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Can Pancreatic Cancer be Controlled by Cellular Immunity?

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Running head: Can Pancreatic Cancer be Controlled

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Abstract

Pancreatic cancer is aggressive and poorly understood with a five-year survival rate of only 9% because most cases are diagnosed at a late stage. Because of ambiguous symptoms, pancreatic cancer is very difficult to detect early, and often when it is finally diagnosed, the cancer has already metastasized or has become too robust to cure. Current treatments are largely ineffective, so there is a great need for a novel approach to targeting this debilitating disease. To determine whether an intracellular peptide target expressed only in pancreatic cancer cells can be utilized to control or eradicate pancreatic cancer tumors by T cell immunity, an altered form of the gastrin/cholecystokinin receptor (CCK2R) associated with aggressive pancreatic cancers was targeted. Altered splicing of the CCK2R mRNA encodes a variant receptor, CCK2i4SVR, which contains a 69 amino acid insertion within the third intracellular loop. Our group has shown that a synthetic peptide corresponding to twenty amino acids of the loop elicits a T cell response in mice. The goal of this research was to determine whether murine cells hyper-expressing the intron-containing CCK2i4SVR variant could elicit similar T cell responses. To achieve particularly high levels of receptor, CCK2i4SVR was transiently transfected by an optimized method into murine pancreatic cancer cells that already expressed a basal amount of the human, variant receptor, and, 48 hours later, these cells were irradiated and used to immunize C57Bl/6 mice. Intron-specific T cell responses were monitored by ELISPOT, and it was unclear whether the super-transfected cells elicited an intron-specific T cell response.
Importance

Though pancreatic cancer is the third leading cause of cancer-related deaths, there are no early detection methods, and treatment options are extremely limited. A mutant form of the CCK receptor that retains a 69 amino acid insertion into the third intracellular loop is found in cancerous pancreatic cells but not normal ones; because it is associated with tumorigenesis, it can be a target for treatment. A T cell response was elicited in mice immunized with a synthetic peptide corresponding to 20 amino acids of the insertion sequence. Since cellular vaccines can elicit a higher quality T cell response than synthetic peptides, the development of a cellular vaccine targeting this mutated CCKR is a next step in combatting this aggressive cancer.

Introduction

Pancreatic Cancer

Advanced pancreatic cancer is one of the most robust and least understood cancers. The survival rate of those diagnosed living beyond five years is only 5% (Hidalgo 2010, Siegel et al 2014). Current chemotherapeutic methods have failed to significantly increase survival rate (Ryan et al 2014). Many other recalcitrant cancers have become less dismal with the advance and application of target-specific therapies to cancer specific receptors (Ma and Adjei 2009, Maitland and Schilsky 2011). Immune-based therapies have also been shown as effective against resilient cancers, specifically melanoma. One reason the survival and response to therapies has been so poor for pancreatic cancer is because the current chemotherapeutic methods are not target specific. Tumor growth associated with pancreatic cancer has been attributed to
the over-expression of certain types of receptors, specifically the CCK receptor (Smith et al 1993, Smith et al 1994).

**CCK and Gastrin**

The cholecystokinin (CCK) and gastrin families of peptides are hormones that bind to central and peripheral CCK receptors. They are secreted and mobilized in the GI tract in the physiological response to a normal meal. CCK and its receptors are also found in the central nervous system where they have many regulatory functions corresponding to anxiety, satiety, analgesia, and dopamine-mediated behavior (Wank 1995). Even though CCK and its receptors are widely distributed and have a vast range of functions, only two primary subtypes of receptors exist, CCK-A receptor and CCK-B receptor. The CCK receptors are G-protein coupled receptors. These transmembrane proteins bind to a signal on the outside of a cell, causing a conformational change. This conformational change initiates an intracellular signal transduction pathway. When the signal binds to the protein, it is enabled to activate an associated G protein by exchanging a bound GDP for GTP. These receptors mediate the actions of both CCK and gastrin—gastrin receptors are identical to CCK-B (Wank 1995). CCK-A receptors are found predominantly in the GI system and specific parts of the central nervous system and have a high affinity for CCK. In contrast, CCK-B receptors are found predominantly in the central nervous system and specific parts of the GI system and have high affinity for both CCK and gastrin (Wank 1995).

