ADVANCED TRANSITIONAL CELL CARCINOMA TREATMENTS VIA EXPRESSION-TARGETED GENE DELIVERY AND MINICELL TECHNOLOGY

AN ABSTRACT
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ABSTRACT

The objective of this project is to develop novel treatments, using expression-targeted gene therapy and minicell technology, to replace current methodologies used in the clinic for the treatment of carcinomas, especially transitional cell carcinoma of the bladder. The expression-targeted gene therapy procedure involves cancer-specific DNA elements (promoters) to drive the expression of engineered suicide genes to induce apoptosis in cancer cells. Minicells, non-living, miniature versions of bacteria (which lack a chromosome), were found inhibition of tumor recurrence and growth through targeted toxicity and an induced immune response that is similar to that induced by Bacille Calmette-Guerin (BCG), but without the risk of infection.

The osteopontin promoter ($p_{\text{opn}}$) was selected via traditional methods by comparing endogenous gene expression between normal and cancerous cells. The $\text{opn}$ gene is expressed in far greater amounts in cancer cells, so it was reasoned that the $p_{\text{opn}}$ promoter would be more active in cancer cells as well. Reporter constructs using $p_{\text{opn}}$ were transfected into both cancerous and normal cell types, with maximum $p_{\text{opn}}$-driven reporter intensity in the cancer cells showing up as strong (102.7%) compared to $p_{\text{cmv}}$-driven positive controls. Further enhancements to targeting and expression were obtained through the incorporation of single-nucleotide polymorphisms (SNPs) in the promoter
sequence. Further investigations to confirm a correlation between endogenous \textit{opn} mRNA levels and $^p\text{opn}$-driven reporter expression produced a surprising lack of correlation ($R^2=0.24$). However, taking into account \textit{opn} mRNA splicing variants, a strong negative correlation was determined between mRNA levels of the variant \textit{opn-a} and $^p\text{opn}$-driven transgene activity ($R^2=0.95$).

Three novel cancer-specific promoter $^p\text{ran}$, $^p\text{brms}1$ and $^p\text{mcm}5$ were identified through a new screening logic. The activities of those promoters were verified to be much higher in the tested cancer cell lines than the current gold standard used to target gene expression to cancer cells: the promoter of human telomerase reverse transcriptase ($^p\text{hTERT}$).

A constitutively active, apoptosis-inducing analog of caspase 3, referred to as Reverse Caspase3 (RevCasp3), was engineered via gene recombination and cloned into expression-targeted plasmid constructs. These constructs showed excellent activity in inducing apoptosis within the cancer cells tested. Moreover, $^p\text{ran}$-RevCasp3 constructs were shown to have significant, cancer-specific killing action within both human and murine cells \textit{in vitro}.

The therapeutic effects of minicell constructs known as VAX-IP were tested within our orthotopic, murine model of transitional cell carcinoma of the bladder. In trials
focused on the prevention of tumor growth and tumor implantation, bell-shaped curves were produced by data reflecting the relation between drug dose and tumor burden. The median and average bladder weights, used as a surrogate for tumor burden, decreased with increasing doses of VAX-IP minicells administered via intravesical, transurethral delivery. Activity was lost at high doses of VAX-IP minicells. Compared with the sham-treated group, 1x10^8 VAX-IP minicells, delivered at 24 hours post-surgery with repeated administrations every 7 days for a total of four treatments, yielded a significant survival advantage to the treated animals (P=0.03).
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TABLE OF CONTENT

CHAPTER 1  INTRODUCTION .............................................................................................................. 1

1.1 TRANSITIONAL CELL CARCINOMA .................................................................................. 2
  1.1.1 The structure of bladder .......................................................................................... 2
  1.1.2 Transitional cell (urothelial) carcinoma ................................................................. 3
  1.1.3 Routine treatments and existing problems ......................................................... 4

1.2 GENE THERAPY .................................................................................................................. 4
  1.2.1 Taxonomy of Gene Therapy Applications .............................................................. 6
  1.2.2 Expression-based gene therapy ............................................................................. 7
  1.2.3 Interference-based gene therapy .......................................................................... 8

1.3 GENE DELIVERY METHODS .......................................................................................... 11
  1.3.1 Viral delivery methods ............................................................................................ 12
    1.3.1.1 Retrovirus ....................................................................................................... 13
    1.3.1.2 Adenovirus .................................................................................................... 14
    1.3.1.3 AAV ............................................................................................................... 15
    1.3.1.4 Other assistance method for viral vectors .................................................... 16
  1.3.2 Non-viral delivery methods ..................................................................................... 17
    1.3.2.1 Chemical gene delivery vector ...................................................................... 17
    1.3.2.2 Physical gene delivery ................................................................................... 18
      1.3.2.2.1 Electroporation and Nucleofection ....................................................... 18
      1.3.2.2.2 High intensity focused ultrasound ...................................................... 19
    1.3.2.2 Gene-activated matrices (GAMs) ................................................................ 20

1.4 EXPRESSION-TARGETED CANCER GENE THERAPY ................................................... 22

1.5 TRANSLATION OF EXPRESSION-TARGETED CANCER GENE THERAPY INTO CLINICAL PRACTICE ....................................................................................................... 24

1.6 MINICELL TECHNOLOGY .................................................................................................. 25

1.7 GOALS OF DISSERTATION .............................................................................................. 26

CHAPTER 2  CANCER-SPECIFIC PROMOTER $^{\rho}$OPN AND SINGLE-NUCLEOTIDE POLYMORPHISMS FOR TARGETED CANCER GENE THERAPY ............................................................... 29

2.1 BACKGROUND ............................................................................................................... 30

2.2 MATERIALS AND METHODS ...................................................................................... 33
  2.2.1 Cell lines and Cell Culture ..................................................................................... 33
  2.2.2 Plasmid Construction ........................................................................................... 34
  2.2.3 Transfection ......................................................................................................... 35
  2.2.4 Stable endogenous hOPN mRNA level measurement ....................................... 36
  2.2.5 Statistics .............................................................................................................. 37

2.3 RESULTS ......................................................................................................................... 39
  2.3.1 $^{\rho}$opn lengths ..................................................................................................... 39
  2.3.2 Species cross-reactivity ......................................................................................... 45
CHAPTER 3  CANCER-SPECIFIC PROMOTERS FOR EXPRESSION-TARGETED GENE THERAPY: $^p$RAN, $^p$BRMS1, AND $^p$MCM5

3.1 BACKGROUND .................................................................................................................. 70
3.2 MATERIAL AND METHODS .......................................................................................... 73
   3.2.1 Cell culture .................................................................................................................. 73
   3.2.2 Promoter Cloning ....................................................................................................... 75
   3.2.3 RevCasp3 cDNA construction .................................................................................. 78
   3.2.4 RevCasp3 activity assay ........................................................................................... 79
   3.2.5 Cells Viability Assay ............................................................................................... 80
   3.2.6 Statistics .................................................................................................................... 81
3.3 RESULTS .......................................................................................................................... 82
   3.3.1 Scheme of searching logic ....................................................................................... 82
   3.3.2 Evaluation of promoter activity ............................................................................... 83
   3.3.3 Verification of RevCasp3 expression and activity ...................................................... 86
   3.3.4 Demonstration of $^p$ran-RevCasp3 targeting and efficacy ........................................ 92
3.4 DISCUSSION .................................................................................................................... 98
   3.4.1 The importance of building a cancer-specific promoter library ................................ 98
   3.4.2 Traditional pathway to look for cancer specific promoter .......................................... 99
   3.4.3 Our logic to find cancer-specific promoters ............................................................ 100
   3.4.4 Why $^p$ran, $^p$brms1 and $^p$mcm5 were selected .................................................. 100
   3.4.5 The pros of our searching strategy ........................................................................... 102
   3.4.6 The cons of our searching strategy ........................................................................... 103
   3.4.7 Why the RevCasp3 was selected ............................................................................. 103
   3.4.8 The basal level activity of $^p$ran, $^p$brms1 and $^p$mcm5 and toxicity of $^p$ran-RevCasp3 for normal cells .......................................................... 107
3.5 CONCLUSION ................................................................................................................... 109

CHAPTER 4  VERIFYING MINICELLS THERAPEUTIC EFFECT IN THE ORTHOTOPIC MODEL OF MURINE TRANSITIONAL CARCINOMA

4.1 BACKGROUND ................................................................................................................. 111
4.2 MATERIALS AND METHODS ....................................................................................... 114
   4.2.1 Generation of VAX-IP ......................................................................................... 114
   4.2.2 Cells ....................................................................................................................... 115
4.2.3 Animals .................................................................................................................. 115
4.2.4 MB49 tumor implantation ....................................................................................... 116
4.2.5 Treatment of established MB49 tumors in tumor growth experiments ...... 117
4.2.6 Treatment of established MB49 tumors on survival .................................. 117
4.2.7 Treatment for impeding tumor cell implantation .................................... 118

4.3 RESULTS .................................................................................................................. 119

4.3.1 Effect of VAX-IP treatment on well established MB49 tumor growth in vivo.
................................................................. 119
4.3.2 Effect of VAX-IP Treatment on Survival ................................................... 123
4.3.2 Effect of VAX-IP Treatment on impeding tumor cell implantation ........ 127

4.4 DISCUSSION ......................................................................................................... 130

4.5 CONCLUSION .................................................................................................... 134

CHAPTER 5 FUTURE WORK: CANCER-SPECIFIC AND STRESS-INDUCIBLE PROMOTER
FOR EXPRESSION-TARGETED GENE THERAPY: \( ^p \)TXNL1, \( ^p \)GRP94, \( ^p \)POLH AND \( ^p \)ZNF394 . 135

5.1 INTRODUCTION TO FUTURE WORK ................................................................. 136
5.2 MATERIAL AND METHODS ............................................................................... 140

5.2.1 Cell culture ...................................................................................................... 140

5.2.2 Promoter Cloning ............................................................................................ 141

5.2.3 EGFP Reporter Indication Assay .................................................................. 143

5.3 PRELIMINARY DATA ........................................................................................ 145

5.4 DISCUSSION ..................................................................................................... 149

LIST OF REFERENCES ................................................................................................. 152
**LIST OF TABLES**

**Table 2.1** – Cell lines and culture media used in opn promoter study.

**Table 2.2** – Effect of mutations at -443, -155, and -66 upon opn promoter transgene expression.

**Table 3.1** – Cell lines and culture media used in ran, mcm5, brms1 promoter study.

**Table 3.2** – Promoter names and cloning sites in ran, mcm5, brms1 promoter study.

**Table 5.1** – Cell lines and culture media used in tnxl1, grp94, polh, znf394 promoter study.

**Table 5.2** – Promoter names and cloning sites in tnxl1, grp94, polh, znf394 promoter study.
LIST OF FIGURES

Figure 1.1 – The illustration of bladder structure and associated tumors (American Cancer Society, 2014).

Figure 1.2 – Promoter and Transcriptional Machinery (Godbey, 2014)

Figure 2.1 – a) Promoter/exon combinations used for plasmid construction.

b) Deletion analysis using the promoter regions from part a. Promoter activity represented by relative intensity.

c) Histogram graph of HFF-1 cells after transfection reported by FACS flow cytometry to show how relative intensity was obtained.

d) Deletion analysis using the promoter regions from part a). Promoter activity represented by transfection efficiency.

e) Four quadrants graph of PC-3 cells (sham treated and \(^{134}\)GFP treated) to show how transfection efficiency was obtained.

Figure 2.2 – a) Comparison of transfection efficiencies obtained for delivered transgenes driven by the human versus murine \(^{\text{human}}\) \(^{\text{Popn}}\) promoter in both human and murine cells.

b) Alignment of the human and murine \(^{\text{Popn}}\) sequences (-134 to -33).

Figure 2.3 – Strength of transfections using the \(^{\text{human}}\) \(^{\text{Popn}}\)-134 in normal versus cancer cell lines, normalized to \(^{\text{Pcmv}}\)-driven controls.

Figure 2.4 – Schematics of the six promoter/exon combinations constructed to study the
SNP effect upon promoter strength, and the locations/identities of the included SNPs.

**Figure 2.5** – Strength of reporter expression using the promoters described in fig. 2.4.

**Figure 2.6** – Endogenous mRNA levels of hOPN, as determined by real time PCR.

**Figure 2.7** – Assessment of possible correlations between endogenous mRNA levels and $^{p}\text{opn-134}$-driven reporter strength.

**Figure 3.1** – the scheme of cancer-specific promoter searching logic

**Figure 3.2**– $^{p}\text{ran}$, $^{p}\text{brms1}$ or $^{p}\text{mcm5}$ activities in normal and cancer cell lines.

**Figure 3.3** – The morphologies and apoptotic statuses of PC-3 cells following transfection.

**Figure 3.4** – Quantification of cell numbers by flow cytometry following transfection.

**Figure 3.5**– a) Cell viability following transfections with $^{p}\text{ran}$-RevCasp3.

b) Floating cells increase normalized to the negative control.

**Figure 3.6** – Verification of human derived promoter and RevCasp3 possesses activity through murine species.

**Figure 4.1** – Effect of VAX-IP treatment on well-established MB49 tumor growth *in vivo*.

**Figure 4.2** – Effect of VAX-IP Treatment on Survival.

**Figure 4.3** – Effect of VAX-IP Treatment on impeding tumor cell implantation.

**Figure 5.1** – Promoters activities between normal and cancer cell lines.
Chapter 1

Introduction
1.1 Transitional Cell Carcinoma

1.1.1 The structure of bladder

The bladder is an organ which stores the urine to allow urination with intention. The bladder wall is composed of the following four layers (fig. 1.1) (American Cancer Society, 2014) (from innermost to outmost): the transitional epithelium, lamina propria (connective tissue), muscularis propria (muscle layer), fatty connective tissue (the fatty layer).

Figure 1.1 The illustration of bladder structure and associated tumors (American Cancer Society, 2014)
1.1.2 Transitional cell (urothelial) carcinoma

Bladder cancer is one of the most prevalent forms of cancer in the United States. It has been estimated that there will be 74,690 new urinary bladder cancer cases and 15,580 resulting deaths in the United States in 2014 (Howlader N, 2014). The disease is of special concern for males, for whom bladder cancer has been the fourth most prevalent types of cancer. There are four types of cancer occurring in the bladder: transitional cell (urothelial) carcinoma, squamous cell carcinoma, adenocarcinoma small cell carcinoma, and sarcoma. Among them, transitional cell (urothelial) carcinoma is the most common cancer type, accounting for 90% of bladder cancer cases (American Cancer Society, 2014).

