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Prophylactic vs. Therapeutic: DNA vaccine to metastatic colorectal cancer using molecular marker guanylyl cyclase-C

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Abstract

The role of heat-stable enterotoxin receptor guanylyl cyclase C (GCC) is well known for its aberrant chloride conductance in the intestinal epithelium in response to bacterial infection. Recently, its use as a molecular marker to detect metastatic colon cancer has grown, as has its use as oral hormone therapy for tumor suppression. This study investigates the preliminary stages of a DNA dendritic cell-based vaccine development specific to targeting guanylyl cyclase in systemic tissues. Construction of the plasmid as well as its transduction to Class I MHC molecules in antigen-presenting dendritic cells is examined. Future exploration includes a comparative assessment of prophylactic and therapeutic treatments.

Introduction

Guanylyl Cyclases

Few classes of enzymes are as diversified and interconnected as the guanylyl cyclase family. Guanylyl cyclases (GCs) catalyze the conversion of 5′GTP to cGMP, a common second-messenger molecule with regional differing expression. Increasingly in recent years, various members of the family have been the target of pharmaceutical therapeutic treatments due to their potential as versatile receptors and downstream modifier. In the mammalian body, four soluble subunits, α₁, α₂, β₁, and β₂ and seven single membrane-spanning integral subunits: GC-A, GC-B, GC-C, GC-D, GC-E, GC-F, GC-G, are expressed. However, only three of the four soluble subunits and five of the seven single membrane-spanning subunits are active in the human body. Particular differentiated tissues, such as the lung, brain, kidney and vasculature, harbor higher concentrations of soluble GCs than other tissues in the body. Soluble GCs are the receptors for nitric oxide in smooth muscle vasculature, and following activation conduct platelet aggregation inhibition and vasodilatation.

In contrast to nitric oxide for soluble GCs, peptides are recognized as the predominant activators of transmembrane GCs. GC-A is expressed vigorously in the brain, liver, testis, vasculature, adrenals, kidneys, lungs, and adipose tissues and less so in the heart and mesentery. Its activity prevents cardiac hypertrophy, hypertension, and ventricular fibrosis. Activation occurs by atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP); hormonal activation occurs by phosphorylation. GC-B is expressed principally in reproductive tissues,
bones, lungs, heart, brain, fibroblasts and vasculature smooth muscle\textsuperscript{16,23,24}. However, it is best known for inducing growth and oocyte maturation\textsuperscript{25,26}. In those respective tissues, C-type natriuretic peptide (CNP) activates GC-B in a paracrine manner\textsuperscript{27}. GC-D, activated by carbon dioxide, bicarbonate\textsuperscript{28-30}, uroguanylin and guanylin \textsuperscript{31}, is well established in the nasal epithelium and influences food preference\textsuperscript{32}. GC-E is found exclusively in the pineal gland, while GC-E and GC-F are both found in the retina\textsuperscript{33}. While extracellular hormones sufficiently activates the other membrane-spanning guanylyl cyclases, GC-E and GC-F rely on guanylyl cyclase activator proteins (GCAPs) for regulation\textsuperscript{34-36}. Upon activation, GC-E is required for recovery of the dark state after phototransduction\textsuperscript{37,38}. The two cyclases also support rod and cone nourishment, thereby preventing blindness, as well as phototransduction for sensory input\textsuperscript{39}. Finally, GC-G is located primarily in skeletal muscle, bone, lungs and the testes\textsuperscript{40-42}. However, recently its chemosensory function in Grueneberg ganglion neurons in the nasal epithelia upon activation by bicarbonate was identified\textsuperscript{43}.

\textbf{Guanylyl Cyclase-C}

Guanylyl Cyclase C (GCC) is a transmembrane receptor structurally analogous to GC-A and GC-B\textsuperscript{44} that binds endogenous peptides uroguanylin (UGN) and guanylin (GN), intracellular peptide cGMP, as well as the bacterial heat-stable enterotoxin (STa)\textsuperscript{45-47}. Expression in humans is restricted to intestinal mucosal cells\textsuperscript{48,49}. Yet in other species, its presence has been identified in the perinatal liver\textsuperscript{50}, lung, gall bladder, testes\textsuperscript{51}, mesentery, atrium, and kidney\textsuperscript{16,52}.

