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3D Porous Chitosan–Alginate Scaffolds as an *In Vitro* Model for Evaluating Nanoparticle–Mediated Tumor Targeting and Gene Delivery to Prostate Cancer

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Abstract

The cationic nanoparticles (NPs) for targeted gene delivery is conventionally evaluated using 2D *in vitro* cultures. However, this does not translate well to corresponding *in vivo* studies because of the marked difference in NP behavior in the presence of the tumor microenvironment. In this study, we investigated if prostate cancer (PCa) cells cultured in three-dimensional (3D) chitosan-alginate (CA) porous scaffolds could model cationic nanoparticle-mediated gene targeted delivery to tumors *in vitro*. We assessed *in vitro* tumor cell proliferation, formation of tumor spheroids, and expression of marker genes that promote tumor malignancy in CA scaffolds. The efficacy of NP targeted gene delivery was evaluated in PCa cells in 2D cultures, PCa tumor spheroids grown in CA scaffolds, and PCa tumors in a mouse TRAMP-C2 flank tumor model. PCa cells cultured in CA scaffolds grew into tumor spheroids and displayed characteristics of higher malignancy as compared to those in 2D cultures. Significantly, targeted gene delivery was only observed in cells cultured in CA scaffolds whereas cells cultured on 2D plates showed no difference in gene delivery between targeted and non-target control NPs. *In vivo* NP evaluation confirmed targeted gene delivery indicating that only CA scaffolds correctly modeled NP-mediated targeted delivery *in vivo*. These findings suggest that CA scaffolds serve as a better *in vitro* platform than 2D cultures for evaluation of NP-mediated targeted gene delivery to PCa.

Graphical Abstract

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Keywords

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INTRODUCTION

Prostate cancer (PCa) is the second leading cause of cancer death among the male population in the United States with approximately 29,000 deaths each year.¹ Current treatment for PCa often involves radical prostatectomy, radiation, and hormone therapy.² Surgery is highly invasive and might increase the risk of metastasis, whereas radiation therapy generates systemic toxicity.^{3, 4} Additionally, PCa can develop resistance to hormone therapy, leading to the relapse of fatal hormone refractory cancer.^{5, 6} Substantial efforts have been devoted to identifying novel treatment modalities, among which gene therapy is particularly appealing as it holds a number of advantages over traditional therapies, including the ability to overcome drug resistance, reduced systemic toxicity, and most importantly, improved target specificity.⁷ Several clinical trials that used suicide genes, immunomodulatory genes, and tumor suppressor genes have demonstrated the potential of gene therapy for PCa.^{6, 8} Nevertheless, translation of potential therapeutics into the clinic remains challenging due to the lack of a safe and efficient delivery strategy.^{9, 10}

Nanoparticles (NPs), including gold NPs, quantum dots, liposomes, and polymer-based nanovectors, have been extensively studied as gene delivery vehicles.^{11, 12} Superparamagnetic iron oxide NPs have shown significant promise as gene delivery vehicles that can be tracked using magnetic resonance imaging.^{13, 14} Typically, NP development is initiated with *in vitro* analysis using two-dimensional (2D) cell cultures followed by extensive *in vivo* evaluation in animals prior to clinical trials. However, successful NP delivery observed in 2D cell culture studies does not usually translate well to *in vivo* studies due to the intrinsic limitation of 2D monolayer cultures that fail to account for the extracellular barriers that are present *in vivo*.¹⁵

To bridge the gap between *in vitro* and *in vivo* analyses, three-dimensional (3D) cell cultures are being developed because they can provide a more realistic model of the *in vivo* tumor microenvironment than cells in 2D culture.^{16, 17} There are many types of 3D cell culture systems that are mostly scaffolds composed of natural materials such as collagen, gelatin, and polysaccharides, as well as synthetic materials such as polystyrene, polycaprolactone, and poly(lactic-co-glycolic acid).^{18–21} Of note, we have demonstrated that 3D porous scaffolds composed of a polyelectrolyte chitosan–alginate (CA) complex are able to mimic the tumor microenvironment and improve the tumorigenic potential of cultured cancer cells, including PCa.^{22–26} Both chitosan and alginate are FDA approved for

broad tissue engineering applications in view of their biocompatibility, biodegradability, and limited immunogenicity.^{27, 28} Their structure resembles glycosaminoglycans (GAGs), which are essential components of the extracellular matrix (ECM),²⁹ which makes these polymers promising choices for *in vitro* modeling of the tumor microenvironment.