Gastrin is an important regulator of the release of gastric acid from the stomach and plays an important role in growth of the GI tract. Gastrin and CCK are involved with growth of GI cancers as well as other malignancies through activation of the CCK-B
Can Pancreatic Cancer be Controlled

receptor (Smith et al 2016). Gastrin expression is high in the embryonic pancreas but not in the adult pancreas. Interestingly, when the adult pancreas becomes cancerous, gastrin is re-expressed. By an autocrine mechanism, this re-expression stimulates the growth of a malignant tumor cells. One strategy to combat this malignancy is to decrease the expression of gastrin in the cancerous tissue. Another is to interfere with the interaction between gastrin and the CCK receptor with receptor-specific anitbodies or receptor antagonists. One such antagonist is the PNB-028 which binds the isoform of CCK-A preferentially over CCK-B, inhibiting the proliferation of CCK-A-expressing pancreatic cancer cells without affecting the proliferation of noncancerous cells (Ponnusamy et al 2016). This antagonist was also shown to be effective in inhibiting the growth of pancreatic cancer xenographs in immunocompromised mice (Ponnusamy et al 2016).

Another study identified a single-nucleotide polymorphism (SNP) in the CCK receptor as a potential biomarker for pancreatic cancer, observing that patients with pancreatic cancer possessed the SNP but that patients with other GI tract malignancies did not (Alsubai et al 2016). Other research suggests that the CCK-B receptor is also involved with some aspects of pancreatic cancer. The CCK-B receptor is the major receptor type in the human pancreas, but the receptor increases in abundance even more during pancreatic cancer (Smith et al 1998). Gastrin-stimulated tumorigenesis is mediated through this CCK-B receptor, not the CCK-A receptor (Smith et al 1995). In cancerous cells, the over-expression of the CCK-B receptor results in the increased production of gastrin mRNA and peptides.
Several mutations to the CCK-B receptor have been described. One is the alternative splicing of the entire fourth intron which results in a 69 amino acid insertion within an intracellular loop (Figure 1A; Smith et al 2002).

This portion of the receptor is involved in GTP signal transduction and cell proliferation. Resulting from a single nucleotide polymorphism (C > A) in position 32 of the intron 4 of the CCK-B receptor gene variant, this mutation occurs in 40% of patients suffering from pancreatic cancer but is not observed in healthy individuals (Smith et al 2012).

Some research suggests that the C to A mutation renders the receptor constitutively active where it promotes tumorigenesis even in the absence of gastrin (Hellmich et al 2000). Transfection of DNA encoding a corresponding mutant receptor into pancreatic cancer cells has been shown to accelerate cell growth in culture, while reducing the expression of this mutant receptor inhibits cell growth (Smith et al 2004).

Because the CCK-B receptor is a G-protein coupled receptor, when CCK or gastrin binds to and activates it, the receptor changes conformation and promotes the exchange of GDP for GTP. Regulating this receptor in pancreatic cancer cells may result in apoptosis and halt cell proliferation (Fino et al 2012). Gastrin stimulation of the CCK-B receptor activates Akt phosphorylation (Todisco et al 2001), but down-regulation of the CCK-B receptor in human pancreatic cancer cells inhibits phosphorylation of protein kinase B (Fino et al 2012). Down regulation of the CCK-B receptor in certain types of pancreatic cancer cells (Fino et al 2012) reduces the expression of the X-linked inhibitor of apoptosis protein (XIAP). XIAP usually inhibits cell death by apoptosis, so reducing its expression would increase rate of apoptosis of cancer cells. XIAP is typically
Can Pancreatic Cancer be Controlled

upregulated in pancreatic cancer (Lopes et al 2007, Satoh et al 2001). Beyond pancreatic cancer, CCK receptors have been characterized as “abundantly-expressive” in gastric cancer, colon cancer, and lung cancer (McWilliams et al 1998, Singh et al 1986, Smith et al 1996). Although both CCK-A and CCK-B receptor types are present, like in some pancreatic cancers, only the CCK-B receptor is identified as the cause of the proliferation of malignant tissue (Moody and Jensen 2001).