Tansitional cell carcinoma, as its name suggests, is the type of cancer where cells of the transitional epithelium become transformed. Among transitional cell carcinomas, there are also two subtypes based on the growth mode: papillary carcinoma and flat carcinoma (fig. 1.1). In addition to the bladder, transitional cells are also found in the kidneys, ureters, and urethra. Transitional cell carcinomas are also found in these structures.
1.1.3 Routine treatments and existing problems

Surgery is required for about 90% of bladder cancer cases (Qian et al., 2013). However, surgery is a poor solution for the condition, with recurrence rates at 1 year in the range of 15–61%. The recurrence rate at 5 years is 31–78% (van der Heijden and Witjes, 2009). To combat tumor recurrence, patient follow-up is extremely important. The current standard follow-up therapies include intravesicular delivery of the mycobacterium Bacillus Calmette-Guerin (BCG), chemotherapy, and radiation therapy. BCG immunotherapy presents an advantage over various chemotherapy drugs in reducing cancer recurrence, as has been shown in multiple clinical trials (Sylvester et al., 2005). It has therefore been used as the first choice for follow-up treatments. However, the recurrence rate with BCG is still high. In addition, patients also suffer a potential risk of infection because BCG is a live, pathogenic bacterium. Thus, there is a need for a more effective and safer treatment to reduce the recurrence of bladder tumors without subjecting patients to the risk of sepsis.

1.2 Gene Therapy

In the past, an accepted definition of gene therapy has been *the delivery of genes into cells to alter cellular function*. Such delivery is typically achieved via encapsulating genetic material inside a viral capsid, complexing the DNA with chemical agents such as cationic lipids or polymers, or via a physical means that deliver genes into cells via forces such as electric (as in electroporation) or puncture and pressure (as in microinjection).
The delivery of RNA via retroviruses was accepted as gene therapy under the above-definition because the RNA was converted into cDNA via reverse transcriptase (followed by the insertion of the cDNA into the host genome via integrase), so genes were indirectly being delivered.

In the case of micro-RNA (miRNA), we must consider the entire system of delivery and response to classify the technology as gene delivery. The miRNA technology involves delivery of “genes” that encode miRNA into the cell for integration into the genome. The unit that is delivered will be transcribed, although the transcription product will not undergo translation. The primary miRNA transcription product will be modified into pre-miRNA, which will be recognized by a RNA-induced silencing complex in the cytoplasm and subsequently participate in the knock-down or silencing of specific gene by reducing or preventing its translation. The initial delivery involved a gene (despite the fact that the sequence will not be used to produce a functioning protein), and cellular function is altered as a result, so miRNA technology can come under the heading of gene therapy.

However, other technologies that utilize RNA interference, such as small interfering RNA (siRNA), again bring up the question of the role of RNA-delivery under the heading of gene delivery. Certainly, in this case the delivered RNA is not a gene, and polynucleotides are being delivered as opposed to polydeoxynucleotides. However, as
with miRNA translation is being impeded, and is not translation a required part of gene expression? If one were to alter the definition of gene therapy to the delivery of material to alter cellular function via manipulation of gene expression, then RNA interference would come under the definition, but again so would the delivery of certain proteins.

One could deliver transcription factors to cells to alter gene expression patterns, but this example of protein delivery would not typically be considered gene therapy, despite a point of view that the action of the delivered transcription factors is a direct therapy directed toward host genes themselves. This would be a literal view of gene therapy: therapy for genes. However, this action would not be considered gene therapy by current views. It would qualify as molecular therapy, but not gene therapy.

As can be seen by the discussion just presented, there is some ambiguity in the field of gene therapy at the moment. For the purposes of this thesis, the following definition of gene therapy will be used: the delivery of polynucleic acids, deoxy or otherwise, to alter cellular function via manipulation of gene expression.

### 1.2.1 Taxonomy of Gene Therapy Applications

Using the above definition, gene therapy applications can be divided into two groups.
The first is the expression-based group, in which polynucleotides (transgenes) are delivered into cells and undergo transcription and translation following the central dogma of molecular biology. The second group is the interference-based group, wherein the delivered polynucleotides directly or indirectly affect target molecules (e.g., mRNA) to alter cellular behavior. Aptamers, ribozymes, and RNA interference (RNAi), which includes siRNA and miRNA, all belong to this group.

1.2.2 Expression-based gene therapy

DNA and RNA have each been used for gene therapy. RNA has been delivered via retroviruses, but mRNA has also been used to deliver a genetic message into cells. The delivery of mRNA is less popular for gene therapy because it is more difficult to engineer and is more easily degraded than DNA when all factors, such as the prevalence of degradative enzymes both inside and outside the cell, are taken into account. Molecules of mRNA are also strongly immunogenic, although chemical modification has been used to reduce its immunogenicity while also increasing their stability. (Kormann et al., 2011)

There are, however, advantages to consider with regards to mRNA delivery. Unlike DNA, which must be delivered to the nucleus for transcription, mRNA does not require transcription and can therefore be delivered directly to the cytoplasm. This can improve transfection efficiency as well as avoid the oncogenic risk associated with the incorporation of a foreign gene into the host genome (McIvor, 2011, Wiehe et al., 2007, ...)
Because of its polyanionic makeup, naked DNA will seldom enter cells because of the electrostatic repulsion between DNA and the negatively charged cell membrane. Non-viral vectors such as polymers or liposomes are typically cationic, which will not only help to condense the DNA (Cavallcaro et al., 2008, Wagner et al., 1991) but also allow the resulting gene delivery complex to adhere to the cell exterior.

The choice of using a viral versus a non-viral gene delivery method is not always straightforward. Advantages of non-viral gene delivery include a lack of immunogenicity, quicker gene amplification due to the use of plasmids, which can be replicated via prokaryotes such as *E. coli*, and lower cost of production. In contrast, virally mediated gene delivery is associated with much higher transfection efficiencies. Transduction via retrovirus and adeno-associated virus (AAV) can also yield permanent gene delivery (Inagaki et al., 2008, Bakowska et al., 2003, Miao et al., 2000, Janovitz et al., 2014, Ciuffi, 2008).

### 1.2.3 Interference-based gene therapy

There are several inhibition mechanisms in the interference-based group, including those associated with antisense oligonucleotides, aptamers, and ribozymes (Dias and Stein,
2002, Grassi et al., 2010, Yao et al., 2009). RNAi approaches have been perhaps the most studied of the interference-based approaches. After the mechanism of RNA interference was described by Fire and Mellow in 1998 (Fire et al., 1998), scientists took advantage of the technique to help prove the function of a plethora of genes. Others have tried to use RNAi to convert diseased cells back to a normal state by silencing or reducing the expression of specific genes. The validity of this approach has been supported by preclinical research, and multiple phase I and II trials using RNAi have been completed (Kanasty et al., 2013).

Two kinds of RNA molecules are at the center of RNAi, small interfering RNA (siRNA) and microRNA (miRNA). The target for both RNAi molecules are cellular mRNA and therefore translation is inhibited. Although they share similar structure and function, the two classes of molecules have some distinct characteristics (Weaver, 2011, Carthew and Sontheimer, 2009). First, genes encoding precursors for miRNA exist in wild-type cells. In contrast, whether siRNA is an endogenous or exogenous molecule is still under debate, but popular belief has siRNA originating from a primordial cellular defense mechanism against virally delivered double-stranded RNA. Another distinction is that siRNA-mediated down-regulation of gene expression involves cleavage of the target mRNA, but miRNA can interfere with translation by binding to miRNA-response-elements within the 3’ untranslated region of mRNA to repress the translation machinery. In addition, miRNA can operate without being perfectly
complementary to the target mRNA, whereas siRNA must be perfectly
complementary (Weaver, 2011, Carthew and Sontheimer, 2009).

Mature siRNA or RNA precursors can be synthesized chemically and transferred into cells via non-viral gene delivery vectors. They can also be produced within the cell following the delivery of and appropriately engineered DNA plasmid or virus. Pros and cons for these two methods of siRNA production are similar to those for the delivery of DNA versus mRNA already mentioned. Efficacy may be reduced when DNA is delivered because of the longer pathway in getting to the nucleus for transcription, or it may be increased due to the siRNA being transcribed off of the delivered DNA, which may produce a larger total number of siRNA molecules or have them present for a longer period of time due to continual (albeit temporary) production (Brummelkamp et al., 2002).

Because miRNA is an endogenous mode of cellular regulation, miRNA is preferred as a drug target rather than an agent. Recent studies show a considerable number of apoptotic and metabolic pathways that are regulated by miRNA, including those involved in myogenesis, cardiogenesis, and brain development (Kloosterman and Plasterk, 2006). It may be possible to harness miRNA for regenerative medicine applications by reprogramming somatic cells to become pluripotent cells. By controlling miRNA regulation, activation of pluripotency genes, inhibition of apoptosis, and increasing cell
division rates may be achievable (Sun et al., 2010).

Regulation of miRNA expression can be achieved through up- or down-regulation. To up-regulate miRNA expression, plasmids encoding primary miRNA sequences can be delivered into cells, or exogenously-produced miRNA can be delivered (Davidson and McCray, 2011). Studies have indicated that the latter method has reduced toxicity versus the delivery of precursors (McBride et al., 2008, Boudreau et al., 2009). To down-regulate miRNA, additional RNA molecules have been applied. One kind of chemically synthesized RNA oligonucleotide, called an antagomir, has been reported to have the ability to silence miR-16, miR-122, miR-192 and miR-194 (Krutzfeldt et al., 2005). Recently, another seed-targeting 8-mer “locked nucleic acid” (LNA) oligonucleotide (named tiny LNA) was developed to inhibit not only single miRNAs but also entire miRNA families (Obad et al., 2011).

1.3 Gene Delivery Methods

It is very difficult to deliver polynucleotides into cells without a carrier. The plasma membrane serves as an effective barrier to charged molecules, both large and small. There exist methods to physically deliver polynucleotides across the membrane, such as microinjection, gene guns, or the spontaneous opening of pores through electroporation (Liu et al., 2001, Gao et al., 2007), but these methods are not suited for
large scale gene delivery in three dimensions, such as to an organ (Gao et al., 2007).

Alternatively, the use of both viruses and cationic biomaterials has been investigated for larger scale gene delivery in both \textit{in vitro} and \textit{in vivo} settings, but issues with transfection efficiency and cytotoxicity are of significant concern. The ideal gene delivery vector would transfer genes into cells efficiently, not instigate an immune response, and would not produce any toxic effects to cells (Sitharaman, 2011). Gene delivery in a targeted fashion is also desired for cell- or tissue-specific applications. While there exists an array of gene delivery agents that adhere to one or two of the above desired criteria, to date the ideal gene delivery agent has not been discovered.

1.3.1 Viral delivery methods

Viruses can be used as vectors through the removal of virulence genes and insertion of engineered genes into the viral genome. Several types of viral vectors, like adenovirus, adeno-associated virus (AAV), retrovirus, herpes virus, and baculovirus have been deeply investigated for use in gene therapy, and some have been tested in clinic trials. Retrovirus, adenovirus, and AAV have been the most commonly used viruses in gene delivery research. Each of these entities is associated with its own set of advantages and disadvantages. For instance, while the adenoviral vector has a relatively large capsid that allows for the delivery of larger genes into cells, these viruses are known to be immunogenic. Retroviral vectors possess reduced immunogenicity and can
potentially induce persistent transgene expression through (random) integration into the host genome, but at the risk of knocking out a vital gene such as a housekeeping gene or tumor suppressor gene or activating an oncogene. As an example, retroviral delivery of the \textit{c-myc} transgene to generate induced pluripotent stem cells caused approximately 20% of the daughter cells to transform into tumor cells (Okita et al., 2007). Specific serotypes of AAV (\textit{e.g.} AAV2 (Janovitz et al., 2014), AAV6 (Towne et al., 2009), and AAV8 (Inagaki et al., 2008)) currently appear to elicit only modest immunogenic responses (Mingozzi and High, 2013), although their gene-carrying capacity is relatively small.

1.3.1.1 Retrovirus

To combat the issue of random cDNA integration causing death or transformation of the host cell, intergrase-defective retroviral vectors (IDRV) have been created through mutation of the integrase domain of the retroviral genome (Philpott and Thrasher, 2007, Apolonia et al., 2007, Banasik and McCray, 2010, Yu et al., 2008). However, while the disadvantage of random integration is avoided with these vectors, the IDRV are lacking the key retroviral advantage of stable transduction. Also, without genomic integration of cDNA, the amount of transgene expression per cell will be diluted over time, as is the case with any transient gene delivery method. Therefore, an ideal approach to have the transgene to be expressed permanently without the risk of random integration is to insert the gene at a target position in the host cell genome. Combined with homologous
recombination, the site-specific gene integration could be achieved via IDRV. The inserted cDNA could be designed with a homologous sequence motif in both 5’ and 3’ side. After the gene is delivered into cells, the gene could be theoretically inserted into the target site by homologous recombination. This approach has been used to successfully insert genes into the predetermined position, but the frequency for gene insertion was low. A recent study shows that just $0.83 \pm 1.32\%$ homologous recombination could be achieved using only the homologous sequence (Okada et al., 2009). To improve the gene editing rate, DNA endonucleases has been incorporated into the IDRV system (Lombardo et al., 2007). In this updated approach, two components made up the system. The first was a zinc finger nuclease (ZFN) used to identify and cleave a specific sequence in the interleukin 2 receptor, gamma gene. The second component was a sequence homologous to the target site in the host genome, used to flank a phosphoglycerate kinase promoter (PGK)-GFP expression cassette. After co-transduction, the PGK-GFP gene was successfully edited into IL2RG region in stem cells. Compared with transduction via donor-IDRV alone, the integration efficiency in human cell lines was increased from 2% to 50% (and from 0.3% to 5% in embryonic stem cells).

### 1.3.1.2 Adenovirus

Helper-dependent adenovirus, also named “gutless adenovirus”, is a third-generation adenoviral vector (Zhang and Godbey, 2006, Vetrini and Ng, 2010). As opposed to the
first and second generations, the only components of the viral genome contained within the 3\textsuperscript{rd}-generation vectors are the packaging sequence and inverted terminal repeats.

The packaging and replication of such viruses is dependent upon helper viruses, which provide other essential viral gene sequences. Through this pathway, the possibility of viral self-replication in the host is highly reduced. At the same time, the deletion of viral genes from these viruses makes room for more engineered genetic material, allowing the researcher to deliver up to 37kb of customized DNA. Compared to IDRV, helper-dependent adenovirus can be used to insert much larger fragments of DNA into a specific site via homologous recombination. A recent report describes how multiple mutations in a long-range gene locus were corrected through the use of this technique in induced pluripotent stem cells (Liu et al., 2011).

1.3.1.3 AAV

Viruses often possess short generation periods and high mutation rates, thus naturally producing new serotypes that may have benefits for the gene therapist. AAV serotype 8 (AAV8) and AAV serotype 9 (AAV9) are good examples. AAV8 and AAV9 were originally isolated from rhesus monkey tissue and human tissue, respectively (Inagaki et al., 2006, Nam et al., 2007). Compared with the widely used AAV2, the AAV8 and AAV9 serotypes not only yield robust transduction efficiencies (Inagaki et al., 2006, Broekman et al., 2006, Klein et al., 2008), but also strong tropism for neuronal, skeletal muscle, and cardiac tissues (Inagaki et al., 2006, Bish et al., 2008, Wang et al.,
Long-term expression of delivered genes is another advantage associated with these serotypes (because of genomic integration), with AAV8- and AAV9-mediated transductions resulting in transgene expression for more than 799 days and 200 days, respectively (Sarkar et al., 2006). Another advantage to these serotypes is that humans have lower levels of preexisting immunity toward them (Boutin et al., 2010), making initial transductions less likely to generate the harsh side effects that are associated with the secondary immune response.

