried out a cell-based assay to monitor turnover of the cell surface protein after co-incubation with the untargeted MSV or ESTA-MSV. A drop of cell surface E-selectin was detected 30 minutes after treatment with ESTA-MSV, but not with the untargeted MSV. However, the protein level came back within the next 30 minutes (Supplementary Fig. 5). This result indicates that the cell surface E-selectin will not be depleted during the process of receptor-mediated endocytosis, and that affinity binding between ESTA and E-selectin will not be affected during a repetitive treatment schedule in an *in vivo* study setting.

3.8 Therapeutic efficacy of STAT3 siRNA on breast cancer bone metastasis

We treated mice bearing bone metastatic MDA-MB-231 tumors with ESTA-MSV-delivered siRNA targeting the human STAT3 gene. We selected STAT3 as the target for siRNA-mediated therapy, since the JAK2/STAT3 pathway played an important role on growth of breast cancer stem cells, and inhibition of this pathway resulted in decrease in the number of cancer stem cells and subsequently the growth of primary breast cancer in murine tumor models [26]. In addition, STAT3 is a pivotal factor in tumor associated angiogenesis [27], as well as osteoclastogenesis [28] which plays an essential role in tumor cell metastasis to bone [29]. The siRNA was packaged into a PEG-PEI nanoparticle, and loaded into the nanopores of ESTA-MSV. Treatment of MDA-MB-231 cells with ESTA-MSV/STAT3 siRNA resulted in knockdown of STAT3 expression in the next five days (Fig. 7A) and a significant reduction in mammosphere formation efficiency (Fig. 7B), a parameter commonly applied to test mammary stem/progenitor cells [30]. The extent of reduction was comparable to a previous report on mammosphere formation efficiency in SUM159 cells with stable knockdown of the STAT3 gene [31].

To examine knockdown efficiency *in vivo*, we treated mice bearing middle stage metastatic MDA-MB-231 tumors in the bone with MSV/Scr siRNA, MSV/STAT3 siRNA, ESTA-MSV/Scr siRNA, or ESTA-MSV/STAT3 siRNA. Mice were sacrificed three days after treatment, and bone marrow cells and tumor cells from the femur were isolated by mild tissue digestion. Flow cytometry was carried out to identify human MDA-MB-231 cells that were stained positive with the human leukocyte antigens (HLA)-ABC antibody (Fig. 7C). STAT3 expression in the tumor cells was compared in the different treatment groups. Treatment with the control scramble siRNA (MSV/Scr and ESTA-MSV/Scr) had no effect on STAT3 expression in tumor cells. Only 10% of tumor cells showed knockdown of STAT3 expression in mice treated with the untargeted MSV/STAT3 siRNA. ESTA-MSV/STAT3 treatment, however, resulted in knockdown of STAT3 expression in about half of the human tumor cells (Fig. 7C).

In a follow-up study, we treated mice bearing MDA-MB-231 bone metastasis with weekly treatments of ESTA-MSV/STAT3 and monitored disease progression. In the control groups,

mice were treated with either PBS or ESTA-MSV/scramble siRNA. Mice in the control groups developed signs of late-stage bone metastasis such as paralysis of the limbs or sudden loss of weight beginning at day 30, and half of the mice succumbed to tumor growth in the bone by day 36 (Fig. 7D). In comparison, ESTA-MSV/STAT3 siRNA treatment extended life by 9 days over the ESTA-MSV/Scramble siRNA or 11 days over the PBS control based on 50% survival time. A statistical significance on the survival benefit from the ESTA-MSV/STAT3 siRNA treatment (25% extension of life) was achieved (Fig. 7D).

4 Discussion

Breast cancer bone metastasis has traditionally been treated with radiation to slow tumor growth and palliative care to manage bone loss-related symptoms. However, mounting evidences have demonstrated that ionizing radiation induces reprogramming of cancer cells [32,33]. The surviving cancer cells are enriched with cancer stem cells that are more resistant to treatment. Although the palliative treatment drugs delay cancer progression by inhibiting osteolysis in metastatic breast cancer, they have little impact on the lethal seeds of the disease, i.e., tumor cells. As a result, the growth of cancer cells reshape the local microenvironment, and the stromal cells produce the parathyroid hormone-related protein, transforming growth factor- β , and other cytokines/chemokines to support cancer cell growth, contributing to a vicious cycle for tumor growth and bone destruction [34–36]. As long as the tumor cells stay intact inside the organ, local bone loss will continue until the patient succumbs to the disease.