**T cell Immunology**

Two subsets of T cells control and provide effector functions for memory-driven, antigen-specific immune responses. CD4+ are commonly referred to as helper T cells while CD8+ are called cytotoxic or killer T cells. Each T cell clone recognizes a specific peptide sequence that is presented on a major histocompatibility complex (MHC) (Abbas et al 2010). When the peptide is presented on the MHC, the T cell receptor docks with both the presented peptide and with the MHC molecule itself. Upon docking, CD4+ cells secrete cytokines which trigger or enhance immune functions of other arms of the immune system and other CD4+ cells. However, as soon as CD8+ cells dock to the peptide and MHC molecule, they secrete substances that trigger a cell death cascade in the target cell, resulting in apoptosis (Abbas et al 2010). Often the peptide that triggers an T cell immune response is presented as a result of an infection or detrimental mutation. Since CD4+ and CD8+ cells are different in function and must maintain specificity in binding, two different classes of MHC molecules present peptides to CD4+ vs CD8+ T cells. MHC I molecules present peptides that can be produced in any host cell to CD8+ killers. CD8+ T cells express a co-receptor, CD8, that binds to a non-polymorphic region of the MHC I molecule. Most all nucleated host cells can be
targeted by CD8+ killer T cells via this mechanism. MHC II molecules present peptides to CD4+ which, then, also expresses a co-receptor specific for a non-polymorphic region of the MHC II molecule (Abbas 2010). When this co-receptor binds to the MHC II molecule, signaling cytokines are released which regulate the rest of the immune system. Only certain subsets of phagocytic host cells express MHC II molecules.

**Pancreatic Cancer and Immunology**

In patients with pancreatic cancer, the over-expression of gastrin stimulates tumor growth by a type of autocrine mechanism—tumor cells become auto-signaled for growth (Smith et al 1996). Further, a mutation of the KRAS gene in the presence of the CCK-B receptor has been linked to pancreatic tumorigenesis (Kore 2010). The effect of the KRAS mutation seems to be quelled when gastrin binding is reduced. CCK$_{24SV}$, an isoform of the CCK receptor that contains an extra 69 amino acids encoded by a retained intron, is found in both pancreatic and colon cancer and can be targeted as a potential marker for T cell response (Ding et al 2002). Because of the unspliced fourth intron that is absent in noncancerous tissue, this isoform is associated with the regulation and proliferation of pancreatic cancer (Hellmich et al 2000).

The CCK$_{24SV}$ must be targeted by a T cell mechanism rather than with antibodies because of the intracellular nature of the protein domain encoded by the retained intron. Antibodies functionally bind to the membrane of a cell or to molecules present on the outside of the cell. Since the loop of interest is inside the cell, antibodies would be ineffective in targeting the mutation. T cells also bind to MHC molecules on the cell surface, but the mechanism of antigen presentation is such that the mutation of interest,
even if it exists intracellularly can be targeted specifically. A peptide that corresponds to an infectious agent or detrimental mutation is presented to a T cell on a corresponding MHC molecule. In the case of the CCK<sub>24SV</sub> receptor, peptides corresponding to portions of the 69 amino acid intron-encoded sequence can be presented, eliciting a T cell response to and/or direct recognition of the mutated tumor cells.

MHC molecules only present a select subset of peptides which contain appropriate anchor residues and other T cell receptor contact residues. Anchor residues at specific locations on the peptide bind the peptide to the MHC molecule and are necessary for a peptide to be presented. T cell receptor contact residues are necessary to elicit the response of a T cell. Both of these residues increase the specificity of the MHC/T cell interaction. A synthetic peptide that corresponds to loop amino acids 281-300 in intron 4 (Figure 1B) has been shown to elicit a significant T cell response in mice. Thus, the cancer-linked CCK<sub>24SV</sub> receptor can be targeted by way of a peptide that corresponds to the intracellular loop insertion.