We hypothesized that PCa cells cultured in 3D CA scaffolds can better replicate the response to targeted gene delivery *in vivo* and thus can be used in preclinical studies to reduce the number of animals required for preclinical tests. In this study, we assessed *in vitro* tumor cell proliferation, formation of tumor spheroids, and expression of marker genes that promote tumor malignancy in CA scaffolds. We then assessed the NP-mediated gene transfection in PCa cells in CA scaffold culture systems as compared with those in PCa tumors *in vivo*.

EXPERIMENTAL SECTION

Materials

All chemicals and reagents were purchased from Sigma-Aldrich and all tissue culture reagents were purchased from Life Technologies unless otherwise specified.

Plasmid DNA Preparation

The plasmid pDsRed-Max-N1 vector containing red fluorescent protein (RFP) encoding DNA under the control of the cytomegalovirus (CMV) promoter was purchased from Addgene,³⁰ and was propagated in DH5 α *E. Coli* and purified using the Plasmid Giga Kit (Qiagen).

Scaffold Synthesis

CA scaffolds were prepared as described previously.^{23, 31} Briefly, 4 wt % CA solutions were casted in molds and lyophilized prior to being cut in 2 mm thick disks and crosslinked in 0.2 M CaCl₂ solution. The CA scaffolds were then washed with excess deionized water and sterilized in 70% ethanol overnight. The scaffolds were then washed three times with Dulbecco's phosphate-buffered saline (DPBS), immersed in 500 mL DPBS, and shaken on an orbital shaker at 100 rpm overnight to remove remaining ethanol.

NP Synthesis

PEG-grafted-chitosan (CP) copolymer and NPs were synthesized using the methods reported previously.^{13, 32, 33} Briefly, CP-coated iron oxide NPs were prepared in presence of CP by co-precipitation of ferrous and ferric chlorides with ammonium hydroxide followed by buffer exchange into thiolation buffer (0.1 M sodium bicarbonate, pH 8.0, 5 mM EDTA) through S-200 Sephacryl resin (GE Healthcare). PEI (1.2 kDa) was conjugated to NPs through the formation of a thioether bond between 2-iminothiolane (Traut's Reagent, Molecular Biosciences) modified NPs and succinimidyl iodoacetate (SIA, Molecular Biosciences) modified PEI, then NPs purified to remove excess PEI through size exclusion chromatography using S-200 Sephacryl resin equilibrated with 20 mM HEPES buffer (pH 7.4). NPs were then complexed with RFP encoding plasmid DNA at a NP:DNA weight ratio of 10:1 by mixing NPs and DNA in reaction buffer (20 mM of HEPES, pH 7.4) for 30 min. Chlorotoxin (CTX, Alamone Laboratories) was conjugated to DNA-loaded NPs using NHS-

PEG₁₂-maleimide (Thermo Scientific). Finally, unreacted CTX was washed away using S-200 Sephacryl resin equilibrated with 20 mM of HEPES buffer (pH 7.4) to form NP-CTX.

Cell Culture

Mouse TRAMP-C2 (TC-2) PCa cells were purchased from American Type Culture Collection. TC-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂. The medium was changed every 2–3 days. For CA scaffold culture, 50,000 cells were seeded on each scaffold in 12-well plates and allowed to grow for 10–14 days to form tumor spheroids before conducting transfection. The medium was changed every 3–4 days. TC-2 cells were seeded at 50,000 cells per well in 12-well plates as 2D control for all *in vitro* assays, and they were subcultured every 4 days once they reached confluency.

RFP expressing TC-2 cells (TC-2^{RFP}) were generated by transfecting TC-2 cells with pDsRed-Max-N1 plasmid using Lipofectamine 2000 following the manufacture's protocol (Life Technologies). Three days after transfection, the regular DMEM medium was replaced by DMEM containing 1 mg/ml G418 disulfate (TOKU-E). Cells were maintained in the selective medium for three weeks prior to sorting with a BD FACS Aria II cell sorter (BD Biosciences). The stably transfected TC-2^{RFP} cells were cultured in a complete medium supplemented with 0.5 mg/ml G418 disulfate.

Live Cell Imaging

TC-2^{RFP} cells grown on 2D and in CA scaffolds were imaged at 10× and 20× magnification with a phase contrast fluorescence microscope (Nikon ECLIPSE TE2000-S).

