Development of an In Vivo Assay for Antibody-Conjugated Gold Nanoparticles Targeted to Human Pancreatic Tumor Xenografts Using an Ex Ovo Avian Embryo Culture System

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Development of an *In Vivo* Assay for Antibody-Conjugated Gold Nanoparticles Targeted to Human Pancreatic Tumor Xenografts Using an *Ex Ovo* Avian Embryo Culture System

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Abstract

Pancreatic cancer therapies remain limited in scope and patient prognoses remain poor. To effectively improve pancreatic cancer outcomes, a highly targeted therapeutic is necessary. A bioconjugated gold nanoparticle (AuNP) provides such a therapeutic platform. Effective targeting of the nanoparticle is possible through the linkage of antibodies to target key antigens highly expressed on the surface of cancerous tissue. Previous work using RT-PCR and Western blotting has established the over-expression of the membrane glycoprotein A33 (gpA33) in the CAPAN-2 pancreatic cancer cell line and the absence of gpA33 in the BxPC-3 pancreatic cancer cell line. This differential protein expression allows for an experimental paradigm in which uptake of AuNP conjugated with anti-gpA33 antibodies can be quantified and compared in vivo between CAPAN-2 and BxPC-3 xenografts. Avian embryos are used as the model animal system and cultured ex ovo. BxPC-3 and CAPAN-2 cell lines were cultured and injected into the chorioallantoic membrane (CAM) of day 8 embryos to induce formation of human tumor xenografts. Antibody-conjugated AuNP were delivered intravenously into the CAM vasculature. Xenografts were allowed to develop for seven days in vivo and subsequently excised, paraffin embedded and analyzed histologically and immunohistochemically to confirm expression patterns of gpA33 in sections of CAPAN-2 and BxPC-3 xenografts. Dot blot and Western Blot analyses demonstrated the presence of targeting antibodies within CAPAN-2 tissue and the absence of targeting antibodies in BxPC-3 tissue. This thesis project validates the in vivo targeting capability of an antibody-conjugated AuNP platform and lays the foundation for its use in the treatment of pancreatic adenocarcinoma.
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Introduction

Pancreatic Cancer

Pancreatic adenocarcinoma is an aggressive disease with a poor prognosis. For those diagnosed, there is a <5% five-year survival rate (Brand et al., 2010). Pancreatic cancer is responsible for approximately 30,000 deaths each year in the United States alone (Li et al. 2004). Only 15-20% of patients have resectable tumors, and only 20% of those patients survive beyond five years post-treatment (Li et al., 2004). Current chemotherapy for metastatic pancreatic adenocarcinoma therapies includes the anti-metabolite gemcitabine (2’,2’difluorodeoxycytidine), fluorouracil and platinum agents, such as oxaliplatin, cisplatin, and carboplatin (Yu et al., 2010). However, these approaches have proven ineffective at advanced stages of the disease. Because pancreatic cancer has proven difficult to diagnose at early stages due to the inconsistency of presenting symptoms, these therapies have been unsuccessful at lowering mortality rates (Danovi et al., 2008).

More targeted therapies for pancreatic cancer will lead to higher survival rates and will minimize collateral damage to other organs and systems in the body. Most current therapies fail to differentiate between normal and cancerous cells, causing extensive damage and devastating side-effects (Danovi et al., 2008). The goal of targeted therapy is to disrupt only the cellular processes necessary for the proliferation of cancer cells (Danovi et al., 2008). Such innovative therapies include those that target specific cell surface receptors, ligands, transcriptional factors or mutant genes through the use of small interfering RNAs (siRNAs), oncolytic viruses and nanoparticles (Yu et al., 2010). Additionally, targeted therapies can be personalized to specific patients, whereby specific chemotherapeutic agents used can be substituted as the disease progresses and becomes resistant to individual agents.
Gold Nanoparticles (AuNPs) as Chemotherapeutic Platforms

Advancements in nanotechnology have allowed for research into the use of nanoparticles as vehicles for cancer diagnosis and therapy (Yu et al., 2010). Due to their small size, nanoparticles can enter cells and organelles with ease and have the potential to disrupt protein function or destroy DNA (Patra et al., 2010). The large surface area to volume ratio of the particles enables the particles to carry a multitude of chemotherapeutic agents (Yu et al., 2010). The development of gold-coated nanoparticles (AuNPs) has led to promising treatment options for myriad reasons. Gold is a biocompatible material that has been shown to be resistant to oxidative corrosion in vivo (Bhattacharyya et al., 2011). Equally important is the ability to modify the surface chemistry of these AuNPs due to the strong affinity between gold and thiols, disulfides, phosphine, and amines (Bhattacharyya et al., 2011). This allows for conjugation of both therapeutic and targeting molecules, such as monoclonal antibodies (mAb).

In order to maximize circulation time in the bloodstream and avoid uptake by the reticuloendothelial system, nanoparticles should measure less than 100nm in diameter (Nie et al., 2007). Additionally, a hydrophilic coating on the particle prevents plasma protein adsorption and any other nonspecific binding events (Nie et al., 2007). Hydrophilic elements that may serve this purpose include polyethylene glycol (PEG), poloxamine, and poloxamers (Nie et al., 2007). PEG coatings have been shown to be biocompatible and non-immunogenic (Lipka et al., 2010). Additionally, PEG coatings reduce cytotoxicity of nanoparticles in vitro and lower renal clearance (Lipka et al., 2010).