In normal physiological activity, cGMP concentrations increase after the ligands uroguanylin and guanylin bind, phosphorylating the cystic fibrosis conductance receptor, and increasing chloride flow into the lumen\textsuperscript{45,53,54}. Enterotoxins released by diarrheagenic bacteria such as \textit{Citrobacter freundii, Escherichia coli, Vibrio cholerae, Yersinia enterocolitica,} and \textit{Vibrio mimicus} bind to the GC-C receptor, inducing chloride and water flow into the lumen, resulting in diarrhea\textsuperscript{54,55}. Inhibition of guanylyl cyclase-C signaling prevents chloride and subsequent water secretion, invoking potential for therapeutic strategies in developing countries where diarrhea is an endemic issue\textsuperscript{56}.

\textbf{Guanylyl Cyclase-C and Tumorigenesis}

GC-C regulates intestinal epithelial proliferation at the G1/S phase intersection of the mitotic cycle and prevents somatic hypermutation\textsuperscript{57}. Elimination of GC-C results in a decrease in the proportion of differentiated cells, a marked increase in growth along the crypt-villus axis, increased migration, and an increase in the rate of hypermutation\textsuperscript{57,58}. Additionally, expression of uroguanylin\textsuperscript{39} and guanylin\textsuperscript{60,61} is significantly decreased. This apparent decrease in uroguanylin and guanylin signaling to GC-C at the site of adenoma is likely responsible for carcinogenesis\textsuperscript{57,58,60,62}. GC-C in both primary and metastatic tumors is not only apparent\textsuperscript{63,64}, but vigorously overexpressed due to a lack of its endogenous ligands uroguanylin and guanylin\textsuperscript{57,58,62,65,66}. Astoundingly, a lack of GC-C is associated with a considerable decrease in the number of adenomas and an increase in apoptosis in the proximal intestine\textsuperscript{67}. 
Guanylyl Cyclase C Related Therapeutics

In the past two decades, therapeutic approaches to targeting colorectal cancer metastasis have focused on functional STa receptors such as GC-C, due to its exclusive presence in the intestinal epithelium and its abnormal overexpression in tandem with adenocarcinoma indicators \(^{63,65}\). One of the most direct approaches is administration of GC-C via oral hormone therapy due to evidence of its tumor suppressing activity \(^{62,65,66,68,69}\). Although GC-C prevents metastasis in the intestinal epithelium by way of colon cancer cell MMP-9 regulation \(^{70}\), the presence of GC-C in extraintestinal organs detected by reverse transcriptase PCR is considered a more sensitive and specific indicator of metastasis than histopathology alone \(^{64,71}\). In stage II colorectal cancer patients, the presence of guanylyl cyclase C mRNA in lymph nodes is consistent with recurrence \(^{71,72}\). Stage II cancers in patients without GCC mRNA lymph node presence did not recur \(^{72}\).

Dendritic Cell Based Immunotherapy

Dendritic cells (DCs) are antigen-presenting cells, first described in 1973 by Steinman and Cohn, that sample various antigens in the surrounding environment \(^{73}\). Their presence is significantly more distinguished in epithelial tissues, such as the skin, lungs, and intestinal lining, where they monitor for foreign pathogens \(^{74-77}\) and activate B cells and T cells much more effectively than other cell types such as B lymphocytes, T lymphocytes, and macrophages \(^{78,79}\). In particular, dendritic cells present phagocytosed antigen on Class II Major Histocompatibility Complexes (MHC) to immature T-cells \(^{79,80}\). MHC Class I molecules are typically loaded with peptides excised from internal cytosolic antigens \(^{81}\), however they can also present peptides from phagocytosis of bacteria \(^{82}\), and apoptosis of other cells \(^{83}\).