Self-complementary AAV (scAAV) is another promising AAV vector. For wild-type or recombinant single-strand DNA AAVs (ssAAV), the limiting step of transduction is the production of a complementary DNA strand against the delivered single-stranded DNA. The scAAV alleviates this barrier via a dimerized genome, which greatly improves transduction efficiency (Petersen-Jones et al., 2009, McCarty, 2008).

### 1.3.1.4 Other assistance method for viral vectors

Ultrasound waves could transmit through the body. If using microbubbles to cover the viruses and then sending to target body position. After applying the ultrasound, the bubbles could break and virus will be released at the targeted site (Bekeredjian et al., 2003). Meanwhile, the ultrasound waves may also induce transient permeabilization of the cell membrane, which could help viruses to enter cells. Thus, ultrasound assistance has been used to increase the transduction efficiency of retroviral, adenoviral, AAV, and
herpes viral vectors both \textit{in vitro} and \textit{in vivo} (Shintani et al., 2011, Xie et al., 2010, Zheng et al., 2009, Howard et al., 2006).

### 1.3.2 Non-viral delivery methods

Non-viral gene delivery includes physical delivery methods such as electroporation, and chemical delivery methods using carriers such as chitosan, polyethylenimine, liposomes, dendrimers, and inorganic nanoparticles (Balazs and Godbey, 2010, Xiujuan et al., 2011). Larger-scale non-viral gene delivery typically utilizes the chemical delivery methods, which are noted to yield lower transfection efficiencies versus the viral delivery methods. However, non-viral gene delivery presents advantages over viral methods in terms of low immunogenicity and gene recombination rate. Chemical gene delivery vehicles are relatively easy to synthesize on a large scale, which makes them especially practical for clinical application. Improvement in transfection efficiencies have been accomplished via modification of traditional non-viral vectors (Deng et al., 2013, Albertazzi et al., 2012), such as conjugation with viral particles (Kim et al., 2013) or other ligands which can help the main factor to be transported into host cells, such as HIV-1 twin-arginine translocation peptide (Peng et al., 2014).

#### 1.3.2.1 Chemical gene delivery vector

Liposomes have been used to achieve excellent delivery of transgenes into cells.
Transfection efficiencies to human MSCs using Lipofectamine 2000™ (Invitrogen) have been reported in the 33% - 74% range (Boura et al., 2013), which is comparable with results obtained using nucleofection as well as viral vectors. Combination of cationic liposomes with superparamagnetic iron oxide nanoparticles has resulted in transfection efficiencies similar to those of retroviral vector systems, in induced pluripotent stem cells (Park et al., 2012).

The efficiency of polyethylenimine (PEI)-mediated gene delivery into cells has also been investigated. For example, different nitrogen residues of PEI per oligonucleotide phosphate (N:P) ratios were examined for delivery to human adipose-derived stem cells (Ahn et al., 2008). In that system, liposome-mediated transfection efficiency using Lipofectamine was about 9%, while the maximal transfection efficiency obtained with PEI was 19% (at an 8:1 N:P ratio). Transfection efficiency was further enhanced by the conjugation of ligands, such as hyaluronic acid, to allow for targeting of the aimed cells. Branched PEI has also been conjugated with hyaluronic acid to bind CD44, CD54, and CD168 receptors on human MSCs, which improved transfection efficiencies from (12.0%±4.2%) to (33.6%±13.9%) (Saraf et al., 2008).

1.3.2.2 Physical gene delivery
1.3.2.2.1 Electroporation and Nucleofection

Electroporation is a physical gene delivery technique that has been used for years.
Electroporation utilizes a transient electrical field to induce a leaky state in the plasma membrane, thus allowing DNA to enter the cell through the pores created. However, transcription of the delivered genes must occur in the nucleus, and further delivery of the DNA from the cytosol to the nucleus is a limiting factor for this method of gene delivery.

Nucleofection is an advanced electrophermeabilization technique that has improved transfection efficiencies in primary cells (Aslan et al., 2006). Used in combination with specifically designed buffers and electropulse programs, DNA is delivered directly into the nucleus rather than into the cytoplasm. A recent study compared lentiviral transduction, nucleofection, traditional electroporation, and lipofection to evaluate their gene-transfer efficiencies in human embryonic stem cells (Cao et al., 2010). The lentiviral and nucleofection methods produced significant transgene expression in these cells which are notorious for being difficult to transfet. For human MSCs, the transfection efficiencies as high as 73.7% have been reported when using nucleofection (Aluigi et al., 2006), which was even higher than what was observed for AAV vectors (65%) in the same study (Stender et al., 2007).

1.3.2.2 High intensity focused ultrasound

High intensity focused ultrasound (HIFU) is an advanced ultrasound technique which has been applied to clinical practice. The HIFU waves can be concentrated on a target, producing energy levels at the focal point that are several orders of magnitude
higher than in surrounding areas. For a diagnostic ultrasound transducer, time-averaged wave intensities are about 0.1-100mW/cm$^2$, with compression and rarefaction pressures of 0.001-0.003 MPa. In contrast, HIFU sends ultrasound waves with intensities of 100-100,000w/cm$^2$, with peak compression pressures and rarefaction pressures of up to 30 MPa and 10 MPa, respectively (Dubinsky et al., 2008). Such a non-invasive high energy concentrated ultrasound shows great potential on tumor ablation (Chen et al., 2012, Hoang et al., 2014). More importantly to the subject of \textit{in vivo} gene delivery, HIFU can serve as an efficient tool to help gene delivery vector to pass the blood-brain barrier (BBB). The blood-brain barrier is a major obstacle to drug and gene delivery to the central nervous (CNS) system for the treatment of neurodegenerative disorders (Bicker et al., 2014). Combined with microbubbles, HIFU can transiently and locally disrupt the blood-brain barrier to allow macromolecules to pass into center nervous system (McDannold et al., 2012). Using this methodology, viral vectors (Hsu et al., 2013), liposomes (Aryal et al., 2013), and even stem cells (Burgess et al., 2011) have been successfully transported into the central nervous system. Thus, HIFU is a very promising and powerful technique for gene delivery in this highly protected region of the body.

1.3.2.2 Gene-activated matrices (GAMs)

The concept of GAMs was established by Bonadio and coworkers in 1999 (Bonadio et al., 1999). GAMs are simply substrates that have been incorporated with plasmids,
either with or without complexation with gene delivery vehicles. GAMs can be implanted into the body to allow for gene delivery over extended time periods in a fashion of controlled release. GAMs are useful for regenerative medicine because they can be used to assist with cell differentiation and tissue development.

The first generation of GAM was based on the bovine type I collagen matrix, being loaded with naked plasmids to aid in bone reformation (Bonadio et al., 1999). More-recent studies have focused on the properties of the materials used as GAM substrates (e.g. porous chitosan-gelatin (Guo et al., 2006) or fibrin (Wang et al., 2009)), improvements in gene delivery obtained by incorporating non-viral gene delivery vectors (e.g. calcium phosphate (Endo et al., 2006), poly(ethylenimine) (Wang et al., 2009), or chitosan (Peng et al., 2009)). The applications of GAMs have also been extended to include regeneration of vascular tissues (Geiger et al., 2007), neurons of dorsal root ganglia (Gonzalez et al., 2006), and periodontal ligament (Peng et al., 2009), among others.

Viral vectors can also be incorporated into GAMs. Such incorporation provides several significant advantages over local injections and systemic administration of viruses: the amount of plasmid and vector administered can be reduced, transduction efficiencies can be increased (by concentrated transductions), and positive transduction can be maintained for longer periods of time. For example, lentiviruses have been successfully
incorporated into poly(lactide-co-glycolide) scaffolds to yield extended periods of gene expression both \textit{in vitro} and \textit{in vivo} (Shin \textit{et al., 2010}). The efficacy of GAMs has also been demonstrated in clinical trials. Recently, a phase I/II clinical trial report presented on patients with non-healing neuropathic diabetic foot ulcer were treated with GAMs enriched with a replication-defective adenovirus encoding human platelet-driven growth factor-B and a bovine collagen substrate, with 10 of 12 patients recovering by 3 months (Mulder et al., 2009). However, because of the extended time periods over which gene delivery complexes are made available through GAMs, extremely high gene delivery efficiencies are not necessary for successful transfection. Combined with the prospect of having GAMs serve as depots for virus release, the non-immunogenic nature of non-viral gene delivery agents make them perhaps more appealing for use with GAM implants.

1.4 Expression-targeted Cancer Gene therapy

Expression-targeted cancer gene therapy is a promising technology that could be applied to treat transitional cell carcinoma. The fundamental idea such treatment is that plasmids driven by cancer-specific promoters will be used to drive the expression of toxic transgenes. After delivery into the cell, the toxic gene would be highly expressed in cancer cells, while normal cells would experience little or no effect. The result would be induced cancer cell death with few or no side-effects.
To develop expression-targeted gene therapy, it is critical to discover promoters associated with strong specificity for and high activity in the target cells. Promoters are functional units in the genome, serving at the binding site for RNA polymerase and the assembly site for the transcriptional machinery. In eukaryotes, RNA polymerase II will bind to the promoter site and proceed with synthesis of RNA that will eventually be modified to messenger RNA. To complete transcription, the polymerase also needs other proteins to form a complex, including transcription activators, chromatin modification and remodeling proteins, coactivators, and basal transcription factors, as sketched in figure 1.2.

![Promoter and Transcriptional Machine](Godbey, 2014)

Figure 1.2 Promoter and Transcriptional Machine (Godbey, 2014)

It has been shown that promoters such as Cox2 (Dobek et al., 2011, Zhang and Godbey, 2011, Zhang et al., 2009, Zhang et al., 2008, Godbey and Atala, 2003), Rad51 (Hine et al., 2012, Hine et al., 2008), and Survivin (Wang et al., 2007, van Houdt et al.,...
could potentially be used for expression-targeted applications in clinical practice. Our laboratory has used the Cox2 promoter drive the expression of pro-apoptotic genes. Our laboratory has also shown that this treatment regimen is more effective than BCG as well as the anti-angiogenic drug Celecoxib (Zhang and Godbey, 2011). We are confident that expression-targeted gene therapy will be an effective treatment for transitional cell carcinomas of the bladder. However, due to the diversity of accumulating mutations in the general population, it is hard to conclusively show that one promoter will provide a function that is universal to all patients. Thus, searching for more promoters to achieve a targeted, therapeutic effect is an important task in the development of expression-targeted gene therapies.

1.5 Translation of Expression-targeted Cancer Gene Therapy into Clinical Practice

The ultimate aim of the research described herein is an expression-targeted treatment for transitional cell carcinoma of the bladder following tumorectomy. Bladders would be treated via transurethral, intravesical therapy using PEI-mediated gene delivery as described in (Zhang and Godbey, 2011), or by using cationic lipids as the gene delivery vehicle as reported in (Wu et al., 2003). Because multiple types of tumors share similar gene expression profiles, expression-targeted gene delivery could be of value in a wide array of clinical situations using the promoters that are detailed in this
thesis. The best vehicle for systemic gene delivery applications is still under intense scrutiny, but the promoters described herein could be used in both viral and non-viral applications because of their relatively small fragment size (less than 1,000 base pairs). Not only are viral and non-viral vectors strong candidates for the in-vivo research that necessarily must take place in small and large animal models before the therapy can be taken into the clinic, but also transfected stem cells (especially mesenchymal stem cells (MSCs)) (Reiser et al., 2005) could be used to treat tumors via systemic gene therapy, such as for metastatic cancer. It was found that MSCs can selectively migrate to tumor sites via the vasculature (Shah, 2012). This approach uses a pro-drug and an activator. The activator is coded for by an expression-targeted transgene, which has been delivered to MSCs. Once the transfected MSCs migrate to tumor sites, it is believed that the expression-targeted gene – which utilizes at least one of the promoters described in this thesis – will be expressed, thereby activating the pro-drug and killing the tumor cells through bystander effects (Nazari, et al., 2011 and Elmageed, et al. 2014).

1.6 Minicell Technology

Minicell technology is also a promising application for follow-up procedures after resection of bladder tumors. Minicells are non-living, miniature versions of bacteria (which lack a chromosome). They do possess that same cell exteriors as the parent bacterium, and as such are immunostimulatory. However, because they lack a chromosome, they are unable to reproduce and therefore cannot present the same
complications as their living counterparts. Modification of the minicells has rendered the able to enter into tumor cells (in a targeted manner) and cause their death via membrane disruption (unpublished data). We have shown, through a collaboration with Vaxion Therapeutics, that the modified-minicell technology is superior to BCG therapy not just because of the lack of living microbes in stimulating an immune response, but also because of the direct, targeted cell killing that has been achieved. The drug is undergoing aggressive investigation as it is prepared for Phase I clinical trials.

1.7 Goals of Dissertation

The objective of this study is to develop novel treatments to complement or replace Bacille Calmette-Guerin (BCG) and chemotherapy drugs for treating transitional cell carcinoma of the bladder. The treatments would involve the use of cancer-specific DNA elements (promoters) to drive suicide genes to induce cancer cells into apoptosis without bystander effects. Furthermore, minicells would prevent tumor recurrence and growth by a targeted toxicity and an induced immune response similar to live BCG, but without the risk of infection.

Chapter 1 presents a brief introduction to the background and concepts behind these studies. In this part, the definition of transitional cell carcinoma, gene therapy, expression-targeted cancer therapy, and minicell are presented. Modern methods used
for gene delivery are comprehensively introduced.

Chapter 2 demonstrates the specificity and efficacy of the Osteopontin promoter ($p_{opn}$) in driving genes used to treat multiple types of carcinomas. This promoter was selected using what we term “traditional methods”, which compare endogenous gene transcription levels. In normal versus cancer cells. Promoter specificity and efficacy were determined via differences in reporter expression in cancer versus normal cell types.

Chapter 3 introduces a different screening logic for locating cancer-specific promoters. Three novel, cancer-specific promoters – $p_{ran}$, $p_{brms1}$ and $p_{mcm5}$ – were identified with this method. The cancer-specificities of these promoters were demonstrated via reporter gene expression in several types of cancerous and normal cell types. A gene encoding a constitutively active apoptotic intermediate, RevCasp3 (an engineered form of caspase 3), under the control of $p_{ran}$, was constructed and expressed to kill cancer cells in a targeted fashion. When transfected by $p_{ran}$-RevCasp3, the cancer cell line PC-3 underwent apoptosis, verified to be due to caspase 3/7 (presumably our RevCasp3 analog). The $p_{ran}$-RevCasp3 was seen to act in a targeted fashion, with few harmful effects detected in normal cells.