Target Antigen Glycoprotein A33

To establish the targeting capability of a mAb-conjugated AuNP, the antigenic profiles of various pancreatic cancer cells lines were assessed. Two pancreatic adenocarcinoma lines,
CAPAN-2 and BxPC-3, were found to differ in the expression of glycoprotein A33 (gpA33). Previous work in the Webb lab has established the presence of gpA33 in the CAPAN-2 line and the absence of gpA33 in the BxPC-3 line both in vitro and in vivo through RT-PCR and Western blotting. This differential protein expression allows for an experimental setup in which the uptake of AuNP conjugated with anti-gpA33 antibodies can be assessed and potentially quantified in vivo between CAPAN-2 and BxPC-3 derived xenografts.

gpA33 is found in more than 95% of human colon cancers and select pancreatic cancers and is largely absent from all other human tissue (Ackerman et al., 2008). It is 43kD and contains three domains: a polar intracellular domain containing 62 amino acids, a hydrophobic trans-membrane domain containing 23 amino acids, and an extracellular domain of 213 amino acids (Heath et al., 1996). Select portions of the extracellular domain are highly conserved among members of the immunoglobulin superfamily, such as the presence of both a V-type domain and C₂-type domain that is commonly found in the CD2 subfamily (Figure 1). Because of this homology to the CD2 group, gpA33 has been assigned to the JAM (junctional adhesion molecule) family of immunoglobulin-like molecules, and is postulated to be involved in cell-cell interactions, although the function of gpA33 has yet to be deciphered (Bazzoni, 2003). The N-terminus of the intracellular region begins with four cysteine residues; this feature is homologous to certain members of the seven trans-membrane G protein-coupled receptor family and may indicate that the molecule is bound by palmitoylation (Heath et al., 1996, 1997).
Figure 1. Putative structure of gpA33 membrane antigen (modified from Scalan et al. 2006).

To effectively target the AuNPs to antigens highly expressed in cancerous tissues, mAbs are linked to the particles. Previous work has shown that mAb 31.1 is directed against gpA33, resulting in an antigen-antibody complex that forms at the cell membrane and becomes endocytosed (Arlen et al. 1998; Webb et al., 2009). A chemotherapeutic payload can be administered to the tumor cells by adequate accumulation of the AuNPs in the targeted tissue. mAbs must be properly linked to the AuNPs such that the antigen-binding Fab portion is facing outward and accessible to the targeted antigen on the surface of the tumor cell. Orientated conjugation of the mAb to the AuNPs is achieved using a short PEG linker terminated at one end by a dithiol and a hydrazine group at the other. Attachment is achieved by the SH-group attraction to the gold surface and chemically linking the hydrazine to the carbohydrate associated with the Fc portion of the mAb (Kumar et al., 2008).
A multitude of therapeutics may be linked to the particles to deliver a therapeutic payload, such as the aforementioned anti-metabolite gemcitabine. Boron Neutron Capture Therapy (BNCT) is the current chemotherapeutic agent being pursued by the Webb lab. Non-radioactive $^{10}\text{B}$ is linked to the AuNP construct through binding of a biotinylated synthetic peptide (poly-GL) carrying large numbers of $^{10}\text{B}$ atoms held in place on the AuNPs by anti-biotin mAbs (Figure 2). Once an adequate amount of AuNPs have accumulated in the cancerous tissue, and a high degree of particle localization has been achieved, the $^{10}\text{B}$ can be irradiated with low energy thermal neutrons; this results in an emission of alpha particles and lithium recoil (Wu et al., 2006). These emissions would ideally result in the specific destruction of cells that have internalized the targeted AuNP construct.

![Figure 2. Graphic of AuNP construct.](image_url)
In Vivo Analysis

The avian embryo was chosen as the in vivo system in which AuNP targeting was analyzed. The avian embryo chorioallantoic membrane (CAM) is an ideal region in which to graft human cancer cells. The membrane itself, a fusion of the chorion and allantois, is the outermost portion of membrane beneath the egg shell (Valdes et al., 2003). Rapid angiogenesis occurs within the CAM during development, a feature that allows for grafted cells to proliferate and become incorporated into the blood vascular system to form human tumor xenografts within a matter of a week to ten days. The membrane primarily functions in gas exchange; it is also involved in the transport of Na\(^+\) and Cl\(^-\) from the allantoic sac to the embryo and the transport of Ca\(^{2+}\) from the shell to the embryonic vasculature (Valdes et al., 2003). The membrane is an immunoprivileged site because the lymphoid system is not yet fully developed, thus allowing foreign cells and tissue to grow and develop without threat of an immune response from the host system (Dohle et al., 2009).

Because tumors have a “leaky” vasculature, a consequence of rapid growth and dysfunctional angiogenesis, intravenously injected mAb-conjugated AuNPs have heightened exposure to cancerous tissue (McDonald and Baluk, 2002) on the basis of a phenomenon known as the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). Endothelial cells lining tumor blood vessels are unevenly distributed and do not form an even monolayer that in non-cancerous tissue provides an adequate barrier (McDonald and Baluk, 2002). This increased accessibility to components of the bloodstream as a result of enhanced permeability allows the cancerous tissue to thrive due to the heightened availability of oxygen and nutrients. Moreover, the EPR effect can also function to maximize interactions between antigens highly expressed in cancerous tissue and antibodies conjugated to nanoparticles (McDonald and Baluk, 2002).
We have taken advantage of a shellless, or *ex ovo*, avian embryo culture system developed by John Lewis that allows for a high degree of accessibility to the CAM for manipulation and imaging (Leong et al., 2010) (Figure 3). The embryo and its surrounding CAM are able to develop normally *ex ovo* in a warm, humid environment (Leong et al., 2010). This culture method provides greater access to the CAM for the purposes of grafting human cancer cells and also access to the blood vascular supply by injecting mAb-conjugated AuNPs intravenously. Additionally, in comparison to other animal models for assessing *in vivo* targeting of engrafted human tumors (e.g., immune-deficient mice), this avian culture method is relatively inexpensive and simple to maintain (Leong et al., 2010). Imaging or excision and analysis of tumor tissue to compare AuNP uptake in targeted and non-targeted tissue post-intravenous delivery of AuNPs can be achieved easily within a few days.