Immature DCs use two mechanisms to capture the antigens which they present: constitutive macropinoctyosis and micropinocytosis of large volumes of fluid, as well as macrophage-mannose receptor (MMR) mediated capture. Macro and micropinocytosis are insatiable mechanisms of antigen capture, while MMR mediated capture is satiable and more selective to specific antigens \(^{84}\). Activation occurs by Toll-like receptors (TLR), cytokine receptors, TNF-receptors, FcR receptors, and sensors for cell death \(^{85}\). Indeed, tumor Necrosis Factor alpha (TNF-α) or soluble CD40 ligand (CD40L) causes a notable increased surface expression of MHC Class I and MHC Class II molecules, B7 and ICAM-1 \(^{86}\).

Upon DC activation and maturation, MHC Class II molecules present peptide fragments to CD 4+ helper T-cells, while MHC Class I molecules present peptide fragments to CD 8+ cytotoxic T-cells \(^{85}\). T-cell activation typically requires a cell activation signal, provided by inflammatory cytokines TNF-α or IL-1, or bacterial components such as LPS. The signal induces MHC and co-stimulation receptor surface expression and cell migration to secondary lymphoid organs for T cell priming \(^{87}\).

In recent years, a growing emphasis has been placed on dendritic cell based immunotherapy due to its potential to reduce tumor size, and prevent recurrence by generating immunological memory in a therapeutic approach. On the other hand, it would be equally beneficial to prevent metastasis in the first place through a prophylactic approach \(^{88}\).
**Materials and Methods**

**pEF1-VP22-mGCC-S1 Plasmid Construction**

**Digestion with Enzymes Not1, XBa**

pEF1-VP22 vector plasmid and pCR-II-TOPO-mGCC-S1 insert plasmid were obtained. Two overnight digestion mixtures were prepared with 5 μL 10X enzyme buffer, 5 μL pEF1-VP22, 2.5 μL XBa, 2.5 μL Not1, and 35 μL water for a total volume of 50 μL. Digestions were loaded onto a 50 mL 0.7% agarose gel and run for approximately an hour at 80V. The gel was stained, destained, and excised under ultraviolet light. Excision of pEF1-VP22 and mGCC bands with desired lengths was completed with sterile scalpels. The weight in milligrams was obtained of the gel bands. Plasmids then underwent purification by centrifugation using the ProMega DNA Centrifugation by Purification protocol. 10 mL Membrane Binding Solution was added per 10 mg of gel. The gel was incubated at 56°C for 10 minutes to dissolve the gel. Concentrations of pEF1-VP22 and mGCC-S1 insert were determined using photometry.

pEF1-VP22 underwent dephosphorylation after purification to ensure successful ligation. 1 μL shrimp-alkaline phosphatase, 5 μL 10X enzyme buffer, 20 μL pEF1-VP22, and 24 μL water for a total volume of 50 μL. Dephosphorylation mixture was incubated at 37°C. After 1 hour, the dephosphorylation enzymes were inactivated, and the vector isolated.

Dephosphorylation inactivation occurred with placement of pEF1-VP22 dephosphorylation mixture at 65°C for 15 minutes. 50 μL of 25:1 Phenol / Chloroform and 50 μL dephosphorylation were combined to create a new mixture that was emulsified via spinner. The 100 μL consequential mixture was transferred to a prepared 2 mL phase lock gel and centrifuged at 14,000 rpm for 5 minutes. A 1:1 volume of chloroform / isoamyl was added to the mixture, and emulsified for 10 minutes via spinner. The 200 μL consequential mixture was centrifuged at 14,000 rpm for 5 minutes. The aqueous phase containing the pEF1-VP22 plasmid was harvested.

The pEF1-VP22 vector was precipitated from the aqueous phase by adding 2.5x volume -20°C ethanol and incubated in a -70°C ice bath for 15 minutes. 0.1x volume ammonium acetate salt was added. The mixture was centrifuged at 16000 rpm for 15 minutes, and ethanol supernatant discarded. 1 mL 70% ethanol was added and the entire mixture centrifuged at 16000 rpm for 15 minutes and supernatant discarded. The process was repeated. The pellet was dried and resuspended in nuclease free water. Concentration of pEF1-VP22 was measured with photometry.
Figure 1 - Plasmid map of vector pEF1-VP22 used for ligation. Highlighted in red boxes are digestion sites for Not1 at 915 bp and XBa at 927 bp.