Chapter 4 evaluates whether minicell constructs produce positive outcomes in the treatment of murine transitional cell carcinoma. The efficacy was determined by three
The first study set tested whether minicells yield a therapeutic effect on existing bladder tumors. The second set verified whether any survival advantage is given to tumor-bearing mice receiving minicell treatments. The third set examined whether minicells can impede the implantation of tumor cells.

Chapter 5 discusses future plans for developing expression-targeted gene therapy, with preliminary data. For expression-targeted gene therapy, we hope to identify a promoter library that can be used to target cancer cells via promoters that are related stress genes. Four promoters – $^{p}txnl1$, $^{p}grp94$, $^{p}polh$ and $^{p}znf394$ – were used to produce very promising data. Their cancer-specificity has been verified by comparing reporter gene expression in our collection of cancer and normal cell lines. Future research will focus on verifying whether genes under the control of these promoters can be activated by specific stress events.
Chapter 2

Cancer-specific Promoter \( p_{opn} \) and Single-nucleotide Polymorphisms for Targeted Cancer Gene Therapy
2.1 Background

Chemotherapy and radiotherapy are two common treatments for patients with cancer. However, these treatment modalities are associated with serious side-effects. Drugs and radiation produce toxic effects in rapidly dividing cells, which include not only cancer cells but also some healthy, untransformed cells such as blood cells, mucosal cells such as those lining the alimentary canal, and cells within hair follicles. As a result, patients undergoing chemotherapy or radiation treatments may suffer symptoms like anemia, diarrhea, and hair loss (DeVita et al., 2008). There is a pressing need to develop novel treatment methods that are specific to cancer cells.

Expression-targeted gene therapy is a promising technology that could someday replace chemotherapy and radiotherapy. The underlying mechanism of this form of gene delivery rests upon plasmids that are driven by tissue-specific DNA elements (promoters and/or enhancers) that are used to drive the expression of the delivered transgenes in only targeted cells. The goal of the research presented here was to produce plasmids that are highly expressed in cancer cells while normal cells experience no response, with the result of induced cancer cell death without side-effects.

Osteopontin (OPN), also known as secreted phosphoprotein 1, plays an important
role in cancer invasion and metastasis (Wai and Kuo, 2004, Rodrigues et al., 2007, Anborgh et al., 2010, Shevde et al., 2010). OPN mediates adhesive cell-matrix interaction by binding integrin receptors and CD44 glycoproteins. The binding of OPN to integrin could either up-regulate expression or activate the activity of urokinase-type plasminogen activator, as well as a series of proteases (such as matrix metalloproteinase-2), which could transform plasminogen to plasmin, resulting in the degradation of the extracellular matrix (Shevde et al., 2010, Tuck et al., 1999, Das et al., 2005). Several reports have indicated that OPN may also be involved in angiogenesis (Hirama et al., 2003, Malyankar et al., 2000, Senger et al., 1996, Chakraborty et al., 2008, Infanger et al., 2008, Takahashi et al., 2002). Furthermore, OPN may also promote cancer cell survival during transport through the circulatory system by suppressing apoptosis, allowing replication by anchorage-independent growth, and allowing the cells to escape recognition and attack by the immune system (Zhang et al., 2003, Thalmann et al., 1999, Wai et al., 2006). Perhaps due to its involvement with invasive and metastatic processes, OPN is over-expressed in malignant cancers. Investigations of lung cancer have shown OPN RNA and protein levels are much higher in primary tumors versus normal lung tissue (Chambers et al., 1996). In gastric cancer, 72.5% of primary tumors and 60% of lymph node metastases have been found to have high OPN mRNA levels as compared to cells of normal mucosa (Ue et al., 1998). According to a recent survey investigation by Weber et al., OPN is also highly expressed in breast, glioma, head and neck, liver, oral, and ovarian cancers (Weber et al., 2010). Because of the high OPN
expression levels in aggressive cancers and paucity of OPN in untransformed cells, regulatory elements of the \textit{opn} gene are attractive candidates for expression-targeted gene therapy.

Herein, investigations into the potential of the osteopontin promoter (\(^{p}\text{opn}\)) for cancer-specific expression-targeted gene therapy are presented. The specificity and activity of transgene expression are assessed for several iterations of promoter length in an attempt to define the necessary portion of the \textit{opn} promoter element for regulated gene expression. Issues surrounding single nucleotide polymorphisms (SNPs) and their effect on promoter activity and specificity are also addressed. An interesting investigation into whether there exists a correlation between endogenous \textit{opn} transcription levels and transgene expression under the control of \(^{p}\text{opn}\) was also performed. Regarding the correlation, a mechanism which addresses hOPN variants regulation is suggested.
2.2 Materials and Methods

2.2.1 Cell lines and Cell Culture

A total of eight cell types, including five human and three murine lineages, were used in these investigations. Identification of the cells and the growth media used are presented in Table 2.1. All cells were maintained in a humid incubator at 37°C and 5% CO₂.

### Table 2.1 – Cell lines and culture media used for the presented investigation

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Species</th>
<th>Description</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF-1</td>
<td>Homo sapiens</td>
<td>Normal foreskin fibroblast cells</td>
<td>DMEM with 15% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>HASMC</td>
<td>Homo sapiens</td>
<td>Normal aortic smooth muscle cells</td>
<td>SmGM-2 BulletKit</td>
</tr>
<tr>
<td>HTB-1</td>
<td>Homo sapiens</td>
<td>Urinary bladder transitional</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>HTB-5</td>
<td>Homo sapiens</td>
<td>carcinoma cells</td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>Homo sapiens</td>
<td>Prostate adenocarcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>MFC</td>
<td>Mus musculus</td>
<td>Normal murine fibroblast cells</td>
<td>RPMI 1640 with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>NOR-10</td>
<td>Mus musculus</td>
<td>Normal muscle fibroblast cells</td>
<td>DMEM with 20% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>MB-49</td>
<td>Mus musculus</td>
<td>Bladder Carcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
</tbody>
</table>

DMEM - Dulbecco’s Modified Eagle’s Medium  
RPMI 1640 - Roswell Park Memorial Institute (RPMI) 1640 Medium  
FBS - Fetal Bovine Serum  
P/S - Penicillin/Streptomycin  
SmGM-2 BulletKit(Lonza)
2.2.2 Plasmid Construction

Plasmids using pEGFP-N1 as a base vector

Some of the plasmids made with cancer-specific promoters were constructed using pEGFP-N1 (Clontech, Palo Alto, CA, USA) as the vector plasmid. The deletion analysis experiments on $^p$opn used this vector. The human opn promoter portions used included base ranges (-922 to +125), (-299 to +125), and (-134 to +125) (fig. 1a). The opn promoters were obtained via PCR performed on whole-genome extractions of human foreskin fibroblasts (HFF-1). For determining species cross-reactivity, the murine opn promoter (-543 to -33) was cloned from the genome of the murine bladder cancer cell line MB49. For these experiments, control plasmids included pEGFP-N1 with the original $^p$cmv promoter and a promotorless version of the same vector. All plasmid promoter sequence identities were verified by DNA sequencing (ACGT, Inc, Wheeling, IL).

Plasmids using pGL3 as a base vector

The pGL3 vector (Promega, Madison, WI, USA) was selected for SNP experiments because it lacks the SV40 enhancer that is present in pEGFP-N1. The luciferase reporter exon was replaced by the enhanced green fluorescent protein exon (GFP) from pEGFP-N1. Six common SNP haplotypes of $^p$opn were then cloned into the modified plasmid, replacing the original promoter.
The origins of the $^p$opn SNP variants were as follows:

-443T -155GG -66G  (-922 to +125)  HFF-1 wild-type genome

-66G  (-134 to +125)  HFF-1 wild-type genome

-443C -155G -66T  (-922 to +125)  HTB-5 wild-type genome

-443C -155GG -66G  (-922 to +125)  site directed mutagenesis (PCR)

-443C -155GG -66T  (-922 to +125)  site directed mutagenesis (PCR)

-66T  (-134 to +125)  site directed mutagenesis (PCR)

The plasmids pGL3-GFP with $^p$cmv (from pEGFP-N1) and a pro-motorless pGL3-GFP served as positive and negative controls. All plasmid promoter identities were verified by DNA sequencing.

2.2.3 Transfection

Cells were transfected using branched poly(ethylenimine) (PEI) having a weight-average molecular weight of 25 kDa (Sigma-Aldrich, St. Louis, MO, USA). Before transfection, cells were plated into six-well plates at 100,000 cells per well and cultured overnight in growth medium. After 16 hours, media were replaced with FBS-free culture media followed by transfection with PEI/DNA complexes made at a
7.5:1 N:P ratio using 3.6 µg of DNA per dose. Transfection media were replaced with growth media after 2 hours. GFP expression was measured after 48 hours via flow cytometry (FACSCantoII, BD Biosciences, San Jose, CA, USA). Fluorescence intensities were noted, being defined as the mean intensity of the strongest fluorescing 10% of cells within the population. Transfection efficiency was defined as the ratio of GFP positive cells versus the total number of cells. For each cell line, the reported relative intensity and normalized transfection efficiency were normalized with those obtained from positive control transfections (\(^{p} cmv\)-driven GFP expression).

2.2.4 Stable endogenous hOPN mRNA level measurement

The stable gross \( opn \) transcription levels (including pre-mRNA and all three mRNA variants \( opn-a \), \( opn-b \), and \( opn-c \)) were measured via real-time PCR. Cells were plated into six-well plates at the density 100,000 cells per well. After reaching the late log phase of growth (~70% confluence), cells from 2-3 wells were combined and mRNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Next, cDNA was synthesized from the collected mRNA samples via an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), with results being analyzed via qPCR using the following primer set: forward 5’- ATG GCC GAG GTG ATA GTG TGG TTT -3’, reverse 5’- ACT GGA TGT CAG GTC TGC GAA ACT -3’. The internal control used was 18s rRNA. Quantification was performed using the \( 2^{-\Delta\Delta C_t} \) method: Fold change =
where $\Delta \Delta Ct = \Delta Ct_{\text{other cell}} - \Delta Ct_{\text{HFF-1}} = [Ct_{\text{opn}} - Ct_{\text{18s}}]_{\text{other cell}} - [Ct_{\text{opn}} - Ct_{\text{18s}}]_{\text{HFF-1}}$ (Yuan et al., 2006).

Individual hopn mRNA variant levels were analyzed by RT-PCR. Each variant was amplified by PCR based on cDNA templates as just described, then analyzed using gel electrophoresis. Primer sets were selected based upon published sequences (Hahnel et al., 2012):

- hOPN-a: forward 5’- ATC TCC TAG CCC CAC AGA AT -3’, reverse 5’-CAT CAG ACT GGT GAG AAT CAT C-3’;
- hOPN-b: forward 5’- ATC TCC TAG CCC CAG AGA C-3’, reverse 5’- AAA ATC AGT GAC CAG TTC ATC AG-3’;
- hOPN-c: forward 5’- TGA GGA AAA GCA GAA TGC TG-3’, reverse 5’-GTC AAT GGA GTC CTG GCT GT-3’.

Gel images were recorded by camera and the maximum intensity of each band was quantified with Gel-Pro Analyzer imaging software.

2.2.5 Statistics

Groups of data were first analyzed by one-way ANOVA. If the test indicated there
was a significant difference within the group of means, the Holm-Sidak post hoc test was applied to compare each pair within the group. Significant differences are defined as having $p<0.05$. 
2.3 Results

2.3.1 $p_{opn}$ lengths

There is some debate regarding the $opn$ promoter region and whether there exists a negative regulatory sequence in the near upstream region of the transcriptional start site. Specifically, some researchers have reported that the (-134 to +125) region yields the strongest transcriptional activity, and that there is a negative regulatory element upstream of -142 (Liu et al., 2004, Hijiya et al., 1994). However, others have found no such negative elements in this region (Wang et al., 2000). The conflicting results may be due to the use of different cell types. For the purposes of expression-targeted gene therapy, negative regulatory elements would only serve to dampen or eliminate expression of the delivered transgene, so it was necessary to revisit the possibility of a negative regulatory element in the near upstream region of $opn$ in multiple cell types.
Figure 2.1 – a) Promoter/exon combinations used for plasmid construction. Three $P_{opn}$ promoter lengths – (-922 to +125), (-299 to +125), and (-134 to +125) – were built into separate reporter plasmids. b) Deletion analysis using the promoter regions from part a). Promoter activity represented by relative intensity. c) Histogram graph of HFF-1 cells after transfection reported by FACS flow cytometry to show how relative intensity was obtained. Fluorescence intensities were noted, being defined as the mean intensity of
the strongest fluorescing 10% of cells within the population.  

**d)** Deletion analysis using the promoter regions from part a). Promoter activities are implied by transfection efficiencies.  

**e)** Four quadrant graph of PC-3 cells (sham-treated and $p_{opn}^{134}$-GFP-treated) to show how transfection efficiency was obtained. Transfection efficiency was defined by the percentage of Q2 and Q4 occupied in whole population. Gate was defined using the saline-treated group. These were analyzed in four human cell types: HFF-1 (normal fibroblast), PC-3 (human prostate cancer), HTB-1 (human bladder cancer), and HTB-5 (human bladder cancer). Data are expressed as “Relative Intensity”, meaning promoter-driven GFP intensity was normalized to a positive controls using $P_{cmv}$-driven GFP. One-way ANOVA, performed separately for each cell type, indicates there is no significant difference between the average fluorescence intensities obtained using the different (non-zero) lengths of $p_{opn}$ to drive reporter expression.  

Error bars represent one standard deviation. “*” indicates that promoterless controls were significantly different from the other promoters used for the cell line (ANOVA + Holm-Sidak post-hoc test, n≥3, P<0.05).
Analysis of upstream *opn* regions commenced with the DNA segment located from bases (-922 to +125) in the wild-type gene. Two subsets of this region were also investigated: (-299 to +125) and (-134 to +125). These three segments were used to drive the expression of the GFP exon in plasmid constructs. We refer to these three plasmids as $P_{opn}^{−922}$-GFP, $P_{opn}^{−299}$-GFP, $P_{opn}^{−134}$-GFP to denote the DNA ranges used for each promoter (fig. 2.1a). These plasmids were delivered into four cell lines – HFF-1 (normal fibroblast), PC-3 (prostate cancer), HTB-1 (human bladder cancer), and HTB-5 (human bladder cancer) – and the resulting fluorescence intensities measured by flow cytometry (fig. 2.1b). No significant differences between the average fluorescence intensities obtained with each plasmid were observed for any of the given cell lines $(P>0.05)$, although each plasmid yielded GFP expression significantly more intense than did the promoterless negative control (ANOVA and posthoc test, $P<0.05$). These results indicate that there is no significant difference in the activity of the three tested $P_{opn}$ regions, implying that there is no active negative control element within 922 bases upstream of the *opn* transcriptional start site.

Among the three tested promoter lengths, $P_{opn}$ (-134 to +125) was selected for the next study. There was no significant difference between the average fluorescence intensities obtained with the three different sizes, so using the smallest promoter was reasoned to allow for a greater number of plasmids to be delivered in experiments for a
given mass of DNA.