Figure 3. Explanted avian embryo on developmental day 8.

This project serves to demonstrate the targeting capacity of a mAb-conjugated AuNPs *in vivo*. The *ex ovo* avian embryo culture is an ideal system in which to determine the efficacy of AuNP targeting in this early stage project. After the establishment of gpA33 expression in CAPAN-2 human tumor xenografts and the absence of gpA33 in BxPC-3 xenografts, the system
was used to study AuNP uptake in both targeted and non-targeted tissue. Results showed AuNP uptake in CAPAN-2 tissue and a lack of AuNP uptake in BxPC-3 tissue, a promising result that demonstrates that the conjugation of mAb effectively targets chemotherapeutic AuNPs to cancerous tissue and provides the basis for continuing studies on the use of BNCT for treatment of pancreatic cancer.


Materials and Methods

Preparation of Tumor Cells for Bolus Injections

CAPAN-2 and BxPC-3 cell lines, obtained from human pancreatic adenocarcinoma were acquired from American Type Culture Collection (ATCC). The lines were cultured in RPMI-1640 Medium (Sigma #R6504) at 37˚C and 5% humidity. The medium was supplemented with 1x antibiotic/antimycotic solution (100 units/mL penicillin, 1 mg/mL streptomycin sulfate, 2.5 µg/mL amphotericin B; Sigma #A5955), 0.2% sodium bicarbonate (Sigma #S8791), and 10% fetal bovine serum (FBS; Hyclone #SH30070.03).

To prepare cells for bolus injections, a ~70% confluent T75 flask (~6 x 10⁶ cells) of each cell line was used. Cells were trypsinized, neutralized with medium, and centrifuged at 1000rpm for 3 min (Sorvall TC 6). The cells were washed with 1X PBS and resuspended in 2mL PBS and placed on ice. Extra PBS was set aside for the injection process to dilute the suspension as was necessary to avoid clogging the apparatus. Grafting was done immediately following cell preparation.

Ex Ovo Avian Embryo Culture (adapted from method of Lewis et al 2010)

Embryos were incubated with rotation for 4 days at 38˚C (100˚F) and 60% humidity (Sportsman 1202). On developmental day 4, embryos were explanted using a dremel tool with a circular blade (Dremel Cut-Off Wheels No. 409). The dremel blade and the egg shell exterior were sterilized prior to explantation with 70% ethanol. Eggs were placed horizontally 10 minutes before explantation to allow for the embryo to rotate to the top of the yolk. Polystyrene weighing boats (VWR, cat. no. 12577-01) and the shell exterior were sterilized with 70% ethanol prior to the explantation process. With the dremel tool on a medium-high setting, two shallow cuts were made below the horizontal equator on either side of the shell (Figure 4).
Gentle pressure was applied to pull apart the two halves of the shell and expel the egg contents intact into the weighing boat (Figure 5). The weighing boats were covered with square petri dishes (VWR, cat. no. 25378-115) and cultured without movement (Sportsman 1550 hatching incubator) at 38°C and 72% humidity in plastic boxes perforated with ½” holes to act as humidity chambers. The chorioallantoic membrane (CAM) was allowed to develop for 4 days before bolus injections of tumor cells into the membrane were performed.
Bolus and Intravenous Injection in the Chorioallantoic Membrane

Bolus injections into the CAM may be performed on developmental days 8 or 9 to create tumor xenografts. The microinjector was prepared by attaching an 18-gauge needle to a 1-mL syringe. The cell suspension was loaded into the syringe before 2-3 inches of Tygon tubing (R-3603 laboratory tubing, 1/32-inch diameter, 3/32-inch outer diameter, 1/32-inch wall thickness) is attached to the 18-gauge needle so that the tubing reaches the base of the needle. The glass needle (Vertical pipette puller, Sutter Instrument Co. Model P-30; Heat: 980, Pull: 850) was then attached to the tygon tubing. The needle was trimmed such that a blunt, rather than beveled, tip was created since a beveled tip is too sharp and results in penetration through rather than into the layers of the CAM. The base of the needle was broken such that the entire length of the needle measured between 1-1.5 inches. Care was taken to remove all air bubbles before the grafting process.

The workspace was sterilized with 70% ethanol and a heating pad, on a medium setting, was used to keep the embryos warm during the time outside the incubator. Grafting was performed close to a blood vessel to ensure sufficient and rapid vascularization of the cell bolus. The needle was angled at 45° above the CAM surface. Gentle pressure was applied with the
microinjector until an invagination of the CAM was visible under the dissecting scope (Figure 6).

**Figure 6. Drawing illustrating Xenografting technique.**

Once the needle was within the membrane, the cell suspension was injected slowly until the aggregation of cells within the membrane was apparent. Any excess cell suspension remaining on the CAM was removed with sterile Kimwipes®. As soon after the grafting procedure as possible, the embryos were returned to the hatching incubator. The CAM requires an additional 2 days of development before intravenous injections may be performed (Figure 7).

