Published in final edited form as:

J Control Release. 2014 June 28; 184: 67–78. doi:10.1016/j.jconrel.2014.03.049.

Cationic lipid guided short-hairpin RNA interference of annexin A2 attenuates tumor growth and metastasis in a mouse lung cancer stem cell model

Terrick Andey^{a,1}, Srujan Marepally^{a,1}, Apurva Patel^a, Tanise Jackson^a, Shubhashish Sarkar^b, Malaney O'Connell^b, Rakesh C Reddy^c, Srikumar Chellappan^d, Pomila Singh^b, and Mandip Singh^{a,*}

^aDepartment of Pharmaceutics, College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, 1520 South Martin Luther King Jr. Blvd., Tallahassee, FL 32307, USA

^bDepartment of Neurosciences and Cell Biology, University of Texas Medical Branch, Galveston, TX, USA

^cIICT, Hyderabad, Andra Pradesh, India

^dDepartment of Tumor Biology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

Abstract

The role of side populations (SP) or cancer stem-like cells (CSC) in promoting the resistance phenotype presents a viable anticancer target. Human-derived H1650 SP cells over-express annexin A2 (AnxA2) and SOX2, and are resistant to conventional cytotoxic chemotherapeutics. AnxA2 and SOX2 bind to proto-oncogenes, c-Myc and c-Src, and AnxA2 forms a functional heterotetramer with S100A10 to promote tumor motility. However, the combined role of AnxA2, S100A10 and SOX2 in promoting the resistant phenotype of SP cells has not been investigated. In the current studies, we examined for the first time a possible role of AnxA2 in regulating SA100A10 and SOX2 in promoting a resistant phenotype of lung tumors derived from H1650 SP cells.

The resistance of H1650 SP cells to chemotherapy compared to H1650 MP cells was investigated by cell viability studies. A short hairpin RNA targeting AnxA2 (shAnxA2) was formulated in a liposomal (cationic ligand-guided, CLG) carrier and characterized for size, charge and entrapment and loading efficiencies; CLG carrier uptake by H1650 SP cells was demonstrated by fluorescence microscopy, and knockdown of AnxA2 confirmed by qRT-PCR and Western blot. Targeting of xenograft and orthotopic lung tumors was demonstrated with fluorescent (DiR) CLG carriers in mice. The therapeutic efficacy of CLG-AnxA2, compared to that of placebo, was investigated after 2 weeks of treatment in terms of tumor weights and tumor burden *in vivo*.

*Corresponding author at: Department of Pharmaceutics, Florida A&M University, 1520 South Martin Luther King Jr. Blvd., Dyson Pharmacy Bldg. Room 021, Tallahassee, FL 32307, USA. Tel.: +1 850 345 2754; fax: +1 850 599 3813. mandip.sachdeva@gmail.com (M. Singh).

¹Equally contributing authors.

Compared to mixed population cells, H1650 SP cells showed exponential resistance to docetaxel (15-fold), cisplatin (13-fold), 5-fluorouracil (31-fold), camptothecin (7-fold), and gemcitabine (16-fold). CLG carriers were nanoparticulate (199 nm) with a slight positive charge (21.82 mV); CLG-shAnx2 was of similar size (217 nm) with decreased charge (12.11 mV), and entrapment and loading efficiencies of 97% and 6.13% respectively. Fluorescence microscopy showed high uptake of CLG-shAnxA2 in H1650 SP cells after 2 h resulting in a 6-fold reduction in AnxA2 mRNA expression and 92% decreased protein expression. Fluorescence imaging confirmed targeting of tumors and lungs by DiR-CLG carriers with sustained localization up to 4 h in mice. CLG-shAnxA2 treatment of mice significantly reduced the weights of lung tumors derived from H1650 SP cells and tumor burden was reduced to only 19% of controls. The loss in tumor weights in response to CLG-shAnxA2 was associated with a significant loss in the relative levels of AnxA2, SOX2, total β -catenin and S100A10, both at the RNA and protein levels. These results suggest the intriguing possibility that AnxA2 may directly or indirectly regulate relative levels of β -catenin, S100A10 and SOX2, and that the combination of these factors may contribute to the resistant phenotype of H1650 SP cells. Thus down-regulating AnxA2 using RNAi methods may provide a useful method for targeting cancer stem cells and help advance therapeutic efficacy against lung cancers.

Keywords

Annexin A2 shRNA; Lung cancer stem cell; Tumor resistance; Liposome delivery; RNA interference; Gene delivery

1. Introduction

Cancer chemotherapy is associated with growing evidence of tumor resistance to conventional cytotoxics. This is further compounded by serious adverse effects which limit the range of their applications and efficacies. With a total predicted annual incidence and mortality of 228,190 and 159,480 people, respectively, and a 5-year survival rate of only 3.0% for patients with metastatic (distant) lung cancer [1], innovative therapies and approaches are necessary.

A side population (SP) of tumor cells with stem cell-like properties, also known as cancer stem cells (CSCs), have been proposed to play a critical role in metastatic progression of cancers and their resistance to commonly used chemotherapeutic agents [2]. CSCs are enriched in embryonic stem cell markers (pluripotent factors), such as alkaline phosphatase, CD9, CD15, CD24, integrin β 1, integrin α 6, c-Myc, E-cadherin, Nanog, Oct3/4, and SOX2 [3]. A major challenge in targeting these cells is overcoming the development of drug resistance; the CSCs are enriched with several drug-resistant markers including efflux pumps (P-glycoprotein, MRP1-6, and ABCG2) and self-renewal factors (Oct4, SOX2, Nanog) which make them unresponsive to chemotherapy [4, 5]. SP cells from various non-small cell lung cancer (NSCLC) cell lines, including H1650 MP, and tumor explants isolated and characterized at the Moffitt Cancer Center suggest the involvement of the transcription factor SOX2 in the maintenance of stemness of H1650 SP cells [6].

H1650 SP cells also overexpress the cell surface associated annexin A2 (CS-AnxA2), which is implicated in mediating many cell survival processes including angiogenesis, cell motility and migration [7,8]. The importance of AnxA2 in cancer promotion is underscored by its post-translational binding to the mRNA of the nuclear oncogene c-Myc [9]. CS-AnxA2 and membrane associated AnxA2 also interacts with S100A10 and SNARE proteins to regulate cytoskeletal organization and endosome fusion/exocytosis respectively [10]. Cytosolic AnxA2 is phosphorylated by Src at its tyrosine 23 site subsequent to membrane translocation where it mediates cancer cell motility and invasion [11, 12]. However, to our knowledge, an argument for the role of AnxA2 *in vivo* in promoting the resistance phenotype in CSCs has not been made. In order to test this hypothesis, an appropriate delivery system for RNA interference (RNAi) is needed.

RNA interference was demonstrated in mammalian cells [13] and the use of RNAi as molecular therapies to specifically target genes and oncogenes involved in tumorigenesis is a vibrant enterprise in oncology. Advancement in RNAi is evident from the successful human trials with RNAi, targeting VEGF and kinesin spindle protein (KSP) in patients with primary and metastatic tumors [14]. A 2012 Phase 1 clinical trial of a similar small interfering RNA (siRNA), ALN-VSP02 targeting colorectal cancer with liver involvement (<http://clinicaltrials.gov/show/NCT01158079>) in 15 patients showed promising results. Notwithstanding these successes, the challenges to successful RNAi delivery are many; safety, stability, and successful delivery of exogenous small interfering/short hairpin RNA (si/shRNA) and specificity in tissue targeting must be addressed.

Lipid-based nanoparticle delivery systems have demonstrated efficacy as carriers of si/shRNA; however, the benefits of these delivery systems must be balanced with their safety [15]. Cationic lipid based delivery systems have been used for RNAi and their efficiency in incorporating and delivering si/shRNA to target tissues has been shown; carriers, therefore, provide a good platform for *in vivo* RNAi delivery. The usual route for administering liposomal RNAi is intravenous (i.v.). But intraperitoneal (i.p.) administration of carriers also shows systemic bioavailability, which is ~95% of that by i.v. [16], with successful uptake of the therapeutic RNA/DNA molecules [17,18]. The primary aim of this study is to establish the significance of AnxA2 in contributing to the resistant phenotype of CSCs. Our hypothesis is that a cationic lipid based delivery vehicle will enhance the uptake and efficacy of shAnxA2, and inhibit the growth of lung tumors derived from CSC/SP populations in a mouse model. We tested this hypothesis by formulating and optimizing a cationic lipid guided carrier for the delivery of shAnxA2 (CLG-shAnxA2) to orthotropic lung tumors in mice. We also investigated the effects of shAnxA2 on the modulation of molecular markers involved in metastatic progression.

