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Increasing the Stability and Expression of Green Fluorescent Protein in Pancreatic Cancer Cells for Metastasis Assays

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ABSTRACT

Pancreatic cancer is the fourth leading cause of cancer death in the United States, with only 11% of patients diagnosed with metastatic disease surviving the first year. Even so, few studies exist to characterize the mechanism of metastasis in this highly aggressive form of cancer. Consequently, it is imperative that techniques, such as tagging cells with green fluorescent protein (GFP), are developed to track cancerous cells over the course of their spread. Unfortunately, the existing GFP vector contains a cytomegalovirus promotor, which is readily silenced by cells and decreases the vector’s usefulness for long-term studies in vivo. It was hypothesized that the insertion of the enhanced GFP gene downstream from a beta-actin promotor with CMV enhancers would increase expression and long-term stability of GFP in pancreatic cancer cells. The enhanced GFP gene was removed from the pEGFP-N1 vector and then ligated into pCAGEN.puro. Murine pancreatic cancer cells were transfected with either pCAGEN.puro-EGFP or pEGFP-N1. Fluorescence intensity and stability were assessed through flow cytometry, revealing that the beta-actin promoter resulted in a greater percentage of cells fluorescing after two months of culture than the cells with GFP expressed under a CMV promoter. Assessment of tumorigenicity of two novel clones in mouse models reveals that one clone in particular shows promise for use in metastasis assays due to its stable fluorescence, high growth rate, and ability to report the presence of metastases and single cells or micrometastases disseminated in the lungs. Ongoing studies are confirming the increase in intensity and stability in human pancreatic cancer lines.
INTRODUCTION

Pancreatic Cancer

Pancreatic cancer is particularly known for its aggressiveness. It’s the fourth most common cause of cancer-related death in the western world, and it’s expected to pass breast cancer in mortality rate (Hariharan et al. 2008; Yachida et al. 2013). Most patients that are diagnosed die within five years. According to a study that used Surveillance, Epidemiology and End Results (SEER) data between 1977 and 2001, 37.8% of patients with local disease survive the first year, and only 11.4% of those with metastatic disease live that long (Shaib et al. 2006).

The localized disease produces few characteristic symptoms, and metastasis occurs either before or quickly after the first symptoms (e.g. jaundice) appear. (Wolfgang et al. 2013). In fact, almost half of patients are diagnosed after metastasis, and this statistic hasn’t changed over time as new diagnostic tests have been developed (Shaib et al. 2006). Because of this late diagnosis, curative therapy is nearly impossible, and incidence almost equals mortality (Hariharan et al. 2008). Metastasis is associated with 90% of pancreatic cancer, and, left untreated, metastatic pancreatic cancer results in mortality within an average of 3-5 months (Yachida et al. 2013; Hariharan et al. 2008).

Even with the weighty implications of metastasis on survival rates, “the mechanisms of metastasis formation are relatively understudied compared with that of carcinogenesis, deregulation of cellular pathways, or treatment resistance” (Yachida et al. 2013).

Cell Tracking

Cell tracking is imperative in the process of understanding the spread of cancer, the role of specific genes in the process and the effect of therapeutics on metastatic tumors. Indirect
tracking of cancer cells, is typically accomplished with the transfection of a plasmid DNA that encodes a reporter gene (Zhang et al. 1996). The most commonly used reporter genes include β-galactosidase, chloramphenicol acetyltransferase, luciferase from the firefly *Photinus pyralis* or the sea pansy *Renilla reniformis*, and green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Coralli et al. 2001). Both the β-galactosidase and luciferase techniques require injection of substrates or cofactors, limiting their usefulness in living organisms (Zhang et al. 1996). Thankfully, green fluorescent protein embodies a simpler alternative.

**Green Fluorescent Protein**

Shimomura discovered green fluorescent protein (GFP) in 1961 while isolating the luminescent blue aequorin protein from *Aequorea victoria* (Shimomura 2005). He found that this unique protein fluoresced green when excited by blue or ultraviolet light, and this fluorescence can be easily detected with a fluorescent microscope (Zhang et al. 1996; Hoffman 2001).