**Figure 7.** A. Inoculation of CAPAN-2 and BxPC-3 cells into the chorioallantoic membrane (CAM) on developmental day 8. B. Developing xenograft on developmental day 10 (circled). Images from the lab of John Lewis, London Regional Cancer Program.
For intravenous injection into the CAM vasculature, the microinjector was assembled as described above. The needle was pulled such that the tip was very long and tapered (Sutter P-97, Heat: 473, Pull: 50, Velocity: 100, Time: 250) and was trimmed to create a beveled tip such that it readily perforated the CAM and cleanly entered a blood vessel. AuNP conjugated with targeting antibodies were prepared according to the method of Kumar et al. (2008) and were delivered intravenously as a suspension of \(\sim 10^{12}\) particles/ml in sterile PBS. Veins can be distinguished from the arteries by the brighter red color of the hemoglobin when compared to the arteries. Injections were made into veins that were 2 branch points removed from a main vessel.

Successful entry into a vein was determined through the visualization of a small pooling of blood adjacent to the injection site. Gentle pressure was applied to the microinjector apparatus to release the appropriate volume, approximately 100\(\mu\)L, of AuNP. After adequate delivery of AuNP, the needle was held in the vessel for a minimum of 30 seconds to minimize hemorrhaging of the vessel and to allow time for clotting at the wound site. The needle was removed slowly and gently, and any excess AuNP or blood was removed from the surface of the CAM with a sterile kimwipe. Embryos were returned to the incubator immediately after IV injections.

**Xenograft Processing and Analysis**

**A. Histological Staining and Immunohistochemistry**

Xenografts were excised and fixed in 4% formaldehyde in PBS at RT for 1 day in small glass vials prior to dehydration in a graded alcohol series: 70% ethanol, 80% ethanol, 95% ethanol, and 100% ethanol for 10’ each. Grafts were cleared in Histoclear® (3 x 15’) and then placed in a 1:1 ethanol:Histoclear® bath overnight in a 65°C oven. Following the overnight incubation, grafts were placed in molten paraffin (2 x 30’ held at 65°C). After the infiltration
with paraffin wax, grafts were transferred in fresh molten paraffin wax in small plastic molds made from the end of pipettors (SAMCO® Transfer Pipets, cat. no. 232) and moved to room temperature to solidify.

The paraffin blocks were trimmed and attached to wooden supports with melted wax. Microtome sections were cut at 5-10µm and the paraffin ribbons were placed in a 40-45°C water bath before being mounted onto coated slides (VWR Superfrost® Plus, cat. no. 48311-703). The slides were allowed to air-dry for a minimum of 20 minutes at room temperature before they were baked on a slide warmer at 45-50°C for 30 minutes. Slides were stored in a dust-free box. Deparaffinization was accomplished through immersion of the slides in Histoclear® (3 x 10’), before rehydration 100% ethanol (2 x 5’), 95% ethanol (1 x 5’), 80% ethanol (1 x 5’), 70% ethanol (1 x 5’) followed by rinsing the slides in distilled water for 5’. Hematoxylin and eosin staining was performed using the AccuMax™ H&E Staining Protocol.

Immunohistochemistry was performed using the Epitomics® Immunohistochemistry Protocol for Paraffin-Embedded Tissue. A rice cooker was used as the “decloaking” chamber for the antigen retrieval portion of the protocol. The slides were heated in the rice cooker for 20 minutes under the ‘White Rice’ setting. Before applying the peroxide block solution, borders were drawn around each section using the Liquid Blocker Super PAP Pen. Primary rabbit polyclonal antibody directed against human gpA33 (Sino Biological, Inc.) was applied (1:1000) to each section and allowed to incubate overnight at room temperature in a humidified chamber created through the placement of damp filter paper in a covered glass petri dish. Following overnight incubation, the goat anti-rabbit IgG HRP-conjugated secondary antibody was applied (Thermo Scientific, supplied in kit ready to use). Counterstaining with hematoxylin was omitted from the protocol to maximize HRP stain visibility.
**B. Protein Extraction**

Each xenograft was placed in 0.5mL mPER lysis buffer containing HALT protease inhibitor cocktail (Thermo Scientific Product #78501BNDL) with 5-10 1.4mm stainless steel beads (Next>>>Advance) per xenograft. The tissue was homogenized in the Next>>>Advance Bullet Blender™ for 10 minutes at setting 7. The tissue extract supernatant was separated from the beads and stored at -20°C.

**C. Pull-down assay with protein A conjugated magnetic beads**

20µL magnetic beads conjugated with 30 mg/ml Protein A (inVentrogen, Dynabeads Protein A) were added to 1mL whole tumor lysates and mixed by pipetting and then incubated for 10 minutes at RT on a shaker to allow binding of IgG-conjugated GNP. The mixtures were then placed on a magnetic rack until a pellet formed along the wall of the tube adjacent to the magnet. The lysate supernatant was carefully removed and discarded. The magnetic beads were thoroughly resuspended in 10µl 50mM glycine (pH 2.8) and the captured protein (IgG + GNP) eluted for 4 minutes on a heated shaker (70°C). The mixtures were again placed in the magnetic rack and the supernatant was transferred and neutralized with 1M Tris buffer (pH 7.5) using a 20:1 ratio of eluate:Tris buffer. The eluted, neutralized samples were spotted directly onto nitrocellulose membrane pre-wetted with transfer buffer for dot blot analysis.