2. Materials and methods

2.1. Chemicals

Non-specific and gene-specific short-hairpin RNA (shRNA) were purchased from Open Biosystem (RHS4430, Thermo Scientific, Pittsburgh, USA). AnxA2 shRNA was provided in a GIPZ lentiviral vector containing a neomycin mammalian selection marker. Out of 5 clones screened, V3LHS_636112 clone was found to downregulate AnxA2 most effectively

(~70–80%). Empty vector without shRNA was used as a control. Primer sequences (sense 5'-AGACGCTGGGAAGAAGGCTTCCT-3' and antisense 5'-TGTGCATTGCTGCGGTTGGTCA-3') for targeting AnxA2 (shAnxA2) were developed in our laboratory at the University of Texas Medical Center.

The non-specific shRNA and primers possessed neither relevant homologies nor functional physiology and were used as negative controls. L- α -phosphatidylcholine (L- α -lecithin), cholesterol, laminin, and poly-D-lysine were procured from Sigma-Aldrich (St. Louis, MO); AC-2 cationic amide lipid was a kind gift from Dr. Arabinda Chaudhuri (Indian Institute of Chemical Technology, Hyderabad); 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG(2000)) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) was purchased from Life Technologies (Grand Island, NY). Anti-antibodies against AnxA2, S100A10, β -Catenin, NF- κ B, c-Myc, Slug and SOX2 were procured from Cell Signaling (Danvers, MA). FITC-conjugated control siRNA, primary antibodies against MMP7, anti-rabbit, anti-goat, anti-mouse secondary antibodies, and ImmunoCruz® staining ABC staining system were purchased from Santa Cruz Biotechnology (Dallas, TX). SYBR Gold stain was obtained from Life Technologies. All other chemicals used were of reagent grade.

2.2. Cell lines

H1650 mixed population cells (MP) and side population cells (SP)/cancer stem-like cells (CSCs) were generously donated by Dr. Srikumar Chellappan of the H. Lee Moffitt Cancer Center and Research Institute (Tampa, FL). H1650 MP cells were maintained in DMEM:F12 media fortified with 10% fetal bovine serum (FBS) and a 2% penicillin/streptomycin/neomycin (PSN) cocktail. H1650 SP cells were cultured in DMEM:F12 base medium enriched with fibroblast and epidermal growth factors (10 μ g/ml) and 2% PSN cocktail. Cells were maintained at 37 °C under an atmosphere of 95% air and 5% CO₂. H1650 SP cells were cultured on a basement membrane-coated matrix consisting of immobilized laminin on a poly-D-lysine layer.

2.3. H1650 SP cell viability

H1650 MP or SP cells were seeded in a 96-well format (1×10^4 per well) and incubated for 16–18 h. Treatment was carried out for 72 h with different concentrations of cisplatin, docetaxel, gemcitabine, fluorouracil and camptothecin. The cells were washed with PBS 2 $\times$ and fixed in 0.1 ml glutaraldehyde solution (0.025% w/v) and incubated at 37 °C for 30 min. Glutaraldehyde was aspirated and 0.1 ml crystal violet solution (0.01% w/v) was added and incubated at room temperature for 15 min. Crystal violet solution was aspirated followed by 2 washes with PBS; the plates were air-dried and disodium hydrogen phosphate solution was added to dissolve the crystal violet. The absorbance of crystal violet was read at 540 nm and the cell viability was calculated as a percentage of the control. Determinations of cell viability were made at least 3 $\times$ and the data presented as mean $\pm$ SD.

2.4. Preparation of cationic ligand guided carriers for delivering shAnxA2

Cationic ligand guided (CLG)-carriers were prepared by the ethanol-injection (EI) method incorporating L- α -phosphatidylcholine, cholesterol, AC-2 cationic amide, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lisamine rhodamine B sulfonyl), and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) in a molar ratio of 3:3.5:1.5:1.1 ratio. The lipids were dissolved in ethanol (total lipid content of 10 mM). The lipid mixture was injected into 20 mM HEPES buffer (pH 7.4) under stirring at room temperature (RT). Excess ethanol and unbound dye were removed by dialyzing across a concentration gradient in PBS (pH 7.4) or 20 mM HEPES buffer (pH 7.4), respectively, using a Fisherbrand® Dialysis tubing with molecular weight cut-off of 6000–8000 for 2 h at RT. The carriers (i.e. EI-carriers) were filter-sterilized by passing through a 0.2 μ m filter. Carriers were characterized for hydrodynamic particle diameter and zeta potential.

CLG carriers were prepared by the thin-film hydration (TFH) method incorporating L- α -phosphatidylcholine, cholesterol, Ac-2 cationic amide, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lisamine rhodamine B sulfonyl), and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) in a molar ratio of 3:3.5:1.5:1.1 ratio. Geleol mono- and diglycerides NF (at a final concentration of 1 mg/ml of vehicle) was dispersed with the lipids in a 1:1 volume mixture of methanol and chloroform, respectively (total lipid content of 10 mM), and dried to a thin layer of film, under nitrogen stream. The film was hydrated for 8 h with 4 ml distilled water to obtain a total lipid content of 2.5 mM. The CLG carrier was bath-sonicated for 15 min and passed through a 0.2 μ m syringe-filter 5 times; and purified by dialysis.

Amide linker based cationic lipid in combination with L- α -phosphatidylcholine, cholesterol, and pegylated-phosphoethanolamine (PE-PEG 2000) at molar ratios of 3:3.5:1.5:2 was dispersed in a methanol– chloroform mixture (1:1 volume ratio) (total lipid content of 10 mM), in a round-bottom flask and dried to a thin film. The thin film was hydrated for 8 h with 4 ml distilled water containing AnxA2 shRNA, mock shRNA or FITC-conjugated control siRNA at a total lipid content of 2.5 mM. The CLG carrier preparation (i.e. CLG-carrier) was bath-sonicated for 15 min and passed through a 0.2 μ m syringe-filter 5 times (Fig. 1).

2.5. Characterization of shRNA-loaded carriers

The formulations were characterized for hydrodynamic size, zeta potential, entrapment efficiency, and serum stability. The CLG carriers were diluted to a final lipid concentration of 0.25 mM in PBS (pH 7.4) and hydrodynamic diameter and zeta potential were determined by dynamic light scattering and surface charge measurements using Nicomp 380 ZLS (Particle Sizing System, Santa Barbara, CA). CLG carrier encapsulating AnxA2 shRNA was centrifuged at 20,000 $\times g$ at 4 °C for 30 min. The supernatant was collected and stained with SYBR gold dye at an optimized concentration of 1:10,000 of the stock in DMSO (at a final volume of 25 μ l). Fluorescence reading of the shRNA–dye complex mixture was taken at excitation and emission wavelengths of 495 nm and 590 nm respectively. The amount of shRNA was interpolated from a standard plot of fluorescence intensity with an amount of nucleic acid and the entrapment efficiency was calculated as follows:

$$\text{Entrapment efficiency, EE(\%)} = 100 \times (\text{shRNA}_{\text{sta}} - \text{shRNA}_{\text{rec}}) / \text{shRNA}_t$$

where $\text{shRNA}_{\text{sta}}$ is the starting amount of AnxA2 shRNA and $\text{shRNA}_{\text{rec}}$ is the corresponding amount of AnxA2 shRNA recovered from the supernatant.

Efficiency of loading of shAnx A2 by CLG carriers was determined from interpolated weights of shRNA obtained from SYBR gold fluorescence intensities as follows:

Loading efficiency, LE (%) = $100 \times [(\text{weight of loaded shAnx A2} - \text{weight of shAnx A2 in supernatant}) / \text{weight of CLG carrier}]$

All experiments were done in triplicates.

2.6. Cellular uptake of shRNA-loaded carriers by H1650 SP cells

Cellular uptake of plasmid-loaded carriers was investigated in monolayers of H1650 SP cells using CLG carriers of FITC labeled siRNA in a 96-well format. A 10-fold dilution of the formulation was made in serum-free DMEM: F12 to a final volume of 200 μl . Cells were inoculated with formulation for 2 h followed by replacement of treatment media with regular media. Cellular uptake of CLG carriers of FITC labeled siRNA was determined by fluorescence microscopy using an Olympus BX40 fluorescence microscope connected to a DP71 camera (Olympus, Japan).