Cell Proliferation Assay

The Alamar blue (AB) assay was used to evaluate cell proliferation on both 2D cultures and CA scaffolds following the manufacture's protocol (Life Technologies). Briefly, cells were cultured as described above. Cells were washed with DPBS three times before adding 10% AB solution in the complete growth medium to the wells. The samples were incubated at 37°C for a predetermined time for each culture method (3 hr for 2D cultures and 4.5 hr for CA scaffolds). Then the AB solution was transferred to a 96-well plate, and the fluorescence at an excitation wavelength of 556 nm and an emission wavelength of 586 nm was measured on a SpectraMax M5 microplate reader (Molecular Devices). The cell number was calculated according to standard curves generated separately for 2D cultures and CA scaffold cultures.

Evaluation of CTX Binding Efficiency *In Vitro*

Flow cytometry was used to assess the binding efficiency of CTX in PCa cells. Alexa Fluor 647 (AF647, Life Technologies) was conjugated to CTX according to the manufacturer's protocol. In brief, 160 µg of AF647 was mixed with 0.5 ml of 1 mg/ml CTX, and reacted for 1 hr at room temperature (pH 8.0) to form the CTX-AF647 conjugates. Unreacted fluorophore was removed using Zeba spin desalting columns (Thermo Scientific) and the number of AF647 per CTX was quantified by UV-Vis. TC-2 cells on 2D culture were

incubated with CTX-AF647 conjugates at CTX concentrations of 1 μ M, 2 μ M and 4 μ M. Free AF647 was used at the fluorophore concentrations equivalent to that on CTX-AF647 conjugates. Incubation was conducted for 1 hr at 37°C, and then cells were washed and collected for flow cytometry analysis. At least 10,000 cells from each condition were analyzed. Experiments were repeated three times.

***In Vitro* Gene Transfection**

For 2D conditions, 24 hr after plating TC-2 cells at a concentration of 50,000 cells/ml (0.5 ml/well) in 12 well plates, transfection was performed by replacing the cell culture medium with 0.5 ml of NP:DNA complex-containing medium (2 μ g plasmid DNA per well). After 4 hr of transfection, the complex-containing medium was removed and replaced with the regular cell culture medium. For cells growing in CA scaffolds, the medium was removed and 50 μ l of NP:DNA complex was added drop-wise onto the tops of scaffolds with 4 μ g plasmid DNA per well. 2 ml of fresh medium was added to each well 4 hr later.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted from TC-2 cells cultured in CA scaffolds for 12 days using the Qiagen RNeasy kit. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. Mouse β -actin served as the house-keeping gene. SYBR Green PCR Master mix (Bio-Rad) was used for template amplification with a primer for each of the transcripts in a Bio-Rad CFX96 real-time PCR detection system. Quantitative amplification was monitored by the level of fluorescence reflecting the cycle number at the detection threshold (crossing point) using a standard curve. Thermocycling for all targets was carried out in a solution of 20 μ l containing 0.5 μ M primers (Integrated DNA Technologies) and 4 pg of cDNA from the reverse transcription reaction under following conditions: 95°C for 2 min, 40 cycles of denaturation (15 sec, 95°C), annealing (30 sec, 58°C), and extension (30 sec, 72°C). The primers are listed in Table 1. Relative gene expression was analyzed using the $2^{-\Delta\Delta C(T)}$ method.

Animal Model

All animal studies were performed in accordance with the University of Washington Office of Animal Welfare guidelines for the use of animals, and all procedures were reviewed and approved by the Institutional Animal Care and Use Committee. For the flank syngeneic model, 8-week-old female black-6 mice (Jackson Laboratories) were anesthetized using 1.5% inhaled isoflurane and one million TC-2 cells were injected into the right flank of the animal in Matrigel matrix (BD Biosciences). Imaging was performed using an IVIS Lumina II system (PerkinElmer) to monitor RFP expression.

Scanning Electron Microscopy (SEM)

Samples for SEM analysis were fixed with 2.5% glutaraldehyde in fully supplemented media for 30 min at 37°C. The samples were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C. The samples were dehydrated in a series of ethanol washes (0%, 30%, 50%, 70%, 85%, 95%, 100%), with each wash performed twice.

The samples were critical point dried, mounted, and sputter coated with platinum, and then imaged with a JSM-7000F SEM (JEOL).

