Published in final edited form as:

Biomaterials. 2013 February ; 34(7): 1810–1819. doi:10.1016/j.biomaterials.2012.11.038.

Prevention of nodal metastases in breast cancer following the lymphatic migration of paclitaxel-loaded expansile nanoparticles

Rong Liu^a, Denis M. Gilmore^a, Kimberly Ann V. Zubris^b, Xiaoyin Xu^c, Paul J. Catalano^d, Robert F. Padera^e, Mark W. Grinstaff^{b,*}, and Yolonda L. Colson^{a,*}

^aDepartment of Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

^bDepartments of Biomedical Engineering and Chemistry, Boston University, Boston, MA 02215, USA.

^cDepartment of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

^dDepartment of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Boston, MA 02115, USA.

^eDepartment of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

Abstract

Although breast cancer patients with localized disease exhibit an excellent long-term prognosis, up to 40% of patients treated with local resection alone may harbor occult nodal metastatic disease leading to increased locoregional recurrence and decreased survival. Given the potential for targeted drug delivery to result in more efficacious locoregional control with less morbidity, the current study assessed the ability of drug-loaded polymeric expansile nanoparticles (eNP) to migrate from the site of tumor to regional lymph nodes, locally deliver a chemotherapeutic payload, and prevent primary tumor growth as well as lymph node metastases. Expansile nanoparticles entered tumor cells and paclitaxel-loaded eNP (Pax-eNP) exhibited dose-dependent cytotoxicity in vitro and significantly decreased tumor doubling time in vivo against human triple negative breast cancer in both microscopic and established murine breast cancer models. Furthermore, migration of Pax-eNP to axillary lymph nodes resulted in higher intranodal paclitaxel concentrations and a significantly lower incidence of lymph node metastases. These findings demonstrate that lymphatic migration of drug-loaded eNP provides regionally targeted delivery of chemotherapy to both decrease local tumor growth and strategically prevent the development of nodal metastases within the regional tumor-draining lymph node basin.

© 2012 Elsevier Ltd. All rights reserved.

***Corresponding Authors:** Yolonda L. Colson, M.D., Ph.D., Division of Thoracic Surgery, Mark W. Grinstaff, Ph.D., Department of Surgery, Brigham and Women's Hospital, 75 Francis Street PBB 544, Boston, Massachusetts 02115. Departments of Biomedical Engineering and Chemistry, 590 Commonwealth Avenue, Room #518, Boston University, Boston, Massachusetts 02215. ycolson@partners.org or mgrin@bu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

nanoparticle; drug delivery; polymer; metastases; breast cancer; lymphatic

Introduction

Breast cancer is the most commonly diagnosed solid organ malignancy in women worldwide, affecting over 1.5 million women in 2010 [1, 2]. Although patients with disease localized to the breast exhibit an excellent long-term prognosis, the development of metastases in the regional lymph nodes is associated with a significant decrease in survival. The NSABP B-04 clinical trial was a landmark randomized controlled study designed to assess differences in outcome for women with nonpalpable axillary nodes as a function of locoregional therapy [3]. Among women treated with radical mastectomy there was a 40% incidence of occult metastases in the axillary nodes and 18% of patients had clinical evidence of tumor growth in the axilla following total mastectomy where axillary lymph nodes are not removed. Whereas, these results established the importance of assessing regional lymph node status, subsequent studies dampened enthusiasm for aggressive lymphadenectomy by highlighting the associated complications and long-term morbidity of nerve damage, increased infectious risk, pain and lymphedema [4, 5]. Consequently, NSABP-32 investigated the differences in clinical outcome in over 5000 women with operable, clinically node negative breast cancer randomly assigned to regional node assessment via sentinel lymph node (SLN) biopsy with subsequent axillary node dissection vs. SLN biopsy alone [6]. Among patients with histologically negative SLN(s), nearly twice as many regional node recurrences were noted in the SLN biopsy alone cohort indicating that occult nodal disease was left behind when an axillary node dissection was not performed [4]. Although regional nodal recurrence was the first clinically evident site of treatment failure in only 21 patients, in-depth histologic analysis of all previously determined negative SLN samples (3887 patients) demonstrated occult metastatic disease, i.e. missed nodal disease, in 15.9% of “negative SLN”. Patients with occult nodal metastases exhibited a nearly 3-fold increase in regional recurrence and statistically significantly worse outcomes in terms of overall survival, disease-free survival, and distant-disease-free interval ($p < 0.04$ for each) [7]. Importantly, these differences were noted despite complete surgical resection and adjuvant treatment with standard radiation therapy, chemotherapy, and endocrine regimens as deemed appropriate based on tumor characteristics. Given that the majority of patient received either adjuvant chemotherapy, endocrine or other systemic therapy and at least tangential external radiation therapy (XRT) to the SLN biopsy site and portions of the level I and II axillary lymph node chains, it is of interest that the number of node-negative patients that presented with locoregional failure was essentially equivalent to the number that presented with distant metastases [7]. Furthermore, although the 10-year risk of locoregional first recurrence following breast conserving therapy can be decreased from 25% to 7-8% with the addition of local radiotherapy, significant concerns of increases in 15-year mortality due to resultant cardiac disease has called for new approaches to prevent locoregional recurrence in these patients [8, 9].

Current regimens for systemic chemotherapy are hampered by systemic side effects that limit the total dose and concentration of drug that can be delivered locally to the desired target tissues, i.e. tumor bed and draining lymph node basins. Furthermore, limited vascular access to regional nodes and rapid systemic clearance results in virtually no persistent drug delivery to regional nodes of patients with breast cancer, even within 6 hours of administration [10]. These limitations suggest that a local drug-delivery system that can deliver chemotherapeutic drug to the primary tumor site and regional lymph nodes via

lymphatic pathways may result in a significantly more efficacious means to control locoregional disease with less morbidity than axillary node dissection or radiation.

To this aim, we investigated an expansile nanoparticle (eNP) local drug delivery system previously shown to exhibit superior anti-cancer efficacy in the treatment of peritoneal carcinomatosis [11]. Paclitaxel-loaded eNP (Pax-eNP) demonstrated increased tumor affinity and resulted in both decreased local tumor burden and prolonged survival *in vivo*. Recently, we have shown that eNP are also capable of lymphatic trafficking *in vivo* suggesting that eNP may provide the means to concentrate drug delivery within the regional lymph node basins draining sites of tumor [12]. We hypothesized that locally administered nanoparticles at the site of the original tumor would be capable of migrating to regional lymph nodes, delivering the chemotherapeutic payload, and preventing primary tumor growth as well as lymph node metastases. Therefore, in the current study we investigate the utility of Pax-eNP to control both local tumor growth and occult lymphatic metastases in a sensitive bioluminescent murine model of metastatic triple negative breast cancer.