2.3.2 Species cross-reactivity

Human and murine $^p$opn were compared to determine any species cross-reactivity between the promoters. The human $opn$ promoter region from (-134 to +125), and the murine region from (-543 to +33) (Craig and Denhardt, 1991), were obtained via PCR performed on genomes obtained from human and murine cells. Transfections were performed in five human and three murine cell lines, and it was found that using the human version of $^p$opn to drive transcription was statistically no more or less effective than using the murine version any of the tested cell types (fig. 2.2a). To understand why the human and murine analogs of $^p$opn have similar functionality in either species, the DNA sequences were compared using the Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov). It was found that the (-134 to -33) promoter region is 87.1% conserved between the two species (fig. 2.2b). Because of the similarity between the promoters, both in DNA sequence and in results obtained, the remainder of the experiments described herein utilized the human analog of $^p$opn.
**Figure 2.2 – a)** Comparison of transfection efficiencies obtained for delivered transgenes driven by the human *versus* murine \(^{\text{P}}\text{opn}\) promoter in both human (cell names underlined) and murine (names not underlined) cells, including both normal (left of dotted line) and cancerous (right of dotted line) cell lineages. Error bars represent one standard deviation. No significant difference was observed between the two promoters for any single cell type (t-test, \(n \geq 3, P \geq 0.05\)). **b)** Alignment of the human and murine \(^{\text{P}}\text{opn}\) sequences (-134 to -33).
2.3.3 $P_{opn}^{134}$ specificity and efficacy

The potential for using the human $P_{opn}^{134}$ to target cancer cells was further analyzed by comparing transfections of each of the four cancer cell lines to all four of the “normal” cell lines (HFF-1, HASMC, MFC, NOR-10) (fig. 2.3a). With the exception of HTB-5 cells, the transfected cancer cells yielded significantly higher fluorescence intensity versus the normal cells. The maximum intensity of $P_{opn}$-driven expression in the four tested normal cell types was only 11.6% of the average value obtained using $P_{cmv}$ to drive transcription, while the expression in the three statistically different cancer cell types ranged from 26.2% to 102.7% that of CMV-driven expression. To lend a different perspective, transfection efficiencies are also presented (fig. 2.3b). In this case, all of the cancer cell lines expressed $P_{opn}$-driven transgenes to a significantly greater degree than did normal cells. It is interesting to note that the cancer-cell selectivity was demonstrated present between the cancerous MB49 and the non-cancerous MFC and NOR-10 cell lines. These cells were murine cells, but the human-derived (-134 to +125) promoter was used to drive expression in them. It is also interesting to note that the strongest activity seen was in MB-49 cells, with expression intensities on par with the ubiquitously strong $P_{cmv}$. 
Figure 2.3 – Strength of transfections using the $^{134}$opn in normal versus cancer cell lines, normalized to $^{\text{cmv}}$-driven controls. Error bars represent one standard deviation. “*” indicates there is a significant difference between the given cancer cell line and the complete set of four tested normal cell lines (ANOVA + Holm-Sidak post-hoc test, n≥3, P<0.05).  

a) Relative intensity of reporter expression.  

b) Transfection efficiencies, normalized to $^{\text{cmv}}$-driven controls.
At this point the vector backbone was changed to pGL3 to reduce the amount of fluorescence obtained in normal cells. The pEGFP vector contained a 72 bp region of tandem repeats of the SV40 enhancer. Switching to a plasmid that used our inserted promoter as the sole control region removed most of the background transfection. Without the SV40 enhancer, transfection efficiency in HFF-1 cells was lowered from 30.8% to 8.4% of that obtained in \(^{\text{p}}_{\text{cmv}}\)-driven controls, and the relative intensity of transfected HFF-1 was lowered to only 1.9% of the brightness observed for the same untargeted controls. Unfortunately, reporter expression was also lowered, although to a lesser extent, in cancer cells, too. This issue is addressed in the next section, regarding single-base mutations.

### 2.3.4 SNPs

We hypothesized that one or multiple SNPs could increase the cancer-specific efficacy of the osteopontin promoter. To allow for a greater number of SNP possibilities, the longer promoter region (from -922) was re-examined. There exist three \(^{\text{p}}_{\text{opn}}\) SNPs of interest in the literature, and two of them occur upstream of -134. It was reasoned that revisiting the longer promoter would be acceptable in light of the lack of significant differences between transfection data obtained using the \(^{\text{p}}_{\text{opn}}^{\text{-134}}\) and \(^{\text{p}}_{\text{opn}}^{\text{-922}}\) regions (fig. 2.1b). Five additional versions of \(^{\text{p}}_{\text{opn}}\) were therefore created to reflect the most common SNPs existing in the first 500 bp upstream of the transcriptional start site.
in the human population (Giacopelli et al., 2004). This was accomplished by site-directed mutagenesis performed on the $^p_{opn}$ obtained from cancer and untransformed human cell genomes and verified by sequencing. The mutations therefore generated and the fragment sizes used are shown in Figure 2.4. Promoter activities were then tested for their ability to drive GFP expression in the human cell lines HFF-1, PC-3, HTB-1, and HTB-5 cells (fig. 2.5).

**Figure 2.4** – Schematics of the six promoter/exon combinations constructed to study the SNP effect upon promoter strength, and the locations/identities of the included SNPs.
The effectiveness of each SNP was not consistent for all cell types (fig. 2.5). In normal fibroblasts, the four versions of $^{\text{P}^{\text{opn}}}^{922}$ were not found to yield significantly different transfection results from one another (ANOVA), but the shorter $^{\text{P}^{\text{opn}}}^{134}$ with SNP -66T yielded significantly higher reporter intensity versus transfections using -66G. In PC-3 cells, $^{\text{P}^{\text{opn}}}^{922}$ with (-443C -155GG -66T) yielded intensities significantly higher than both (-443C -155GG -66G) and (-443C -155G -66T). For HTB-1 cells, all SNP combinations with T at position -66 were shown to have higher activity than -66G, no matter the promoter length ($^{\text{P}^{\text{opn}}}^{922}$ or $^{\text{P}^{\text{opn}}}^{134}$). Similar results were also found for HTB-5 cells.
Figure 2.5 – Strength of reporter expression using the promoters described in figure 2.4. Fluorescence intensity was measured by flow cytometry. The raw transfection efficiencies achieved with $^{P}cmv$-driven GFP for each cell line were 42.1% ± 4.8 for HFF-1, 66.0% ± 4.6% for PC-3, 32.0% ± 0.8% for HTB-1, and 36.2% ± 6.6% for HTB-5. Error bars represent one standard deviation. “*” indicates there is a significant
difference between the mean represented by the given data bar paired with the mean
indicated by the horizontal bracket(s) above (\( [\underline{\text{ }}] \)) (ANOVA + Holm-Sidak post-hoc
test, \( n \geq 3, \ P < 0.05 \)). White bars: (-922 to +125) promoters. Dark bars: (-134 to
+125) promoters.  

\( a \) HFF-1 cells  \( b \) PC-3 cells  \( c \) HTB-1 cells  \( d \) HTB-5 cells.
Furthermore, when the original $p_{opn}^{\text{-922}}$ underwent PCR directed mutagenesis to produce (-443C -155GG -66T), $p_{opn}^{\text{-922}}$ activity in the cancer cell types increased (mRNA concentrations increased with a fold change of 1.2 ~ 3.5) while there was a decrease in reporter activity in normal controls (fold change = 0.7). This was the case for both transfection efficiency and reporter intensity (normalized transfection efficiencies are given in table 2.2). These data indicate that SNP combinations could serve as a new direction for the improvement of both promoter efficacy and selectivity in cancer cells.

Table 2.2 – The effect of mutations at -443, -155, and -66 upon transgene expression.

<table>
<thead>
<tr>
<th></th>
<th>$p_{opn}^{\text{134}}$ Wild-type</th>
<th>$p_{opn}$, SNP Mutations -443C, -155GG, -66T</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF-1</td>
<td>8.4% ± 1.3%</td>
<td>5.8% ± 1.9%</td>
</tr>
<tr>
<td>PC3</td>
<td>36.9% ± 3.3%</td>
<td>45.5% ± 8.7%</td>
</tr>
<tr>
<td>HTB-1</td>
<td>17.6% ± 4.2%</td>
<td>35.1% ± 4.7%</td>
</tr>
<tr>
<td>HTB-5</td>
<td>4.8% ± 8.2%</td>
<td>16.6% ± 4.1%</td>
</tr>
</tbody>
</table>

Values reflect average normalized transfection efficiencies using the indicated $p_{opn}$. The three indicated SNPs improved transfection efficacy in the three cancer cell lines, but there was a reduction in transgene expression in the normal control cell line (HFF-1).
2.3.5 Transgene expression versus endogenous transcription levels

Levels of \textit{opn} RNA (including all pre-mRNA and mRNA variants) were measured by real-time PCR for the human cell types HFF-1, PC-3, HTB-1, and HTB-5. Data for the cancer cell lines were normalized to HFF1 values using the $2^{-\Delta\Delta C_t}$ method (Yuan et al., 2006). Surprisingly, the endogenous \textit{opn} RNA levels were highest in the two cell types that responded the most poorly to $^p\textit{opn}$-driven transfections (fig. 6a). This is counterintuitive because the underlying principle of expression-targeted gene delivery is that using the promoters/enhancers from actively transcribed genes to drive the expression of delivered transgenes will yield high transgene transcription. By extension, one might expect that cells that show high levels of endogenous \textit{opn} RNA would strongly express $^p\textit{opn}$-driven reporter genes. However, HTB5 cells with weak fluorescent intensity had relatively high amounts of \textit{opn} RNA while bright PC-3 and HTB-1 cells had relatively low amounts of \textit{opn} RNA. This observation seemed very weak at first. The graph of relative reporter intensity versus endogenous \textit{opn} RNA levels had an $R^2$ value of 0.24 (fig. 2.6b).
Figure 2.6 – Endogenous mRNA levels of hOPN, as determined by real time PCR. The fold-difference reported is versus HFF-1 controls.
There are three known variants of \textit{opn} mRNA. To investigate whether variant identity had any bearing upon \textit{opn} activity, the levels of individual \textit{opn} mRNA variants – a, b, and c – were detected by RT-PCR. Total mRNA was extracted in four independent runs and each sample was analyzed three separate times by PCR. The PCR run yielding the strongest band is shown in Figure 7 for each of the four runs. It was determined from the data presented in Figure 7a that all three \textit{opn} mRNA variants are present in HFF-1 cells. For PC-3 cells, \textit{opn}-c is typically the dominant band. For HTB-1 cells, all three variants were found, but \textit{opn}-c is the dominant species. For HTB-5 cells, \textit{opn}-a and \textit{opn}-b were the dominant variants. Another way to state these results is that both \textit{opn}-a and \textit{opn}-b were the dominant variants in HFF1 and HTB5 cells.
Figure 2.7 – Assessment of possible correlations between endogenous mRNA variants levels and $^P$opn-134-driven reporter strength. For these comparisons, the identity of the base at -66 was determined for each individual cell type, and cells were transfected with reporter plasmids that were driven by an $^P$opn having the same SNP. (The SNP at -66 is: G for HFF-1, PC-3, and HTB-1 cells; T for HTB-5 cells) Error bars represent mean±SD.

a) Gel images of endogenous opn variant levels as determined by PCR.  b) Quantified and normalized (to internal 18S control) endogenous opn variant levels versus reporter intensities. Relative intensity of reporter expression is normalized to $^P$cmv-driven controls. Error bars represent mean±SD.
The bands representing transcription levels of the mRNA variants were quantified via imaging software, with values normalized to 18s rRNA values to yield relative transcription amounts. A linear regression was then fit to the resulting values to determine whether there was a correlation between the \(^{\text{p} \text{opn}^{134}}\)-driven transgene activity (measured by relative fluorescence intensity) versus endogenous levels of each SNP (measured by the normalized image intensity read from the gel) (fig. 7b). Note that the \(^{\text{p} \text{opn}^{134}}\) used were not identical for each transfection. The promoter used for each cell type had the same polymorphism at -66 as was determined to be native to that cell type. For example, the SNP of wild type PC-3 at position -66 is G, so the intensity of PGL3-66G was selected for comparison. The SNP of wild-type HTB-5 at position -66 is T, so the intensity of PGL3-66T was selected. Results of such regression lines (fig. 7b) revealed no correlation at all for \(opn\)-c (\(R^2 = 0.00\)). However, a negative correlation was observed for \(opn\)-b (\(R^2 = 0.83\)), and a strong negative correlation (\(R^2 = 0.95\)) was observed for \(opn\)-a.
2.4 Discussion

This work was performed to investigate the potential of \( ^*\text{opn} \) to serve as a cancer-specific promoter for expression-targeted gene therapy. Promoter lengths and combinations of single-nucleotide polymorphisms were considered in terms of transcription and expression activities and cell specificities. The relations between \( ^*\text{opn} \)-driven gene expression and endogenous \textit{opn} transcription levels, including gross and individual cell-specific variants, were also assessed.

2.4.1 SNPs

SNPs in \( ^*\text{opn} \) have been reported to be associated with various diseases, including diabetes (Marciano et al., 2009), chronic hepatitis (Shaker et al., 2012), urolithiasis (Liu et al., 2010a) and various types of cancer (Chen et al., 2010, Xu et al., 2012, Chiu et al., 2010, Zhao et al., 2012, Schultz et al., 2009a, Dong et al., 2012). The most widely reported SNPs in \( ^*\text{opn} \) are 443 T/C, -155 GG/G and -66 T/G. Our data show that, at the -66 position, the promoter activity with a T at -66 is stronger than the G polymorphism, consistent with findings published by Giacopelli, \textit{et al} (Giacopelli et al., 2004). They reasoned that the -66 position is associated with the transcription factor Sp1, and that a thymine in this position will allow for higher binding affinity with Sp1 than would a guanine. Furthermore, abnormal Sp1 expression has been linked with multiple types of cancer (Li and Davie, 2010), which makes the SNP of -66T a useful component of cancer
cell targeting via $^{\text{P}}\text{opn}$.

At the -155 position, the insertion of an additional guanosine residue has been associated with the binding of the transcription factor RUNX2 (Giacopelli et al., 2004). RUNX2 expression and function have been correlated with progression and metastasis of prostate (Akech et al., 2010) and breast (Onodera et al., 2010) cancers, which is consistent with the fact that PC-3 cells were sensitive to the addition of a second guanylate at position -155 in $^{\text{P}}\text{opn}$ (-155GG) in our investigations (fig. 2.5b).