Histological Analysis

CA scaffolds containing TC-2 tumor spheroids were fixed in 4% formaldehyde for 24 hr and placed in 30% sucrose until fully saturated. Afterwards, the scaffolds were cut into small pieces and frozen in optimal cutting temperature compound (OCT) embedding medium (Sakura) at -80°C . The frozen sections ($12\text{ }\mu\text{m}$ thickness) were stained with hematoxylin and eosin (H&E) and photographed under a Nikon ECLIPSE TE2000-S microscope.

Statistical Analysis

All of the data was statistically analyzed to express the mean $\pm$ standard deviation (SD) of the mean. Statistical significance was set at $p < 0.05$ and tested with Student's *t*-test.

RESULTS AND DISCUSSION

Characterization and Growth Features of TC-2 Prostate Cancer Cells Cultured in 2D and 3D

A 3D culture system should offer an environment in which the alterations of tumor cell phenotype are similar to those associated with tumor progression.^{41, 42} It is therefore critical to assess the development of a tumor-like structure in CA scaffolds to evaluate their potential as an *in vitro* model for cancer research. Cell growth patterns and morphological changes were monitored after mouse TC-2^{RFP} PCa cells were cultured in 2D TCPs and 3D CA scaffolds. TC-2^{RFP} tumor spheroids were observed in CA scaffolds (Figure 1). In contrast, TC-2^{RFP} cells in 2D cultures in TCPS exhibited characteristics of adherent epithelial cells. The morphology of TC-2^{RFP} cells was also observed with SEM (Figure 2 and Figure S1). TC-2 cells in 2D cultures grew as flat monolayers (Figure 2a and b), whereas they formed tumor spheroids in the pores of the porous CA scaffolds (Figure 2c and d) and grew to $>100\text{ }\mu\text{m}$ in diameter in 12 days (Figure 2d and Figure S1). This was further confirmed by H&E staining of a cross section of the scaffold, where tumor spheroids were located both inside (Figure 3a–b, and Figure S2a) and towards the exterior (Figure 3c–d, and Figure S2b) of CA scaffolds. The ability of PCa cells to form tumor spheroids in 3D cultures demonstrated that CA scaffolds provided a better mimic of the *in vivo* tumor environment than 2D cultures.

Although cancer research has been mainly conducted using cells cultured as monolayers on TCPS, the distinct physicochemical properties of 3D cell culture platforms can efficiently bridge the simplified *in vitro* 2D models and complex *in vivo* models.^{15, 17} Various engineering methods have been developed to facilitate tumor spheroid formation. Tumor cells can form aggregates when confined in “hanging drops”, in rotating-wall vessels on orbital shakers, or centrifuged in well plates with round or conical bottoms.^{43–46} However, many of these systems were designed for cells to self-aggregate to form spheroids generating loose pack of cells without the distinct features of tumors. In the current study, TC-2 cells were trypsinized and resuspended as single cell suspensions before seeding onto CA scaffolds. CA scaffolds have pore sizes of $100\text{--}300\text{ }\mu\text{m}$.³¹ Observed under the microscope, cells were well distributed throughout the entire scaffolds after seeding.

Therefore, we speculated that unlike the other 3D culturing systems, the naturally formed tumor-like spheroids observed in CA scaffolds originated from single PCa cells. We proposed that cell-cell interactions, production of ECM, and cell proliferation from the exterior to the spheroid center in CA scaffolds are more representative of what occur in the tumor milieu *in vivo*. We have previously demonstrated that in addition to maintaining tumor cell morphology, CA scaffolds can also increase tumor cell malignancy.^{22, 23, 26} This further emphasized the important role of CA scaffolds as an *in vitro* tumor culture system that can better represent the tumor microenvironment than 2D TCPS.

Prostate Cancer Cell Proliferation in CA Scaffolds

To further evaluate the behavior of cancer cells in the CA culture system, we monitored the growth kinetics of the naturally formed TC-2 tumor spheroids. Cell proliferation in 2D and CA cultures was determined using the AB assay. TC-2 cells in 2D cultures had a high proliferation rate and became highly confluent in three days (Figure 4), which corresponds with the growth kinetics of most established cancer cell lines on conventional 2D culture systems.^{47, 48} In contrast, TC-2 cells cultured in CA scaffolds showed a greatly reduced growth rate, better matching the *in vivo* growth of tumors.¹⁷ It was speculated that PCa cells in 2D cultures were exposed to sufficient nutrients and oxygen whereas CA scaffolds had limited nutrient and oxygen transport and removal of cell wastes, which is similar to the *in vivo* milieu as tumors must recruit blood vessels before they can begin to proliferate rapidly. In fact, this agrees with previous studies that the 3D microenvironment provided by porous scaffolds promotes malignancy of tumor cells *in vitro* and *in vivo*, which contributes to the decreased cell proliferation and a better mimic tumor *in vivo* conditions than the rapidly proliferating cells in standard 2D culture.^{17, 49} The current hurdle of PCa drug development lies in the lack of predictability of tumor behavior, which is due to the discordance between traditional cell culture and the *in vivo* microenvironment. The features of TC-2 cell growth in CA scaffolds better reflect the *in vivo* physiological situation in human cancer tissues than conventional 2D cultures.