2. Materials and methods

2.1 Nanoparticle preparation

Paclitaxel-loaded expansile (Pax-eNP) and unloaded expansile (unloaded eNP) nanoparticles were prepared using a miniemulsion polymerization technique [13]. Encapsulation efficiency of 5% Pax-eNP (w/w of polymer) was assessed via HPLC and was greater than 95% for all studies. For studies involving eNP trafficking, Rhodamine-B (Sigma-Aldrich, St. Louis, MO) was covalently attached to the NP at 0.02% w/w loading and prepared using the corresponding acrylate monomer. In addition, to separately identify the location of the nanoparticle monomer and the paclitaxel payload, Rhodamine-B labeled eNP were prepared with 0.02% w/w Oregon Green 488 conjugated paclitaxel (Life Technologies Corp., Carlsbad, CA) as the payload (OGPax-Rho-eNP).

2.2 Cell culture and in vitro cell viability assay

MDA-MB-231-luc-D3H2LN (Caliper Scientific, Hopkinton, MA), was chosen for these studies as it is a firefly luciferase transfected human breast cancer cell line that is “triple negative” for estrogen, progesterone and her-2neu receptors, multi-drug resistant and highly metastatic to regional lymph nodes. It was maintained at 37°C in 5% CO₂ using MEM media containing 10% fetal bovine serum, streptomycin (100 µg/mL), and penicillin (100 units/mL). Anti-tumor cytotoxicity of Pax-eNP, unloaded eNP, and paclitaxel resuspended in standard cremaphor/ethanol solution (Pax-C/E) were assessed using the MTS in vitro cytotoxicity assay (CellTiter 96® Aqueous One, Promega, Madison, WI) as described previously [11]. Briefly, tumor cells were cultured in 96-well plates at 3.0×10^3 cells/well for 24 hours. Culture media was removed and replaced with media containing Pax-eNP, unloaded eNP, or Pax-C/E (Pax control). Cell viability was measured after three days of treatment exposure with the percent viability calculated as absorbance relative to control wells (cell with culture media). A total of 4- 6 wells were used per treatment per concentration for each experiment and all assays were repeated a minimum of four times.

2.3 Microscopic and established breast cancer model in mice

All animal experiments were approved and conducted in accordance with the guidelines for humane care and use of laboratory animals from the Institutional Animal Care and Use Committee. Orthotopic breast cancers were induced with inoculation of two million MDA-MB-231-luc-D3H2LN cells into the 4th mammary fat pad in 6-8 wks female Nude mice (Harlan, Indianapolis, IN). Local treatment with Pax-eNP, unloaded eNP or 4 mg/kg Pax-C/E were injected at the site of tumor inoculation on the same day (Day 0) for the microscopic

tumor model and on Day 7 for studies utilizing the established tumor model. 100ug Pax-eNP, the paclitaxel dose equivalent of 4 mg/kg local Pax-C/E, was used for all studies with efficacy compared against a standard dose of 12 mg/kg systemic Pax-C/E administered intraperitoneally. Tumor size was measured twice per week with a digital caliper and at the time of sacrifice, with the estimated tumor volume calculated as $\pi \times (\text{length} \times \text{width} \times \text{height} / 6)$. Mice were euthanized 5½ weeks after treatment for both models or when tumors reached 2 cm, skin necrosis developed, body mass dropped 15% within one week, or animals became moribund.

2.4. Bioluminescent imaging for detection of lymph node metastases

Axillary lymph node bioluminescent imaging was performed at the designated time point, 5 1/2 weeks after treatment for both models, or when early termination required due to the size or necrosis of tumor. Mice received 150 mg/kg D-Luciferin (Caliper Life Sciences, Hopkinton, MA) prior to euthanasia. Axillary lymph nodes (LNs) were immediately harvested and submerged in 1.5 mg/mL luciferin in PBS in black 96-well plates and imaged for 3 minutes with a Caliper IVIS-100 bioluminescence camera (Caliper Life Sciences, Hopkinton, MA). In initial animal studies, a threshold of positive (i.e. metastatic) nodes was set as the mean + two $\times$ standard deviations greater than signals of nodes from non-tumor control animals. Subsequent studies utilized the absolute bioluminescent signal at photons/sec for statistical analyses.

2.5 Confocal microscopy

For *in vitro* cell imaging, MDA-MB-231-luc-D3H2LN were seeded at 2.0×10^4 cells on a poly-lysine (Sigma) treated glass coverslips (No. 1.5, Fisher Scientific, Pittsburg, PA) and placed with a 24-well plate for 48 hours at 37 C in 5% CO₂. Cells were incubated with Rho-eNP loaded with or without OGPax. Coverslips were washed with HBSS three times, fixed in 2% paraformaldehyde (Fisher Scientific, Pittsburg, PA) for 10 minutes, and washed three times with HBSS. Cells were stained with Hoechst 33342 (Life Technologies, Carlsbad, CA) and Alexa Fluor® 488 conjugated wheat germ agglutinin (WGA 488, Life Technologies, Carlsbad, CA) for identification of the nucleus and cell membranes respectively, and mounted on slides with Antifade Prolong Gold® (Life Technologies, Carlsbad, CA) mounting media in room temperature overnight. Slides were stored at -20°C until analysis. Images were obtained with Zeiss LSM 510 inverted confocal laser scanning microscope with Plan-Apochromat 10x/0.45 or C-Apochromat 40x1.2 W corr lens (Carl Zeiss Microscopy, Thornwood, NY).

Confocal imaging of labeled eNP that have migrated *in vivo* to axillary LNs was performed after the injection of 100 µL of Rho-eNP or OGPax-Rho-eNP in the 4th mammary fat pad of naïve or tumor-bearing mice. Mice were sacrificed and axillary LNs were harvested 4 or 10 days after injection, placed in Tissue-Tek O.C.T. compound (Fisher Scientific) and frozen in liquid nitrogen. Each node was cryo-sectioned at 5 m intervals and sections examined for fluorescence via confocal microscopy after nuclear staining with DAPI dihydrochloride (Life Technologies, Carlsbad, CA). Adjacent tissue sections were stained with hematoxylin and eosin for general histologic evaluation. Volocity 6.0.1 software (PerkinElmer Inc., Waltham, MA) was utilized to quantify colocalization with calculation of the co-efficient parameter “MX”, which indicates percentage of Rho-eNP that contains OGPax.