We have developed a platform to deliver a large quantity of therapeutics to the bone marrow. Such therapeutics could be the traditional chemotherapy drugs for killing of cancer cells, gene-silencing agents for suppression of gene expression in key signal transduction pathways for cancer cell survival, or even a radiation sensitizer to facilitate radiation therapy. In the current study, we have demonstrated enrichment of E-selectin-targeted delivery of MSV in the bone marrow (Fig. 3 and 4), and effective knockdown of gene expression by the released STAT3 siRNA in metastatic MDA-MB-231 tumor cells (Fig. 7C). To the best of our knowledge, this represents the first study on successful delivery of large payloads of a therapeutic siRNA to the bone marrow for the treatment of metastatic breast cancer. Interestingly, the JAK2/STAT3 signaling is preferentially activated in breast cancer stem cells comparing to the bulk of non-cancer stem cells in a tumor tissue by IL-6 [26], a cytokine overexpressed in the MDA-MB-231 tumor cells [37]. Knockdown of STAT3 expression in these cells could potentially block the activity of the downstream factors essential for cancer stem cell survival. Our mammosphere formation assay on cancer stem cell activity and *in vivo* efficacy study on animal survival (Fig. 7B, 7D) have demonstrated the essential role of STAT3 on MDA-MB-231 tumor growth.

Except for the direct cancer stem cell inhibition effect, the ESTA-MSV particles were also anticipated to deliver STAT3 oligos to stromal cell, and indirectly inhibited tumor growth. JAK2/STAT3 pathway acts as a critical pathway on tumor associated angiogenesis and tumor cell bone metastasis related osteoclastogenesis [27–28]. Although the knockdown of STAT3 expression could not eliminate the cancer stem cell activity completely in our cell based experiment (Fig. 7B), the anti-STAT3 treatment indeed gave promising improvement

on the survival of tumor bearing mice (Fig. 7D). It indicated that our strategy might provide further effects on the inhibition of tumor angiogenesis or osteoclastogenesis induced bone metastasis, which was proved by other studies [38,39].

E-selectin is differentially expressed inside the bone marrow vasculature. Its expression level is 16-times as high in the microvessels close to the endosteal region as in the central sinusoid vessels [14]. Coincidentally, we have observed a tendency of ESTA-MSV accumulation next to the endosteum (Fig. 6A). Since this region has been implicated as an important niche for normal and malignant hematopoietic stem cells [40], targeted delivery of therapeutics should be an effective approach to eliminate the roots of tumor growth. Radiation treatment can also trigger a dramatic increase in E-selectin expression in the endosteal region [14], which might facilitate E-selectin targeted delivery in patients undergoing radiation therapy. Since the ESTA-MSV particles can exit the vasculature and enter the perivascular region efficiently (Fig. 3), this approach might also be applicable to delivery of agents aimed for modulation of the tumor microenvironment, an important area for development of cancer therapeutics [36,41].

Bone is home to leukemia and multiple myeloma. Besides metastatic breast cancer, bone is also a major organ for metastasis of several other solid cancer types including prostate, lung, kidney, and thyroid [42]. In addition, recent studies have also revealed the supporting roles of bone marrow-derived progenitor cells on tumor metastasize to other major organs such as the lungs [43,44]. Furthermore, it has been speculated that cancer cells might not home to the remote organs for future metastasis immediately after they leave the primary tumor. Instead, they migrate to the bone marrow where they go through a selection and enrichment process by acquiring more mutations to increase their metastatic potential [45]. Taken together, effective management of tumor growth in the bone and modulation of the bone marrow microenvironment will have a huge impact on prevention and treatment of multiple cancers. Our targeted delivery system can serve as a crucial technology platform in the development of cancer therapy.

In summary, expression of the endothelial surface protein E-selectin is enhanced by tumor cell-secreted cytokines. Bone marrow enrichment of therapeutics can be achieved by affinity targeting of the overexpressed E-selectin. We have demonstrated knockdown of expression and therapeutic efficacy on breast cancer bone metastasis by targeted delivery of STAT3 siRNA. This strategy can be applied to treat not only bone metastasis of breast cancer and other solid cancers, but also cancers of the hematopoietic origin such as leukemia and multiple myeloma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of ESTA-MSV loaded with PEG-PEI/siRNA polyplexes

A. SEM images of APTES-MSV (left panel) and ESTA-conjugated MSV (right panel). The size of silicon particles was 1 μm in diameter and 400 nm in height. Scale bar: 500 nm. B. Characterization of PEG-PEI/siRNA polyplexes. Zeta potential and hydrodynamic size changed with increased N/P ratio. C. Agarose gel electrophoresis to separate siRNA from polyplexes. N/P ratios for each sample were provided above each lane. D. Loading capacity and loading efficiency of PEG-PEI/siRNA polyplexes into ESTA-MSV. E. PEG-PEI/Alexa555-siRNA loaded ESTA-MSV was visualized under a Nikon Eclipse 80i fluorescent microscope. Scale bar: 5 μm . F. Time-dependent siRNA release from ESTA-MSV in FBS at 37°C (n = 3).