This simple discovery possesses a plethora of benefits. Because the fluorophore is a part of the protein’s primary structure, the need for substrate addition is eliminated, reducing preparation time compared to luciferase and eliminates perfusion variables. When comparing β-galactosidase-stained and GFP-tagged tumors, Zhang et al. found that 60% of the appropriately transfected cells were GFP positive, while only 40% were β-gal positive (Zhang et al. 1996). Thus, GFP also exhibits better sensitivity than other choices. Additionally, it possesses a high ratio of tumor fluorescence compared to the autofluorescence of background tissues, and any remaining autofluorescence can be minimized through the use of a proper filter (Hoffman 2001; Hoffman 2009). At high resolution, it is possible to visualize cell outlines and some morphological traits (Hoffman 2001). GFP is species-independent, permitting its use in both
bacteria for amplification and in a variety of model mammals (Zhang et al. 1996). More significantly, it has even been stably transduced in human cell lines, allowing for more accurate representations of “tumor growth, angiogenesis, dormancy, dissemination, invasion, metastasis, and progression through all stages in vivo” (Hoffman 2001).

Imaging fluorescent proteins enables researchers “to visualize whether a cell that reaches a distant organ will proliferate, arrest, or die, and to establish the factors that influence this process” (Hoffman 2009). As stated by Hong, “The development of transgenic mice which express fluorescent proteins has revolutionized cancer research and enabled the study/imaging of many important biological processes (not limited to cancer) that previously could only be studied in vitro” (Hong et al. 2010).

Despite the numerous benefits of GFP in tracking metastasis, it is not without its limitations. Wild-type GFP directly extracted from a jellyfish has several undesirable qualities including its lower intensity under blue light and poor expression in a few specific mammals. Fortunately, an enhanced GFP variant (EGFP) was constructed by using two point mutations on the fluorophore to make it 35 times brighter and by using human-favored codons instead of “jellyfish codons” to increase expression (Zhang et al. 1996). This enhanced protein is now used in most GFP-related studies.

**Promoter Stability**

A cause for concern with reporter genes is their stability of expression in vivo. Chan et al., have recognized this need and have completed investigations to determine the best promoter for transgene expression. Long-term transfections often utilize a viral promoter to drive robust constitutive expression during extended culture. The cytomegalovirus (CMV) promoter was a
frequent choice in the aforementioned sources due to its strong activity in many mammalian cell lines (Chan et al. 2008). Even though the CMV promoter is commonly used, its effectiveness has been shown to fluctuate at various cell-stages, and it can be repressed by p53 (Chung et al. 2002; Liu et al. 2014). Unfortunately, pEGFP-N1 (Clontech) utilizes a CMV promoter, limiting its usefulness in long-term studies. On the other hand, β-actin is a ubiquitously-expressed gene found in most vertebrate cells, and, as such, its promoter is unlikely to be silenced by the cell (Liu et al. 2014). Realizing this, Niwa et al. connected the promoter driving the chicken β-actin gene (Ac) to a CMV immediate early enhancer to create the CAG promoter in pCAGGS (Niwa et al. 1991). GFP activity controlled by a CMV promoter has been found to only fluoresce at 1% of a CAG promoter’s level in human embryonic cells after 36 hours (Chung et al. 2002). Additionally, one study determined that pCAGGS vectors enabled fluorescence for four months (Liu et al. 2014). It has also been shown that chicken β-actin promoters are better for long-term gene tests than CMV promoters (Chan et al. 2008).

It was hypothesized that a chicken β-actin promoter with CMV immediate-early enhancers will increase long-term stability and high expression of EGFP in tumors of pancreatic cancer. To test this, a pEGFP-N1 vector was digested with restriction endonucleases so that the EGFP gene could be inserted downstream of a β-actin promoter in pCAGEN.puro. Subsequently, the plasmid was transfected into mammalian pancreatic cancer cells so that the intensity and percentage of fluorescing cells could be measured with flow cytometry. The transfected cells were assayed in vivo to assess their efficacy in model organisms.
MATERIALS AND METHODS

Transformation of E. coli

DH5α cells (0.2 mL) in 0.1 mL TMC were transformed with approximately 1 ng of DNA using heat shock. Tubes were incubated on ice, put in a 37°C bath for 2 minutes, and then incubated at room temperature for 10 minutes before 2xYT media was provided for a 30 minute cycle on the 37°C shaker. Subsequently, the cultures were plated onto 1x YT the supplemented with Ampicillin (100 µg/mL) or Kanamycin (50 µg/mL) and incubated at 37°C overnight.