2.6 Paclitaxel tissue concentration

Both left and right side axillary LN were harvested and weighed four days following mammary fat pad injection of 4 mg/kg Pax-eNP or Pax-C/E into female Nude mice. Paclitaxel tissue concentrations were measured using High-performance liquid chromatography (HPLC) by Apredica Inc. (Watertown, MA). Briefly, paclitaxel was

extracted from the tissue using acetonitrile incubation for 30 min, centrifuged for 5 min at 14k RPM and the supernatant analyzed by LC/MS/M using an Agilent 6410 mass spectrometer coupled with an Agilent 1200 HPLC and a CTC PAL chilled autosampler, all controlled by MassHunter software (Agilent). After separation on a C18 reverse phase HPLC column (Agilent, Waters, or equivalent) using an acetonitrile-water gradient system, peaks were analyzed by mass spectrometry (MS) using ESI ionization in MRM mode. The limit of detection was 13.5 ng/g tissue.

2.7 Statistics

Data were presented as mean $\pm$ standard error unless specified in the text. A standard mixed effects regression model was fit to the cube-root of the tumor volume to assess growth trends in time, adjusted for animal (random effect with a first order autoregressive error structure) and side of tumor (fixed effect). Doubling times were computed from 3 weeks after treatment for both tumor models. All computations were done in SAS v9.2 for Unix (SAS Institute, Inc., Cary, NC) or Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA). All significance tests and quoted *p*-values are two-sided with *p* < 0.5 as significant.

3. Results

To maximize drug delivery to regional lymph nodes with the ultimate goal of preventing nodal metastases in breast cancer, we studied whether Pax-eNP could 1) enter breast cancer cells and result in exhibit tumor cytotoxicity comparable to paclitaxel alone; 2) result in effective anti-tumor efficacy against microscopic and established tumors *in vivo*; and 3) migrate to regional lymph nodes and deliver paclitaxel within the nodes. Therefore, each of these criteria was assessed along with subsequent efficacy against metastatic nodal disease.

3.1 In vitro cellular uptake studies

Intracellular uptake of eNP within triple negative human breast cancer cells was investigated using Rhodamine-B labeled fluorescent eNP (Rho-eNP) prepared by incorporating Rhodamine-B directly into the polymer backbone at the time of eNP preparation. After 8 hours of Rho-eNP co-incubation with the human breast cancer cell line MDA-MB-231-luc-D3H2LN [14], confocal microscopy demonstrated that Rho-eNP had accumulated within the cytoplasm in the majority of tumor cells with intracytoplasmic location verified via z-scan imaging (Fig. 1A). Flowcytometric analysis quantified the intracellular presence of Rho-eNP within MDA-MB-231-luc-D3H2LN showing evidence of significant uptake within 2 hours and nearly universal uptake over 24 hours (Fig. 1B). As expected, Rhodamine-B signal was not detectable when cells were treated with media alone in the absence of Rho-eNP. These results indicate that eNP quickly enter tumor cells but are not rapidly exocytosed, thereby allowing intracellular drug delivery to occur over a much longer period of time than the few hours typically reported for free paclitaxel [15].

3.2 In vitro cytotoxicity studies

To assess whether eNP-mediated delivery of paclitaxel is effective, Pax-eNP were first assessed for cytotoxicity against the aggressive triple negative breast cancer cell line MDA-MB-231-luc-D3H2LN *in vitro*. Efficacy was compared to increasing concentrations of free paclitaxel suspended in the standard Cremophor-ethanol carrier (Pax-C/E) and against unloaded eNP as a negative control. Using a standard *in vitro* cytotoxicity assay, Pax-eNP and free Pax-C/E demonstrated nearly identical dose-dependent cytotoxicity curves against MDA-MB-231-luc-D3H2LN as evident in Fig 1C. The half maximal inhibitory concentrations (IC50s) of Pax-eNP and Pax-C/E after 72 hours of exposure were 6.7 ng/mL (4.7 – 9.6 ng/mL, 95% Confidence Interval) and 2.3 ng/mL (1.4 – 4.0 ng/mL, 95% Confidence Interval), respectively. As with all other tumor cell lines previously tested,

unloaded eNP are not intrinsically toxic to MDA-MB-231-luc-D3H2LN [11, 13], thereby confirming that the anti-tumor cytotoxicity exhibited by Pax-eNP is due to paclitaxel release and not polymer toxicity. Although Pax-C/E is statistically more effective than Pax-eNP in the *in vitro* assay, both Pax-C/E and Pax-eNP were statistically superior to unloaded eNP controls at paclitaxel concentrations 10 ng/mL ($p < 0.05$).

3.3 In vivo tumor studies

In vivo anti-tumor efficacy of local administration of Pax-eNP was investigated using two different xenogeneic murine models - one to approximate microscopic residual disease and the other against established *in situ* tumors. As a model of aggressive microscopic residual disease, MDA-MB-231-luc-D3H2LN tumor cells were injected into the mammary fat pad of athymic nude mice with subsequent treatment administered on the same day (Day 0) at the same site (Fig. 2A). The ability of locally injected Pax-eNP to prevent primary tumor growth of a microscopic tumor burden was assessed over the next 5 1/2 weeks. Efficacy of locally injected Pax-eNP (100 μ g, $n = 16$), was compared to an equivalent dose of 4 mg/kg Pax-C/E injected within the mammary fat pad ($n = 12$), a standard dose of 12 mg/kg systemic intraperitoneal Pax-C/E ($n = 12$) or a saline control ($n = 12$). All treatments for this phase of the study were administered at the time of initial tumor cell inoculation on Day 0. As shown in Fig. 2B, *in vivo* growth rates of the primary tumor following Pax-eNP injection were significantly slower than all other treatments including the equivalent dose of Pax-C/E administered locally or the 3-fold higher Pax-C/E dose given systemically (all $p < 0.0001$). In addition, despite administration of only a single dose of Pax-eNP, 75% of Pax-eNP treated mice had no clinical evidence of tumor at the end of the 5 1/2 week study compare to 33% following treatment with local Pax-C/E, 8% following systemic Pax-C/E, and 0% in the saline control group. Among those mice that did develop tumors, the tumor doubling times in mice treated with saline, local Pax-C/E or systemic Pax-C/E were significantly shorter than those treated with Pax-eNP where doubling time was not met for the duration of the study (7.1 days, 5.7 days, and 7.4 days respectively vs. > 38 days in Pax-eNP treated mice; Fig. 2C).

To further test the efficacy of Pax-eNP in a more clinically relevant *in vivo* model of established orthotopic tumors, injected MDA-MB-231-luc-D3H2LN cells were allowed to grow within the mammary fat pad for 7 days prior to the local administration of saline ($n = 12$), Pax-eNP ($n = 16$), or the equivalent dose of 4 mg/kg Pax-C/E ($n = 15$) vs. the intraperitoneal administration of 12 mg/kg Pax-C/E ($n = 12$) as a systemic chemotherapy control (Fig. 3A). Among all four treatment groups, the Pax-eNP treated mice exhibited smaller tumors and significantly slower tumor growth than any other group (Fig. 3B, $p < 0.0001$ Pax-eNP vs. all other treatments). The average tumor doubling time in Pax-eNP treated mice with established tumors was 17.8 days compared to 7.8 days in saline treated or 7 days with either Pax-C/E treatment regardless of total dose or the route of administration (Fig. 3C).