Our data did not reflect any differences in $^{\text{P}}\text{opn}$ activity for tested SNPs at -433 in human bladder and prostate cancer cell lines. However, findings published elsewhere have indicated that $^{\text{P}}\text{opn}$ with -443C is more active than -443T in cell lines of human melanoma (Schultz et al., 2009b) and gastric cancer (Zhao et al., 2012) origin. Conversely, a separate report states that -443T is the stronger polymorphism in hepatocellular carcinoma (Dong et al., 2012). We hypothesize that transcription factor binding at -443 plays different expressional and functional roles in different types of cancer. These different, yet non-conflicting findings indicate that customized gene therapy, where SNPs of a patient’s cancer are considered on an individual basis, may provide greater clinical benefit.

In the area of expression-targeted cancer research, SNPs have attracted little
attention to date. It is a straightforward task to find the names of specific genes/proteins in reports concerned with promoter selection, but sequence details and useful SNPs within the promoter are typically either omitted or not addressed at all. It is known that transgene promoter selectivity can depend upon cis- or trans-regulatory elements. For cis-regulatory elements (promoters), the promoter sequence must be recognized by transcription factors. SNPs within a promoter region can affect the affinity of a transcription factor for the altered promoter. Thus, when investigations focus on expression-targeting, it is important to verify and assess the actual gene sequence used for any SNP mutations. Furthermore, even for promoters which have been shown to have excellent cancer-targeting specificity, single-base changes could further improve specificity and efficacy.

### 2.4.2 Screening for promoters for expression-targeting

Traditionally, potential promoters are selected by noting either endogenous mRNA levels, to imply transcription rates, or protein levels, to demonstrate expression. Microarrays, qPCR, and Western blots are tools often employed to find which gene’s mRNA or protein levels are up-regulated in cancer cells. Promoters are identified and copied from genomic DNA then cloned into a plasmid vector. After delivering the vector into both cancer and normal cells the reporter gene expression is measured. If the expression presents strong selectivity, the promoter is a good candidate for cancer-related
expression-targeted gene therapy.

However, our data show that the traditional approach can be misleading. There was no positive correlation between gross endogenous opn mRNA levels and \(^{p}\)opn-driven reporter expression. We observed that the cells with the highest endogenous opn mRNA levels had the weakest reporter expression. In contrast, the cells that were most responsive to \(^{p}\)opn-driven transgene expression had the lowest amount of total endogenous opn mRNA (which included all three splicing variants). This unexpected result could be due to methylation of the endogenous opn gene, a distant transcription repressor, or even miRNA. The first two of these possibilities would cause lower transcription of the host opn gene with no effect on transcription of the delivered plasmids, which lack methylation and should be unaffected by genomic repressor sites. Similarly, while anti-opn miRNA could serve to lower host opn mRNA levels, there would be no effect upon the mRNA transcribed off of the delivered plasmids (Bhattacharya et al., 2010). Therefore, one should not make predictions about the strength and efficacy of engineered genes based solely on endogenous levels of mRNA or proteins in host cells.

2.4.3 Alternative splicing

There have been three variants of opn mRNA identified in humans: opn-a, opn-b
and opn-c. The opn-a variant represents the full-length mRNA, which contains the complete set of 7 exons. The opn-b variant has been spliced to exclude exon #5 and opn-c has been spliced to remove exon #4. It has been reported that the opn variants function differently in promoting cancer cell growth (Goparaju et al., 2010, Yan et al., 2010, He et al., 2006), but the regulatory mechanisms associated with the variants are still unclear.

In some cells, a huge variety of the mRNA band intensity was presented in multiple repeats (fig. 2.7a). For example, within HTB-1 cells, for opn-a and opn-b variants, two obvious bands were presented in #2 and #4 repeats, but they are disappearing in #1 and #3 repeats. The reason of those differences may be that the cells were in different states of the cell cycle. Potentially, #2 and #4 repeat was in a same state. Meanwhile, #1 and #3 repeat was in another state. Thus, different state may cause different variant expression. Future efforts will be spent on applying serum deprivation method to synchronize the cell cycle to investigate whether cell cycle will affect OPN variant expression.

Our data show that there is a strong correlation (although negative) between \( ^{\text{p}}\text{opn} \) activity and mRNA levels of the opn-a variant in the cells tested. To explain the negative correlation, we offer three hypotheses. The first hypothesis considers that degradation of \( ^{\text{p}}\text{opn} \)-a mRNA correlates with promoter activity, contributing to a decrease
in endogenous hOPN-a protein levels. Micro-RNA may play a function here. A report published by Bhattacharya et al. indicates that Micro-RNA-181a decreases OPN expression in hepatocellular carcinoma cell lines (Bhattacharya et al., 2010). Stronger promoter activity could therefore be associated with greater expression of a specific miRNA that targets opn-a, causing greater opn-a degradation. The result would be a decrease in OPN-a mRNA with a concomitant increase in \( P_{\text{opn}} \)-driven transgene expression, just the negative correlation that we observed.

A second hypothesis to explain the negative correlation involves a recruitment-coupling mechanism (Braunschweig et al., 2013). Recruitment-coupling refers to the case where alternative RNA splicing requires the recruitment of splicing factors to the transcription site by the transcriptional machinery. A good example of this was published in 2012, with the splicing factor hnRNP L being shown to interact with the transcriptional co-activator MED23 to regulate alternative mRNA processing at the core promoter on a global, genome-wide level (Huang et al., 2012). In the \( P_{\text{opn}} \) system we report on here, the strong activity of the promoter in cancer cells could be increased because of a higher utilization of transcription factors and mediators at the core promoter. This, in turn, could induce more splicing factors to reduce the number of complete exon sets (i.e. the opn-a variant) while increasing the levels of the spliced variants. This scenario could explain the negative correlation between \( P_{\text{opn}} \) activity and opn-a transcription.
A third hypothesis to explain the negative correlation is based on the observation that could cause a reduction in mRNA levels without the involvement of miRNA. While exons are removed to produce the opn-b and opn-c alternate forms, other forms (either inactive or unknown-to-date) of opn RNA could be produced. The result would be less opn mRNA overall, with levels of the unspliced version being reduced the most. Again, such splicing would have no effect on the expression of $^{p}$opn-driven transgenes, so reporter expression could rise while opn-a,b levels are reduced. Furthermore, there is another degradation mechanism that high endogenous OPN protein expression may also suppress the promoter activity. More efforts will be spent here to figure out which mechanism is the main reason in this negative correlation.
2.5 Conclusion

The results presented show no significant differences in promoter activity for the three selected promoter lengths (-922 to +125, -299 to +125, and -134 to +125), indicating that there is no transcriptional repressor region within 922 bases upstream of the transcriptional start site. It was also found that $P_{opn}$ provides transfection selectivity in that transgene expression was largely restricted to cancer cells. Certain SNPs were found to affect expression-targeted efficacy and selectivity, and the effects were different for different cell types. These SNPs were verified against each wild-type SNP for individual cultures. Finally, there was a strong inverse relation between $P_{opn}^{922}$ activity (in driving transgene expression) and mRNA levels of the $opn$-a variant. The data regarding SNPs and alternative splicing have implications for how future searches for expression-targeting promoters should be conducted.
Chapter 3

Cancer-specific Promoters for Expression-targeted Gene Therapy:

\( p_{\text{ran}}, p_{\text{brms1}}, \) and \( p_{\text{mcm5}} \)
3.1 Background

In the early days of gene therapy research, having a gene delivered into a cell with subsequent expression by the cell was a great achievement. As the technology developed, it became evident that greater precision was needed. If, for example, one wanted to deliver genes into cancer cells to somehow effect their demise, then having the gene expressed by normal cells would not only be disadvantageous, it could be deadly. Targeted expression of delivered genes has since been used in cancer research for screening (Browne et al., 2011), imaging (Ahmed et al., 2002, Bhang et al., 2011) and treatment (Zhang and Godbey, 2011). While some have achieved targeting through the attachment of molecules to the exterior surfaces of the gene delivery complexes themselves, others have targeted transgene expression at the transcriptional level. This latter approach, referred to as expression-targeting, uses specific transcriptional control elements, such as promoters or promoter elements, to drive the transcription of the delivered genes in the targeted cells. The optimal control element would have high specificity for the targeted cells as well as a high efficacy in terms of the amount of protein that is expressed as a result of the transfection.

Traditionally, potential promoters for expression-targeting have been selected by examining either the levels of mRNA levels produced off of the endogenous gene or the levels of the protein product to imply transcription rates of the host gene. The
promoters of genes whose mRNA or protein levels are up-regulated in transformed cells
as compared to normal cells are often – and sometimes incorrectly – assumed to be
cancer-specific regulatory elements suitable for expression-targeted gene therapy. This
method has been used to successfully identify cancer-specific promoters such as $^{\text{p}}h\text{TERT}$
(Majumdar et al., 2001), $^{\text{p}}\text{psa}$ (Bhang et al., 2011) and $^{\text{p}}\text{cox2}$ (Godbey and Atala, 2003).

However, according to fundamental transcriptional regulation mechanisms (and our
own experience), the above method is not an efficient approach for identifying potential
promoters for expression-targeting. The transcription of endogenous genes is regulated
by several mechanisms working together. Native mRNA levels for a given gene in a
cancer cell can be up-regulated not simply because of stronger promoter activity, but also
because of changing chromatin structure (Wolffe, 2001), methylation of negative control
elements (Baylin, 2005) or distant enhancers (Kulaeva et al., 2012). The strength of a
promoter can also be masked by miRNA activity that reduces the amount of mRNA
associated with the gene (Jansson and Lund, 2012). In gene delivery, transgene
expression is much more straightforward, with transcriptional activity being directly
controlled by the promoters/enhancers included in the cloned plasmid being delivered.
There is not necessarily a strong relation between mRNA or protein levels associated
with an endogenous gene and the activity of an engineered plasmid (Landolin et al.,
2010). The use of traditional methods of screening promoters therefore may prove to be
very expensive in terms of time and resources invested for the amount of protein product
levels or cellular specificity yielded.

Using a view of promoter selection that takes into account the above concerns
(which will be interpreted in the Discussion), three promoters were identified and
harnessed for novel use in cancer-specific expression-targeted gene delivery: $p^{ran}$,
$p^{brms1}$, and $p^{mcm5}$. The most successful of the three promoters, $p^{ran}$, was then used to
drive the expression of the pro-apoptotic protein Reverse Caspase 3 (RevCasp3) to
selectively kill cancer cells.
3.2 Material and Methods

3.2.1 Cell culture

All cells were maintained in a humid incubator maintained at 37 °C and a 5% (v/v) \( CO_2 \) atmosphere. A total of 10 cell types were included in the investigations, in which six originated from normal tissue and four types from cancer tissue. The selected cell lines spanned human and murine species. The details of each line with its growth media were presented in Table 3.1.
### Table 3.1 - Cell lines and culture media.

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Species</th>
<th>Description</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF-1</td>
<td>Homo sapiens</td>
<td>Normal foreskin fibroblast cells</td>
<td>DMEM with 15% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>HASMC</td>
<td>Homo sapiens</td>
<td>Normal aortic smooth muscle cells</td>
<td>SmGM-2 BulletKit (Lonza)</td>
</tr>
<tr>
<td>HAEC</td>
<td>Homo sapiens</td>
<td>Normal aortic endothelial cells</td>
<td>EGM-2 BulletKit (Lonza)</td>
</tr>
<tr>
<td>PrEC</td>
<td>Homo sapiens</td>
<td>Normal prostate epithelial cells</td>
<td>PrEGM BulletKit (Lonza)</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>Homo sapiens</td>
<td>Normal prostate epithelial cells</td>
<td>K-SFM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Homo sapiens</td>
<td>Mammary gland/breast adenocarcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>PC-3</td>
<td>Homo sapiens</td>
<td>Prostate adenocarcinoma cells</td>
<td>RPMI 1640 with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>Bg-1</td>
<td>Homo sapiens</td>
<td>Ovarian carcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>MFC</td>
<td>Mus musculus</td>
<td>Normal murine fibroblast cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>MB49</td>
<td>Mus musculus</td>
<td>Bladder Carcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
</tbody>
</table>

**PS:** DMEM - Dulbecco’s Modified Eagle’s Medium

RPMI 1640 - Roswell Park Memorial Institute (RPMI) 1640 Medium

FBS - Fetal Bovine Serum

P/S - Penicillin/Streptomycin

K-SFM - Keratinocyte Serum Free Medium Kit (Invitrogen)
3.2.2 Promoter Cloning

All tested promoters were obtained via PCR amplification (Table 2). The templates came from whole-genome extracts of human foreskin fibroblasts (HFF-1). The identification and verification of the amplified promoters were carried out by DNA sequencing.

Table 3.2 - Promoter names and cloning sites

<table>
<thead>
<tr>
<th>mRNA ID</th>
<th>Gene Name</th>
<th>Start*</th>
<th>End*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_006325</td>
<td>RAN, member RAS oncogene family (RAN)</td>
<td>129921589</td>
<td>129922635</td>
</tr>
<tr>
<td>NM_001024957</td>
<td>Breast cancer metastasis suppressor 1 (BRMS1)</td>
<td>65869043</td>
<td>65870090</td>
</tr>
<tr>
<td>NM_006739</td>
<td>Minichromosome maintenance complex component 5 (MCM5)</td>
<td>34125258</td>
<td>34126212</td>
</tr>
</tbody>
</table>

*Start and End designations refer to positions obtained from the Human Genome Browser – HG-18 assembly.

Promoters were amplified via PCR using modified nested polymerase chain reaction method and we named it as “Matryoshka doll” method. In this method, a longer fragment spanning the desired range is first amplified based on the whole genomic template. Then, using the newly amplified PCR fragments as a template, the promoters within the specific range (table 3.2) (with added restriction sites) are amplified during the second PCR run. The primer details are as follows:
\( ^{p_{\text{ran}}} \)

First round:
Forward- ATT TGC GTC ACT GGG GTT CC
Reverse- GAG CGG AGG ATG AAA CGG GG

Second round:
Forward- (EcoR I) GTG TGT GAA TTC ACG CGT CCA GAC TGC AAA CA
Reverse- (Age I) ATA TAT ACC GGT GGT CGC GAT ACC TTC CAG AA

\( ^{p_{\text{brms1}}} \)

First round
Forward- CAC GAC GGA GAT TCC CTG AG
Reverse- CCG CAT GCC CAT GAA CAA AA

Second round
Forward- (EcoR I) GTG TGT GAA TTC TCC CTC CCC TAA TCT GAG AA
Reverse- (Age I) ATA TAT ACC GGT ACG ACG GAG ATT CCC TGA GA

\( ^{p_{\text{mcm5}}} \)

First round
Forward- GCA GTG TGT TAG GGT GCG A
Reverse- CTG GTA GAC GCG TGT GAG
Second round

Forward- (EcoR I) GTG TGT GAA TTC CAG TGT GTT AGG GTG CGA GA

Reverse- (Age I) ATA TAT ACC GGT ACT AGC CTC ACC TCT GGT TTT C

EGFP Reporter Indication Assay

All plasmids with the EGFP reporter gene were constructed using the PGL3 vector (Promega, Madison, WI, USA) as a backbone. The luciferase reporter exon was replaced with the enhanced green fluorescent protein exon (EGFP) from the plasmid pEGFP-N1 (Clontech, Palo Alto, CA, USA). Promoters to drive EGFP expression were inserted into the MCS of the vector. The promoter and EGFP elements of each constructed plasmid were verified by DNA sequencing.