2.7. Small-hairpin RNA interference of AnxA2 in H1650 SP cells

H1650 MP or SP cells were seeded (1×10^6 per well) in a 6-well plate format and incubated for 16–18 h. At about 90% confluency, the media was replaced with serum-free media. Lipofectamine (7.5 μl) or carrier (15 μl) was diluted to 150 μl with serum-free media. AnxA2 shRNA (1.0 and 2.5 μg) was diluted to 150 μl with media. The lipofectamine/carrier and AnxA2 shRNA mixtures were mixed and vortexed for 30 s. The samples were incubated at room temperature for 5 min. The mixture (250 μl) was added to the 6-well plate (in a total volume of 2 ml of media) and incubated for 4 h. The media was replaced with regular media and incubated for 20 h. The cells were detached and protein and RNA were extracted using RIPA lysis buffer and Trizol respectively per manufacturer's protocol. Protein and mRNA expression of AnxA2 was assayed by Western blot and real-time qRT-PCR respectively using β -actin as internal control.

2.8. In vivo tumor targeting and delivery of carrier

Male Nu/Nu mice weighing 28–35 g (Harlan Laboratories, Indianapolis, IN) were obtained for use in a protocol for *in-vivo* experiments approved by the Animal Care and Use Committee (ACUC), Florida A & M University. The animals were acclimated to laboratory conditions for 1 week prior to experiments and were maintained on standard animal chow and water *ad libitum*. The room temperature was maintained at 22 ± 1 °C and the relative humidity of the experimentation room was kept in the range of 35–50%. Animals were randomized and grouped according to treatment.

H1650 SP cells at 80–90% confluency were harvested using accutase (Sigma-Aldrich, St. Louis, MO) [19] and washed 2× with PBS. Cells were counted (1×10^6) and suspended as single cells in 50 μ l serum-free DMEM:F12 media and diluted in matrigel (3 mg/ml) to a final volume of 300 μ l on ice. Cell suspension in matrigel (300 μ l) was injected on the right flank of mice using a pre-chilled 24-gauge needle and syringe. Animals were maintained under standard husbandry for xenografts to develop.

At tumor volumes approximating 150 mm³ (by day 7), a single intraperitoneal treatment of DiR-Rhodamine-labeled carrier was administered. Animals were sacrificed at 5, 10, 15, 30, and 45 min and the xenograft tumors were excised. Tumors were covered in aluminum foil and stored at –80 °C subsequent to imaging.

Mice were placed under isoflurane-induced anesthesia under aseptic conditions. The left lateral chest was doused with iodine and cleaned with an alcohol swab. A small lateral incision (~5 mm) was made to the left chest in the plane of the left fore-limb just below the scapula. A (H1650 MP or H1650 SP) cell suspension-filled B-D® 1 ml latex free syringe connected to a 27-gauge Surflo® winged infusion set was used to deliver an inoculum of 1×10^4 cells (in a 0.1 ml volume of serum-free DMEM:F12 base media) through the sixth intercostal space into the left lung. Incisions were closed with surgical skin clips and animals were observed for full motor and cognitive recovery. Mice were maintained for 30 days for development of lung tumor was verified by dissection of a random mouse for anatomical observation.

2.9. Annexin A2 shRNA-loaded CLG carriers for intraperitoneal administration

Mice with orthotopically-implanted lung tumors (from H1650 MP and H1650 SP cells) were placed into 2 sets (H1650 MP group and H1650 SP group) of 3 groups according to treatment ($n = 5$) and administered A) no treatment, B) CLG carrier, and C) CLG-shAnxA2. CLG carriers were given in a volume of 0.1 ml by intraperitoneal injection to deliver 15 μ g/kg weight of shAnxA2. Treatments were administered daily for 7 days followed by a final dose of DiR-labeled carrier injection on the 8th day 2 h before the animals were sacrificed. Mice were sacrificed under CO₂-induced hypoxia followed by cervical dislocation. The animals were dissected and the lungs, heart, liver, kidney and spleen were collected and stored on ice or at –80 °C for imaging or storage respectively. Imaging of tissues for distribution of fluorescent carriers was carried out using an In Vivo-FX Pro™ imager (Carestream, CT).

2.10. Imaging of xenograft and orthotopic tumors

Trafficking of DiR-rhodamine-labeled carriers to xenograft and orthotopic tumors were monitored using an In Vivo-FX Pro™ imager and filtered for rhodamine (excitation 511 nm; emission 535 nm) and DiR (excitation 750 nm; emission 780 nm). Areas of fluorescent saturation and tissue contrast indicated CLG carriers' localization. Tumor occurrence was presented as total flux efficiency in perfusion/second (p/s) normalized against the total surface area of tissue.

2.11. Assessment of lung tumor regression

Evidence of tumor growth inhibition was determined on the basis of the histopathology, weight and volume of tumor. Molecular variations within and/or between the treatment groups versus control were assessed by immunohistochemical staining of tissue sections as well as Western blot and qRT-PCR analyses of samples. Tumor volume was estimated using the formula,

$$\text{Tumor volume (mm}^3\text{)} = \left((\text{Width, W})^2 \times (\text{Length, L}) \right) \times 0.5$$

2.12. Western blot analysis of lung tumors

Tumor tissues were homogenized in PBS and centrifuged at 14,000 $\times g$ for 15 min. The homogenate was washed in PBS and centrifuged 2 $\times$ and the pellet were suspended in RIPA lysis buffer supplemented with protease inhibitor; the homogenate was frozen at -80°C for 2 h. The frozen tissue was probe-sonicated for 1 min and incubated on ice for 1 h. The mixture was centrifuged at 14,000 $\times g$ for 15 min and the supernatant was collected. The protein content was determined by the BCA method and 50 μg of total protein was separated by SDS-PAGE on a Mini-Protean TGX gel (Bio-Rad Laboratories Inc., Hercules, CA). The proteins were blotted on a nitrocellulose membrane and blocked overnight with 3% BSA in PBST. The blots were probed for AnxA2, S100A10, SOX2, β -catenin, MMP7, c-Myc, and slug; and β -actin was used as an internal control.

2.13. Statistical analysis

Results obtained were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.). Statistical significance in differences in cell viability and migration was determined by unpaired t -test ($***P < 0.0001$); qRT-PCR data by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test ($***P < 0.05$); tissue distribution of fluorescent DiR-carriers was analyzed by two-way ANOVA followed by Bonferroni test ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$); mRNA and protein expression by one-way ANOVA followed by Tukey's post-test ($***P < 0.05$); and tumor weight and lung weight ($*P < 0.05$) by one-way ANOVA followed by Tukey's post-test ($***P < 0.0001$; $**P < 0.05$). Results are presented for at least three determinations and presented as mean $\pm$ SD.

3. Results

3.1. H6150 SP cells are resistant to chemotherapy

Fig. 2 shows a plot of the IC_{50} of different chemotherapeutic agents tested in H1650 MP and SP cells. The IC_{50} of docetaxel against MP and SP cells was 8.70 ± 0.13 nM and 133.75 ± 13.65 nM, respectively. The IC_{50} of cisplatin, fluorouracil, camptothecin, and gemcitabine against MP cells was 11.03 ± 0.36 μM , 2.63 ± 0.22 μM , 6.48 ± 0.31 μM , and 2.68 ± 0.30 μM , respectively; the IC_{50} increased against H1650 SP cells to 142.01 ± 20.24 μM , 81 ± 1.44 μM , 48.51 ± 0.30 μM , and 42.68 ± 4.00 μM , respectively.

3.2. Preparation and characterization of carriers

Nanoparticulate unilamellar vesicles incorporating or entrapping a fluorescent probe or shRNA, respectively, were prepared by the ethanol-injection (EI) or thin film-hydration (TFH) method as described above. The hydrodynamic diameter, polydispersity index, zeta potential and entrapment efficiency of the carriers are presented in Table 1. EI-carriers had a particle size and zeta potential range of 93.36–94.18 nm and 16.17–18.20 mV respectively. The particle size and zeta potential for TFH-carriers range from 93.01 to 94.83 nm and 17.20 to 18.92 mV, respectively. CLG-carriers show normal distribution in size (97.86 ± 1.11 nm) and zeta potential (18.47 ± 0.51 mV); incorporation of shAnxA2 into CLG-carriers (i.e. CLG-shAnxA2) resulted in a particle size of 98.83 ± 0.65 nm. Surface charge decreased to 12.11 mV for CLG-shAnxA2. The entrapment efficiency of shAnxA2 in CLG-carriers was 96.18% with an average loading of 6.13%.