DNA Extraction

DNA was extracted from bacteria using a QIAspin Miniprep or EndoFree Plasmid Maxi Kit (Qiagen). Plasmid concentration was determined through the use of a NanoDrop (Thermo Scientific). Extracted DNA was stored at 4°C.

Restriction Digests and Gel Electrophoresis

Restriction digests were performed in a total volume of 50µL using 2 µg DNA and 10 units of enzyme per manufacturer’s protocol (New England BioLabs). Reactions were incubated at 37°C overnight and then kept at 4°C until gel electrophoresis could be performed.

Gels were cast with 1% agarose in TAE with 0.2 µg/mL ethidium bromide and then submerged in a container of TAE with ethidium bromide (0.2 µg/ml). Undigested DNA was run as a control and one lane contained 10 µL Hi-Lo DNA Marker (Minnesota Molecular) as the standard ladder. Samples were loaded with 5x sample buffer and resolved at 110 V and 550 mA for 1 hour and 20 minutes.
Gel Purification

Bands were excised from the gel and DNA purified using QIAquick Gel Extraction Kit (Qiagen). Product concentration was determined by NanoDrop.

Ligation Reactions

Ligation reactions were performed according to Promega’s T4 Ligase protocol. Reactions were prepared at vector:insert ratios of 1:1, 1:3, and 3:1. All were placed in the thermocycler to incubate at 15°C.

Cell Culture

PANC02 murine pancreatic cancer cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS). After transfection, media was supplemented with either 5µg/mL puromycin or 400 µg/µL G418. Cells were incubated at 37°C in an incubator with 5% CO₂. Passaging was accomplished through a phosphate buffered saline (PBS) rinse followed by a 4-6 minute incubation with 0.25% trypsin/EDTA (Gibco).

Kill Assays

Appropriate selective antibiotic concentrations were assessed through kill assays. PANC02 cells were incubated in media supplemented with 0, 1, 2, 3, 4, or 6 µg/mL of puromycin. Another 6-well plate contained wells of PANC02 cells incubated in media supplemented with either 0, 100, 150, 200, 250, or 300 µg/mL of G418. One additional assay was performed to confirm the results, and this included a control well, 300 µg/mL neomycin,
350 µg/mL neomycin, 4 µg/mL puromycin, 5 µg/mL puromycin, and 6 µg/mL puromycin. Chosen concentrations contained a minimal number of viable cells after 48 hours of selection.

Transfection and Clonal Isolation

pCAGEN.puro-EGFP and pEGFP-N1 were each stably transfected into PANC02 cells using Lipofectamine 2000 (Invitrogen) per manufacturer’s recommended protocol. At 24 hours, an initial screen of the cells was conducted by fluorescence microscopy. For stable transfection, cells were passaged 1:10 at 24 hours. At 48 hours post-transfection, cells were placed on appropriate selective media – puromycin for pCAGEN.puro-EGFP and neomycin for pEGFP-N1.

Stably-transfected colonies with greater intensity as observed by fluorescence microscopy were transferred to individual wells of a 24-well plate with a P1000 micropipette. This process was performed for 18 colonies of cells transfected with pCAGEN.puro-EGFP and for 12 colonies transfected with pEGFP-N1. Once attached to the plate surface, each individual colony was trypsinized and dispersed to permit even growth across the well. Upon reaching confluency, clones were transferred into wells of increasing surface area. The use of selective media was discontinued after the first month of culture. Clones were frozen for cryostorage (20% FBS and 10% dimethylsulfoxide in PBS).

Fluorescence Microscopy

Cells were observed using a Nikon TS-100 inverted scope equipped with fluorescence and a FITC filter set. Photographs were taken of each clone and those that were deemed to be
the most intense were cultured over the course of two months. Photographs were obtained of the clones at passage 6 and around passage 12.