3.4 In vivo lymph node migration and paclitaxel delivery studies

Given the efficacy of Pax-eNP against the primary human breast tumor *in vivo*, we sought to determine if Pax-eNP could also be used to deliver chemotherapy via the local lymphatics to the regional draining LNs, namely the axillary LN basin. To answer this question, Rho-eNP were injected into the mammary fat pad of athymic mice and four days later animals were sacrificed and regional axillary LNs were harvested and processed for confocal microscopy. As shown in Fig. 4, Rho-eNP were readily visible within the ipsilateral axillary LN in 85% of mice examined (11 of 13), confirming that Rho-eNP are able to enter the lymphatic channels, migrate to the regional draining LNs in a short time interval, and remain in the nodal microenvironment for several days. As expected for particles gaining access to the LN

via the afferent lymphatics, Rho-eNP are present within the sinusoidal spaces where nodal micrometastases are commonly described. In contrast, Rho-eNP were not visible on confocal examination of brachial nodes, establishing that the presence of Rho-eNP within the draining axillary LNs is due to migration via directed lymphatic channels from the site of injection, as opposed to systemic distribution of Rho-eNP throughout the body.

Having demonstrated that eNP can traverse the lymphatic channels to the axillary LNs, we encapsulated Oregon Green labeled paclitaxel within Rho-eNP (OGPax-Rho-eNP) to assess whether paclitaxel could be delivered via eNP to the axillary LNs. Given that lymphatic drainage and the kinetics and duration of this regional chemotherapy delivery system may differ in the setting of an established tumor burden and regional lymphatic metastases, MDA-MB-231-luc-D3H2LN tumor xenografts were allowed to grow within the mammary fat pad of nude mice for 14 days before dual-labeled OGPax-Rho-eNP were injected peritumorally ($n = 4$). Animals were sacrificed at 4 or 10 days after eNP injection and the regional LNs were harvested and analyzed using confocal microscopy. Fig. 5A demonstrates the LN architecture with nuclear staining of an ipsilateral axillary LN harvested 10 days after the peritumoral mammary fat pad injection of OGPax-Rho-eNP. The intranodal location of paclitaxel and Rho-eNP within the same LN is documented by the presence of OGPax shown in green (Fig. 5B) and rhodamine signal shown in red (Fig. 5C), respectively, on the same histologic image. The co-localization of paclitaxel and Rho-eNP signals appears yellow in the merged image shown in Fig. 5D. Among eNP visualized within LNs *in situ* $76\% \pm 10\%$ contained OGPax, thereby establishing that paclitaxel is directly delivered to the draining axillary LNs by eNP rather than via lymphatic migration of free drug released from local particles. Furthermore, OGPax-Rho-eNP were abundant within the ipsilateral axillary nodes at both 4 and 10 days, suggesting that paclitaxel delivered via Pax-eNP accumulate within the draining nodes and remain available for drug release for at least several days. Persistence of intranodal paclitaxel following Pax-eNP delivery is of particular interest since the standard half-life of systemically administered paclitaxel has been shown to be only 7 hours at which time $< 0.5\%$ of the administered dose remains within the local tissues [16].

To quantitatively compare the intranodal concentration of paclitaxel as a function of delivery method, ipsilateral regional axillary LNs were harvested four days after the mammary fat pad injection of the 4 mg/kg paclitaxel equivalent of Pax-C/E vs. Pax-eNP and were subject to HPLC analysis. Fig. 5E demonstrates that significantly more paclitaxel is present within the regional LNs, even several days later, when paclitaxel is delivered via Pax-eNP (1575 ± 868 ng/g) as opposed to Pax-C/E (171 ± 31 ng/g, $p < 0.05$).

3.5 In vivo lymph node metastases studies

The above results demonstrate that the local peritumoral injection of eNP loaded with the chemotherapeutic agent paclitaxel, results in the delivery of paclitaxel, for at least several days, to the tumor-draining regional LNs at greatest risk for metastases. Therefore, we investigated whether the higher intranodal drug levels found with Pax-eNP therapy translated into a decreased incidence of nodal metastases. Given the known aggressiveness of MDA-MB-231-luc-D3H2LN for regional LN metastases, this triple negative human breast cancer cell line was chosen to test this hypothesis. A peritumoral injection of 4 mg/kg paclitaxel, given either as Pax-C/E ($n = 8$) or Pax-eNP ($n = 12$), was administered 7 days after inoculation of tumor cells within the mammary fat pad of nude mice (Fig. 6A). Efficacy in the prevention of nodal metastases was compared with controls of unloaded (i.e. empty) eNP ($n = 11$) and 12 mg/kg systemic Pax-C/E administered intraperitoneally ($n = 8$). Animals were followed for 6 1/2 weeks following tumor injection (i.e. 5 1/2 weeks after treatment) or until the size of the primary tumor mandated sacrifice of the animal. At the time of euthanasia, for either reason, axillary LNs from both sides of all animals were assessed via bioluminescent imaging for the presence of occult microscopic tumor cells.

Nodal metastases with significant tumor burden were readily detectable via bioluminescent imaging in animals treated with saline, unloaded eNP, local Pax-C/E or Pax-IP (Fig. 6B), but not in animals treated with Pax-eNP. As shown in Fig. 6C, all tumor-bearing animals treated with unloaded eNP demonstrated evidence of ipsilateral nodal metastases at the time of sacrifice, with many animals requiring early sacrifice for large primary tumors. This is in marked contrast to the 67% of Pax-eNP treated animals that did not exhibit any evidence of nodal metastases even with bioluminescent imaging at the 6 1/2 week timepoint. The administration of an equivalent 4 mg/kg dose of Pax-C/E locally within the mammary fat pad or the standard 12 mg/kg dose of systemic Pax-C/E IP appeared less effective than Pax-eNP at preventing regional LN metastases, with twice the overall incidence of nodal metastases (75% and 62.5% respectively, vs. 33% for Pax-eNP). Pax-eNP treated animals were the only group to demonstrate a statistically significant decrease in the incidence of nodal metastases vs unloaded eNP controls ($p < 0.005$). There was no evidence that systemic Pax-C/E (even at 12 mg/kg) was superior to local Pax-C/E or Pax-eNP given peritumorally at 4 mg/kg. To directly compare the efficacy of Pax-eNP as a function of paclitaxel delivery, additional tumor-bearing animals were treated one week after tumor inoculation, with the local peritumoral injection of an equivalent 4 mg/kg paclitaxel dose administered as Pax-C/E or Pax-eNP ($n = 10/\text{group}$). Five and a half weeks later, the incidence of occult nodal disease was quantified via the degree of bioluminescent signal detected within the ipsilateral regional lymph nodes. As shown in Fig. 6D, bioluminescent signal from harvested LNs was significantly greater in animals treated with Pax-C/E ($29.7 \pm 7.9 \times 10^4$ photons/sec) vs. animals that received Pax-eNP ($8.1 \pm 4.3 \times 10^4$ photons/sec, $p < 0.05$), confirming the presence of significant tumor burden within regional nodes of Pax-C/E treated animals. The distribution of bioluminescent signals among these animals confirms the bimodal population of positive and negative nodes characterized on initial imaging, with the incidence of significant metastatic disease being less frequent among animals treated with Pax-eNP vs. Pax-C/E (20% vs. 60%, respectively). Therefore, when comparing the incidence of metastatic nodal disease among all animals treated locally with 4 mg/kg paclitaxel, Pax-eNP treatment ($n = 22$) results in significantly less metastatic nodal disease than in animals treated with Pax-C/E ($n = 18$) (27% vs. 67%, $p < 0.025$).