**Materials and Methods**

**Cell Culture of PANC02 and Related Lines**

PANC02 (P+24) pCAGEN.neo-CCKCR<i>i</i>3/UTR/SNPA cl.12 (P20), PANC02 (P+24), pCAGEN.neo-CCKB<i>i</i>3/UTR cl.7 (P14) and PANC02 (P+27) cell lines were removed from a liquid nitrogen freezer and thawed in a warm water incubator at 37° C. The PANC02 (P+27) cells were cultured in 10 mL RPMI 1640 media + 10% FBS on 100 mm cell culture dishes. The PANC02 (P+24) pCAGEN.neo-CCKCR<i>i</i>3/UTR/SNPA cl.12 (P20) and PANC02 (P+24) pCAGEN.neo-CCKB<i>i</i>3/UTR cl.7 (P14) cells were cultured in 10 mL RPMI 1640 media + 10% FBS + 200 mg/mL G418 on 100 mL cell culture
dishes. When cells were 80-90% confluent, they were passaged by phosphate-buffered saline (PBS) and 0.25% Trypsin EDTA in a laminar flow hood. After the media was aspirated, 5 mL of PBS was pipetted onto the plate and subsequently aspirated. 2 mL Trypsin was added, and the plate was incubated at 37°C for five minutes. The cells were trituated by pipetting, and 8 mL of media were added to quench the Trypsin, creating a total volume of 10 mL in the plate. 1 mL of this was added to a new plate already containing 9 mL of media to create a 1:10 dilution. The 1:10 dilution would take 3-4 days to reach 80-90% confluence for all three cell lines.

Transfection Optimization

Using 10 mL trypsinized and quenched cells from an 80% confluent 100 mm cell culture dish, 0.259 mL, 0.432 mL, 0.605 mL and 0.691 mL were added to different wells on a 12-well plate to achieve 30%, 50%, 70% and 90% confluence, respectively. 0.5 mL of media was added to each well, and the plate was incubated at 37°C overnight. 24 hours later, pCAGEN.puro-EGFP (1.187 μg/μL) and either FuGene or Lipofectamine 2000 were added to specific wells according to the manufacturer’s protocol. 24 hours later, the cells were imaged by fluorescent microscopy, and the transfection efficiencies of the different conditions were analyzed.

IP Immunizations Using Irradiated Tumor Cell Lines

PANC02 (P+24) pCAGEN.neo-CCKCR*i3/UTR/SNPA cl.12 cells were pipetted into four 100 mm cell culture plates so that they would be at 80% confluence after overnight incubation at 37°C. 24 hours later, all four plates were transfected with Lipofectamine 2000, one with the pCAGEN.puro-EGFP plasmid and three with the pCAGEN.neo-CCKCR*i3/UTR/SNPC plasmid. 24 hours later, transfection efficiency of the
pCAGEN.puro-EGFP plasmid was analyzed by fluorescent microscopy, and the cells from the other three plates were trypsinized, quenched, combined and distributed equally to T175 flasks, each already containing 30 mL of media. After incubating at 37°C for three days, the T175 flasks were washed with PBS, trypsinized with 8 mL 0.25% Trypsin EDTA and quenched with 12 mL of media. All contents were combined into two 50 mL conical centrifuge tubes. These tubes were centrifuged at 200 rpm for five minutes (Jouan CR412 table top refrigerated centrifuge; M4 swinging bucket rotor with appropriate inserts; 4°C). After the media of both tubes was aspirated, both cell pellets were resuspended in a combined 24 mL of media. 2 mL of media were removed for RNA analysis. The cell concentration of the remaining 22 mL was counted by hemacytometer using trypan blue. The 22 mL cell suspension was irradiated for 1052 seconds in the Gammacell at Penn State Hershey College of Medicine Department of Health Physics. The suspension was centrifuged at 200 rpm for five minutes (4°C). After aspirating the media, the cell pellet was washed three times with Hank’s salt without phenol red, centrifuging at the same parameters and resuspending each time. After the third wash, the cell pellet was resuspended in 0.6 mL of Hank’s salt without phenol red. 0.2 mL (2 x 10⁷ cells) were injected intraperitoneal into each of three C57Bl/6 mice. Excluding the parallel transfection of pCAGEN.puro-EGFP, this process was repeated so that the second immunization occurred 14 days after the initial injection. 2.5 x 10⁷ irradiated cells were injected intraperitoneal for the second immunization. This protocol was approved by IACUC 2016-08R-F2018. RNA was isolated and purified using an RNeasy Mini Kit according to QIAGEN protocol.