**Flow Cytometry**

Cells were detached using Cellstripper non-enzymatic cell dissociated solution (Mediatech). Cells were pelleted and resuspended in 1 mL of PBS and then maintained on ice. Each sample was transferred to a polystyrene tube and vortexed before flow cytometry (Special Order BD LSR II). Data from 30,000 cells were recorded at a low setting for most samples; 250,000 events were collected at a high setting for the transient transfections. Cells were excited with 488 nm light and detection occurred at 525 nm. Analysis was conducted with BD FACS Diva Software.

**In vivo Tumor Assessment**

Sixteen male C57Bl/6 mice were used to determine the tumorigenicity, metastatic potential, and fluorescence of the pCAGEN.puro-EGFP clones. All protocols were approved by the Messiah College IACUC. Parental PANC02 cells at the 30th and 31st passage were used as a control. PANC02-pCAGEN.puro-EGFP clone 15 (passage 12) was chosen for its high percentage of cells fluorescing after two months of cell culture, and PANC02-pCAGEN.puro-EGFP clone 17 (passage 6) was chosen for its high fluorescence intensity. The parental line and each clone were detached and counted by hemocytometer. After centrifugation at 4°C and 900 rpm for 5 minutes, the media was aspirated and the cells were resuspended in Hank’s buffered saline solution to the desired concentration.
Mice were anesthetized intramuscularly in the left hind leg with ketamine hydrochloride (100 mg/kg) and Xylazine (10 mg/kg). Incision sites were sterilized with ethanol and betadine, and ophthalmic ointment was applied to the eyes. After surgery, the wounds were closed with wound clips and treated with 1% lidocaine, and the mice were allowed to recover on plate warmers. The surgical staples were removed two weeks post-surgery.

Three routes of cell injection were performed, each with a 27 gauge needle. For subcutaneous tumor growth studies, cells were injected (1 x 10^6 cells in 0.1 mL; n=1 mouse per cell line) at one site in both the right and left dorsolateral flanks. Orthotopic injections (1 x 10^6 cells in 0.1 mL; n=3 mice per cell line) were performed following exposure of the pancreas through a 1 cm incision in the left ventrolateral side. Intrasplenic injection (2 x 10^5 cells in 0.1 mL; n=2 mice per clone) was performed following similar surgical exposure of the spleen.

Each week, the tumors of mice with subcutaneous injections were measured orthogonally with calipers. For the other injection routes, tumor size was approximated via palpation. As tumor diameter approached 1 cm, mice were euthanized via cervical dislocation and necropsied. Fluorescence microscopy was utilized to assess fluorescence and to detect any metastases, tumor burden was visually assessed, and the mass of the primary tumor was measured when possible. The subcutaneous tumors were dissected, fixed in 10% neutral buffered formalin for 24 hours, and placed in 70% ethanol for future use.

Metastases detected from the intrasplenic injections were aseptically excised and plated in 35 mm dishes with 2 mL of RPMI 1640 media with 10% FBS, 5 µg/mL puromycin, and 1x penicillin/streptomycin.
RESULTS

Subcloning the EGFP gene into pCAGEN.puro

The first aim of the experimental design was to create pCAGEN.puro-EGFP (Figure 1), a vector with the EGFP gene downstream from a β-actin promoter. To accomplish this, a double digest was performed with the restriction enzymes EcoRI and NotI and with the plasmids pCAGEN.puro and pEGFP-N1. Performing gel electrophoresis on these products resulted in the separation of the 772 bp EGFP gene from the rest of pEGFP-N1 vector and the linearization of the 5983 bp pCAGEN.puro plasmid (Figure 2). These fragments were then cut from the gel to undergo gel purification. The yield of each fragment purified from the agarose gel was too low for the NanoDrop to accurately measure, so it was assumed that there was a 70% extraction recovery.

The EGFP insert and pCAGEN.puro vector were ligated together using T4 ligase, and the reactions were performed in three different ratios of vectors and inserts. The products of these reactions were transformed into competent bacterial cells and plated with ampicillin. Two colonies were selected from each ratio of insert to vector, and were cultured in 2xYT media with ampicillin. The DNA from each culture was extracted via MiniPrep, and reasonable concentrations were obtained.