4. Discussion

Nanoparticle based drug delivery systems are extensively investigated as a means to increase drug solubility, target specific sites within the body, alter biodistribution, and minimize drug side effects [17-22]. The current study demonstrates that paclitaxel-loaded expansile nanoparticles inhibit the local growth of breast cancer within the mammary fat pad and significantly decrease the development of nodal metastases within the regional lymph node basin that drains the tumor. These results are of clinical importance, as randomized clinical trials have reported a 40% incidence of occult positive nodes in women treated via radical mastectomy alone leading to the practice of axillary node dissection and the resulting clinical problems of lymphedema and increased risk of regional infections. Further advances in clinical care have resulted in breast conserving surgery with the addition of adjuvant radiation therapy for locoregional control in nearly all patients but at the cost of increasing the risk of poor wound healing, chest wall necrosis, and latent secondary malignancies. However, despite these advances and the common use of systemic drug therapy, occult nodal disease continues to be a clinically important problem as evident by the NSABP B-32 clinical trial where the rate of regional node recurrence doubled when regional LNs were not removed via axillary node dissection [6]. It has been postulated that these nodal recurrences occur despite systemic therapy as a result of poor drug delivery to these regional nodes. In support of this hypothesis, drug levels have been shown to be minimal in ipsilateral axillary lymph nodes removed at the time of surgery from patients, even as early as 6 hours after administration of systemic chemotherapy [10]. In addition, intravenous administration of

drug-loaded NPs does not deliver maximal or even sufficient drug to the regional lymph nodes, and the NPs are typically captured by the reticuloendothelial system (RES) and accumulated within the liver and spleen more than within specific regional lymph nodes [23, 24]. We have leveraged these findings to improve the delivery of chemotherapy specifically to the regional draining lymph nodes through the use of Pax-eNP specifically designed to delay particle swelling and resultant drug release until endocytosis within the target cell has occurred. We have found that this mechanism allows sufficient time for lymphatic migration of drug-loaded particles to occur before drug is released, thereby maximizing the local concentration of drug within the regional nodes where it is needed. Furthermore, paclitaxel remains within the nodes for several days as compared to systemically administered paclitaxel, which has been characterized by immediate tissue distribution and rapid clearance to have a biologic half-life of only 7.2 hours [16].

Pax-eNP are pH-responsive polymer expansile nanoparticles designed to expand and release paclitaxel within the mildly acidic pH ($\text{pH} < 5$) found within the cellular endosome of tumor cells [13] and within the microenvironment of some solid tumors [25-27]. Prior *in vitro* studies with Pax-eNP have demonstrated that there is minimal paclitaxel release of drug at physiologic pH, but slow release at $\sim 4\%$ per hour after “pH-triggering” [13]. This is in contrast to non-expansile formulations or conventional poly(lactic-co-glycolic acid) nanoparticles in which drug release occurs quickly with the majority of drug released in a bolus fashion within the first 4-8 hours after administration - long before many nanoparticles may have entered the primary tumor or reached the regional LNs of interest. This limitation is evident in the fact that many nanoparticle systems have demonstrated superior *in vitro* efficacy against various malignant cell lines but this has rarely translated into success *in vivo* [28-31]. The use of pH responsive mechanisms as a means to prolong nanocarrier drug release has been reported for chitosan-silica nanospheres however, despite a two week course of every other day nanosphere injections, only a modest decrease in the growth of local tumor was noted [32]. The clinical importance of developing a nanoparticle capable of intracellular expansion and prolonged drug release are manifest in the ability to accumulate paclitaxel within the cytoplasm of even non-mitotic tumor cells (Fig. 1), and in the superior efficacy evident in the prevention of microscopic and established tumor growth *in vivo* with only a single dose of Pax-eNP (Fig. 2 and 3). The continued intracellular presence of Pax-eNP within breast cancer cells at 24 hours, as evident with confocal imaging and flowcytometric analysis, suggests that eNP swelling delays eNP exocytosis from the tumor cell thereby increasing the duration of intracellular drug exposure and likelihood that the drug will be present during tumor cell division. This mechanism is supported by the superior inhibition of established tumor growth noted following treatment with Pax-eNP at a dose of only 4 mg/kg as compared to the systemic administration of paclitaxel at 12 mg/kg.

In addition to the effective treatment of local tumor, the current data also demonstrate direct migration of Pax-eNP to regional draining lymph nodes at greatest risk of metastasis, without the need for unique peptides or magnet application to assist in intranodal localization as reported with other systems [33, 34]. In order to maximize the concentration of chemotherapy within the tumor-draining lymph nodes, and thus maximize efficacy in the treatment of occult nodal metastases, we envision two essential properties that nanoparticles (NP) must possess. First, NP must be able to migrate via the lymphatic channels in an efficient manner from the site of tumor to the regional lymph node basin. Second, the chemotherapeutic should remain within the NP until the NP has migrated and entered the draining lymph node. Many investigators have demonstrated that lymphatic migration of particles is largely determined by particle size, surface charge, and composition, with lymphatic migration optimized in particles with diameters of 30- 100 nm and a negative surface charge [35, 36]. We have confirmed that these parameters are similar for eNP even within a large animal model, as 50 nm eNP demonstrate more efficient migration to draining