3.3. CLG-shAnxA2 inhibits mRNA and protein expression of AnxA2 in H1650 SP cells

On an average ~40% of SP cells, per mm² area ($n = 3$) were positive for cellular uptake of CLG-shAnxA2 (Fig. 3A). Treatment with CLG-shAnxA2 resulted in 6-fold reduction in AnxA2 mRNA levels compared to that in response to mock shRNA (Fig. 3B). Expression of AnxA2 and NF- κ B was also inhibited by ~92 and 55%, respectively, after treating the H1650 SP cells with a high concentration of CLG-shAnxA2 (3:1); treatment with lower concentrations, however, was not as effective. Important expression of EGFR and VEGF was not altered on treatment with CLG-shAnxA2 (Fig. 3C).

3.4. Tumor-trafficking of DiR-rhodamine-labeled carriers

3.4.1. DiR-rhodamine-labeled carriers prepared by ethanol injection—Dual fluorescence (DiR-Rhodamine)-labeled EI-carriers (i.e. DR-EI-carriers) were initially evaluated in mice bearing xenograft tumors originating from H1650 SP cells. DR-EI-carriers were administered intraperitoneally (i.p.) as a proof-of-principle of tumor targeting. Analysis of the pharmacokinetic profile of tumor localization of DiR-EI-carriers at 5, 10, 15, 30 and 45 min filtered for rhodamine shows optimum flux of 7985 and 6695 expressed in perfusion/second (p/s) at 15 and 30 min, respectively (Fig. 4A and B). Filtering for DiR fluorescence showed enhanced flux at similar times of 9142 and 7318 p/s (Fig. 4C and D).

3.4.2. Optimizing DiR-labeled carriers prepared by thin-film hydration—TFH-carriers incorporating DiR and rhodamine (i.e. DR-TFH-carriers) injected by i.p. resulted in sustained localization of carriers in lung tumors for up to 4 h with a total flux of about 27-fold of free dye (Fig. 5A and B). Specific lung targeting of DR-TFH-carriers was higher in tumor-bearing lungs (Fig. 5A-III, -IV, -V, -VI at 2546, 3198, 4584 and 7025 p/s) compared to lungs with no tumors (Fig. 5A-I and -II at 1018 and 1386 p/s respectively).

3.4.3. In vivo sustained tumor targeting and delivery of carrier—Following successful targeting of carriers after i.p. administration, the tissue distribution of free DiR (control) and CLG-DiR-carriers in lung, spleen, kidney, heart and liver was investigated at 2, 3, and 4 h (Fig. 6A and B). Trace amounts of DiR were present in the lungs of control groups at 2 h (7258 p/s) and 4 h (8129 p/s) (Fig. 6A-I and A-II) with comparable deposition in the liver (4028 and 9875 p/s respectively). Lung targeting of CLG-DiR-carriers showed a

time-dependent increase of 10,864 p/s at 2 h (Fig. 6A-III), 13,215 p/s at 3 h (Fig. 6A-IV) and 16,894 at 4 h (Fig. 6A-V). Comparative distribution of free and CLG-DiR in xenograft tumors and tissues at 4 h (Fig. 6A-II and A-V) showed a 2-fold enhanced localization of CLG-DiR in lungs.

3.4.4. Xenograft tumor and tissue distribution of CLG-DiR-carriers—Tumor distribution of CLG-DiR-carriers was investigated in mice bearing H1650 SP cells as xenograft tumors (Fig. 7A and B) at 2, 3, and 4 h post-injection (Fig. 7A-I, -II, and -III respectively). There was a time-dependent increase of carrier targeting in lung and tumors. Also, uptake of carriers at corresponding time-points (2, 3, and 4 h) was similar between lung (1268, 5698, and 8989 p/s respectively) and xenograft tumors (1698, 7365, and 8456 p/s respectively).

3.5. CLG-shAnxA2 inhibits H1650 MP and H1650 SP lung tumor growth

Physical assessment of the morphology of lung with tumors showed a decrease in the occurrence of tumors nodules following treatment with CLG-shAnxA2 carriers (Fig. 8A). Tumor growth was shown to be more aggressive in mice bearing orthotopic H1650 SP lung tumors compared to H1650 MP tumors (Fig. 8A). And while fold induction of annexin A2 mRNA was similar in CLG-shAnxA2-treated H1650 MP (0.03484 ± 0.0069) and H1650 SP (0.03443 ± 0.0046) tumors, the relative knockdown of annexin A2 protein was higher in the H1650 SP group compared to H1650 MP (Fig. 8B and C). In mice receiving no treatment or receiving CLG-Scr-shRNA carriers in the H1650 SP tumor model, the occurrence of tumor nodules is contiguous compared to mice receiving CLG-shAnxA2 carriers where tumor nodules are reduced (Fig. 8A). In the H1650 SP tumor model (Fig. 9), the average lung weight was 0.25 ± 0.029 g (Fig. 9A) and significant differences were found between the CLG-Scr-shRNA carrier group (0.282 ± 0.025 g) and CLG-shAnxA2 (0.226 ± 0.02 g) ($P < 0.05$). The mean tumor weight of untreated and CLG-Scr-shRNA was 0.1701 ± 0.032 g and 0.1548 ± 0.028 g, respectively (Fig. 9B). Tumor weight was reduced by 72–75% (0.04295 ± 0.015 g) in the CLG-shAnxA2 group. The mean tumor weights were not different between the untreated and Scr-shRNA groups; however, significant differences were observed between the control and CLG-shAnxA2 carrier groups ($***P < 0.001$) (Fig. 9B). The reduction of tumor weight correlates with a decrease in tumor burden after treatment with CLG-shAnxA2 (19.01%) compared to the untreated (69.15%) and CLG-Scr-shRNA carrier (54.98%) groups (Fig. 9C).

3.6. AnxA2 knockdown inhibits expression of oncogenic and stemness factors

Relative levels of AnxA2, S100A10 and SOX2 were measured in tumors resected from different groups of mice as shown in Fig. 10A. AnxA2 protein expression was decreased in mouse lung tumors receiving CLG-ShAnxA2 to 46.20% compared to mouse tumors receiving either no treatment (100%), or receiving CLG-Scr (99.20%) (Fig. 10B). SOX2 expression was reduced in the CLG-shAnx2 treatment group relative to controls (11.07% of control) (Fig. 10C). Relative levels of S100A10 were reduced to only 1.66% of control values in response to treatment of mice with CLG-ShAnxA2 (Fig. 10D). Effect of AnxA2 interference on canonical β -catenin activation was analyzed by measuring the expression of total β -catenin and its downstream targets including c-Myc, MMP7, and Slug (Fig. 11).

CLG-shAnxA2 inhibited the expression of β -catenin by 4.21 ± 0.21 (% of control) with resultant decreased expression of c-Myc (0.00%), MMP7 (3.70%), and S lug (26.02%).

4. Discussion

The discovery of multiple molecular mechanisms and their targeted therapies has opened ways to classify lung cancer into novel 'molecular subtypes' [20]. However cancer involves several altered elements, whose simultaneous regulations in various stages of malignancy make the therapeutic strategies gradually inefficient. Developing effective treatment becomes a daunting task since down-regulation of a single factor may trigger up-regulation of many other pro-proliferative factors in tumor micro-environment. Moreover, the issue of collateral damage would become even worse if the modality is off-targeted. AnxA2 falls in that coveted class of single factors where its over expression is associated with multiple factors such as neoangiogenesis [21], cellular proliferation [22], extracellular matrix organization [23], fibrinolysis and migration [24]. AnxA2 is an important member of a Ca^{+2} binding and cellular membrane-associated family of proteins abundantly expressed in most cancers [25]. We have demonstrated that AnxA2 expression is required for activating both NF- κ B and β -catenin signaling pathways, and for the hyperproliferative effects of progastrin on colonic crypts *in vivo* [26]. Hence down regulation of AnxA2 could lead to alteration of several aberrant mechanisms, and consequently retard tumor growth.