Preparation of Red Cell Depleted Splenocyte Suspensions
Three immunized and three naïve mice were euthanized by cervical dislocation. The left side of the euthanized mouse was rinsed with 70% ethanol. Disinfected forceps and scissors were used to make a 2 cm incision in the outer skin at the base of the ribcage. A few drops of 70% ethanol were dripped onto the exposed inner skin before cutting. Another 2 cm incision was made into the inner skin over the spleen. Using the forceps the grasp the spleen, it was removed from the body, and the scissors were used to cut fatty tissues and vessels off the spleen. The spleen was placed into a sterile 15 mL conical centrifuge tube containing 7 mL of cold, supplemented RPMI(0) media and stored on ice. In a sterile, laminar flow hood, a small wire screen was placed on a 100 mm Petri dish. The screen was rinsed several times with one 5 mL aliquot of RPMI(0) media. The media was aspirated. The contents of the 15 mL conical centrifuge tube were poured onto the screen, with the spleen landing on top of the screen. Using the sterile plunger from a freshly opened 3cc disposable syringe, the spleen was broken, and the splenocytes were dislodged. The plunger was pressed against the spleen and a circular motion was used to complete the dislodging. The cells were dispersed and the debris from the screen was rinsed by pipetting the spleen cell suspension through the screen several times with a 10 mL serological pipet. The splenocyte suspension was placed into a sterile 15 mL conical centrifuge tube with the same 10 mL pipet. The plunger was used again to squeeze any remaining cells through the screen. The plunger and screen were washed with 5 mL RPMI(0) media using a 5 mL serological pipet. Using this pipet, the splenocyte suspension was added to the same 15 mL conical centrifuge tube, creating a total volume of around 12 mL. The tube was stored on ice until the other spleens were processed. Splenocyte suspensions were pelleted
by centrifugation at 1000 rpm for seven minutes (Jouan CR412 table top refrigerated centrifuge; M4 swinging bucket rotor with appropriate inserts; 4°C). The supernatant was aspirated, and the pellets were resuspended in 7 mL sterile Tris Ammonium Chloride solution prewarmed to 37°C using a 5 mL serological pipet. The tubes were incubated at 37°C for five minutes, mixing the contents of the tubes by inversion once during this incubation. 7 mL RPMI(0) were added to each tube, and the solutions were centrifuged at 1000 rpm for seven minutes at 4°C. After aspirating the supernatant, the pellets were resuspended in 5.5 mL RPMI (0) media. The tubes were placed on ice, and the debris was allowed to settle for ten minutes. The cell suspension was transferred into a sterile 15 mL conical centrifuge tube and mixed by trituration. The cell concentrations were counted by hematocrit using trypan blue. The cells were pelleted by centrifugation at 1000 rpm for seven minutes (4°C) and resuspended in enough HL-1 media to have a final living cell concentration of 1x10^7 cells/mL.