ECM and Cell-Matrix Interactions of PCa Cells in CA Scaffolds

In vitro 3D cultures may serve as a more representative model of the *in vivo* system than standard 2D cultures. In fact, the local tumor microenvironment is primarily composed of endogenous ECM.⁵⁰ Collagen and laminin are both important components of the ECM and have been used in producing 3D culture systems.^{51–54} These two ECM related genes in cells cultured in 2D cultures and 3D scaffolds were quantified with qRT-PCR. A 20% (collagen) and 81% (laminin) increase in mRNA expression was observed in cells cultured in CA scaffolds relative to 2D cultures (Figure 5a). This suggests that in addition to resembling the architecture of solid tumors, the CA scaffolds also promoted the expression of ECM genes.

An epithelial-mesenchymal transition (EMT) is a developmental process of cells switching from epithelial to mesenchymal status.⁵⁵ Additionally, the EMT is activated by carcinoma cells during their course of invasion and metastasis.⁵⁶ Recent studies have demonstrated the role of the EMT in facilitating progression and metastasis of PCa as it increases motility and invasiveness of the cancer cells.^{57, 58} We have previously shown that culture of brain and liver cancer cells on CA scaffolds increases their malignancy and chemo-resistance,^{22, 25}

which was caused by transition to a more-stem like state through an EMT.²² Therefore, to better recapitulate the *in vivo* situation *in vitro*, 3D culture systems should be able to induce the EMT in PCa cells. We measured the expression levels of EMT related genes in PCa cells grown in CA scaffolds and 2D cultures.

Snail mediates the EMT through downregulation of cell adhesion molecules, and its expression increases with PCa progression.^{59, 60} A 20% increase in Snail expression was demonstrated in CA scaffolds over 2D cultures as determined by qRT-PCR (Figure 5b), suggesting the CA culture system promoted the EMT of PCa cells.

Slug plays a critical role in the EMT and its upregulation is associated with advanced-stage PCa,^{61, 62} and Twist expression is positively correlated with Gleason grading of PCa, suggesting its role in tumorigenesis.⁶³ Twist is a highly conserved basic helix-loop-helix transcription factor in PCa that promotes the EMT.⁶⁴ The expression patterns of these two gene markers were similar to that of Snail, where the increased expression in CA scaffolds was approximately 34% and 90% higher than in 2D cultures (Figure 5b). Slug can also suppress the proliferation of PCa cells,⁶⁵ which is in line with the decreased proliferation rate of TC-2 cells in CA scaffolds shown above (Figure 4).

Sip1, a member of the δ EF-1 family of two-handed zinc finger nuclear factors, is another protein involved in the EMT of PCa.⁶⁶ Sip1 expression correlates with PCa cell migration and invasion.^{67, 68} Sip1 mRNA expression in TC-2 cells cultured in CA scaffolds increased 2.2-fold as compared to those in 2D cultures (Figure 5b).

In addition to the upregulation of the mesenchymal markers, we also observed a 2.7-fold increase in mRNA expression of an epithelial marker E-cadherin (Figure 5c). High expression of E-cadherin is speculated to occur at the late stage of EMT, when the disseminated mesenchymal tumor cells recapitulate the pathology of their corresponding primary tumors at the metastatic sites.⁶⁹ In fact, E-cadherin upregulation can induce a more stem-like state in PCa cells.^{69, 72, 73} Furthermore, upregulation of E-cadherin has been detected in advanced metastatic PCa, and is considered to be correlated with tumor invasion and poor patient prognosis.^{70, 71}

Overall, the upregulation of these EMT related transcriptional factors induced by culture in CA scaffolds shows the increased migratory potential and the ability of cells to invade an artificial scaffold, a typical phenotypic feature of PCa. Previous studies have demonstrated that the expression levels of these factors were induced by microenvironmental stimuli.⁷⁴ This phenomenon observed in CA cultures suggests the potential of 3D CA scaffolds to act as a mimic of *in vivo* tumor microenvironment.