In the present study, for the first time, we have identified a possible important role of AnxA2 in maintaining stem cell characteristics. In recent studies we discovered that AnxA2 associated with the cell membranes binds with an autocrine oncogenic growth factor, progastrin, causing clathrin-mediated endocytosis of the complex [27], resulting in up-regulation of cancer stem cell markers (DCLK1, CD44) [26]. More recently we have reported that cell surface associated AnxA2 (CX-AnxA2) co-localizes with DCLK1 and CD44 on transformed/tumorigenic stem cells [28]. In preliminary studies we have demonstrated that circulating colon cancer stem cells are also positive for CS-AnxA2, DCLK1 and CD44 [29]. In recent years it has become increasingly evident that CS-AnxA2 plays an important role in proliferation/metastasis of many cancer cells [30,31]. Progression of pancreatic/breast cancer disease was reported to be associated with a switch from cytoplasmic to cell-surface expression of AnxA2 [32]. Exosomes, secreted by cancer cells, contain AnxA2 [33] and may be the source of CS-AnxA2 and soluble-AnxA2 that has been measured in conditioned-medium/serum of cancer cells/patients [34]. Accumulating evidence in literature supports the notion that immortalization/oncogenic transformation appears to be conducive to increasing the presence of CS-AnxA2 on epithelial cells [35], further implicating a critical role of CS-AnxA2 in the proliferative and metastatic potential of cancer cells, as further confirmed by the current studies. In the current study, we demonstrate that the loss of AnxA2 inhibits the expression of pluripotent marker SOX2, which maybe downstream of the loss of relative levels of total β -catenin (Fig. 10).

Although anticancer therapeutics employing RNA interference of oncogenic pathways holds promising potentials for sequence-specific silencing of target genes, their delivery to specific tissues and cells and more importantly, their intracellular release at sites of interest still remain a major challenge. We designed an annexin A2 mRNA-specific plasmid

encoding shRNA in order to inhibit transcription of annexin A2 via RNA interference (RNAi). RNAi or posttranscriptional gene silencing (PTGS) is a mechanism of gene transcript regulation identified as a means of genome conservation (from native or invading exogenous genetic material) [36]. Double-stranded RNA (dsRNA) molecules are processed into duplexes of short double-stranded RNA sequences (21–25 nucleotides) or small interfering RNA (siRNA) by Dicer, an RNase III enzyme [37]. A multi protein RNA-induced silencing complex (RISC) incorporates the siRNA and unwinds it by the action of a helicase to produce antisense strands [38]. The antisense strand pairs with a complementary cognate mRNA transcript with resultant degradation of the mRNA [38]. Endogenous or synthetic siRNA bypass Dicer with consequent mRNA degradation [38]. Unlike siRNA, short hairpin RNA (shRNA) is synthesized in the cell by DNA-directed RNA transfection typically using viral or other vector delivery technologies [39]. Compared to siRNAs, shRNAs are more persistent, require low dosing, have higher specificity, and lower immunogenicity [39].

Different types of vehicles have been used for the delivery of nucleic acids, such as lipid–nucleic acid complexes and polyplexes. However, a major set-back impeding the clinical success of cationic transfection is their low bioavailability in serum and consequently poor *in vivo* efficacy [40,41]. To address these limitations, we developed a novel naturally derived cationic lipid having an amide linker between hydrophilic and hydrophobic moieties. The lipid was synthesized employing an enzymatic reaction for coupling naturally occurring myristic acid with bis(2-aminoethyl)amine and was subsequently subjected to quarternization followed by chloride ion exchange [42]. The amide lipid can be easily formulated into nanoparticles and offers the following advantages: a) facilitates intracellular uptake and promotes endosome release/escape of the genetic payload into cytoplasm, b) has high circulation stability, c) protects genetic materials from enzymatic degradation, d) has high serum compatibility and e) specifically targets to lungs as a result of structural orientation of the amide linker when given intravenously to animals [43].

In order to probe the role of shRNA against AnxA2, in sensitizing lung cancer stem cells, and evaluating its anticancer effects, we developed cationic amide lipid nucleic acid particles carrying plasmids expressing AnxA2 shRNA (CLG-shAnxA2) to target lung tumors. Small particle size and near-neutral surface charge increase cellular uptake of liposomes. While the effect of surface charge on the cellular uptake of liposomes is varied and cell-specific [44–46], small particle size generally enhances uptake [45,47]. Our CLG-shAnxA2 were around 100 nm with a moderate positive surface charge. CLG-shAnxA2 were superior in entrapment efficiency (95.42%) compared to PLGA nanoparticles containing shAnxA2 (57.65%) [49]. Paradoxically, cell surface-localized AnxA2 may have enhanced the uptake of CLG-shAnxA2 through endosomal trafficking [48]. Compared to intratumorally administered shAnxA2 polymeric nanoparticles [49], CLG-shAnxA2 showed superior serum stability, and sustained targeting and localization in lung tumors resulting in significant tumor inhibition.

In order to evaluate the transfection efficacies of CLG-ShAnxA2, cellular uptake studies were performed with H1650 SP cells with FITC-labeled lipid–nucleic acid complexes (Fig. 3A). Significant inhibition of AnxA2 mRNA and protein expression was observed with

CLG-ShAnxA2 (Fig. 3). AnxA2 shRNA-mediated knockdown in H1650 SP cells was further verified by qRT-PCR and Western blot analysis, and showed optimum knockdown at a 3:1 ratio (wt:wt) of lipid to shRNA at a final dose of 0.6 mg/kg (Fig. 3B, C). Although AnxA2 knockdown resulted in decreased NFkB expression *in vitro* (Fig. 3), the expression of EGFR and VEGF were not altered (Fig. 3C) [50].

Studies have shown that side population (SP) cells isolated from non-small cell lung cancer (NSCLC) cell lines exhibit cancer stem cell properties [6]. These side populations are more aggressive and much less amenable to chemotherapy compared to the mixed population cells (Fig. 8). This underlies the failure of conventional cancer therapies resulting in an initial response, with a high rate of relapse at later time points, due to resistance of cancer stem cells. Thus, there is a growing consensus that this intratumoral heterogeneity of cancer cells presents a major challenge to the development of effective cancer therapies [51]. These niches of transformed stem-like side population cells (SP) have the ability to undergo both self-renewal and differentiation into the diverse cancer cell population that constitutes the bulk of the tumor and are believed to drive the chemo-resistance machinery in many tumors [4,52]

Transcriptional factor SOX2 orchestrates the self-renewal and pluripotency of SPs. Recent studies have demonstrated that SOX2 specifically over expresses in all types of lung cancer tissues and regulates transcriptional network of oncogenes [5,53]. The resistance of H1650 SP cells was demonstrated with different chemotherapeutic agents including docetaxel (Fig. 1). Over-expression of SOX2 and AnxA2 by H1650 SP cells was verified by Western blot analysis (data not shown), and the cancer-associated transcriptional and signaling activities of these two markers have been shown [6]. Further, SOX2 modulates the expression of drug efflux pump, ABCG2 in NSCLC SP cells through the activation of β -catenin [6]. SOX2 activates β -catenin by inhibiting its association with E-cadherin (13). The expression of β -catenin leads to the activation of several pro-oncogenic downstream markers including ABCG2, Oct4, Nanog, c-Myc, MMP7, and Slug. Thus knocking down SOX2 can potentially inhibit the activation of β -catenin and lead to the down regulation of ABCG2 and consequently sensitize SPs to chemotherapy [54]. Therefore, we evaluated the role of AnxA2 shRNA in down-regulating stem cell factor SOX2, and found that ~90% of SOX2 expression in SP cells was attenuated on treatment with CLG-shAnxA2. The inhibition of AnxA2 expression correlated with inhibition of S100A10 expression [10]. S100A10 promotes tumor migration and invasion and its inhibition has been shown to reduce tumor growth [55]. AnxA2 and S100A10 interact as a heterotetrameric complex to mediate various physiologic processes [10]; and deficiency of AnxA2 results in low levels of S100A10, due to ubiquitin-mediated degradation [56]. The reduction in SOX2 and total β -catenin expression in both treatment groups (Fig. 10) is suggestive of the inhibition of the wnt/catenin pathway [57].

Tumor targeting ability of the formulations after i.p. injection was evaluated with fluorescently labeled CLG-ShAnxA2 in trafficking to H1650 SP xenograft tumors (Fig. 4). Prolonged tumor targeting was demonstrated in xenografts for up to 4 h. Tumor targeting of fluorescent CLG-ShAnxA2 was time-dependent and localized in the lung (Fig. 5). H1650 SP xenograft tumor-bearing mice were also investigated for distribution of fluorescent CLG-

ShAnxA2 following i.p. injection and demonstrated slow, sustained release and distribution in both lung and tumors for up to 4 h (Fig. 7). Evidence of the aggressive tumor growth behavior of H1650 SP versus H1650 MP cells was shown (Fig. 8). Mice receiving CLG-ShAnxA2 showed significant reduction in tumor growth compared to placebo (Fig. 9); reduced tumor growth in the treatment groups translated into reduced lung tumor burdens as an index of effectiveness of the therapy.