lymph nodes than 100nm particles with a less negative zeta potential [12]. Although nanoparticle drug delivery systems such as crosslinked-chitosan hydrogels loaded with ¹³¹I or ErbB-conjugated chitosane-silica nanospheres have demonstrated decreased local growth of breast cancer implants, neither demonstrated direct delivery of drug to the regional lymph node nor were nodal metastases examined [32, 37]. Several other nanocarrier systems have been investigated specifically with the goal of developing potential approaches for lymphatic delivery but all were limited in terms of histologic analysis and/or anti-tumor efficacy. Lu et al was one of the earliest studies to examine the efficacy of lipid nanoparticles against breast cancer, but P388, a murine leukemia line, was utilized to assess LN metastases with qualitative assessment of response based on LN size rather than tumor burden [38]. One of the most widely studied systems is that of a hyaluronan-cisplatin conjugate, in which non-covalent cisplatin was detected in regional nodes for approximately 48 hours following subcutaneous injection in the mammary fat pad [39]. Although multiple weekly doses of the cisplatin conjugate resulted in an initial delay in growth of established local breast cancer implants, it was not superior to intravenous administration of cisplatin and no data on lymphatic metastases were reported [40]. A recent study of this nanoconjugate in a murine model of locoregional head and neck squamous cell carcinoma demonstrated improved local control of the primary tumor and presumably nearby lymph nodes, although these were not examined separately [41]. Neither study demonstrated histologic evidence of lymphatic migration of the nanoconjugate, nodal uptake, nor the individual presence of drug and polymer within the nearby nodes. Lastly, the delivered drug did not appear to remain within the regional node beyond 48 hours, which is a major shortcoming in the treatment of occult nodal disease and explains the need for multiple weekly doses. In contrast, the current study demonstrates that a single dose of Pax-eNP results in a) the lymphatic migration and nodal deposition of both the eNP nanocarrier and the drug, b) a nearly 10-fold increase in intranodal paclitaxel concentration as compared to Pax-C/E alone, and c) the prolonged intranodal presence of paclitaxel for at least 10 days. To our knowledge this is the longest duration of intranodal nanoparticle-mediated drug delivery with documented efficacy to decrease nodal metastases.

More important than demonstrating intranodal drug delivery, was the assessment of Pax-eNP in the prevention of nodal metastases. Given the lack of tumor-bearing models in swine, we utilized Pax-eNP in an aggressive murine model of triple-negative metastatic breast cancer. We utilized a luciferase-transfected human breast cancer line to increase the sensitivity and allow for detection of occult disease often missed on histologic analysis. Using this sensitive model, a single dose of Pax-eNP at the “subtherapeutic” dose of 4 mg/kg paclitaxel resulted in a decrease in lymph node metastases by over 60% as assessed 45 days after tumor inoculation. To our knowledge, this is the first study to establish superior efficacy of a nanoparticle-mediated delivery system in the treatment of occult nodal disease. Therefore, in addition to treating local disease, the current study has demonstrated the feasibility and superior efficacy of drug-loaded nanoparticle migration to regional draining LNs at greatest risk for developing metastases. Confocal imaging of resected lymph nodes demonstrated prolonged co-localization of paclitaxel and eNP within the lymph nodes, which correlated with significantly higher intranodal HPLC drug concentrations, and a significant decrease in the incidence of lymph node metastases compared to animals treated with local or systemic paclitaxel. A potential limitation of this study is the local administration of the Pax eNPs at the site of the original tumor as opposed to an intravenous administration of Pax eNPs. However, as discussed earlier, intravenous administration of Pax does not afford significant drug levels at the lymph nodes. Likewise, intravenous administration of drug-loaded NPs results in uptake via the RES and a significant amount of drug being distributed across other non-tumor bearing sites rather than concentrated within the lymph nodes, where disease is present. Consequently, development of a locoregional responsive NP delivery system, such as the one described in the manuscript, is potentially

translatable to clinical care since higher concentrations of drug are delivered directly to the regional tumor-draining lymph nodes and the procedure is clinically feasible.

Conclusions

In the current study, local administration of Pax-eNP resulted in the effective inhibition of local tumor control and superior prevention of lymph node metastasis in murine models of microscopic and established triple negative human breast cancer. Given the clinical impact of locoregional recurrence and the morbidity of current surgical and adjuvant therapies, particularly among women with triple negative breast cancer, the marked regional efficacy of only a single dose of Pax-eNP warrants additional studies to further explore nanoparticle pharmacokinetics, clinical feasibility, and biological long-term safety of eNP-mediated *in situ* nodal drug targeting.

Acknowledgments

This work was supported in part by the Center for Integration of Medicine and Innovative Technologies (CIMIT #07-004), National Science Foundation (DMR-1006601 and CNS-0958345), the Boston University's Nanomedicine Program and Cross-Disciplinary Training in Nanotechnology for Cancer (NIH R25 CA153955), Brigham and Women's Hospital, and Boston University. The authors wish to express appreciation for the excellent care provided by the staff at the Animal Resources Facility at Dana-Farber Cancer Institute, and to acknowledge the contributions of the Confocal Core Facility at Beth Israel Deaconess Medical Center under the direction of Lay-Hong Ang.

References

1. Forouzanfar MH, Foreman KJ, Delossantos AM, Lozano R, Lopez AD, Murray CJ, et al. Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. *Lancet*. Oct 22; 2011 378(9801):1461–1484. [PubMed: 21924486]
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA: a cancer journal for clinicians*. Mar-Apr;2011 61(2):69–90. [PubMed: 21296855]
3. Fisher B, Jeong JH, Anderson S, Bryant J, Fisher ER, Wolmark N. Twenty-five-year follow-up of a randomized trial comparing radical mastectomy, total mastectomy, and total mastectomy followed by irradiation. *N Engl J Med*. Aug 22; 2002 347(8):567–575. [PubMed: 12192016]
4. Fleissig A, Fallowfield LJ, Langridge CI, Johnson L, Newcombe RG, Dixon JM, et al. Post-operative arm morbidity and quality of life. Results of the ALMANAC randomised trial comparing sentinel node biopsy with standard axillary treatment in the management of patients with early breast cancer. *Breast cancer research and treatment*. Feb; 2006 95(3):279–293. [PubMed: 16163445]
5. Schrenk P, Rieger R, Shamiyeh A, Wayand W. Morbidity following sentinel lymph node biopsy versus axillary lymph node dissection for patients with breast carcinoma. *Cancer*. Feb 1; 2000 88(3):608–614. [PubMed: 10649254]
6. Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Costantino JP, et al. Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial. *The lancet oncology*. Oct; 2010 11(10):927–933. [PubMed: 20863759]
7. Weaver DL, Ashikaga T, Krag DN, Skelly JM, Anderson SJ, Harlow SP, et al. Effect of occult metastases on survival in node-negative breast cancer. *N Engl J Med*. Feb 3; 2011 364(5):412–421. [PubMed: 21247310]
8. Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans E, et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet*. Dec 17; 2005 366(9503):2087–2106. [PubMed: 16360786]
9. Darby S, McGale P, Correa C, Taylor C, Arriagada R, Clarke M, et al. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of