Figure 2 - Plasmid map of insert pCR-II-TOPO-mGCC-S1 used for ligation. Highlighted are the Not1 and XBa digestion sites at 3 bp and 1402 bp respectively used for the primary approach. Also highlighted is another Not1 digestion site at 1441 bp used in the alternative approach.
Figure 3 - Plasmid map of pEF1-VP22-mtGNAQ-PADRE vector, used in the alternative approach. The mtGNAQ-PADRE was extracted using Not1 digestion sites at 915 bp and 2077 bp.

Figure 4 - Plasmid map of pEF1-VP22-mGCC-S1, the desired plasmid expressed in dendritic cells. The mGCC-S1 segment was inserted using Not1 digestion sites at 915 bp and 2353 bp.
Digestion with Enzyme Not1 Using Alternative Vector

An alternate approach attempted was using the pEF1-VP22-mtGNAQ-PADRE plasmid to construct a vector and pCR-II-TOPO-mGCC-S1 as an insert. Two overnight digestion mixtures were prepared with possible digestion mixtures were prepared with 5 μL 10X enzyme buffer, 5 μL pEF1-VP22-mtGNAQ-PADRE, 5 μL Not1, and 35 μL water for a total volume of 50 μL. Digestions were loaded onto a 50 mL 0.7% agarose gel and run for approximately an hour at 80V. The gel was stained, destained, and excised under ultraviolet light. Excision of pEF1-VP22 and mGCC-S1 bands with desired lengths was completed with sterile scalpels. Plasmids then underwent purification by centrifugation. Concentrations of pEF1-VP22 and mGCC-S1 insert were determined using photometry.

pEF1-VP22 underwent dephosphorylation after purification to ensure successful ligation. 1 μL shrimp-alkaline phosphatase, 5 μL 10X enzyme buffer, 20 μL pEF1-VP22, and 24 μL water for a total volume of 50 μL. Dephosphorylation mixture was incubated at 37˚C. After 1 hour, the dephosphorylation enzymes were inactivated, and the vector isolated. Dephosphorylation inactivation occurred with placement of pEF1-VP22 dephosphorylation mixture at 65˚C for 15 minutes.

The pEF1-VP22 vector was then isolated by gel extraction in a 50 mL 0.7% agarose gel run at 80V for approximately 60 minutes. The vector was purified by centrifugation.

Ligation and Transformation

Two ligation mixtures were prepared, one with purified pEF1-VP22 and mGCC-S1 insert fragments, and the other solely with pEF1-VP22 for self-ligation. The appropriate amounts of insert mGCC-S1 and vector pEF1-VP22 used in ligation were calculated based on a formula shown below:

\[ \text{Formula: ng insert} = \frac{ng \text{ vector} \times k \text{ molar ratio of vector}}{k \text{ size of vector}} \times \text{insert} \]

The insert to vector 3:1 was used for ligation. The ligation mixture was prepared with 1 μL pEF1-VP22, 1 μL mGCC-S1 insert, 1 μL 10X enzyme buffer, 1 μL ligase, 6 μL water for a total volume of 10 μL. The self-ligation mixture was prepared with 2 μL pEF1-VP22, 1 μL 10X enzyme buffer, 1 μL ligase, 6 μL water for a total volume of 10 μL. Ligation occurred overnight at 16˚C.

Electroporation occurred at 1.5 kV with the objective of inserting the pEF1-VP22-mGCC-S1 ligation plasmid into bacteria. Standard electroporation mixtures were prepared for both the pEF1-VP22/mGCC-S1 ligation and the pEF1-VP22 self-ligation. Electroporation mixtures including 1 μL ligation mixture, 4 μL water, 20 μL 10% glycerol and 25 μL ampicillin-resistant bacteria were inserted into cuvettes. Immediately after electroporation, 600 μL SOC was added to the cuvettes, and the entire mixture transferred to a bacterial tube for incubation at 37˚C for 1 hour. After incubation, 100 μL of bacteria containing pEF1-VP22-mGCC-S1 ligation plasmid was plated onto ampicillin-resistant agar and incubated at 37˚C overnight. Within 24 hours,
plates were checked for colony growth. Ligation of pEF1-VP22 with the mGCC-S1 insert was confirmed with comparison to self-ligation. The ligation would contain colonies, whereas the self-ligation would not.