Target-specificity of CLG-shAnxA2 was confirmed by decrease in AnxA2 expression in lung tumors. While concurrent knockdown of VEGF has been demonstrated in prostate tumors by AnxA2 silencing [49], *in vitro* investigation with CLG-shAnxA2 in H1650 SP cells showed associated loss in expression of NFkB with AnxA2 knockdown [26], while relative levels of VEGF and EGFR remained unchanged (Fig. 2). AnxA2 silencing also resulted in decreased expression of S100A10/p11, therefore, interrupting the cellular trafficking mechanisms vital for survival of tumor cells [55]. AnxA2 and S100A10 form a heterotetrameric complex (AIIIt) that regulates cellular trafficking and fusogenic processes including endocytosis, exocytosis, and fibrinolysis [56]. The decreased expression of S100A10 could have resulted from degradation by ubiquitylation as a result of its dissociation from and/or inability to participate in the AIIIt complex [56]. Altogether, these aberrant expression patterns could have resulted in inhibition of AIIIt-mediated cancer metastasis and invasion [58].

Also, for the first time, we have demonstrated that knockdown of AnxA2 results in decreased expression of the stemness transcription factor, SOX2. SOX2 transcriptional activities modulate the expression of many oncogenic markers [53,54]. A tentative association of annexin A2 as a transcriptional target of SOX2 has been made [59]; however, the decreased expression of SOX2 by annexin A2 silencing is difficult to explain outside the possibility of a feedback loop between these two markers. Of significance however, is our observation that pluripotency, self-renewal, invasion, and metastasis are abolished by annexin A2 silencing by CLG-shAnxA2 resulting in inhibition of tumor growth.

5. Conclusion

We demonstrate for the first time that down-regulation of AnxA2 in lung tumors can effectively target lung tumor stem-like cells. We have designed a serum compatible and lung selective cationic transfection amide based cationic lipid-guided carriers with AnxA2 shRNA (CLG-shAnxA2). We have demonstrated that CLG-ShAnxA2 are stable, uniform nanoparticles by conducting chemical characterizations, which effectively down-regulated the expression of AnxA2 *in vitro* and *in vivo*. Nanoparticles carrying AnxA2 shRNA retarded tumor growth by modulating multiple mechanisms such as down regulating the activation of tumorigenesis factor β -catenin, and altering stemness factors. In summary, the present findings demonstrate for the first time that shRNA against AnxA2 profoundly influences stemness factors such SOX2 and β -catenin and efficiently retards tumor growth. The strategy of down-regulating AnxA2 expression in tumor cells could open up a window for altering tumor heterogeneity and eradicating drug resistant side populations, in order to improve the outcomes of chemotherapy treatment.

References

1. Howlader, N., et al. SEER Cancer Statistics Review, 1975–2010. National Cancer Institute; Bethesda, MD: 2013.
2. Goldberg SB, et al. Chemotherapy with erlotinib or chemotherapy alone in advanced non-small cell lung cancer with acquired resistance to EGFR tyrosine kinase inhibitors. *Oncologist*. 2013; 18(11): 1214–1220. [PubMed: 24072220]
3. Cheng Y, et al. Physiological beta-catenin signaling controls self-renewal networks and generation of stem-like cells from nasopharyngeal carcinoma. *BMC Cell Biol*. 2013; 14(1):44. [PubMed: 24073846]
4. Prince ME, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 2007; 104(3):973–978. [PubMed: 17210912]
5. Xiang R, et al. Downregulation of transcription factor SOX2 in cancer stem cells suppresses growth and metastasis of lung cancer. *Br J Cancer*. 2011; 104(9):1410–1417. [PubMed: 21468047]
6. Singh S, et al. EGFR/Src/Akt signaling modulates Sox2 expression and self-renewal of stem-like side-population cells in non-small cell lung cancer. *Mol Cancer*. 2012; 11:73. [PubMed: 23009336]
7. de Graauw M, et al. Annexin A2 phosphorylation mediates cell scattering and branching morphogenesis via cofilin activation. *Mol Cell Biol*. 2008; 28(3):1029–1040. [PubMed: 18070928]
8. Morel E, Parton RG, Gruenberg J. Annexin A2-dependent polymerization of actin mediates endosome biogenesis. *Dev Cell*. 2009; 16(3):445–457. [PubMed: 19289089]
9. Vedeler A, et al. Multiple roles of annexin A2 in post-transcriptional regulation of gene expression. *Curr Protein Pept Sci*. 2012; 13(4):401–412. [PubMed: 22708494]
10. Umbrecht-Jenck E, et al. S100A10-mediated translocation of annexin-A2 to SNARE proteins in adrenergic chromaffin cells undergoing exocytosis. *Traffic*. 2010; 11(7):958–971. [PubMed: 20374557]
11. Zheng L, et al. Tyrosine 23 phosphorylation-dependent cell-surface localization of annexin A2 is required for invasion and metastases of pancreatic cancer. *PLoS One*. 2011; 6(4):e19390. [PubMed: 21572519]
12. Deora AB, et al. An annexin 2 phosphorylation switch mediates p11-dependent trans-location of annexin 2 to the cell surface. *J Biol Chem*. 2004; 279(42):43411–43418. [PubMed: 15302870]
13. Elbashir SM, et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. 2001; 411(6836):494–498. [PubMed: 11373684]
14. Tabernero J, et al. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. *Cancer Discov*. 2013; 3(4):406–417. [PubMed: 23358650]
15. Silva AH, et al. Solid lipid nanoparticles induced hematological changes and inflammatory response in mice. *Nanotoxicology*. 2013; 8(2):212–219. [PubMed: 23451884]
16. Chaudhury A, et al. Potent therapeutic activity of folate receptor-targeted liposomal carboplatin in the localized treatment of intraperitoneally grown human ovarian tumor xenograft. *Int J Nanomedicine*. 2012; 7:739–751. [PubMed: 22359453]
17. Landen CN, et al. Intraperitoneal delivery of liposomal siRNA for therapy of advanced ovarian cancer. *Cancer Biol Ther*. 2006; 5(12):1708–1713. [PubMed: 17106249]
18. Singhanian A, Wu SY, McMillan NA. Effective delivery of PEGylated siRNA-containing lipoplexes to extraperitoneal tumours following intraperitoneal administration. *J Drug Deliv*. 2011; 2011:192562. [PubMed: 21773042]
19. Bajpai R, et al. Efficient propagation of single cells accutase-dissociated human embryonic stem cells. *Mol Reprod Dev*. 2008; 75(5):818–827. [PubMed: 18157870]
20. West L, et al. A novel classification of lung cancer into molecular subtypes. *PLoS One*. 2012; 7(2):e31906. [PubMed: 22363766]
21. Ling Q, et al. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *J Clin Invest*. 2004; 113(1):38–48. [PubMed: 14702107]