Figure 2. Murine models of human breast cancer bone metastasis

A. Growth of MDA-MB-231 and MCF-7 bone metastatic human breast cancers was monitored by bioluminescence at 20, 30, and 40 days after tumor inoculation. Tumor growth was arbitrarily divided into early, middle, and late stages based on bioluminescent intensity. B. Histological analysis of MDA-MB-231 tumor growth inside the femur and spine. The dense tumor nodules were stained lighter than the bone marrow cells by H&E staining, and the tumor-bone marrow boundary can be distinguished based on cell intensity and H&E staining. Black arrows point to the tumor nodules. C. Histological analysis of MCF-7 tumor growth inside the femur and spine. Bone fracture inside the spine in the late-stage tumor is pointed with a red arrow. Black arrows point to the tumor nodules.

Figure 3. Accumulation of MSV particles inside the bone marrow

A. Histological analysis on bone marrow distribution of free MSV particles. Left panel: H&E staining of bone marrow. Right panel: E-selectin staining (in green) for E-selectin expression inside the bone marrow. Nuclei were stained in blue with 4', 6'-diamidino-2-phenylindole (DAPI), and Cy5-labeled MSV particles were in red, and pointed with arrows. B. Histological analysis on bone marrow distribution of ESTA-MSV particles. Left panel: H&E staining of bone marrow. Right panel: E-selectin staining for E-selectin expression inside the bone marrow. Nuclei were stained in blue by DAPI, and Cy5-labeled ESTA-MSV particles were in red, and pointed with arrows. C. Fluorescent microscopic analysis on ESTA-MSV particle distribution inside and outside the bone marrow. Endothelial cells were stained in green, and nuclei were stained in blue. The Cy5-ESTA-MSV particles were in red.

Figure 4. Bone marrow-targeted delivery of ESTA-MSV in mice bearing metastatic breast cancer

Murine models of MDA-MB-231 and MCF-7 human breast cancer bone metastasis were dosed with Cy5-MSV or Cy5-ESTA-MSV. Mice were sacrificed 20, 30, or 40 days later, and tissue blocks were processed for particle distribution analysis. A. Accumulation of Cy5-MSV or Cy5-ESTA-MSV particles inside the bone marrow of mice bearing metastatic MDA-MB-231 tumor. The number of red fluorescent particles was counted under a fluorescent microscope, and number of MSV particles per view was the average of red fluorescent dots in 20 microscopic views ($n = 3$ mice per group per time point). B. Accumulation of Cy5-ESTA-MSV particles inside the bone marrow of mice bearing MCF-7 tumor ($n = 3$ mice per group per time point). C. Flow cytometry analysis of the CD31⁺E-selectin⁺ subpopulation in cells isolated from bone marrow bearing MDA-MB-231 (left panels) or MCF-7 tumors (right panels) ($n = 3$ mice per group). D. E-selectin staining of a tissue block from the bone marrow of a mouse with early stage MCF-7 metastatic tumors. Small tumor nodules can be visualized by H&E staining, and pointed with arrows. E. E-selectin staining of a tissue block from the bone marrow of a mouse with late stage MCF-7 metastatic tumors. Two large tumor nodules can be visualized by H&E staining, and are pointed with arrows. * indicates $P < 0.05$, and ** indicates $P < 0.01$.

Figure 5. Analysis on stimulation of E-selectin overexpression by tumor cell culture media
A. Flow cytometry analysis on E-selectin expression in HMVEC cells treated for 8 hours with 25 ng/mL TNF- α , 50% MDA-MB-231 growth medium, or 50% MCF-7 growth medium. B. Western blot analysis on activation of key pathways that regulate E-selectin expression.

Figure 6. Differential distribution pattern of ESTA-MSV in tumor and adjacent bone marrow
 A. Fluorescent microscopic analysis of Cy5-ESTA-MSV inside the femur with metastatic MDA-MB-231 tumor. Nuclei were in blue, and Cy5-ESTA-MSV particles were in red. Particles close to the endosteum are pointed with arrows. B. Fluorescent microscopic analysis of Cy5-ESTA-MSV inside the spine with metastatic MCF-7 tumor. C. Penetration of Alexa555-siRNA into tumor nodules inside the bone marrow. Regions of high fluorescent intensity inside the tumor can be visualized.

Figure 7. Targeted delivery of STAT3 siRNA and efficacy evaluation

A. Western blot analysis on knockdown of STAT3 expression. B. Evaluation of mammosphere formation efficiency (MSFE) in cells treated with MSV/siRNA (5 wells per group). C. Flow cytometry analysis on STAT3 expression in MDA-MB-231 human breast cancer cells isolated from the bone marrow of tumor mice treated with MSV/siRNA or ESTA-MSV/siRNA (3 mice per treatment arm). D. Kaplan-Meier plot on animal survival in mice bearing MDA-MB-231 metastatic tumors treated with ESTA-MSV/siRNA (8–9 mice per treatment arm).