Analyzing Plasmid Delivery in CA Scaffolds

Our previous studies demonstrated that CTX conjugated NPs are able to enhance delivery specifically to brain tumors by increasing the distribution and cell uptake of the NP throughout the tumor.^{13, 75} It has also been reported that CTX is able to target PCa through specific interactions with cell surface receptors MMP-2 or Annexin A2.^{76, 77} To determine if CTX can target TC-2 PCa cells, the cells growing on 2D cultures were incubated with CTX-

AF647 conjugate and free AF647 fluorophore at different concentrations for 1 hr and the binding of CTX-AF647 to PCa cells was assessed using flow cytometry. Here the free fluorophore served as the control to determine the background cellular uptake. Little binding of the free fluorophore to PCa cells was observed at the three fluorophore concentrations tested (Figure 6a and b). On the other hand, the binding of CTX-AF647 conjugates to PCa cells at the three concentrations of CTX with the fluorophore amounts equivalent to the three free fluorophore concentrations was substantially higher than free AF647 and demonstrated strong dose-dependence. PCa cells treated with CTX-AF647 exhibited an approximate 3-, 4-, and 6-fold higher fluorescence intensity at the three fluorophore concentrations, respectively, compared to the untreated PCa cells, while free AF647 showed less than 1 fold increase in fluorescence intensity for all three concentrations of fluorophores as compared to the untreated cells (Figure 6b).

To reveal the difference in mimicking the tumor microenvironment between 2D cultures and CA scaffolds for tumor targeted NPs as gene delivery carriers, PCa cells cultured in both 2D cultures and CA scaffolds were treated with RFP encoding plasmid DNA loaded NPs with (NP-CTX) or without CTX (NP-control) conjugated. The transfection efficiency was evaluated three days post-inoculation of NPs by measuring RFP fluorescence in solubilized cells using a microplate reader (Figure 7a and b). NP-CTX showed no cell targeting effect in 2D cultures whereas an 80% increase in RFP fluorescence intensity was observed in NP-CTX treated cells cultured in CA scaffolds as compared with NP-control treated cells. This is consistent with the fluorescence microscopy images that show more RFP expressing TC-2 cells in CA scaffolds treated with NP-CTX than those treated with NP-control, while a similar percentage of RFP positive cells in 2D cultures was detected with both treatments (Figure 7c). Perls' Prussian blue staining also revealed that NP-CTX has higher affinity to TC-2 tumor spheres than NP-control, whereas cellular uptake is similar between NP-control and NP-CTX in 2D cultures (Figure 7d). In accord with our previous findings,^{13, 75} CTX promoted the distribution of NP inside the tumor, suggesting the high penetration capacity of NP-CTX in solid tumor.

The lack of tumor cell targeting of NP-CTX in 2D cultures was likely due to the direct interaction between each cell and the positively charged NPs and to the absence of other materials in 2D cultures. The uptake of NP-CTX by each tumor cell was caused by both receptor-mediated (CTX and tumor cell surface receptors) and absorptive-mediated (positively charged NPs and negatively charged cells) endocytosis. Absorptive-mediated endocytosis dominates cellular uptake of NPs when NPs are highly positively charged, which has been observed with several other cationic gene delivery agents.^{78, 79} When NPs were incubated with PCa tumor spheroids in CA scaffolds, positively charged NPs interact with cells as well as CA scaffolds. The tumor-targeted NP-CTX had high affinity to the PCa cells through specific interactions with cell surface receptors MMP-2 or Annexin A2,^{76, 77} whereas non-targeted NP-control had similar affinity to the cell surface, CA scaffold, and ECMs produced by tumor cells through electrostatic interactions. Thus, the cellular uptake of NP-CTX in CA scaffolds was via both receptor- and adsorptive-mediated endocytosis while the cellular uptake of NP-control was via adsorptive-mediated endocytosis only. Additionally, the higher malignancy of PCa cells in 3D CA scaffolds as compared to 2D cultures likely caused increased expression of MMP-2⁸⁰ so provided a higher number of

cell surface receptors for NP-CTX to attach to. Thus, NP-CTX would have stronger affinity to PCa cells in tumor spheroids than PCa cells in 2D cultures.^{81–83}