To confirm that the extracted plasmids were truly pCAGEN.puro-EGFP, it was necessary to perform a diagnostic digest. XmaI was chosen as the appropriate restriction enzyme because it would create a distinct banding pattern for the differentiation of pCAGEN.puro (the control) and pCAGEN.puro-EGFP. Based on the known sequence of pCAGEN.puro, this vector was expected to produce fragments of 5382 and 620 bp after digestion with XmaI. On the other hand, if pCAGEN.puro-EGFP possessed the predicted sequence, it should produce fragments of 4220,
1915, and 620 bp. The presence of a fragment near 2000 bp (Figure 3) is indicative of the successful construction of pCAGEN.puro-EGFP. All but one of the candidate colonies (1:1 ratio of insert to vector) was positive. One of the successful cultures with the 1:1 ratio (Figure 3, Lane 1A) was selected for ongoing use.

Stable Transfection of pCAGEN.puro-EGFP and pEGFP-N1 into PANC02 Cells

PANC02 cells were transfected with pCAGEN.puro-EGFP (CAG promoter). As a control for comparing promoter stability and expression, pEGFP-N1 (CMV promoter) was transfected in parallel.

At 24 hours post-transfection, fluorescence was observed in both transfected lines. Initially, both transfections appeared to show comparable levels of fluorescence, although the transfection efficiencies varied. Following passaging and three days growth in selective media, fluorescence microscopy was utilized again to assess the progress of the transfected colonies (Figure 4). The colonies seem to vary in intensity, even among the same transfection. However, it was noted within the pEGFP-N1 colonies (~4-10 cells), cells exhibited differing intensities (Figure 4, right). Meanwhile, the colonies with pCAGEN.puro-EGFP were more homogenous in their fluorescence intensity.

After culturing several clones for each plasmid, photographs (2 second exposure) were taken so that intensity could be assessed (Figure 5). Both promoters resulted in heterogeneity within the clones, but the cells expressing EGFP under a CAG promoter were certainly brighter than those with a CMV promoter. In fact, only clone 11 of pEGFP-N1 appears to be fluorescing at an intensity comparable to the pCAGEN.puro-EGFP clones. Clones 7, 12, 15, and 17 with the
CAG promoter and clones 7, 8, and 11 with the CMV promoter were selected for long-term culturing.

Fluorescence microscopy was utilized again after the first month of culture, and the resultant photographs are shown in Figure 6. Five second exposures were utilized due to decreased intensity of fluorescence across all clones. Once again, the β-actin promoter clearly exhibits greater expression of EGFP. It is also apparent that a lower proportion of the cells that contain the CMV promoter are still fluorescing. This can be clearly demonstrated with clone 11; the photograph depicts a confluent plate, but less than ten cells can be seen fluorescing (Figure 6A).

Flow Cytometry of Long-Term Cultures

Flow cytometry data was collected for four clones transfected with pCAGEN.puro-EGFP and three clones transfected with pEGFP-N1, all of which had been continuously passaged over the course of two months. The results are depicted in two different forms in Figures 7 and 8. Overall, the clones utilizing a CAG promoter for EGFP expression yielded an average of 67% of cells still fluorescing two months post-transfection whereas those with a CMV promoter only yielded an average of 11% (Table 1). Thus, the CAG promoter appears to increase the stability of expression. The pCAGEN.puro-EGFP clones possessed variations in their level of intensity, and clone 7 was particularly heterogeneous. Cells of clone 17 produced the greatest fluorescence intensity, but clone 15 created the greatest percentage of cells that retained EGFP expression. Meanwhile, clones containing pEGFP-N1 were significantly less stable in their fluorescence. The most stable clone (clone 7) only produced 30% fluorescent cells, and those that did fluoresce
did so at a very low intensity. Thus, pCAGEN.puro-EGFP appears to better sustain EGFP expression at a higher intensity than pEGFP-N1.