**D. Dot Blot**

The nitrocellulose membrane (Thermo 0.2µm pore, 8 x 12cm, Product #77012) was presoaked in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) before application of 10 µl samples (xenograft protein extract or protein A recovered antibody-conjugated AuNP). The blot was then allowed to incubate in blocking solution (5g Blotting-Grade Blocker, Bio-Rad 170-6404, in 100mL of wash buffer, 1x TBS/0.1% Tween 20) for one hour shaking at RT. After rinsing briefly with wash buffer, the blot was shaken at RT for 1hr
with goat anti-human IgG antibody (Millipore) conjugated with HRP diluted 1:10,000 in blocking buffer. Total protein loading was established and normalized by probing duplicate dot blots with a rabbit anti-GAPDH primary antibody at a 1:2000 dilution (Santa Cruz) followed by an HRP conjugated goat anti-rabbit IgG secondary at 1:10,000 dilution (Thermo Fisher). Blots were developed with Luminol reagent (Denville Scientific Inc E2400) and visualized under a chemiluminescence protocol using the BioRad GelDoc XR imager.

E. Western Blot

Neutralized eluates from a pull-down assay performed on xenografts (non-injected control and injected with AuNP conjugated with the rabbit polyclonal anti-A33 antibody) as described above were mixed with non-reducing sample buffer (Thermo #39001) heated to 95°C for 5 minutes and allowed to cool to room temperature (RT) before loading into wells of 10% Precise Protein Gels (Thermo #25201). Electrophoresis was performed in Tris-HEPES-SDS running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS, pH 8; Thermo #28398) at 45 V for 30 minutes then 150 V for 45 minutes, until the loading dye was ~ 0.5cm from the bottom of the gel. Following SDS-PAGE, separated proteins were electrophoretically transferred from the gel to nitrocellulose membrane in pre-chilled transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) at 40 V for 90 minutes. The membrane was then rinsed in distilled water and shaken for 1 hr at RT in blocking buffer (TBS containing 5% w/v BioRad blocker # 170-6404). After rinsing and washing in TBST (TBS containing 0.05% Tween-20), the location and presence of both gpA33 and rabbit IgG were determined by incubating the Western blot first in the anti-gpA33 polyclonal antibody at a dilution of 1:10,000 (~0.2 µg/ml) in blocking buffer overnight at 4°C, followed by thorough washing in TBST, and then a goat anti-rabbit IgG-HRP conjugate secondary antibody (Thermo #32260) diluted 1:5,000 in blocking buffer for 1hr at RT.
After rinsing in TBST and patting dry with a clean paper towel, the membrane was sprayed with luminol chemiluminescent substrate (Denville Scientific E2400), allowed to sit for ~1 min and then again blotted dry and imaged in a thin plastic folder using a BioRad GelDoc XR imager.
Results

In order to determine the targeting capability of antibody-conjugated gold nanoparticles (AuNP) to cancerous tissue in vivo, an ex ovo avian embryo culture was established for the growth of human tumor xenografts. CAPAN-2 and BxPC-3 pancreatic adenocarcinoma lines differ in expression of glycoprotein A33 (gpA33) and were checked for this phenotype prior to use (Figure 8).

![Image of Dot blot analysis](image.png)

**Figure 8. Establishment of gpA33 expression in CAPAN-2 and BxPC-3 cell lines.** Dot blot analysis was used to detect gpA33. Rabbit polyclonal anti-gpA33 (Sino Biological Inc., 1:10,000) and Goat anti-Rabbit, HRP-conjugate (Thermo Scientific, 1:5000), respectively, were used to detect gpA33. GAPDH was used as a loading control for protein detection in both samples.

These cell lines were grafted by injection of live cells into the chorioallantoic membrane (CAM) of chicken embryos, followed several days later by intravenous delivery of AuNP targeted against gpA33. The specificity of AuNP targeting was established by biochemical assays performed on xenograft extracts.

The efficacy of this in vivo targeting system is highly dependent on thorough vascularization of grafted tumor tissue. The extent of vascularization was validated by direct
light microscopy of grafts as they developed in the CAM (Figure 9 A, B below), and also following intravenous injection of fluorescent lectin to further highlight vascular endothelium (Figure 9C).

**Figure 9. Xenograft vascularization in vivo.** (A) CAPAN-2 xenograft after 5 days of development in vivo. (B) CAPAN-2 xenograft after 8 days of development in vivo. (C) Multiple 5 day old BxPC-3 xenografts highlighted (arrows) by intravenous injection of fluorescent lectin.

Conventional histological analysis revealed that BxPC-3 xenografts adopt a compact and highly ordered, pancreatic tissue-like organization, whereas the CAPAN-2 grafts are far more disorganized and loosely arranged (Figure 10).
Figure 10. Paraffin-embedded sections of xenografts stained with Hematoxylin and Eosin. (A) BxPC-3 xenograft, 10X. (B) CAPAN-2 xenograft, 10X. (C) BxPC-3 xenograft, 20X. (D) CAPAN-2 xenograft, 20X. Note how the xenografts shown in A and B are positioned between layers of the CAM (arrows).

Immunohistochemistry (IHC) was used to confirm the expression patterns of gpA33 in CAPAN-2 and BxPC-3 xenografts in vivo. Previous RT-PCR and Western blotting analyses performed in the Webb Lab on xenograft extracts had demonstrated both transcription and translation of the gpA33 gene by CAPAN-2, but not BxPc-3 cells, in vivo (data not shown). IHC of sections from xenografts grown in the CAM were in accord with these previous findings (Figure 11). Significant staining of gpA33 is seen in the CAPAN-2 sections, whereas BxPC-3 sections lack any evidence of antibody reactivity, thus confirming the robust nature of this antigen phenotype in vivo.
Figure 11. Immunohistochemical staining in xenograft sections to evaluate expression of gpA33. Paraffin-embedded sections of BxPC-3 (A) and CAPAN-2 (B) xenografts after 8 days of growth in vivo were reacted first with a rabbit polyclonal antibody directed against human gpA33, followed by goat anti-rabbit HRP secondary antibody. The presence of antibody was visualized with DAB and viewed here at 10x magnification.