- individual patient data for 10,801 women in 17 randomised trials. *Lancet*. Nov 12; 2011 378(9804): 1707–1716. [PubMed: 22019144]
10. Chen J, Wang L, Yao Q, Ling R, Li K, Wang H. Drug concentrations in axillary lymph nodes after lymphatic chemotherapy on patients with breast cancer. *Breast Cancer Res*. 2004; 6(4):R474–477. [PubMed: 15217515]
 11. Colson YL, Liu R, Southard EB, Schulz MD, Wade JE, Griset AP, et al. The performance of expansile nanoparticles in a murine model of peritoneal carcinomatosis. *Biomaterials*. Jan; 2011 32(3):832–840. [PubMed: 21044799]
 12. Khullar OV, Griset AP, Gibbs-Strauss SL, Chirieac LR, Zubris KA, Frangioni JV, et al. Nanoparticle migration and delivery of Paclitaxel to regional lymph nodes in a large animal model. *Journal of the American College of Surgeons*. Mar; 2012 214(3):328–337. [PubMed: 22225645]
 13. Griset AP, Walpole J, Liu R, Gaffey A, Colson YL, Grinstaff MW. Expansile nanoparticles: synthesis, characterization, and in vivo efficacy of an acid-responsive polymeric drug delivery system. *Journal of the American Chemical Society*. Feb 25; 2009 131(7):2469–2471. [PubMed: 19182897]
 14. Jenkins DE, Hornig YS, Oei Y, Dusich J, Purchio T. Bioluminescent human breast cancer cell lines that permit rapid and sensitive in vivo detection of mammary tumors and multiple metastases in immune deficient mice. *Breast Cancer Res*. 2005; 7(4):R444–454. [PubMed: 15987449]
 15. Yen MS, Juang CM, Lai CR, Chao GC, Ng HT, Yuan CC. Intraperitoneal cisplatin-based chemotherapy vs. intravenous cisplatin-based chemotherapy for stage III optimally cytoreduced epithelial ovarian cancer. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics*. Jan; 2001 72(1):55–60. [PubMed: 11146078]
 16. Sparreboom A, Scripture CD, Trieu V, Williams PJ, De T, Yang A, et al. Comparative preclinical and clinical pharmacokinetics of a cremophor-free, nanoparticle albumin-bound paclitaxel (ABI-007) and paclitaxel formulated in Cremophor (Taxol). *Clin Cancer Res*. Jun 1; 2005 11(11): 4136–4143. [PubMed: 15930349]
 17. Moghimi SM, Hunter AC, Andresen TL. Factors controlling nanoparticle pharmacokinetics: An integrated analysis and perspective. *Annu Rev Pharmacol Toxicol*. 2011; 52:481–503. [PubMed: 22035254]
 18. Davis ME, Chen Z, Shin DM. Nanoparticle therapeutics: An emerging treatment modality for cancer. *Nat Rev Drug Discov*. 2008; 7:771–782. [PubMed: 18758474]
 19. Wang AZ, Langer RS, Farokhzad OC. Nanoparticle delivery of cancer drugs. *Annu Rev Med*. 2011; 63:185–198. [PubMed: 21888516]
 20. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nature Rev Drug Discov*. 2005; 4:145–160. [PubMed: 15688077]
 21. Blanco E, Hsiao A, Ruiz-Esparza GU, Landry MG, Meric-Bernstam F, Ferrari M. Molecular-targeted nanotherapies in cancer: Enabling treatment specificity. *Mol Oncol*. 2011; 5:492–503. [PubMed: 22071376]
 22. Li T, Hu W, Liu Y, Huang G, Sumer BD, Gao J. Shape-specific polymeric nanomedicine: Emerging opportunities and challenges. *Exp Biol Med*. 2011; 236:20–29.
 23. Ernsting MJ, Tang WL, MacCallum NW, Li SD. Preclinical pharmacokinetic, biodistribution, and anti-cancer efficacy studies of a docetaxel-carboxymethylcellulose nanoparticle in mouse models. *Biomaterials*. Feb; 2012 33(5):1445–1454. [PubMed: 22079003]
 24. Wang X, Zhao G, Van S, Jiang N, Yu L, Vera D, et al. Pharmacokinetics and tissue distribution of PGG-paclitaxel, a novel macromolecular formulation of paclitaxel, in nu/nu mice bearing NCI-460 lung cancer xenografts. *Cancer chemotherapy and pharmacology*. Feb; 2010 65(3):515–526. [PubMed: 19593566]
 25. Yang Q, Wang S, Fan P, Wang L, Di Y, Lin K, et al. pH-Responsive Carrier System Based on Carboxylic Acid Modified Mesoporous Silica and Polyelectrolyte for Drug Delivery. *Chemistry of Materials*. 2005; 17(24):5999–6003. 11/01 2012/07/26.
 26. Thistlethwaite AJ, Leeper DB, Moylan DJ 3rd, Nerlinger RE. pH distribution in human tumors. *International journal of radiation oncology, biology, physics*. Sep; 1985 11(9):1647–1652.