**Flow Cytometry of Short-Term Cultures**

To provide a more rounded picture of early expression levels in a mixed culture, since colony-picking introduces bias on the part of the researcher, additional transient and stable transfections were performed with each plasmid less than a week prior to flow cytometry at Penn State Hershey College of Medicine. Only one plate of each transfection was sampled. Unfortunately, the stable transfectants were not provided with adequate time to create colonies in the selective media, so the data may not be representative of the percentages present when a mixed culture reaches confluency. Even so, the percentages are reported in Table 1, and show that 4% more cells were fluorescing in clones with the CMV promoter, contrary to earlier results. This is not a statistically significant difference, however, and may simply reflect a slight difference in an overall poor transfection efficiency. A larger sample size of independent transfections with increased transfection efficiency, is necessary to better compare the two promoters upon initial transfection. Although pCAGEN.puro-EGFP produced less cells fluorescing, those that did fluoresce were concentrated at a higher intensity (Figure 9).

The transient transfection products were also assessed through flow cytometry to better quantify any differences there may be in the transfection efficiency of the two plasmids. Both transfections only produced 0.1% fluorescence (Table 1), so once again, a greater sample size is required.
In Vivo Tumor Assessment

Two clones of PANC02-pCAGEN.puro-EGFP were assessed in vivo – in an ectopic tumor model (subcutaneous injection), orthotopic model (intrapancreatic) and an experimental metastasis assay (intraspelenic injection). Orthogonal measurements were collected weekly for the three mice with subcutaneous tumors (two tumors per mouse). The mean tumor diameter was defined as the square root of the product of the greatest and smallest orthogonal measurements. The calculated values for each tumor were averaged to determine the mean tumor diameter. Figure 10 displays the results of these measurements over the duration of the study. As expected, the untransfected parental PANC02 cell population achieved the greatest tumor growth rate due to its genetic heterogeneity. Although clone 15 of the PANC02 cells transfected with pCAGEN.puro-EGFP started off with a smaller tumor diameter, it still managed to exhibit a growth rate that was only slightly reduced from that of the parental line. Clone 17, on the other hand, did not grow particularly quickly, not even reaching 1 cm by the end of the 8-week study.

The mice with orthotopic injections only survived 2-5 weeks; 5 of 9 mice across all 3 groups died rapidly, with little warning (no cachexia, limited posture or behavioral changes). As a result, comparison of growth rate would not be insightful. Similarly, a comparison of primary tumor mass would not be fruitful due to the staggered times of necropsy based upon the timing of mouse death or euthanasia. Interestingly, primary tumor size and mass was greatly variable despite the fact that the tumor was the apparent cause of death for these mice. The tumor burden for clone 17 was relatively light, whereas the ribs were not visible in the parental and clone 15 mice due to the high tumor burden of the dorsal abdominal cavity wall.
All mice with intrasplenic injections were necropsied on the sixth week, although dissection of tumors for mass determination was difficult due to high dissemination. Tumor burden also appeared greatly elevated when there was a suspected leakage during injection. A comparison of growth rate is depicted in Figure 11. Once again, clone 15 outperformed clone 17, although it should be noted that these tumor measurements are palpated approximations rather than direct measurements obtained with calipers.

Photographs were taken of the parental PANC02 tumors under the fluorescent scope (Figure 12A). Without experience, identifying tumor tissue may be difficult, and it would be necessary to undergo laborious histological work to confirm the presence of cancerous cells. Figure 12B depicts that clone 15 of PANC02-pCAGEN.puro-EGFP successfully produced fluorescent tumors that aid in the expedient identification of tumors and metastases. The fluorescence was even detectable with the bright-field light on and an exposure of 0.8 seconds. Furthermore, the fluorescence also enabled the detection of single-cells or micrometastases in the liver and even in the lungs of the intrasplenically-injected mice (Figure 13). Unfortunately, clone 17 exhibited very little tumor fluorescence and those few sites with faint fluorescence were markedly heterogeneous in their expression (Figure 12C).
DISCUSSION

Despite the aggressiveness and low survival rates associated with pancreatic cancer, its mechanism of metastasis is poorly understood. To better track cancerous cells as they spread, a method of stably expressing GFP in the cells of interest is necessary. To reach this goal, pCAGEN.puro-EGFP was successfully constructed. In this vector, the EGFP gene is downstream from a chicken β-actin promoter with CMV immediate-early enhancers, which was predicted to be more effective in long-term studies than a CMV promoter.