22. Balch C, Dedman JR. Annexins II and V inhibit cell migration. *Exp Cell Res.* 1997; 237(2):259–263. [PubMed: 9434621]
23. Chiang Y, et al. Specific down-regulation of annexin II expression in human cells interferes with cell proliferation. *Mol Cell Biochem.* 1999; 199(1–2):139–147. [PubMed: 10544962]
24. Liu J, et al. Nuclear annexin II negatively regulates growth of LNCaP cells and substitution of ser 11 and 25 to glu prevents nucleo-cytoplasmic shuttling of annexin II. *BMC Biochem.* 2003; 4:10. [PubMed: 12962548]
25. Vishwanatha JK, et al. Enhanced expression of annexin II in human pancreatic carcinoma cells and primary pancreatic cancers. *Carcinogenesis.* 1993; 14(12):2575–2579. [PubMed: 8269629]
26. Sarkar S, et al. Annexin A2 mediates up-regulation of NF-kappaB, beta-catenin, and stem cell in response to progastrin in mice and HEK-293 cells. *Gastroenterology.* 2011; 140(2):583–595 e4. [PubMed: 20826156]
27. Sarkar S, Kantara C, Singh P. Clathrin mediates endocytosis of progastrin and activates MAPKs: role of cell surface annexin A2. *Am J Physiol Gastrointest Liver Physiol.* 2012; 302(7):G712–G722. [PubMed: 22241862]
28. Kantara C, et al. Circulating tumors cells (CTCs), positive for cancer-stem-cell (CSC) markers and cell-surface annexinA2, are significantly increased in mice bearing metastatic versus primary colon cancer tumors. *Gastroenterology.* 2012
29. Sarkar S, et al. Progastrin overexpression imparts tumorigenic/metastatic potential to embryonic epithelial cells: phenotypic differences between transformed and nontransformed stem cells. *Int J Cancer.* 2012; 131(7):E1088–E1099. [PubMed: 22532325]
30. Diaz VM, et al. Specific interaction of tissue-type plasminogen activator (t-PA) with annexin II on the membrane of pancreatic cancer cells activates plasminogen and promotes invasion in vitro. *Gut.* 2004; 53(7):993–1000. [PubMed: 15194650]
31. Sharma M, Ownbey RT, Sharma MC. Breast cancer cell surface annexin II induces cell migration and neoangiogenesis via tPA dependent plasmin generation. *Exp Mol Pathol.* 2010; 88(2):278–286. [PubMed: 20079732]
32. Singh P. Role of Annexin-II in GI cancers: interaction with gastrins/progastrins. *Cancer Lett.* 2007; 252(1):19–35. [PubMed: 17188424]
33. Esposito I, et al. Tenascin C and annexin II expression in the process of pancreatic carcinogenesis. *J Pathol.* 2006; 208(5):673–685. [PubMed: 16450333]
34. Valapala M, Vishwanatha JK. Lipid raft endocytosis and exosomal transport facilitate extracellular trafficking of annexin A2. *J Biol Chem.* 2011; 286(35):30911–30925. [PubMed: 21737841]
35. S S, et al. AnnexinA2 is increasingly expressed and released into serum of patients positive for colonic growths in relation to disease progression: diagnostic implications. *Gastroenterology.* 2011; (Supplement-1):S-341.
36. Agrawal N, et al. RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol Rev.* 2003; 67(4):657–685. [PubMed: 14665679]
37. Cheng JC, Moore TB, Sakamoto KM. RNA interference and human disease. *Mol Genet Metab.* 2003; 80(1–2):121–128. [PubMed: 14567961]
38. Ambesajir A, et al. RNA interference: a futuristic tool and its therapeutic applications. *Saudi J Biol Sci.* 2012; 19(4):395–403. [PubMed: 23961202]
39. Rao DD, et al. siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev.* 2009; 61(9):746–759. [PubMed: 19389436]
40. Li S, et al. Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Ther.* 1999; 6(4):585–594. [PubMed: 10476218]
41. Zelphati O, et al. Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim Biophys Acta.* 1998; 1390(2):119–133. [PubMed: 9507083]
42. Rajesh M, et al. Dramatic influence of the orientation of linker between hydrophilic and hydrophobic lipid moiety in liposomal gene delivery. *J Am Chem Soc.* 2007; 129(37):11408–11420. [PubMed: 17718562]

43. Srujan M, et al. The influence of the structural orientation of amide linkers on the serum compatibility and lung transfection properties of cationic amphiphiles. *Biomaterials*. 2011; 32(22): 5231–5240. [PubMed: 21501864]
44. Kapoor M, Burgess DJ. Efficient and safe delivery of siRNA using anionic lipids: formulation optimization studies. *Int J Pharm*. 2012; 432(1–2):80–90. [PubMed: 22575754]
45. Su W, et al. PEG/RGD-modified magnetic polymeric liposomes for controlled drug release and tumor cell targeting. *Int J Pharm*. 2012; 426(1–2):170–181. [PubMed: 22266537]
46. Broekgaarden M, et al. Development and in vitro proof-of-concept of interstitially targeted zinc-phthalocyanine liposomes for photodynamic therapy. *Curr Med Chem*. 2013; 21(3):377–391. [PubMed: 23931271]
47. Kulkarni SA, Feng SS. Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. *Pharm Res*. 2013; 30(10):2512–2522. [PubMed: 23314933]
48. Mukerjee A, et al. Efficient nanoparticle mediated sustained RNA interference in human primary endothelial cells. *Nanotechnology*. 2011; 22(44):445101. [PubMed: 21990205]
49. Braden AR, et al. Polymeric nanoparticles for sustained down-regulation of annexin A2 inhibit prostate tumor growth. *J Nanosci Nanotechnol*. 2009; 9(5):2856–2865. [PubMed: 19452941]
50. de Graauw, M., et al. Annexin A2 depletion delays EGFR endocytic trafficking via cofilin activation and enhances EGFR signaling and metastasis formation. *Oncogene*. 2014. <http://dx.doi.org/10.1038/onc.203.219> [Retrieved on November 6, 2013 from <http://www.nature.com/ncurrent/full/ncurrent2013219a.html>]
51. Lara PN Jr, Lau DH, Gandara DR. Non-small-cell lung cancer progression after first-line chemotherapy. *Curr Treat Options Oncol*. 2002; 3(1):53–58. [PubMed: 12057087]
52. Clarke MF, et al. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res*. 2006; 66(19):9339–9344. [PubMed: 16990346]
53. Chen S, et al. SOX2 gene regulates the transcriptional network of oncogenes and affects tumorigenesis of human lung cancer cells. *PLoS One*. 2012; 7(5):e36326. [PubMed: 22615765]
54. Chen Y, et al. The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. *J Biol Chem*. 2008; 283(26):17969–17978. [PubMed: 18456656]
55. Myrvang HK, et al. Protein interactions between surface annexin A2 and S100A10 mediate adhesion of breast cancer cells to microvascular endothelial cells. *FEBS Lett*. 2013; 587(19):3210–3215. [PubMed: 23994525]
56. He KL, et al. Endothelial cell annexin A2 regulates polyubiquitination and degradation of its binding partner S100A10/p11. *J Biol Chem*. 2008; 283(28):19192–19200. [PubMed: 18434302]
57. Cheng, Y., et al. Physiological beta-catenin signaling controls self-renewal networks and generation of stem-like cells from nasopharyngeal carcinoma. *BMC Cell Biol*. 2014. <http://dx.doi.org/10.1186/1471-2121-14-44> [Retrieved on November 26, 2013 from <http://www.biomedcentral.com/1471-2121/14/44>]
58. Rescher U, et al. Annexin 2 is a phosphatidylinositol (4,5)-bisphosphate binding protein recruited to actin assembly sites at cellular membranes. *J Cell Sci*. 2004; 117(Pt 16):3473–3480. [PubMed: 15226372]
59. Eltaraboulsi, W., et al. Annexin A2 is a direct target of SOX2 and a potential therapeutic target in squamous cell lung cancer. American Thoracic Society 2013 International Conference, American Thoracic Society, Philadelphia Pennsylvania; 2013.

Fig. 1.

Preparation of cationic lipid-guided annexin A2 shRNA carriers. Carriers were prepared by thin film hydration using a cationic amide linker (Ac2), L- α -phosphatidylcholine, cholesterol, and pegylated-phosphoethanolamine (PE-PEG 2000) of molar ratios of 3:3.5:1.5:2 at a total lipid content of 10 mM.

Fig. 2.

H1650 side population (SP) cells are resistant to conventional cytotoxics compared to mixed population of parent (MP) cells. (A–B) H1650 MP and SP cells were treated with cisplatin, fluorouracil, camptothecin, gemcitabine, docetaxel, or DMSO for 72 h. Cell viability was determined by crystal violet staining and cell viability in treatment group normalized against DMSO. The concentration of drug treatment resulting in 50% cell death (IC₅₀) was estimated from cell viability data using linear regression analysis. Experiments were repeated three times with three replicates per treatment and results are presented as mean $\pm$ SD. Differences in IC₅₀ of cytotoxics in MP vs SP were analyzed by upaired *t*-test (two-tailed). ****P* < 0.0001.

Fig. 3. Carriers prepared by thin-film hydration (TFH) mediate efficient uptake by H1650 SP cells and mediate knockdown of AnxA2 mRNA and protein expression when incorporated with shAnxA2. (A) H1650 SP monolayers were transfected with FITC-labeled TFH-carriers for 4 h and the efficiency in uptake determined by fluorescence microscopy. (B–C) H1650 SP cells were treated with (B) lipid–nucleic acid complexes of Scr. shRNA or shAnxA2 with lipofectamine 2000 or TFH-carrier; (C) shAnxA2 and TFH-carriers were used at different shAnx2: lipid ratios as shown. Treated cells were incubated for 4 h and the efficiency of TFH-carriers in inhibiting AnxA2 mRNA and protein expression determined by quantitative Reverse Transcription-Real Time PCR (qRT-PCR) using an ABI 7200 thermal cycler (B) and Western blot analysis (C), respectively. Results are presented at mean $\pm$ SD of AnxA2 mRNA fold change normalized against GAPDH. Differences in fold change between treatment groups were analyzed by one-way analysis of variance (ANOVA) (*** P < 0.0001) followed by Bonferroni's multiple comparison test (P < 0.05). *** P vs Scr. ShRNA-liposome/lipofectamine, * P vs shAnxA2.