27. Kallinowski F, Vaupel P. pH distributions in spontaneous and isotransplanted rat tumours. *British journal of cancer*. Sep; 1988 58(3):314–321. [PubMed: 3179183]
28. Coleman RL, Brady WE, McMeekin DS, Rose PG, Soper JT, Lentz SS, et al. A phase II evaluation of nanoparticle, albumin-bound (nab) paclitaxel in the treatment of recurrent or persistent platinum-resistant ovarian, fallopian tube, or primary peritoneal cancer: a Gynecologic Oncology Group study. *Gynecologic oncology*. Jul; 2011 122(1):111–115. [PubMed: 21497382]
29. Sawicki JA, Anderson DG, Langer R. Nanoparticle delivery of suicide DNA for epithelial ovarian cancer therapy. *Advances in experimental medicine and biology*. 2008; 622:209–219. [PubMed: 18546630]
30. Sloat BR, Sandoval MA, Li D, Chung WG, Lansakara PD, Proteau PJ, et al. In vitro and in vivo anti-tumor activities of a gemcitabine derivative carried by nanoparticles. *International journal of pharmaceutics*. May 16; 2011 409(1-2):278–288. [PubMed: 21371545]
31. Werner ME, Karve S, Sukumar R, Cummings ND, Copp JA, Chen RC, et al. Folate-targeted nanoparticle delivery of chemo- and radiotherapeutics for the treatment of ovarian cancer peritoneal metastasis. *Biomaterials*. Nov; 2011 32(33):8548–8554. [PubMed: 21843904]
32. Deng Z, Zhen Z, Hu X, Wu S, Xu Z, Chu PK. Hollow chitosan-silica nanospheres as pH-sensitive targeted delivery carriers in breast cancer therapy. *Biomaterials*. Jul; 2011 32(21):4976–4986. [PubMed: 21486679]
33. Luo G, Yu X, Jin C, Yang F, Fu D, Long J, et al. LyP-1-conjugated nanoparticles for targeting drug delivery to lymphatic metastatic tumors. *International journal of pharmaceutics*. Jan 29; 2010 385(1-2):150–156. [PubMed: 19825404]
34. Yang F, Jin C, Yang D, Jiang Y, Li J, Di Y, et al. Magnetic functionalised carbon nanotubes as drug vehicles for cancer lymph node metastasis treatment. *Eur J Cancer*. Aug; 2011 47(12):1873–1882. [PubMed: 21493061]
35. Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. *Nature biotechnology*. Oct; 2007 25(10):1159–1164.
36. Rao DA, Forrest ML, Alani AW, Kwon GS, Robinson JR. Biodegradable PLGA based nanoparticles for sustained regional lymphatic drug delivery. *Journal of pharmaceutical sciences*. Apr; 2010 99(4):2018–2031. [PubMed: 19902520]
37. Azab AK, Kleinstern J, Doviner V, Orkin B, Srebnik M, Nissan A, et al. Prevention of tumor recurrence and distant metastasis formation in a breast cancer mouse model by biodegradable implant of ¹³¹I-norcholesterol. *J Control Release*. Nov 6; 2007 123(2):116–122. [PubMed: 17854940]
38. Lu B, Xiong SB, Yang H, Yin XD, Chao RB. Solid lipid nanoparticles of mitoxantrone for local injection against breast cancer and its lymph node metastases. *Eur J Pharm Sci*. May; 2006 28(1-2):86–95. [PubMed: 16472996]
39. Cai S, Xie Y, Bagby TR, Cohen MS, Forrest ML. Intralymphatic chemotherapy using a hyaluronan-cisplatin conjugate. *The Journal of surgical research*. Jun 15; 2008 147(2):247–252. [PubMed: 18498877]
40. Cohen MS, Cai S, Xie Y, Forrest ML. A novel intralymphatic nanocarrier delivery system for cisplatin therapy in breast cancer with improved tumor efficacy and lower systemic toxicity in vivo. *American journal of surgery*. Dec; 2009 198(6):781–786. [PubMed: 19969129]
41. Cai S, Xie Y, Davies NM, Cohen MS, Forrest ML. Carrier-based intralymphatic cisplatin chemotherapy for the treatment of metastatic squamous cell carcinoma of the head & neck. *Therapeutic delivery*. Aug; 2010 1(2):237–245. [PubMed: 21339844]

Fig. 1.

Intracytoplasmic translocation and subsequent anti-tumor efficacy of expansile nanoparticles (eNP) against human breast cancer cells. (A) Confocal microscopic images with Z-scans of MDA-MB-231-luc-D3H2LN cells incubated in culture media containing Rhodamine B-labeled eNP (Rho-eNP) for 8 hrs. Red signal: Rho-eNP; blue: nucleus; green: cell membrane with wheat germ agglutinin staining. (B) Flowcytometric analysis of Rho-eNP uptake over 24 hrs. (C) Paclitaxel-loaded eNP prevent MDA-MB-231-luc-D3H2LN cell growth in an *in vitro* cytotoxicity assay ($n = 4$ separate experiments). Both Pax-eNP and Pax-C/E are significantly different than unloaded eNP. * $P < 0.05$ vs. unloaded eNP.

Fig. 2.

Pax-eNP demonstrates superior efficacy in inhibiting growth of microscopic primary tumor burden as compared to local or systemic Pax-C/E delivery. **(A)** *In vivo* experimental design. Mice receive mammary fat pad tumor injection and paclitaxel therapy locally at the same site (equivalent of 4 mg/kg) or via systemic intraperitoneal (IP) injection (12 mg/kg) on the same day of tumor injection (Day 0). **(B)** Pax-eNP significantly inhibits primary tumor growth rate as compared to all other treatment groups. * $P < 0.0001$ Pax-eNP vs. all other groups respectively. ** $P < 0.0001$ Pax-C/E local vs. Pax-C/E IP or Saline, respectively. **(C)** Doubling time of primary tumor volume calculated from 3 weeks following treatment highlights efficacy of Pax-eNP therapy. * doubling time not reached.

Fig. 3.

Local administration of Pax-eNP prevents growth of established primary tumor. (A). *In vivo* experimental design of established tumor and lymph node metastases model. (B). Pax-eNP local delivery significantly decreases tumor growth rate compared to an equivalent dose of Pax-C/E injected locally or even a larger systemic IP dose ($P < 0.0001$ Pax-eNP vs. all other groups) (C). Doubling time of primary tumor volume calculated after 3 wks of treatment confirms superiority of Pax-eNP therapy.

Fig. 4. Representative confocal micrograph of ipsilateral axillary LN confirming nodal migration of Rho-eNP (red) 4 days after nanoparticle injection into the murine mammary fat pads. The cell nuclei of individual cells within the LN are stained blue with DAPI to highlight nodal architecture. **(A)** Visualization at 10× demonstrates presence of eNP throughout the node, including presence within the sinusoids. **(B)** Magnification at 40× better identifies diffused distribution of Rho-eNP in LN parenchyma.

Fig. 5.

Paclitaxel is delivered via eNP within the cortex of regional lymph node (LN). Dual-labeled OGPax-Rho-eNP nanoparticles were injected 10 days prior to harvest and confocal analysis of the ipsilateral axillary LNs. Confocal images demonstrate nodal architecture with nuclei seen as blue (**A**), and the intranodal presence of Oregon green labeled paclitaxel viewed as green (**B**) and rhodamine-labeled eNP viewed as red (**C**). In the merged image (**D**), yellow color represents the colocalization of OGPax and Rho-eNP within the cortex, demonstrating that the payload drug is carried into the regional LN via eNP and remains present for at least 10 days following administration. (**E**) HPLC analysis confirms that Pax-eNP results in significantly higher intranodal paclitaxel concentration compared to local administration of an equivalent Pax-C/E dose.

Fig. 6.

Peri-tumoral administration of Pax-eNP decreases the incidence of axillary LN metastases.

(A) *In vivo* experimental design of established tumor and lymph node metastases model. (B) Representative bioluminescent images of bilateral axillary LN ex vivo 6 1/2 weeks following tumor inoculation and 5 1/2 weeks following the designated peri-tumoral treatment on the ipsilateral side or systemic paclitaxel IP therapy. Composite grayscale and bioluminescent color scale images demonstrate evidence of occult metastatic disease in all treatment groups except Pax-eNP treated animals. (C) Incidence of LN metastases as determined via detection of positive bioluminescent signal within ipsilateral LN. Pax-eNP was the only treatment to significantly decrease the incidence of lymph node metastases (33%) compared to 100% in the unloaded-eNP group ($P < 0.005$). (D) Quantitative comparison of bioluminescent signal within ipsilateral LN as a function of Pax therapy demonstrates significantly less evidence of metastatic nodal disease in Pax-eNP treated animals, $P < 0.05$.