Branched poly(ethyleniminie) (PEI) with a weight-average molecular weight of 25 kDa (Sigma-Aldrich, St. Louis, MO, USA) was used to deliver plasmids into cells. PEI/DNA complexes were prepared at a 7.5:1 N: P ratio, using 3.6 µg of DNA per dose (Zhang et al., 2008, Dobek et al., 2011). The transfection procedure consisted of plating 100,000 cells into wells of 6-well plates 16 hours prior to the administration of gene delivery complexes. At the time of transfection, the growth medium in each well was replaced by 2 ml of serum-free medium plus 100 µl of gene delivery complexes in normal saline, prepared as just specified. Cells were incubated in the transfection medium for 2 hours at 37°C, after which the medium was replaced by the appropriate
growth medium for that cell type. Analysis of EGFP expression took place at the 48-hour time point.

EGFP expression was measured by flow cytometry (FACSCantoII, BD Biosciences, San Jose, CA, US). Cells were trypsinized with 0.3ml trypsin and then flushed from the plate with an additional 0.7ml of FBS-free medium, and the entire 1 ml was immediately used for flow cytometry. A value for population intensity was recorded, being defined as the mean fluorescence intensity of the strongest 10% cells within the population. Intensity values were normalized to the positive control: $P_{cmv}$-driven EGFP expression.

3.2.3 RevCasp3 cDNA construction

The cDNA of a constitutively active recombinant Caspase-3 was built by swapping the order of the short and long domains (fig. 3.2c) (Srinivasula et al., 1998, Luo et al., 2010). The cDNAs of the two domains were separately amplified from the template $p_{cox2}$-iCasp3 plasmid (described in (Godbey and Atala, 2003)) via PCR. Primer sets were synthesized by using the following primer sequences:

Long domain:

Forward: ATC GAT ACC GGT GGA GAA CAC TGA AAA CTC AGT G (Age I site underlined)
Reverse: ATC GAT GAA TTC TCA GTC TGT CTC AAT GCC ACA GTC CA

(EcoR I site underlined)

Short domain:

Forward: ATG CAT GAG CTC GCC ACC ATG ATT GAG ACA GAC AGT GGT

G (Sac I site underlined. Note the presence of a Kozak sequence)

Reverse: ATG CAT GGT ACC CGT GAT AAA AAT AGA GTT CT (Kpn I site underlined)

After amplification, the two fragments were connected by using the linker sequence

ATC GAT GGT ACC ATG AAG TTG ATG GGG GAT CCC CCA TAC CGG TAT

CGA T (KpnI and Age I sites underlined) and built into a PGL3-GFP plasmid by replacing the GFP exon. Site-directed mutagenesis was next performed to remove the now-unnecessary restriction enzyme sites in the linker sequence. DNA sequencing was used to verify that the gene for this constitutively active caspase, which we refer to as “RevCasp3” (for “reversed caspase 3”) (Srinivasula et al., 1998), had the same sequence as the published cDNA sequence (GenBank: AF052645.1) (Srinivasula et al., 1998).

3.2.4 RevCasp3 activity assay

RevCasp3 activity was quantified by using the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Invitrogen, Carlsbad, CA, US). The basic steps of the
transfection portion of this procedure was identical to the GFP expression assay described above. PC-3 cells were plated into six-well plate at the concentration of 100,000 cells per plate. After 16 hours, cells were transfected with complexes containing either the negative control vector pUC19 (New England Biolabs, Ipswich, MA, US) or $^{\text{ran}}$-RevCasp3 plasmids. After 2 hours of transfection, growth medium with added CellEvent® Caspase 3/7 Green Detection Reagent (1:1000 v/v) was used to replace the transfection media. At 48 hours post-transfection, cell morphologies and green fluorescence were documented by fluorescent microscopy (Olympus DP70). After pictures were recorded, the media, which contained floating cell debris, were collected from each cell well. The remaining attached cells were trypsinized and resuspended in FBS-free media. SYTOX® AADvanced™ Dead Cell Stain was added into each of the two fractions from each well (the fraction containing floating debris and the solution containing trypsinized cells) at a 1:1000 ratio. After 5 minutes, the cell suspensions were processed via flow cytometry and counted with channels for FITC and PerCP-Cy™ 5.5.

3.2.5 Cells Viability Assay

Easily cultured cells, including HFF-1, PC-3, BG-1, MCF-7, MFC, and MB49 cells, were plated into wells of separate 6-well plates and transfected. Cell types requiring expensive media, including HASMC, PrEC, RWPE-1, were plated and transfected in
24-well plates with all volumes and seeding densities reduced proportionately. At 48 hours post-transfection, cell supernatants and attached cells were collected in two distinct fractions as described above and separately analyzed via flow cytometry. The resulting data are reported as ratios of cell number for $^{\text{ran}}$-RevCasp3 transfections versus pUC19 transfections. This normalization was performed to remove any background resulting from PEI-induced toxicity.

### 3.2.6 Statistics

One-way ANOVA was applied to analyze the differences between groups, then the Holm-Sidak Post Hoc Test was applied to compare the differences between selected promoter and the control. $P < 0.05$ was used to define differences as significant.
3.3 Results

Figure 1, the scheme of our cancer-specific promoter searching logic

3.3.1 Scheme of searching logic

![Diagram](Image)

Figure 3.1, the scheme of cancer-specific promoter searching logic

The promoter-search logic is presented in figure 3.1. Landolin and colleagues studied the functional information of promoters that addressed the entire human genome (Landolin et al., 2010). In that paper, the activities of 4,575 promoters were measured in eight cancer cell lines. Full information about each the activity of each promoter was generously provided. For our project, that comprehensive information served as an excellent database and starting point for the selection of cancer-specific promoters. The algorithm for our selection was based on the following process. First, the activity of the 4,575 promoters in all 8 cancer cell lines was ranked. The promoters with the strongest activities were considered. Next, the functions and expression patterns of the corresponding genes in normal and cancer cells were deeply investigated through literature searches. Although some of the promoters were shown to have very strong
activities in Landolin’s report, they were excluded from our final list because of a lack of
evidence to correlate expression with cancer progress. Considering the promoter
activities and gene functions within cancer cells, the most promising promoter candidates
– including $^p$ran, $^p$brms1 and $^p$mcm5 (for which the relationship between gene and cancer
progress will be described in section 3.4.4) – were added to our final list, which were
verified via the following experiments.

3.3.2 Evaluation of promoter activity

Plasmids that utilize the three selected promoters, $^p$ran, $^p$brms1, and $^p$mcm5, to drive
GFP reporter expression were constructed. An initial test of promoter specificity was
performed in the form of PEI-mediated transfections. The resulting data indicate
excellent cancer-cell selectivity and reporter expression levels (versus normal cell types)
(fig. 3.2a). In the normal cell types – HFF1, HASMC, HAEC, and RWPE-1 – the
reporter expressions garnered with the cancer-specific promoters $^p$ran, $^p$brms1 and $^p$mcm5
were very similar to those obtained by using promoterless plasmids. However, the
activities of $^p$ran, $^p$brms1 and $^p$mcm5 in cancer cells were much stronger. In comparison
to fluorescence intensities achieved with the positive control promoter, $^p$cmv, $^p$ran yielded
average fluorescent intensity that were 64.7% - 99.8% of those achieved with
$p$cmv-driven plasmids, $^p$brms1 yielded 58.6% -87.7% of the fluorescence, and $^p$mcm5
yielded 35.5%-55.8%, depending on cell type (fig. 3.2a).
To demonstrate the practical importance of the strength of the three promoters, the activities of $^\text{p} \text{ran}$, $^\text{p} \text{brms1}$ and $^\text{p} \text{mcm5}$ were compared against those of the human telomerase reverse transcriptase promoter ($^\text{p} \text{hTERT}$). $^\text{p} \text{hTERT}$ has been used as a cancer-specific promoter for expression-targeting, with applications that have reached clinical trials (Gu et al., 2002, Kyo et al., 2008, Nemunaitis et al., 2010).

There were no significant differences in reporter expression for cells transfected with genes driven by $^\text{p} \text{hTERT}$ versus any of the three novel promoters in the normal cell types investigated (ANOVA+Post Hoc Test, $N \geq 3$, $P > 0.05$), as none of these cells expressed great amounts of reporter. In each of the cancer cell lines tested (from breast, prostate, and ovarian carcinomas), reporter expression was significantly higher when cells were transfected with genes driven by $^\text{p} \text{ran}$, $^\text{p} \text{brms1}$ or $^\text{p} \text{mcm5}$ versus $^\text{p} \text{hTERT}$ (fig. 3.2b). These results indicate that $^\text{p} \text{ran}$, $^\text{p} \text{brms1}$ and $^\text{p} \text{mcm5}$ are excellent subjects for future applications of cancer-targeted gene therapy. Although there is no statistically significant difference of those three promoters driven expression, the $^\text{p} \text{ran}$ was selected for following apoptosis inducing assay because the average intensity is higher than other two promoters in cancer cells.
Figure 3.2—\(^p\)\textit{ran}, \(^p\)\textit{brms1} or \(^p\)\textit{mcm5} activities in normal and cancer cell lines. **a)** Intensities of reporter expression resulting from transfections having utilized the indicated promoters. The data have been normalized to \(^p\)\textit{cmv}-driven positive controls to remove variations due to cell type. A line has been drawn at 100% to reference \(^p\)\textit{cmv} activity. **b)** Promoter activity, normalized to \(^p\)\textit{hTERT}-driven GFP expression. **"** = significant difference between selected promoter and promoterless control (P<0.05). **"#"** = significant difference between selected promoter and \(^p\)\textit{hTERT} (P<0.05) Error bars indicate standard deviations (n ≥ 3).
3.3.3 Verification of RevCasp3 expression and activity

Apoptosis is tightly regulated in living cells. Normally, caspase proteins exist in cells as inactive, single-chain precursors. Activation, in this case of caspase 3, occurs through proteolytic cleavage of the precursor into two polypeptides, with these newly-freed entities (arising from the “long” and “short” domains of the inactive precursor) coming together to form a heterodimer. The active heterodimer can act to cleave the next target of the cascade (fig 3.3c). For initiator caspases, the target will be another caspase, such as active caspase 8 cleaving (and thereby activating) caspase 10 or 3. Effector caspases will cleave cellular targets. For example, activated caspase 3 will cleave an inhibitor of caspase-activated DNase, resulting in fragmentation of the host genome (Porter and Janicke, 1999).

By modifying the order of the two domains in the caspase 3 gene and linking them as described earlier, the resulting RevCasp3 protein will be active even in the absence of the cleavage event for cellular caspase 3. Expression of plasmids containing the RevCasp3 gene should result in apoptotic death of the expressing cell (without bystander effects).

To verify whether the RevCasp3 constructs can be used to induce apoptosis in cancer cells, *p*ran-RevCasp3 plasmids were cloned and delivered into PC-3 cells.
control for any possible background toxicity due to the act of gene delivery in general, the empty plasmid pUC19 was delivered to cultures in parallel, with sham-treated cells serving as an additional negative control. At 48 hours post-transfection, cell morphologies and densities were observed via light microscopy. There was no obvious indication of toxic effects observed in the pUC19-transfected group. In contrast, the \( ^{\text{p}}\text{ran} \)-RevCasp3-treated group displayed significant changes in cell morphology, granularity, and population density (fig. 3.3a,b). The activity of RevCasp3 was also indicated by a CellEvent Caspase 3/7 detection kit (Invitrogen, Carlsbad, CA, US). The main reagent in the kit consists of a four-amino acid peptide (DEVD) conjugated to a nucleic-acid-binding fluorogenic dye. Activated caspase 3, (including RevCasp3) or 7 can cleave the recognition sequence DEVD to release the dye, which will then bind to nucleic acids and fluoresce brightly upon exposure to \( \lambda_{488} \). For the experiments associated with Figure 3, PC-3 cells were transfected with the empty vector pUC19 or by \( ^{\text{p}}\text{ran} \)-RevCasp3. After 48 hours, caspase 3- (or 7-) positive cells were qualitatively observed via fluorescence microscopy. A trace number of labeled cells were present within pUC19-treated group, while a greater number of cells were labeled in the \( ^{\text{p}}\text{ran} \)-RevCasp3-treated group, which already had a lower total number of cells presumably because of apoptosis induced by the RevCasp3 in the earlier hours of the experiment (fig. 3.3a,b).
Figure 3.3 – The morphologies and apoptotic statuses of PC-3 cells following transfection. PC-3 cells were transfected with either a) the pUC19 null-vector or b) 

\[ \text{ran-RevCasp3} \]
ran-RevCasp3.  *Left images:* light-microscopy was used to qualitatively assess cell morphologies and population densities.  *Right images:* a fluorescent reagent was used to detect the presence caspase 3/7 activity, which includes RevCasp3 activity as well.  c) The scheme for indicating how endogenous caspase 3 to be activated and how RevCasp3 fold automatically into an active form (Luo et al., 2010).
Quantitative analysis of the same pUC19- and ran-RevCasp3-treated cells was then performed via flow cytometry (fig. 3.4). Floating cells debris and attached cells (detached via trypsin) were collected separately and exposed to a second label, with Excitation/Emission = 546/647 nm, to identify dead cells via penetrating dead cells with compromised plasma membranes. (Note that the green label detected active caspases 3/7, which did not necessarily mean that a cell was dead yet. The red dye detected dead cells, even those that did not die from apoptosis.) To aid in determining the FACS gates to use for quantification, sham-treated cells were used in the assay as a control (data not shown). As shown in Figure 3.3, comparison of the number of attached cells revealed far fewer living (unstained) cells in the $^p$ran-RevCasp3-treated group versus the pUC19-treated group (32,000 versus 77,800 events/well, respectively), and there was a number of (green) caspase 3/7-positive cells in the $^p$ran-RevCasp3-treated group but not in the negative control. Meanwhile, there was a greater number of floating cells and debris in the $^p$ran-RevCasp3-treated group versus the pUC19-treated negative control (15,500 versus 1,900 events/well, respectively). It was noted that 87.7% of the events recorded in the floating fraction of the $^p$ran-RevCasp3-treated group were stained either red, green, or both, indicating that these events were associated with dead or dying cells.
Figure 3.4 – Quantification of cell numbers by flow cytometry following transfection with either (top) the pUC19 null-vector or (bottom) $\mu ran$-RevCasp3. Floating cell debris and attached cells were analyzed both separately and together. Quantitative analysis of the same pUC19- and ran-RevCasp3-treated cells was then performed via flow cytometry. Floating cells debris and attached cells (detached via trypsin) were collected separately and exposed to a second label (red) to detect dead cells via permeable membranes. The green label detected active caspases 3/7, which did not necessarily mean that a cell was dead yet. The red dye detected dead cells, even those that did not die from apoptosis. Cells in quadrant 3 are alive and healthy cells. Cells in other quadrants are either dead or dying cells.
Analysis of the combined fractions showed very few labeled cells (dead or dying) in the pUC19-treated group (2.6%), but a large number of dead/dying cells in the \( p_{\text{ran}} \)-RevCasp3-treated group (38.0%). Note that the 38% does not include the cells that were killed early-on and broken up into fragments incapable of binding the fluorescent label.