**ELISPOT Assay**

Six days after the second immunization, Functional Grade purified capture antibody was diluted 1/250 in sterile ELISPOT Coating Buffer. 100 μL was added to each of the 96 wells, and the plate was incubated at 4°C overnight. 24 hours later, the coating antibody was aspirated from the plate, and each well was washed twice with 200 μL ELISPOT Coating Buffer. The plate was blocked with 200 μL/well 1% BSA (w/v) in PBS for one hour at room temperature. After aspirating the blocking solution from the wells, each well was washed twice with 200 μL ELISPOT Coating Buffer. Four peptide solutions were added to the plate to achieve a final concentration of 10 μM in a final volume of 0.2 mL. 50μL of each stock peptide at a concentration of 40μM
in HL-1 medium was added to wells that contained 50μL of HL-1 medium. 50 μL of CR281-300 were added to each well in rows A, B and H. 500 μL of CR272-291 were added to each well in rows C and D. 50 μL of HBc128-140 were added to each well in rows E and F. 50 μL of T581-595 were added to each well in row G. The amino acid sequences of these peptide targets are described in Figure 6. 100 μL of the splenocyte suspension from the first naïve mouse were added to each well in columns one and two. 100 μL of the splenocyte suspension from the second naïve mouse were added to each well in columns three and four. 100 μL of the splenocyte suspension from the first immunized mouse were added to each well in columns five, six, seven and eight. 100 μL of the splenocyte suspension from the second immunized mouse were added to each well in columns nine and ten. 100 μL of the splenocyte suspension from the third immunized mouse were added to each well in columns eleven and twelve. The plate was incubated at 37° C in a 5% CO₂ humidified atmosphere for 24 hours. After 24 hours, the cells and media from the plate were aspirated, and the plate was washed six times with 200 μL/well ELISPOT Wash Buffer. Detection Antibody was added 100 μL/well, and the plate was incubated at room temperature for two hours on a rocker. Detection Antibody was aspirated, and each well was wash six times with 200 μL ELISPOT Wash Buffer, allowing wells to soak for one minute in each wash. 100 μL/well of Avidin-HRP reagent in Assay Diluent (1/250) was added, and the plate was incubated for 45 minutes at room temperature on a rocker. Avidin-HRP was aspirated, and each well was washed first four times with 200 μL ELISPOT Wash Buffer and then three times with 200 μL PBS lacking Tween 20. 100 μL/well of freshly-prepared AEC Substrate Solution was added to each well. Spots were allowed to develop for 35 minutes at room temperature while the plate was
protected from light. The plate was washed six times with distilled water. The plate was protected from light and dried overnight. The next day, spots were counted using a CTL plate scanner (Penn State Hershey). ELISPOT was used to monitor the T cell responses of the mice immunized with irradiated super-transfected murine pancreatic cancer cells (Figures 3 and 4).

**Tumor Growth Studies**

All three cell lines were used to monitor subcutaneous tumorigenesis. Four 100 mm plates at 80% confluence were used for each cell line. After trypsinizing and quenching with media, the four plates of each cell line were combined, creating three suspensions, one for each cell line. Using trypan blue, hemacytometer counts were obtained for each suspension. Cells were centrifuged at 200 rpm for five minutes at 4° C. The media was aspirated, and cell pellets were resuspended in enough HBSS (x1) so that each C57Bl/6 mouse was injected with 2.5 x 10^6 cells. Injections were administered subcutaneously on the left flank of 16 mice. Four mice received PANC02 cells, six mice received PANC02 (P+24) pCAGEN.neo-CCKCR*i3/UTR/SNPA cl.12 cells and six mice received PANC02 (P+24) pCAGEN.neo-CCKBRi3/UTR cl.7 cells. Tumor growth was monitored weekly both by palpation and with calipers.

**Results**

**Transfection Optimization**

To compare the transfection efficiencies of Lipofectamine 2000 and FuGene with PANC02 (P+24) pCAGEN.neo-CCKCR*i3/UTR/SNPA cl.12 cells, an optimization study was completed using the pCAGEN.puro-EGFP (GFP) plasmid. This plasmid enabled transfection efficiencies to be calculated by fluorescent microscopy. One factor affecting
transfection efficiency is the confluence of the cells being transfected, so cultures of different densities were tested in this study. The transfection efficiency of monolayer cultures at various densities (30% and 50% of confluence for Fugene; 70% and 90% for Lipofectamine 2000) were observed utilizing various concentrations of transfections agent according to manufacturers protocol (Figure 2). Lipofectamine 2000 transfected around 50% more efficiently than FuGene. As such, Lipofectamine 2000 was utilized as the transfection reagent for the super-transfection of the murine pancreatic cancer cells with the pCAGEN.neo-CCKCR*i3/UTR/SNPC (1.28 \( \mu \)g/\( \mu \)L) plasmid. This super transfection was done in parallel with a transfection of the same lot of cells with the GFP plasmid so that super transfection efficiency could be monitored (Table 1). The super transfection efficiency was only 27% which is far less than the smaller, pilot study where Lipofectamine 2000 transfected with over 60% efficiency. Though transfection was less efficient than the pilot study suggested it should have been, these supertransfected cells were used in the immunizations of the mice in the next step of this study. 