A biochemical approach was adopted as a means to determine specificity of mAb 31.1-conjugated AuNP targeting using dot blots to detect the presence of IgG in protein extracts of intravenously targeted CAPAN-2 and BxPC-3 xenografts (Figure 12). In addition to CAPAN-2 and BxPC-3 tissue obtained from a targeted CAM, non-targeted tissue and various positive controls were assayed. Initial findings determined there to be a lack of specific binding of the goat anti-human IgG secondary antibody conjugated to HRP (Millipore), since signal was observed on the negative control (see Figure 12E below).
Figure 12. Dot Blot of various CAPAN-2 and BxPC-3 xenograft protein extracts to probe for the presence of mAb 31.1.
(A) Non-targeted CAPAN-2 protein extract with exogenously added AuNP-31.1.
(B) Protein extract of a non-targeted CAPAN-2 xenograft directly injected with AuNP-31.1.
(C) Protein extract from a CAPAN-2 xenograft intravenously targeted with AuNP-31.1.
(D) Protein extract from a BxPC-3 xenograft that was intravenously targeted with AuNP-31.1.
(E) Protein extract from a non-targeted CAPAN-2 xenograft.
(F) 31.1-conjugated AuNPs.

Because the xenografts are inevitably highly vascularized, one possible explanation for this result is cross-reaction between the secondary antibody directed against human IgG and chicken immunoglobulin (IgY) present in the samples despite rinsing with PBS after excision. However, Figure 13 shows a dot blot of chicken serum compared to a sample of the mAb 31.1-AuNP probed with the goat anti-human IgG secondary antibody that eliminates this conclusion.

Figure 13. Chicken serum cross-reactivity dot blot. (A) Chicken serum and (B) mAb 31.1. Chicken serum was obtained from 12 day-old chicken embryos.

In order to increase the specificity and potential sensitivity of the dot blot assay for the presence of antibody-conjugated AuNP in xenografts, an affinity-capture method was devised. Magnetic beads conjugated with Protein A were used to isolate and concentrate the 31.1-conjugated AuNPs from other protein in the extracts prior to spotting on the membrane. Probing
of membranes carrying samples prepared in this way with goat anti-human IgG secondary antibody conjugated with HRP consistently revealed a signal only in CAPAN-2 extracts and not BxPC-3 tissue (Figure 14). These findings demonstrate exclusive targeting of the mAb 31.1-conjugated AuNP only to tissue expressing the gpA33 antigen.

Figure 14. Dot blot of xenograft extracts following magnetic bead pull-down assay. (A) Lysis buffer (mPER containing HALT). (B) Protein extract from a BxPC-3 xenograft targeted intravenously with AuNP-31.1. (C) Protein extract from a CAPAN-2 xenograft targeted intravenously with AuNP-31.1. (D) 8.19 x 10^7 AuNP-31.1. (E) 4.09 x 10^8 AuNP-31.1. (F) 8.19 x 10^8 AuNP-31.1.

It was conjectured that the weak signal (Figure 14) might be due to loss of AuNP during the protein extraction procedure, specifically during the centrifugation step (10k x g 10'). To test this hypothesis, a control experiment was performed through spiking lysates with AuNP-31.1 (5.18 x 10^8) and then comparing the protein-A capture before and after centrifugation. The results of this test (Figure 15) suggest that >50% of the AuNP are lost due to pelleting during the xenograft extraction protocol.
Figure 15. Significant loss of AuNPs during xenograft extraction and recovery. (A) Lysis Buffer (mPer containing HALT). (B) Sample of non-targeted BxPC-3 protein with added AuNP-31.1 before centrifugation. (C) Sample of non-targeted BxPC-3 protein with added AuNP-31.1 after centrifugation. (D) $5.18 \times 10^8$ AuNP-31.1.

Data from three trials of dot blots were compiled and detection intensity between targeted CAPAN-2 and BxPC-3 tissue was compared. Signal intensity in CAPAN-2 tissue, indicative of the presence of human IgG, ranged from ~6-8.5X greater than signal intensity measured in BxPC-3 tissue (Table 1).

Table 1. Ratio of Human IgG Detection Intensity on three dot blots between targeted CAPAN-2 and BxPC-3 xenografts.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>6.88</td>
</tr>
<tr>
<td>Trial 2</td>
<td>8.40</td>
</tr>
<tr>
<td>Trial 3</td>
<td>5.83</td>
</tr>
<tr>
<td>Mean + SD</td>
<td>7.04 ± 1.29</td>
</tr>
</tbody>
</table>