Fig. 4.

DiR and rhodamine dual-fluorescence labeled ethanol injection carriers (DR-EI-carriers) show efficient uptake in H1650 SP tumors in a mice xenograft model of cancer. (A–D) Mice received (I) no treatment or (II–VI) intraperitoneal injection of DR-EI-carriers for (II) 5 min, (III) 10 min, (IV) 15 min, (V) 30 min and (VI) 45 min. Mice were sacrificed and xenografts were resected. *Ex-vivo* fluorescence imaging of resected tumors was done using an In Vivo-FX Pro™ imager with filters for (A) rhodamine (excitation and emission wavelengths of 510 and 535 nm respectively) and (C) DiR (excitation and emission wavelengths of 750 and 780 nm respectively). Fluorescence was quantified using the ImageJ software and normalized against total tumor surface area and time in seconds. Data is presented as mean $\pm$ SD of total flux (percentage of maximum flux) for (B) rhodamine and (D) DiR. Data analysis was done with GraphPad Prism 5.0 and significance in the differences observed were analyzed by one-way ANOVA ($***P < 0.0001$) with Bartlett's test for equal variances ($***P < 0.0001$) followed by Tukey's multiple comparison test ($***P < 0.05$).

Fig. 5.

DiR-labeled carriers were optimized for sustained localization in lung-bearing tumors by the thin-film hydration method of carrier preparation. (A) Mice received (I–II) DiR-solution and (III–VI) DiR-carriers for (I, III) 1 h, (IV) 2 h, (V) 3 h and (II, VI) 4 h by intraperitoneal injection. Mice were sacrificed and lungs were resected for fluorescence imaging using an In Vivo-FX Pro™ imager with filters for DiR (excitation and emission wavelengths of 750 and 780 nm respectively). (B) Fluorescence was quantified using the ImageJ software and normalized against total tumor surface area and time in seconds. Data is presented as mean $\pm$ SD of total flux (percentage of maximum flux) with treatment. Data analysis was done with GraphPad Prism 5.0 and significance in the differences observed were analyzed by one-way ANOVA (*** $P < 0.0001$) with Bartlett's test for equal variances (*** $P < 0.0006$) followed by Tukey's multiple comparison test ($P < 0.05$).

Fig. 6.

A (A–B) The distribution of DiR solution and DiR-labeled carriers in lung, spleen, kidney, heart, and liver after intraperitoneal injection was investigated. (B) Mice received DiR solution or DiR-labeled carriers for (I, III) 2 h, (IV) 3 h, and (II, V) 4 h. Mice were sacrificed and organs were resected for fluorescence imaging using an In Vivo-FX Pro™ (excitation and emission wavelengths of 750 and 780 nm respectively). (B) Fluorescence data was normalized against total organ surface area and duration of treatment. Results were analyzed by GraphPad Prism 5.0 and presented as mean $\pm$ SD of flux (perfusion per second, p/s) with treatment.

Fig. 7.

(A–B) The distribution of DiR-labeled carriers in xenograft tumor, lung, spleen, kidney, heart, and liver after intraperitoneal injection was investigated. (A) Mice received DiR-labeled carriers for (I) 2 h, (II) 3 h, and (III) 4 h. Mice were sacrificed followed by resections of tumor and organs for fluorescence imaging using an In Vivo-FX Pro™ (excitation and emission wavelengths of 750 and 780 nm respectively). (B) Fluorescence data was normalized against total tumor/organ surface area and duration of treatment. Results were analyzed by GraphPad Prism 5.0 and presented as mean $\pm$ SD of flux (perfusion per second, p/s) with treatment.

Fig. 8.

Annexin A2 shRNA carriers inhibit tumor growth in mice bearing H1650 SP and H1650 MP cells as orthotopic tumors. Mice were randomized and grouped into 2 sets (H1650 SP and H1650 MP groups) of 3 groups and given no treatment, Scr-shRNA carrier, and Annexin A2 shRNA carrier daily for 2 weeks. Mice were sacrificed and lungs were resected and assessed for evidence of (A) tumor growth inhibition and inhibition of annexin A2 (B) mRNA and (C) protein expression. Data was analyzed by GraphPad Prism 5.0 for significance in differences observed and presented as mean $\pm$ SD. One-way ANOVA ($***P < 0.0001$, CLG-shAnxA2 vs controls) followed by Tukey's multiple comparison test.

Fig. 9.

Annexin A2 shRNA carriers inhibit tumor growth and tumor burden in mice bearing H1650 SP cells as orthotopic tumors. Mice were randomized and given (I) no treatment, (II) Scr-shRNA carrier, and (III) Annexin A2 shRNA carrier daily for 2 weeks. (A–C) Mice were sacrificed and lungs were resected for assessment of (A) lung weight, (B) tumor weight, and (C) tumor burden. Data was analyzed by GraphPad Prism 5.0 for significance in differences observed and presented as mean $\pm$ SD of (A) lung weight (one-way ANOVA ($*P = 0.0184$) followed by Tukey's multiple comparison test ($*P < 0.05$), (B) tumor weight (one-way ANOVA ($***P < 0.0001$) followed by Tukey's multiple comparison test ($P < 0.05$) and (C) tumor burden (i.e. ratio of tumor weight to lung weight).

Fig. 10.

(A–D) Knockdown of annexin A2 by shAnxA2 carriers inhibits protein expression of markers associated with cancer resistance and progression. Mice bearing H1650 SP tumors as orthotopic lung tumors were given (I) no treatment, (II) Scr. shRNA carriers, and (III) shAnxA2 carriers. Mice were sacrificed and lung tumors were processed for Western blot as described in Materials and methods. Blots were probed for (B) annexin A2, (C) SOX2, (D) S100A10, and β-actin using monoclonal antibodies. Protein expression was estimated and normalized against β-actin as an internal control. Results are presented as percentage mean $\pm$ SD of protein/β-actin ratio with treatment. Data were analyzed using GraphPad Prism 5.0 and differences were analyzed by one-way ANOVA (***) $P < 0.0001$ followed by Tukey's multiple comparison test ($P < 0.05$).

Fig. 11.

(A–F) Knockdown of β-catenin by shAnxA2 carriers inhibits protein expression of downstream targets associated with cancer resistance and progression. Tumor lysates from mice were prepared and subjected to SDS-PAGE as described in Materials and methods. Blots were probed for (A) β-catenin, c-Myc (B) MMP7, Slug, and β-actin using monoclonal antibodies. (C–F) Protein expression was estimated and normalized against β-actin as an internal control. Results are presented as percentage mean ± SD of protein/β-actin ratio with treatment. Data were analyzed using GraphPad Prism 5.0 and differences were analyzed by

one-way ANOVA (** $P < 0.0001$) followed by Tukey's multiple comparison test ($P < 0.05$).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Preparation and characterization of CLG carriers. Carriers were prepared by the ethanol-injection (EI-carriers), thin-film hydration (TFH-carriers) method. TFH-carriers incorporating the Ac2 lung targeting lipid (i.e. cationic ligand-guided carriers, CLG-carriers) were prepared with encapsulated shAnxA2. CLG carriers were characterized for hydrodynamic diameter, polydispersity index (PDI), zeta potential, entrapment efficiency (EE), and loading efficiency (LE). Data are presented as mean $\pm$ SD of at least 3 analyses.

Liposome	Size (nm)	PDI	Zeta (mV)	EE (%)	LE (%)
EI-liposomes	220.02 $\pm$ 13.47	0.194 $\pm$ 0.021	17.86 $\pm$ 1.55	–	–
TFH-liposomes	199.70 $\pm$ 1.88	0.262 $\pm$ 0.069	22.64 $\pm$ 1.19	–	–
CLG-liposomes	198.86 $\pm$ 0.77	0.135 $\pm$ 0.024	21.82 $\pm$ 0.66	–	–
CLG-AnxA2 liposomes	217.50 $\pm$ 1.74	0.179 $\pm$ 0.004	12.11 $\pm$ 0.88	96.81 $\pm$ 0.74	6.13 $\pm$ 0.04