**Mini-Prep of Select Colonies for pEF1-VP22-mGCC-S1 Verification**

Once ligation presence was confirmed with bacterial colony presence, sterile toothpicks were used to transfer select colonies to bacterial tubes containing 3 mL ampicillin-resistant liquid agar for overnight incubation at 37°C. After incubation, bacterial tubes were centrifuged at 4000 rpm for 10 minutes. Supernatant was discarded and bacterial pellet resuspended. Sterile toothpicks were used to swipe each colony onto its respective space on a master plate. Bacteria was resuspended in 600 μL water, and combined with 100 μL cell lysis buffer, subsequently 300 μL neutralization solution and centrifuged at 14000 rpm for 3 minutes. Supernatant was collected and eluted through columns into collection tubes using centrifugation at 14000 rpm. Afterwards, 200 μL of Endotoxin Removal Wash was added to each column and eluted by centrifugation. 400 μL Column Wash was then added and eluted through each column by centrifugation. Then 30 μL of distilled water was eluted through the column by centrifugation. Bacterial colony plasmids were loaded into a 30 mL 0.7% agarose gel and run for approximately 60 minutes at 80 V for DNA length confirmation.

**Dendritic Cell Nucleofection of Green Fluorescent Protein, Red Fluorescent Protein, pEF1-VP22-mGCC-S1 plasmid**

Mouse femurs were harvested and immature dendritic cells flushed out via syringe with RPMI 1640 supplemented with culture medium II (10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine). Cells were centrifuged at 300xg for 10 minutes at room temperature, resuspended until at a density of 1x10⁶ cells / mL and transferred to 24-well plates for incubation at 37°C for six days.

On Day 2, 700 μL was replaced with culture medium I (10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM glutamine and 2000U/ml GM-CSF). On Day 3, all of the culture medium was removed and the wells washed multiple times in 500 μL culture medium I. 1 mL fresh culture medium I was added to each well. On Day 6, loosely adherent immature dendritic cells were harvested by pipetting with culture medium I.

**Maturation of Dendritic Cells**

Mature dendritic cells were generated by stimulating immature dendritic cells with 0.1 μg/ml LPS. 1 mL maturation media replaced 1 mL of cell supernatant. On Day 7, loosely adherent mature dendritic cells were harvested by pipetting with culture medium I.
**Nucleofection**

In preparation for nucleofection, 48-well plates were filled with 400 μL culture medium I and incubated at 37°C. Cells were centrifuged at 300xg for 10 minutes after determining cell density. Supernatant was discarded entirely and the pellet resuspended in 100 μL room temperature Nucleofector Solution. 100 μL room temperature Nucleofector Solution was combined with 5 different DNA plasmids examined for expression: 2 μg pEF1-VP22-mGCC-S1 plasmid from Colony 1, 2 μg pEF1-VP22-mGCC-S1 plasmid from Colony 2, 2 μg pmax pMax-GFP Vector, 2 μg pmax RFP Vector, and 2 μg pEF1-VP22-mtGNAQ-PADRE-S1 as a control. Cell Solution with DNA was transferred to cuvette and run under Nucleofector Program Y-001. 400 μL culture medium I was added to the cuvette immediately after nucleofection. Sample was transferred to a 48-well plate. Nucleofection had occured according to Lonza Amaxa Mouse Dendritic Cell Nucleofector Kit for Immature and Mature Dendritic Cells. Cells were stained with anti-CD11C-PE antibodies, imaged, and analyzed via flow cytometry (FACS Analysis).

**Results**

**pEF1-VP22-mGCC-S1 Construction**

Not1 / XBa double digestion fragment lengths for gel excision were 6722 bp for pEF1-VP22, and 1399 bp for the mGCC-S1 insert. However, the double digest approach did not generate bacterial colonies upon transformation multiple times. On several instances, Not1 / XBa did in fact generate bacterial colonies. The plasmids from these colonies underwent MiniPrep plasmid isolation and digested with enzymes BamH1 and HindIII for 3 hours at 37°C in separate reactions. Upon analysis of the fragment lengths on agarose gel, it was determined there were no viable candidates for further pursuit.