***In Vivo* Delivery of RFP Using NP-CTX**

Abnormal vasculature, elevated interstitial fluid pressure, and ECM are three unique architectural features of solid tumors that restrict mobility of a nanomedicine through malignant tissue.^{81, 82} NPs can escape blood vessels by the enhanced permeation and retention (EPR) effect. Following extravasation from blood vessels, the transport of NP through the extracellular space to reach target cells is hindered by the complex structure of ECM in solid tumors comprising fibrous macromolecules such as collagen and GAGs.⁸³ Without a targeting ligand, positively charged NPs have a strong affinity to both ECM and target cells. Therefore, a targeting ligand is important for enabling the specific attachment of the NPs to the tumor cells. Our studies revealed that CTX enhanced NP-mediated transfection on cancer cells cultured in CA scaffolds.

We expected that the 3D model would serve as a good indicator of gene transfection efficiency *in vivo*. After establishing that the NP-CTX could enhance the delivery of plasmid DNA into cells in CA culture, we evaluated targeted gene delivery of NP-CTX in mice bearing TC-2 PCa tumors. NP-CTX and NP-control complexed with RFP plasmid were injected intravenously through the tail vein. After 48 hr, tumors were collected and imaged using the IVIS Lumina II system. 1.6-fold increase in fluorescence intensity was observed in the NP-CTX treated tumor as compared to cells treated with NP-control, demonstrating the increased transfection efficiency in the tumor as a result of targeting ability of CTX in PCa (Figure 8a and b). Perls' Prussian blue staining corroborated these results and showed accumulation of NP-CTX at a higher level than NP-control in tumor sections (Figure 8c and d). These results showed that NP-control and NP-CTX in PCa tumors behave similar to that in CA scaffolds *in vitro* (Figure 7c and d). Importantly, the increased transfection efficiency observed in the PCa tumor was target specific as RFP expression in liver, kidney, spleen, heart and lung was not affected by the presence of CTX (Figure 9).

Various strategies have been developed to facilitate efficient NP penetration into tumors. Size has been found to be a crucial factor that affects the permeability of nanodrugs.^{84, 85} Additionally, targeting ligands have been shown to improve the delivery of NPs throughout the tumor.^{13, 86, 87} The targeting capacity of CTX to human brain tumors is well established.^{13, 88} Some evidence also indicates that CTX can target PCa *in vivo*.⁷⁶ When conjugated onto NPs, CTX did not facilitate targeted gene delivery in PCa cells cultured on standard 2D TCPS surfaces. Instead, these *in vitro* results falsely predicted CTX is not a good targeting agent to use on the surface of cationic NPs to target PCa even though CTX itself showed high uptake in PCa cells. However, in agreement with the previous studies, our results demonstrate that CTX conjugated NPs had high binding efficiency in PCa tumors and were readily taken up and well distributed throughout PCa tumors while not affecting non-specific uptake in healthy tissues. Importantly, the *in vivo* targeting corresponded well to the *in vitro* NP-CTX delivery in CA scaffolds, revealing that NP-CTX mediated gene delivery in this *in vitro* cell culture model could replicate the *in vivo* targeting effect. It also indicated

that this 3D cell culture model could be applied in the study of penetration and transport of anticancer nanomedicines in order to better predict their targeting efficiency *in vivo*.

CONCLUSION

In the present study, we demonstrated that PCa TC-2 cells cultured in CA scaffolds *in vitro* more accurately predict *in vivo* targeted nanoparticle-mediated gene delivery. TC-2 PCa cells formed tumor spheroids when cultured in CA scaffolds and showed upregulation of ECM and EMT gene markers as compared to standard 2D culture on TCPS. Targeted nanoparticle-mediated delivery of RFP plasmid DNA into TC-2 tumor spheroids was achieved with CTX in cells cultured in CA scaffolds but not on 2D cultures in TCPS. This underscores the utility of the 3D culture when testing positively-charged targeted NPs as the standard 2D culture on TCPS did not show the targeting effect of CTX. Significantly, the *in vitro* targeted transfection results seen using CA scaffolds matched the *in vivo* targeted transfection efficiency observed in PCa tumors. Taken together, our results provide strong evidence that this *in vitro* cell culture system can serve as a useful 3D tumor model that mimics the *in vivo* tumor microenvironment structure and accurately predicts *in vivo* targeting efficiency. Thus, this 3D scaffold culture system should be used in the initial screening of tumor targeted NP delivery vehicles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Optical (top row) and fluorescent (bottom row) images of the tumor cells/spheroids of TC-2^{RFP} cultured in 2D cultures and CA scaffolds for 5 and 12 days. Scale bars represent 100 μm .