Upon initial transfection into cells, both the CAG-promoted and CMV-promoted GFP appeared to fluoresce at comparable levels of intensity, indicating that the initial integrity of pCAGEN.puro-EGFP is similar to that of pEGFP-N1. After several days, colonies transfected with pCAGEN.puro-EGFP exhibited greater homogeneity in expression than those with the CMV promoter, perhaps supporting the hypothesis that CMV promoters are less reliable at different cell cycle stages.

Fluorescence microscopy over the course of cell culture once again provided evidence that the CAG promoter drives greater expression of EGFP in pancreatic cancer cells as compared to a CMV promoter. Flow cytometry quantitatively confirmed these observations. Not only were cells with the CAG promoter fluorescing more intensely, but a greater percentage of the cells were still fluorescing after two months. Meanwhile, an average of 89% of cells transfected with pEGFP-N1 were no longer expressing EGFP.

Because it was established that the chicken β-actin promoter with CMV immediate-early enhancers increases both the expression and intensity of expression of GFP in pancreatic cancer cells in vitro, cells transfected with the plasmid containing the CAG promoter were injected into mice for in vivo analysis. Clone 15 from these cells exhibited comparable growth rate and
tumorigenicity and maintained GFP expression long enough that metastases could be detected through fluorescence microscopy. Fluorescence was also intense enough to permit the detection of micrometastases. This clonal population should enable more accurate characterization of the mechanisms of metastasis thanks to its improved stability in long-term studies. Serial selection, the repeated rescue and reinjection of viable cells from live, fluorescent metastases is currently underway to isolate a subpopulation with a greater metastatic potential. This process would be virtually impossible without fluorescence-tagging.

The implications of these findings are weighty. Traditionally, a CMV promoter has been utilized in experiments that tag pancreatic cancer cells with GFP. But if very few of the cells still express the reporter gene partway through the study, cancerous cells may remain undetected in vivo and metastases grossly underestimated, or tumor tissue could be mistakenly sampled as normal tissue. This could greatly confound the results of an assay seeking to better characterize the mechanisms of metastasis. Furthermore, CMV promoters have been used historically to drive expression of a wide variety of genes studied in cancer. If clonal cell populations in these studies similarly silenced the CMV promoter and became dramatically heterogeneous for expression of the gene-of-interest, RNA and protein analyses would be a highly inaccurate depiction of expression. Both in vitro and in vivo data regarding the role of the gene(s) in tumorigenesis, growth, or metastasis would be similarly faulty. The results of this study call into question the efficacy of the CMV promoter. Thus, expression vectors with the chicken β-actin (CAG) promoter should be considered as an alternative system in such studies due to this promoter’s increased longevity of expression.

Further studies should seek to better validate the findings through the assessment of additional clones via flow cytometry. Progressive flow cytometry of the same clonal cell line
over a greater duration of culture would enable tracking of any drift in gene expression over time. In addition to the murine pancreatic cancer cells utilized in this study, human pancreatic cancer cells have recently been transfected with pCAGEN.puro-EGFP and both \textit{in vitro} and \textit{in vivo} assays should be conducted to determine the stability and intensity of GFP expression in these cells. In the future, these stably-fluorescing clones could be utilized in concert with other genes of interest to better characterize the mechanisms of pancreatic cancer metastasis.
### Table 1: Percent of Cells Fluorescing

<table>
<thead>
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<th>Plasmid</th>
<th>Average Long-Term</th>
<th>Short-Term</th>
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<td>Transient</td>
</tr>
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<td>0.1</td>
</tr>
<tr>
<td>pEGFP-N1</td>
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Figure 1: EGFP was excised from pEGFP-N1 and was ligated into pCAGEN.puro.
The EGFP gene from pEGFP-N1 was removed through digestion at the shown restriction sites and inserted into pCAGEN.puro (top). The resulting plasmid is pCAGEN.puro-EGFP (bottom), which has EGFP downstream from a β-actin promoter. Puromycin resistance is the selectable marker.