Not1 digestion fragment lengths for gel excision were 6969 bp for pEF1-VP22 and 1438 for the mGCC-S1 insert. The alternate approach using the pEF1-VP22-mtGNAQ-PADRE-S1 vector yielded several bacterial colonies. The plasmids from these colonies, upon MiniPrep and digestion with enzymes BamH1 and HindIII, yielded expected fragment lengths. Due to the potential for presence of the desired plasmid, a multi-digest was performed with bacterial colonies 1,2 and 6 from the MiniPrep. The multi-digest consisted of separate reactions with enzymes HindIII, BamH1, Not1, ApaL1, and BglII for clone 1, and HindIII, BamH1, Not1, and BglII for clones 2 and 6. Fragment lengths for Not1 digestion yielded fragment lengths 6969 bp for pEF1-VP22 and 1438 for the mGCC-S1 insert. Colony 1 and Colony 2 had the largest presence of the apparent plasmid pEF1-VP22-mGCC-S1, and were thus used for nucleofection.
**Figure 1** - Gel excision of pEF1-VP22 mGCC-S1 insert (NotI Digestion)

Lane 1 - DNA 1 kb Ladder  
Lane 2 - pEF1-VP22 - S1 (6969 bp)  
Lane 3 - mGCC-S1 (1438 bp)

**Figure 2** - Gel excision of pEF1-vector and VP22 vector and mGCC-S1 insert (NotI/Xba Digestion)

Lane 1 - DNA 1 kb Ladder  
Lane 2 - pEF1 - VP22 - S1 (6722 bp)  
Lane 3 - mGCC-S1 (1399 bp)

**Figure 3**

MiniPrep of ligated clones using NotI digestion/ pEF1-VP22-mtGNAQ-S1 vector.

Upper - Hind III Digestion  
Lane 1 - DNA 1 kb Ladder  
Lane 2 - Clone 1, Lane 3 - Clone 2, Lane 4 - Clone 3, Lane 5 - Clone 4, Lane 6 - Clone 5, Lane 7 - Clone 6, Lane 8 - Clone 7

Lower - Bam H1 Digestion  
Lane 1 - DNA 1 kb Ladder, Lane 2 - Clone 1, Lane 3 - Clone 2, Lane 4 - Clone 3, Lane 5 - Clone 4, Lane 6 - Clone 5, Lane 7 - Clone 6, Lane 8 - Clone 7
Figure 4 - MultiDigest of Clones 1, 2, and 6 from MiniPrep after ligation to confirm presence of desired pEF1-VP22-mGCC-S1 plasmid. Clone 1: Lanes 2-6; Lane 2 - HindIII, Lane 3 - BamH1, Lane 4 - Not1, Lane 5 - ApaL1, Lane 6 - BglII, Clone 2: Lanes 8-11; Lane 8 - HindIII, Lane 9 - BamH1, Lane 10-Not1, Lane 11-BglII, Clone 3: Lanes 13-16; Lane 13-HindIII, Lane 14-Lane-BamH1, Lane 15-Not1, Lane 16-BglII. Of particular interest is Not1, which for all three clones displays the expected size lengths of 6969 for pEF1-VP22-mGCC-S1 and 1438 for the mGCC-S1 insert.

FACS Analysis of RFP, GFP, and CD11C

Protein expression efficacy of dendritic cells nucleofected with pMaxGFP and pMaxGFP was determined by counting fluorescent cells per field 24 hours after nucleofection and ranged from 23.8% (pMaxRFP) to 49% (pMaxGFP). Analysis of cell differentiation revealed that in control cells, 6% demonstrated GFP fluorescence to gate threshold levels and 9% demonstrated phycoerithrine (PE) fluorescence to gate threshold levels. In cells labeled with CD11c-PE antibodies, 4.8% of dendritic cells demonstrated GFP fluorescence to gate threshold levels and 84.8% demonstrated PE (and thus CD11c) fluorescence to gate threshold levels. Analysis of protein expression after nucleofection elucidated that in cells nucleofected with pMax RFP, 0.5% demonstrated GFP fluorescence to gate threshold levels, and 23.8% for RFP fluorescence to gate threshold levels. In cells nucleofected with pMax GFP, 49% demonstrated GFP fluorescence to gate threshold levels and 95.5% demonstrated antibody CD11c-PE fluorescence to gate threshold levels.
**Figure 5** - Panels (from left to right) 1 and 2: pMaxGFP fluorescent expression in dendritic cells (49% efficacy), Panel 3: pMax-RFP expression (23.8% efficacy), Panels 4 and 5: bright field of pMaxGFP expression, Panel 6: magnification of pMaxGFP fluorescent expression.