Figure 2.
SEM images of TC-2 cells cultured in 2D (a–b) and CA scaffolds (c–d) for 5 and 12 days.
Scale bars represent 50 μm .

Figure 3. H&E staining of TC-2 tumor spheroids grown in CA scaffolds for 12 days. Tumor spheres were observed (a–b) inside and (c–d) close to the surface of scaffolds. Scale bars represent 100 μm .

Figure 4. Growth kinetics of TC-2 cells in 2D cultures and in CA scaffolds as determined by the AB assay.

Figure 5.

Expression of (a) ECM and (b and c) EMT gene markers quantified by qRT-PCR. Cells were grown in 2D cultures and CA scaffolds for 12 days prior to collection and analysis. Asterisks indicate significance as determined by Student's *t*-test: * $P < 0.01$ and ** $P < 0.05$. Results are from 3 independent experiments run in triplicate.

Figure 6.

In vitro evaluation of tumor targeting of CTX to TC-2 cells. (a) Flow cytometry analysis of 2D cultured TC-2 cells incubated with free AF647 fluorophore and AF647 conjugated CTX (CTX-AF647) at different concentrations of CTX. (b) Normalized mean fluorescence intensity of TC-2 cells from (a).

Figure 7.

In vitro evaluation of targeted delivery of RFP plasmid to TC-2 cells in 2D cultures and CA scaffolds using NP-control and NP-CTX. (a) RFP transfection of TC-2 cells induced by NP-control and NP-CTX in 2D cultures and CA scaffolds. RFP fluorescence in TC-2 cells was measured using a microplate reader and normalized to total protein content. (b) RFP fluorescence intensity from NP-CTX and NP-control treated TC-2 cells growing in 2D and CA scaffolds acquired from (a) was normalized to the NP-control. Asterisk indicates significance as determined by Student's *t*-test: *P < 0.01. (c) Brightfield and fluorescence images of NP-control and NP-CTX transfected TC-2 cells growing in 2D cultures and CA scaffolds. Scale bars represent 100 μ m. (d) NP-control and NP-CTX were also detected by staining with Perls' Prussian blue. TC-2 cells were counterstained with nuclear fast red. Scale bars correspond to 100 μ m.

Figure 8.

Accumulation and transfection efficiency of NP-control and NP-CTX in tumors. (a) Xenogen imaging of tumors from untreated, NP-control, and NP-CTX treated animals. (b) Quantification of fluorescence intensities (photons/sec/cm²/steradian)/($\mu\text{W}/\text{cm}^2$) of tumors from untreated mice and mice treated with NP-control and NP-CTX. (c–d) Perls' Prussian blue staining indicating the accumulation of NP. Scale bars represent 100 μm .

Figure 9. Xenogen images of harvested livers, kidneys, spleens, hearts, and lungs from untreated mice and mice treated with NP-control or NP-CTX (n = 3).

Table 1

Primers used for qRT-PCR

Genes	Forward	Reverse
<i>β-actin</i> ³⁴	CGGTTCCGATGCCCTGAGGCTCTT	CGTCACACTTCATGATGGAATTGA
<i>Colla1</i> ³⁵	GCAACAGTCGCTTCACCTACA	CAATGTCCAAGGGAGCCACAT
<i>Laminin α5</i> ³⁶	ACCCAAGGACCCACCTGTAG	TCATGTGTGCGTAGCCTCTC
<i>Snai1</i> ³⁷	TCCAAACCCACTCGGATGTGAAGA	TTGGTGCTTGTGGAGCAAGGACAT
<i>Slug</i> ³⁷	CACATTCGAACCCACACATTGCCT	TGTGCCCTCAGGTTTGATCTGTCT
<i>Sip1</i> ³⁸	ATGGCAACACATGGGTTTAGTGGC	ATTGGACTCTGAGCAGATGGGTGT
<i>Twist</i> ³⁹	AATTCACAAGAATCAGGGCGTGGG	TCTATCAGAATGCAGAGGTGTGGG
<i>E-cadherin</i> ⁴⁰	AAGTGACCGATGATGATGCC	CTTCTCTGTCCATCTCAGCG