Figure 2: Double digest of plasmids with EcoRI and NotI produced fragments of the expected size.
The ~750 bp EGFP band from pEGFP-N1 and the the linearized ~6,000 bp backbone of pCAGEN.puro were each gel purified prior to ligation.
Figure 3: Restriction digest displaying successful construction of pCAGEN.puro-EGFP
Colonies of competent *E. coli* cells were transformed with the products of the ligation reactions. Once extracted, the plasmids were digested with *Xmal* and compared to pCAGEN.puro. The new band around 2,000 bp indicated successful ligation in all but one of the colonies.

Figure 4: Preliminary screening suggests that the β-actin promoter may drive more homogenous expression of EGFP.
PANC02 cells transfected with pCAGEN.puro-EGFP (left, two second exposure) show more consistent levels of fluorescence than a colony expressing EGFP under the CMV promoter (right, two second exposure).
Figure 5: Transfectants with β-actin (CAG) promoter exhibit a greater intensity of fluorescence after six passages. PANC02 cells transfected with (A) pCAGEN.puro-EGFP and PANC02 cells transfected with (B) pEGFP-N1 as seen by fluorescence microscopy with a 2 second exposure (100x objective). Each photo represents a distinct clone.
Figure 6: After one month of culture, transfectants with the β-actin (CAG) promoter exhibit both greater intensity of fluorescence and a greater proportion of cells still fluorescing. PANC02 cells transfected with (A) pCAGEN.puro-EGFP and PANC02 cells transfected with (B) pEGFP-N1 as seen by fluorescence microscopy with a 5 second exposure (100x objective). Each photo represents a distinct clone. (C) Both photos depict clone 11 of pEGFP-N1, but the bright field photograph reveals the confluency of the plate. Few cells remain fluorescent.
Figure 7: Dot plots representing flow cytometry results confirm an increase in stability when the CAG promoter is used. Clones engineered with (A) pCAGEN.puro-EGFP yielded an average of 67% of cells fluorescing while those with (B) pEGFP-N1 yielded an average of 11%. Those that did fluoresce with pEGFP-N1 had a lower intensity. Each dot plot depicts data collected for a separate clonal population maintained in culture for two months. The percentage of cells fluorescing is indicated for each clone.

Figure 8: Flow cytometry histograms further confirm the stability of the CAG promoter. Clones engineered with (A) pCAGEN.puro-EGFP are compared to those with (B) pEGFP-N1. The percentage of cells fluorescing is indicated for each clone. Clone 17 for pCAGEN.puro-EGFP produced the greatest intensity, but clone 15 of the same plasmid had the greatest percentage of cells still fluorescing.
Figure 9: Dot plots reveal that the intensity is more concentrated at a particular level in cells stably transfected with pCAGEN.puro-EGFP. (A) Cells expressing EGFP under the CAG promoter clustered at a high intensity as opposed to (B) cells with the CMV promoter which were evenly dispersed in their intensity of fluorescence.

Figure 10: Tumor growth rate of clone 15 of pCAGEN.puro-EGFP is comparable to the parental PANC02 line. The parental population produced the fastest tumor growth. Following an initial lag, clone 15 also grew to reach a substantial size within a similar time frame. Clone 17 exhibited the slowest growth rate, not even reaching 9 mm by the end of the study.
Figure 11: Tumor growth rate of clone 15 of PCAGEN.puro-EGFP is greater than clone 17 for the intrasplenic injections as well. Tumor size was estimated through palpation every week, and the averages were plotted. Clone 15 produced a faster growth rate and a larger primary tumor at the time of necropsy.

Figure 12: Clone 15 produced fluorescent tumors while clone 17 did not result in homogeneously fluorescent tumors. (A) A non-fluorescent tumor of PANC02 cells, denoted by T, would be difficult to confirm without histology. (B) Peritoneal tumor burden 3 weeks after orthotopic injection of clone 15. (C) The primary tumor (T) three weeks after orthotopic injection of clone 17 did not express EGFP. However, a satellite tumor on the dorsal abdominal wall was partially fluorescent (see arrow). Together, these indicate that clone17 cells are losing GFP expression due to genomic instability or promoter silencing.
**Figure 13: Clone 15 enables detection of single-cell metastases.**
Micro-metastases are visible in lung tissue 6 weeks after an intrasplenic injection of clone 15. Without the EGFP tag, these cells would not have been detectable.
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LITERATURE CITED