In addition to omission of the centrifugation step, all subsequent targeting experiments used AuNP conjugated with a polyclonal anti-A33 (pA33) antibody directed against gpA33 in preference to the mAb31.1 to maximize AuNP uptake in targeted tissue and thus enhance the resultant signal indicative of the presence of IgG on the blot. Recent findings in the Webb Lab have demonstrated significantly higher binding affinity of pA33 when compared to that of the mAb 31.1 (data not shown), so it was conjectured that the signal due to presence of targeted AuNP might similarly be enhanced by use of the polyclonal antibody. To assess this difference in uptake in vivo, a comparison was made between xenografts targeted by particles conjugated with the two antibodies. Findings show a modest enhancement of signal from CAPAN-2 tissue targeted with AuNP-pA33 compared to AuNP-31.1 (Figure 16).
Figure 16. Comparison of uptake of AuNP conjugated to mAb 31.1 or polyclonal A33 in targeted and non-targeted xenografts.
(A) Dot blot analysis of protein-A captured AuNPs.
(B) Mean intensity values for dot blot data.
1. Lysis buffer negative control
2. Non-targeted BxPC-3 tissue
3. AuNP conjugated to pA33
4. AuNP conjugated to mAb 31.1
5. Protein extract from CAPAN-2 tissue targeted with AuNP-31.1
6. Protein extract from BxPC-3 tissue targeted with AuNP-31.1
7. Protein extract from CAPAN-2 tissue targeted with AuNP-pA33
8. Protein extract from BxPC-3 tissue targeted with AuNP-pA33
Western Blotting was also used to probe for the presence of IgG and gpA33 in targeted tissue. Magnetic beads conjugated with Protein A were again used to isolate Human IgG in CAPAN-2 and BxPC-3 targeted by AuNP conjugated with rabbit polyclonal anti-gpA33. Both IgG and gpA33 were detected in CAPAN-2 tissue (Figure 17), demonstrating that the magnetic beads isolated IgG bound to gpA33. The elution process likely disrupted this interaction, resulting in the two distinct IgG and gpA33 bands.

Figure 17. Western Blot of CAPAN-2 and BxPC-3 tissue post-purification with magnetic bead pull-down assay. Whole xenograft lysate was purified using the magnetic bead pull-down assay as described in Materials and Methods. Samples were subjected to SDS-PAGE. Detection of gpA33 and IgG was accomplished through Western Blotting with Rabbit polyclonal anti-gpA33 (Sino Biological Inc., 1:10,000) and Goat anti-Rabbit, HRP-conjugate (Thermo Scientific, 1:5000), respectively.
Discussion

This study has demonstrated the targeting capability of an antibody-conjugated gold nanoparticle (AuNP) using an ex ovo avian embryo model. Two human pancreatic adenocarcinoma cell lines that differ in expression of glycoprotein A33 were grafted into the chorioallantoic membrane of 8 day-old explanted chicken embryos and allowed to develop. The xenografts were subsequently targeted intravenously with AuNP conjugated to antibodies targeted against gpA33. Specific uptake of the particles was demonstrated through biochemical means in the targeted tissue, validating the efficacy of this targeting strategy.

Future Directions

Future work should focus on a more precise way to quantify uptake of AuNPs in vivo. The nature of the grafting procedure makes it difficult to calculate the exact volume of AuNP, and hence number of nanoparticles, delivered intravenously; there is inevitably some spillage onto the surface of the CAM. The Dot blot and Western blot analyses used in the present study do not allow for the quantitation of AuNPs detected in tissue. Other methods of detecting AuNPs in targeted and non-targeted tissue may lend themselves to more precise methods of quantifying results. Histological analysis may be useful to probe for the presence of AuNPs in tissue using a silver enhancement method (e.g. Ted Pella prod #15718). Silver enhancement may be used on sectioned tissue as a means to amplify the AuNP signal under light microscopy through enlarging and darkening AuNPs within the tissue. Calculated surface area of staining on the sections can be used to roughly quantitate AuNP presence and compare uptake between tissues.

Alternatively, various imaging programs, such as ImageJ, can be used to quantify and compare signal intensity of AuNPs between tissues. Some fluorescent element, such as anti-
biotin antibodies or quantum dots, along with targeting antibodies, may be conjugated to particles. The program would be used to calculate and compare the strength of the fluorescent signal in CAPAN-2 and BxPC-3 tumors. Imaging would be performed with the xenografts still within the CAM, and thus no tissue processing would be required. Magnetic resonance imaging (MRI) is another imaging method that would allow for a more precise method of calculating AuNP uptake in tissue. Gold-coated iron oxide particles (GoldMag) conjugated to targeting antibodies may be delivered intravenously into the CAM vasculature. CAPAN-2 and BxPC-3 xenografts would then subsequently be excised and suspended in agarose within phantom glass tubes. The extent of relaxivity of each tissue would allow for a determination of the extent of AuNP uptake.

Another aspect of this study that requires further investigation is the outcome of the particles \textit{in vivo} and if and when they become eliminated from the system. It is of interest to determine the ideal time to excise xenografts post-intravenous injection so as to allow for maximum particle uptake while avoiding embryo death and loss of viable xenografts. The investigation of AuNP uptake in tissue excised at various time points would demonstrate the time necessary for maximum uptake to be achieved. Previous work has demonstrated that intravenously injected 40nm AuNPs remain in normal mouse liver tissue for as long as six months before significant elimination is seen (Sadauskas et al., 2009). Particle retention in tissue is high, though the time necessary for complete localization requires further investigation.

Previous findings have shown that PEGylation and decreased particle diameter, were two critical factors in minimizing uptake of the particles by the reticuloendothelial system and maximizing particle uptake in tumor tissue (Zhang et al., 2009). Specifically, the highest degree of retention of AuNP in tumor tissue was observed with 20nm particles coated with PEG_{5000}.
(Zhang et al., 2009). PEGylation of the particles is a critical step to minimize nonspecific uptake of AuNP in vivo. PEG coatings on AuNP have been shown to minimize nonspecific protein adsorption, and their hydrophilic nature minimizes clearance and prolongs circulation time (Gu et al., 2009). Additionally, the PEG functions to minimize particle aggregation in vivo, a critical feature that increases the likelihood of conjugated antibodies interacting with surface antigens (Gu et al., 2009). Size of the particles is another crucial factor for the successful uptake of AuNP, with previous work demonstrating ideal sizes of <50 nm for endocytotic uptake (Chithrani et al., 2006). Larger particles aggregate more readily, a phenomenon that not only reduces endocytotic uptake, but also increases the cytotoxicity of the particles (Cui et al., 2012).