**Immunization Using Supertransfected gamma-Irradiated PANC02 Tumor Cell Lines**

The supertransfected cells were isolated and gamma-irradiated in Gammacell at Penn State Hershey College of Medicine Department of Health Physics. Then, they were washed and injected interperitoneal into three C57Bl/6 mice. Fourteen days later, another immunization of supertransfected, gamma-irradiated cells was administered interperitoneal to each of the same three mice. The spleens of the three immunized mice and of three naïve mice were dissected, and the splenocytes were harvested and purified. ELISPOT was used to monitor the T cell responses of the mice (Figures 3 and
4). The SV40 Tag and HBc peptide served as irrelevant controls to which to compare the T cell response of splenocytes to the target peptides. The amino acid sequence of these peptide targets are described in Figure 6. The 272 peptide was thought to be the target of the mutant receptor for T cells in the past, so it was used to confirm that the 281 peptide was, in fact, the target. No significant T cell response was elicited by the super transfected cells when comparing the reactivity of splenocytes to target peptides against their reactivity to the irrelevant controls.

Tumor Growth Study

Injections three different cell lines were administered subcutaneously on the left flank of 16 mice. Four mice received PANC02 cells, six mice received PANC02 (P+24) pCAGEN.neo-CCKCR*i3/UTR/SNPA cl.12 cells and six mice received PANC02 (P+24) pCAGEN.neo-CCKBRi3/UTR cl.7 cells. Tumor growth was monitored weekly, with some exceptions, both by palpation and with calipers. The averages for each type of tumor were calculated for each day measurements were recorded (Figure 5). Though it started off producing tumors the slowest, the PANC02 (P+24) pCAGEN.neo-CCKCR*i3/UTR/SNPA cl.12 cell line elicited the most exponential trend in tumor growth by the end of this study. The other two cells lines grew exponentially at relatively the same rate as each other, but at a less exponential rate than the CCKCR cell line.

Discussion

The GFP plasmid was used to monitor transfection efficiency because its backbone is structurally very similar to the mutant CCKR plasmid. So, the efficiency with which the GFP plasmid was transfected into murine pancreatic cancer cells was assumed to be the same as that of the mutant CCKR plasmid.
The scale-up transfection was far less efficient than the smaller pilot study. This poor efficiency most likely reduced the induction of a T cell response in the mice used for immunizations. The study anticipated immunizing each mouse with 2-3x10^7 super-transfected cells that hyper-expressed the mutant receptor. However, only 27% of these cells actually received the gene for the mutant plasmid, so the gene dose was significantly lower than intended. This was but one of the possible explanations for the lack of 281-specific T cells in the splenocytes of the immunized mice. It is unknown how transfection with the mutant CCKR affects the expression of the gene in the host cell, so poor expression could be another reason for the dismal T cell response. Overall, it is unclear whether the super-transfected cells elicit an intron-specific T cell response for the mutant CCKR. Going forward, we want to use immunity generated by the 281 peptide to control the outgrowth of the tumors. Also, we want to characterize the nature of 281-reactive T cells (CD4+, CD8+ or both; cytokine profile).