**Figure 6** - FACS analysis of cell differentiation. 6% fluorescence significant for pMax-GFP on left. 9% fluorescence significant for phycoerithrine (PE) on right.
Figure 7 - FACS analysis of cell differentiation. 4.8% fluorescence significant for pMax-GFP on left. 84.8% fluorescence significant for phycoerithrine (PE) on right.

Figure 8 - FACS analysis of protein expression post-nucleofection. 0.5% fluorescence significant for pMax-GFP on left. 23.8% fluorescence significant for pMax-RFP on right.

Figure 9 - FACS analysis of protein expression post-nucleofection. 49% fluorescence significant for pMax-GFP on left. 95.5% fluorescence significant for CDC11c-PE on right.

Discussion

While the reason for its dysfunction remains unclear, the primary double digest with NotI and XBa is not recommended for construction of the desired pEF1-VP22-mGCC-S1 plasmid. Gel excision ascertained pEF1-VP22 and mGCC-S1 were present in ample quantities. However, after multiple ligations and transformations, growth of bacterial colonies was scarce and amorphous. The plasmid strains in the few colonies present, after digestion with enzymes BamH1 and HindIII, did not yield expected fragment lengths.
On the other hand, the alternative use of Not1 with two digestion sites on the pEF1-VP22-mtGNAQ-PADRE vector and the pCR-pCR-II-TOPO-mGCC-S1 insert proved more successful. Gel excision confirmed pEF1-VP22 and mGCC-S1 were present in ample quantities. Upon ligation, clearly defined colonies were present in greater numbers than in the primary approach. From the MultiDigest, it’s apparent that the desired pEF1-VP22-mGCC-S1 plasmid may be present. Not1 digest revealed expected fragment lengths (6969 bp for the vector and 1438 bp for the insert), lending potential to the presence of the DNA vaccine.

FACS Analysis confirmed the presence of viable dendritic cells capable of transducing presented DNA into Class I MHC molecules. Whereas the control cell group exhibited 6% fluorescence for pMax GFP and 9% for PE, cells labeled with CD11c-PE antibodies exhibited 84.8% for PE, a significant difference. Thus, cells labeled with CD11c-PE antibodies would fluoresce significantly more than if unlabeled. Additionally, cells isolated from mouse bone marrow and nucleofected with pMax-RFP were 23.8% effective at transducing presented DNA to the surface. Cells isolated from mouse bone marrow and nucleofected with pMax-GFP were 49% effective at transducing presented DNA to the surface. The same cells were 95.5% effectively labeled with CD11c-PE antibodies. Therefore, cells transduced with DNA would be 46.8% effective at fluorescing CD11c-PE antibodies labeling the antigen expressed in dendritic cells. Efficacy of transduction and antibody labeling could vary upon multiple attempts, however the presence of viable dendritic cells is certain.

With further pursuit, a DNA vaccine capable of targeting guanylyl cyclase in the systemic body could be developed and administered in vitro to dendritic cells harvested from the organism. The harvested dendritic cells could be matured with LPS, nucleofected with the constructed plasmid, and would express guanylyl cyclase as an antigen on class I MHC molecules. Such antigen-presenting dendritic cells would activate killer T cells targeting cells expressing guanylyl cyclase. Such a vaccine would be effective at targeting metastatic adenocarcinoma, known for overexpression of guanylyl cyclase. Such treatments would begin with observing prophylactic treatment effectiveness in preventing metastasis compared to that of therapeutic treatment in destroying microtumors.

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