In addition, the surface charge of AuNP in serum is a critical factor to be considered when optimizing the specificity of nanovehicle targeting to cancer cells (Zhang et al., 2008).

In order to make a bona fide comparison between levels of AuNP uptake between CAPAN-2 and BxPC-3 tissue, it is necessary to establish that there exist equivalent levels of vascularization in the tumors within the CAM. Hematoxylin and eosin staining of the two xenograft types revealed the distinct organization of cells in the two tissues in vivo (Figure 10), which may affect how these tissues become vascularized. Intravenous injection of fluorescent lectin revealed the extent of vascularization of the BxPC-3 tissue through highlighting vessel walls within the xenografts (see Figure 9C). Extent of vessel wall visualization through intravenous injection of fluorescent lectin between CAPAN-2 and BxPC-3 tissue could be compared using fluorescent microscopy to determine relative levels of vascularization. Additionally, immunohistochemistry could be performed on BxPC-3 and CAPAN-2 xenograft sections to probe for the presence of endothelial cell markers (e.g. von Willebrand factor [http://www.abcam.com/Von-Willebrand-Factor-antibody-ab6994.html]). The extent of staining
on xenograft sections would reveal whether or not the two tissues have similar access to the CAM vasculature.

To ensure that differential uptake of targeted AuNP is due to successful targeting, rather than a lack of adequate vascularization in the non-expressing tissue, an alternative +/- targeting system may be established. Such a system would involve a target antigen that is highly expressed in BxPC-3 tissue and not expressed in a different pancreatic adenocarcinoma tissue. Previously published findings demonstrate the expression of mesothelin (MSLN) in a multitude of carcinomas, including a majority of pancreatic adenocarcinomas (Argani et al., 2001). Some of the pancreatic adenocarcinomas that do not express mesothelin include PANC-1, Hs766T, and HPDE (Argani et al., 2001). Demonstrating specific targeting of AuNPs to BxPC-3 tissue and a lack of uptake in PANC-1 tissue would show that the lack of AuNP uptake in BxPC-3 tissue in the current +/-gpA33 system is not due to lack of adequate vascularization. Very recent preliminary investigation of this system in our lab suggests that MSLN is indeed expressed at high levels by BxPc-3 cells, but contrary to some other reports, our CAPAN-2 cells are negative for MSLN.

The premise of this project lies in the localization of particles to targeted tissue such that irradiation from Boron Neutron Capture Therapy (BNCT) affects only cancerous regions. The current method of assessing location of particle accumulation fails to demonstrate that AuNPs are not accumulating in regions within the embryo system other than the grafted tissue. A more thorough investigation can be done through the use of magnetic resonance imaging (MRI). Gold-coated iron oxide particles (GoldMag) can be used for this imaging study. As done with the gold particles used in this study, these particles may be conjugated to targeting antibodies and injected intravenously. The entire embryo set-up can be placed in an MRI, and relaxivity of
different regions can be determined. These biodistribution data would be critical to demonstrate both clearing of normal tissue following nonspecific interactions, as well as optimum loading of targeted xenografts prior to BNCT.

**Future Animal Model Systems**

The next phase of this *in vivo* work will involve orthotopic tumor xenografts in a rodent model. The orthotopic xenograft model is becoming the *in vivo* system of choice due to its better relevance to clinical scenarios (Huynh et al., 2011). For the purposes of this project, pancreatic adenocarcinoma cell lines of interest would be injected directly into the pancreas of the rodent. Injection of the cells into the corresponding organ of origin allows for the study of organ-specific effects on AuNP uptake. This model is hailed for being much more useful and relevant than subcutaneous xenografts methods. Therapies shown to be highly effective against subcutaneous tumors in mice were commonly found to be fairly ineffective against tumors in human patients (Huynh et al., 2011). Subcutaneous tumors develop in a microenvironment wholly different from the organ of origin. As a consequence, they typically fail to develop histology characteristic of the organ, rarely metastasize, and thus behave abnormally due to localized environmental effects. These differences prevent this system from being a truly predictive model.

Though the orthotopic xenograft system is better able to mimic clinical scenarios, there are disadvantages associated with this model as well. Imaging is often required both for the procedure itself, as well as to monitor tumor development within the organ. Subcutaneous injection of tumor cells is relatively straightforward, and the progress of tumor growth merely requires measuring tumor size beneath the skin. Another drawback with the orthotopic system is the highly technical nature of the procedure and the associated recovery time of the rodents.
(Huynh et al., 2011). Nonetheless, this in vivo system is promising in its ability to replicate the conditions seen in cancer patients.

**Conclusions**

The work presented in this study shows the targeting capability of an antibody-conjugated gold nanoparticle in an ex ovo avian embryo culture system. The next phases of the in vivo portion of this project will require more precise methods to quantify AuNP uptake in tissue and possibly the use of another +/- targeting system to account for differences between tissues and further validate the findings of the gpA33 system reported here. Additionally, future work must progress to a rodent model to show the efficacy of this approach in a mammal. These preliminary findings are promising in demonstrating that a long-circulating AuNP can recognize and accumulate in tumor tissue expressing a particular antigen, and thus allow for a therapeutic payload to be delivered in a highly targeted manner.
References