The tumors produced by the three different cell lines all show signs of exponential growth. The tumors in the mice injected with PANC02 (P+24) pCAGEN.neo-CCKCR*i3/UTR/SNPA cl.12 cells grew the most variably. In two of these mice, no tumors were observed at all over the 42 days. In a different one of these mice, tumorigenesis was not observed until day 32. These zero-values for tumor size were used to calculate the averages, but these mice still competed for the highest tumor growth rate. This was because the mice in this group that did exhibit tumorigenesis had the tumors with the most exponential growth rate. The other two cells lines produced tumors in every mouse observable on day 7 and had more consistent tumor growth. The mice injected with CCKBR variant exhibited slightly larger tumors than the mice injected with WT, but
both of these types of tumors grew at relatively similar rates. Even though the CCKCR tumors had more trouble establishing themselves—that is, initiating tumor growth—than the CCKBR and WT variant—which grew in every injected mouse, the CCKCR tumors grew more robustly.

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Figure Legends

**Figure 1. A. WT vs. Mutant CCK2R** The CCK2i4SVR mutant conserves intron 4 through RNA splicing, resulting in a 69 amino acid insertion within an intracellular loop

**B. The amino acid sequence of the CR281 peptide** which corresponds to peptide sequence circled in figure and amino acid domain labeled as 281 and 300 respectively. The amino acids corresponding to positions 272 and 291 are also indicated.

**Figure 2. Various transfection conditions compared.** Since FuGene failed to efficiently transfect in smaller, pilot studies, two different lots of FuGene were used to ensure that, rather than having a bad lot, this reagent was inefficient in transfecting the murine pancreatic cancer cells. Each was tested on cells at densities falling within the suggested range. The absence of the sixth bar (right) indicates no observable transfection with Lipofectamine 2000.

**Figure 3. ELISPOT assay displaying T cell reactivity to peptides.** Columns represent red cell-depleted splenocytes from three immunized (5-12) or two naïve (1-4) mice. Mice were immunized twice (day 0 and day 14) with the irradiated cells transiently transfected with the CCK2i4SVR, and splenocytes were harvested on day 21. Peptides were added to achieve a final concentration of 10 μM: rows A, B and H, 281-300 intron peptide; rows C and D, 272-291 intron peptide; rows E and F, control HBV core 128-140 peptide; row G, control SV40 Tag 581-595 CD4+ peptide.

**Figure 4.** Graph displaying the reactivity of immune splenocytes from both the naïve mice and the immunized mice to target peptides based on number of spots counted in each well. Positive and negative error bars depict standard deviation.

**Figure 5.** Graph showing the growth of tumors from three different cell lines. Mice were injected subcutaneously on Day 0. The average tumor size from all mice injected with a given cell line was calculated for each day measurements were taken. All three show signs of exponential growth.

**Figure 6.** Amino acid sequences of the peptides used as targets in the ELISPOT assay. CR272-291 and CR281-300 were derived from the retained fourth intron in CCK2i4SVR. T529-543 is a CD4+ epitope and was derived from SV40 T antigen and provided a negative control for ELISPOT specificity of intron-specific T cells. HBc128-140 was derived from the Hepatitis B virus core antigen and was used as an irrelevant control.
**Tables**

**Table 1.** Efficiency of transient transfection of CCK2\textsubscript{4SV}R+i3 into murine pancreatic cancer cells.

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<tr>
<th>Cells</th>
<th>Transfected Cells</th>
<th>Transfection Efficiency</th>
<th>Avg. Transfection Efficiency</th>
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Figures

**CCK2R Genomic DNA:**

[Diagram showing genomic DNA with Exon and Intron regions labeled.]

**Transcription**

CCK2R  CCK2_{467},R

69 amino acid insertion

**Figure 1A**

[Diagram showing the extracellular and intracellular domains of CCK2R with labeled amino acids 291, 281, 272, and 300.]

**Figure 1B**

[Diagram showing the intracellular domain of CCK2R with labeled amino acids 291, 281, 272, and 300.]
Can Pancreatic Cancer be Controlled

Figure 2

Figure 3
Can Pancreatic Cancer be Controlled

**Figure 4**

![Bar graph showing average tumor size over time for different treatments.](image)

**Figure 5**

![Line graph showing tumor size progression over time.](image)

**Figure 6**

- GEAELWRATGPAGVGTEMK CR281-300
- GAGPREONLGEAELWRATGP CR272-291
- TPPAYRPPNAPIL Hbc128-140
- WYRPVAFQAQSIQSRT581-595 (SV40)