### 3.3.4 Demonstration of \( p_{\text{ran}} \)-RevCasp3 targeting and efficacy

Multiple cell types were transfected with the \( p_{\text{ran}} \)-RevCasp3 construct, with results compared to treatments using the empty pUC19 control vector. Qualitatively, bright field microscopy revealed that there was a noticeably greater amount of floating debris in the PC-3 and Bg-1 wells receiving the apoptosis-inducing plasmids. A large number of the attached cells in these wells had dense, granular structures.

Similar FACS quantification experiments as described above were again carried out, this time to demonstrate that the targeted \( p_{\text{ran}} \)-RevCasp3 plasmids retain their lethal activity in cancer cells, but are relatively harmless in normal cells. As shown in Figure 3.5a, there is little-to-no cell damage in the normal cell lines. With the exception of HFF-1 cells, which had a viability of 89.3% \( \pm \) 1.4%, there were no significant differences in viabilities in normal cell types exposed to pUC19 versus \( p_{\text{ran}} \)-RevCasp3 treatments.
However, all of the carcinoma cell lines tested were vulnerable to treatment with the $^\text{p}ran$-RevCasp3 plasmids. Versus transfections with the null vector, there was a significant reduction in cell viability in the cancer cell lines after $^\text{p}ran$-RevCasp3 transfections. Consistent with the qualitative observations mentioned above, cell viability was especially impaired in the PC3 prostate cancer (viability = 58.1% ± 15.5%) and BG-1 ovarian cancer (viability = 39.8% ± 11.5%) cell lines.

Targeted cancer cell death was also suggested by analysis of floating debris (fig. 3.5b). In the normal cell types, there was no significant difference between the numbers of events counted in the supernatants of the pUC19-treated and $^\text{p}ran$-RevCasp3-treated cells, with the exception of RWPE-1. However, there were once again significant differences noted in the PC-3 and Bg-1 cells treated with $^\text{p}ran$-RevCasp3 plasmids. In the PC-3 cultures, the targeted, pro-apoptotic plasmids caused the generation of ~3.7 times more debris in the supernatant than did the negative control plasmids. In Bg-1 cultures, the increase in debris was ~6.6 times the amount generated by PEI-mediated transfection of empty vectors. Labeling showed the debris to be from dead and dying cells.
Figure 3.5 – **a)** Cell viability following transfections with $^p$ran-RevCasp3, normalized to pUC19-treated negative controls. **b)** Floating cells increase normalized to the negative control. Error bars represent one standard deviation. “**” indicates there is a significant difference between the $^p$ran-RevCasp3 and pUC19 transfetions (t-test, n=3, P<0.05). “#” indicates a significant difference between the given cell type and the set of normal cell types (One-way ANOVA, n≥3, P<0.05).
Future testing of this expression-targeted gene therapy construct will take place in a murine model. Because of this, additional testing of the human $^p$ran and RevCasp3 gene elements in murine cell lines was required to verify cross-species effectiveness. Murine fibroblast cells (MFC) and the murine bladder carcinoma cell line (MB49) were selected to verify selectivity and activity. A GFP expression assay was performed first. All four of the selected promoters were shown to retain excellent selectivity between normal and carcinoma cells in the murine cell types (fig. 3.6a). In the MB49 cells, the relative intensities of GFP expression following delivery of plasmids driven by $^p$ran, $^p$brms1, $^p$mcm5, and $^p$hTERT were very strong. Those promoters can produce the GFP intensity reaching to 53.6%-65.1% of $^p$cmv-driven transfections. In the normal cells, there was no significant difference in GFP expression between any of the four promoters and the promoterless negative control.

After selectivity was verified, the therapeutic efficacy of $^p$ran-RevCasp3 was tested in the two murine cell types. Figure 3.6b shows a significant reduction in viable cells induced by $^p$ran-RevCasp3 plasmid in the murine cancer cell line, but no obvious cell death in the normal cells. (cell viability 114.1%±9.9% and floating cell debris fold change 108.3±14.4%). But $^p$ran-RevCasp3 cause almost half cell death for MB49 cells(cell viability: 54.4%±2.8%). Meanwhile, more than 3 folders of floating cell debris were found.
Figure 3.6 – Verification of human derived promoter and RevCasp3 possesses across activity through murine species. a) Promoter activities between murine normal and cancer cell lines. Plasmids under the control of $^P_{ran}$, $^P_{brms1}$ and $^P_{mcm5}$ and $^P_{hTERT}$ driven GFP were transfected into murine fibroblasts (MFC) and murine bladder carcinoma cells (MB49). A promoterless plasmid was used as a negative control. The data have been normalized to $^P_{cmv}$-driven positive controls. ** indicates significant differences between MFC and MB49 (t-test, n≥3, P<0.05) b) Murine cell viability assay of transfection with $^P_{ran}$- driven RevCasp3. MFC and MB49 cells were transfected by
\textit{p}ran-RevCasp3 plasmid as well as pUC19 plasmid. The pUC19 plasmid is to serve as a negative control. Error bars represent one standard deviation. “*” indicates there is a significant difference between the cancer cells MB49 and normal cells MFC (Student t-test, n≥3, P<0.05)
3.4 Discussion

This work was performed to look for more cancer-specific gene elements to build a cancer-specific promoter library, based on new searching logic. Three new cancer-specific promoters, including $p_{ran}$, $p_{brms1}$ and $p_{mcm5}$, were identified. Furthermore, the therapeutic selectivity and efficacy of $p_{ran}$ driven reverse Caspase 3 were investigated through in vitro experiments.

3.4.1 The importance of building a cancer-specific promoter library

Not every cancer progress in the same way in every person. Due to the diversity of mutation by cancer accumulation, it is hard to say one promoter will play the function universally for all cancer types and patients. Thus, treatment with a personalized promoters set is potentially more effective for cancer patients. It is a reasonable strategy that tumor cells from patients should be cultured first. And those cancer cells could be transfected by promoters within the cancer-specific library thorough a high-throughput screening assay. Then those promoters which are highly responded by patient's tumor cells could be considered for the following treatments. Furthermore, it is very easy to develop drug resistance by single promoter treatment. Treatments by multiple promoters could kill a wider range of cells and inhibit the drug resistance development. Thus, it is essentially helpful to get a cancer-specific promoter library to provide the applicable candidates.
3.4.2 Traditional pathway to look for cancer specific promoter

The traditional method looking for cancer specific promoters is based on comparing endogenous gene expression between cancer and normal cells. Microarrays, qPCR, and Western blots are tools often employed to find which gene’s mRNA or protein levels corresponding to a candidate gene are up-regulated in cancer cells. Promoters are identified and copied from genomic DNA, then cloned into a DNA vector. After delivering the vector into both cancer and normal cells, the reporter gene expression is measured. If the expression presents strong selectivity, the promoter is a good candidate for cancer-related expression-targeted gene therapy.

However, there is only a weak correlation between the level of endogenous gene expression and the activity of engineered plasmids. This weak relationship was addressed in a recent genome information study (Landolin et al., 2010), where the correlation of 1,188 pairs of gene promoter: endogenous mRNA levels were investigated. The Pearson correlation coefficient between those two parameters was found to be only 0.43. Thus, it is possibly misled by using the endogenous gene expression data to indicate functional regulatory element.
3.4.3 Our logic to find cancer-specific promoters

Our searching logic considered three factors: the direct transgene promoter activity in cancer cell line, gene function in cancer progression, the gene expression in normal tissue. The evidence of direct transgene promoter activity is the most important one. Fortunately, the publication just mentioned measure 4,575 promoters in all 8 cancer cell lines (Landolin et al., 2010). We can easily get a strongest promoter list. Then the next task for us is to identify those promoters are universally strong, like EF1alpha or cancer-specific promoters, like hTERT. Thus, we deeply investigate those gene function based on literature mining. Meanwhile, we also investigate their gene expression in normal tissue from several databases, including the Human Protein Atlas (Uhlen et al., 2010) and Expression Atlas (Kapushesky et al., 2010). Finally, the following promoters, $p_{\text{ran}}$, $p_{\text{brms1}}$ and $p_{\text{mcm5}}$ were selected into investigation.

3.4.4 Why $p_{\text{ran}}$, $p_{\text{brms1}}$ and $p_{\text{mcm5}}$ were selected

Ras-related nuclear protein (RAN, member RAS oncogene family) is a small GTPase belong to Rat Sarcoma (RAS) oncogene family. One of major function of RAN is to transport the RNA and proteins in and out of the nucleus through the nuclear pore complex (Sorokin et al., 2007). Furthermore, RAN is also correlated with cell mitosis by contributing to the spindle formation (Xia et al., 2008). RAN, as its official name, is an oncogene. Cancer cell replication thorough the mitosis may highly depend on the
RAN expression and function. Inhibition of RAN expression in cancer cell could highly prevent mitosis of cancer cell. Contrastly, the mitosis of normal cell would not get any effect with decreased RAN expression (Xia et al., 2008).

Breast cancer metastasis-suppressor 1 (BRMS1), as its official name, is a metastasis suppressor which is initially recognized within breast cancer (Wu et al., 2012, Seraj et al., 2000). BRMS1 does not contribute to the tumorigenicity, but it can repress cancer cell migration (Meehan et al., 2004). BRMS1 prevent cancer migration though inhibition of nuclear factor-κB activity and subsequent conquest of urokinase-type plasminogen activator (Cicek et al., 2005), restoration of cell-cell communication via gap junctions (Saunders et al., 2001), and inhibiting cell apoptosis thorough decreasing osteopontin expression (Wu et al., 2012).

Minichromosome maintenance complex component 5 (MCM5) plays an essential role in the initiation of DNA replication within the cell dividing, by which it affiliate with replication licensing factors (Forsburg, 2004). MCM complex, consisting by MCM 2-7 serve as a primary heterohexameric helicase to unpack the gene in most eukaryotes at the replication fork (Forsburg, 2004). Since the function of MCM is associated with cell dividing, the MCM5 expression is often associated with abnormal cancer cell proliferation.
Within these three types of gene, the expression of RAN and MCM5 are usually positively correlated with cancer progress. RAN is overexpressed in multiple cancer cell types, including renal cell carcinoma (Abe et al., 2008), ovarian cancer (Barres et al., 2010) breast and lung cancers (Yuen et al., 2013, Kelly et al., 2012) and so on. Meanwhile MCM5 has been reported that it is overexpressed in bladder cancer (Brems-Eskildsen et al., 2010, Kelly et al., 2012, Korkolopoulou et al., 2005), oral squamous cell carcinoma, (Yu et al., 2014) skin squamous cell carcinoma (Liu et al., 2007). It would be not too much surprised that $^{\text{p}}$ran and $^{\text{p}}$mcm5 could drive more higher transgene expression in cancer cells. But, interestingly, the endogenous expression of BRMS1 is often negatively correlated with cancer progress (Liu et al., 2006, Smith et al., 2009, Zhang et al., 2006). If we follow the traditional searching method, we may lose the $^{\text{p}}$brms1 at the beginning stage.

3.4.5 The pros of our searching strategy

There are two main advantages of our searching logic. The first one is the searching efficiency was improved. The traditional method is based on the indirect evidence, endogenous gene expression. Those evidences cannot directly reflect the promoter activity. Our logic focuses on the promoter activity directly. Half of the process has been finished with the previous research (Landolin et al., 2010). The
following task for us is just to identify whether those promoters are cancer-specific, or not.

The second advantage is that the selected promoter based on this strategy has strong efficacy. It is one of problems blocking the application of expression-targeted gene delivery that usually the efficacy of the cancer-specific promoter is not always satisfactory (Kanegae et al., 2011). Our promoters were selected from the strongest promoter list, which has assured the efficacy of those promoters at first.

### 3.4.6 The cons of our searching strategy

Our searching strategy also has some potential drawbacks. For examples, we just have a single source to obtain the entire genome promoter activity data. Our searching strategy is highly dependable for the previous research. Meanwhile, the selected promoter may have some basal level activity in normal tissues, because those promoters are selected from the strongest promoter list.

### 3.4.7 Why the RevCasp3 was selected

There are three types of suicide gene system, including gene/pro-drug, direct toxic foreign gene and apoptosis gene. The gene/prodrug system plays function requiring
coadministration of gene and pro-drug. The pro-drug will not have the killing effect until it is activated by transgene. One classic example in this group is the herpes simplex virus thymidine kinase (HSVtk) / ganciclovir (GCV) system (Barese et al., 2012). After HSVtk/GCV treatment, the nontoxic GCV will be phosphorylated by HSVtk into GCV triphosphate. The GCV triphosphate can inhibit the DNA synthesis of replicating cells and therefore induce tumor cell death. One of the advantages of gene/prodrug system is the bystander effect. Not only transfected cells, but also neighbor tumor cells will be killed by the active drug. By the bystander effect, a higher therapeutic efficacy could be achieved. However, the bystander effect will also potentially cause damage for the neighbor normal tissue, since the targets of activated drug are the all replicating cells. Thus, the safety of this system should be carefully evaluated.

The foreign toxin gene is also an effective treatment for cancer suicide gene therapy. Diphtheria toxin A (DT-A) is a good example in this group. Diphtheria toxin is originally synthesized by Corynebacterium diphtheria. Diphtheria toxin is consisted of two subunits, diphtheria toxin A-chain (DT-A) and diphtheria toxin B-chain (DT-B) (Murphy, 2011). DT-A is the main toxin component which is responsible for inactivating elongation factor-2 (EF-2) and blocking RNA translation and consequently leading cell death (Murphy, 2011). DT-B is functional for binding the host cell receptor to process the endocytosis. DT-A is very effective, since just one molecule DT-A can induce cell death (Yamaizumi et al., 1978). Moreover, expressed DT-A can
not enter into other cell without DT-B corporation (Murphy, 2011). So DT-A toxicity can be limited to the host expression cells, which highly increase the safety of the system. However, it has been reported that DT-A can be leaked into the extracellular matrix. Thus, it may induce the host immune response in vivo, since DT-A is a foreign protein for the host body.

The third type of suicide gene system is the apoptosis inducing gene system. Safety is a clear advantage of such system comparing with the gene / prodrug and foreign toxic gene systems. By promoter regulating, those activated apoptosis proteins will be just expressed in cancer cells. Finally, they will be packed into cell debris and carried into the digestion by macrophages. There is no toxic risk to their nearby normal cells. Furthermore, since those bioactive proteins originate from the host cells, there is almost no immunogenicity in such system.

However, cell apoptosis involves a complicated pathway. Thus, it should be carefully considered how to choose the delivered apoptotic proteins. Some research selected the tumor necrosis factor (TNF) family, including TNF-alpha (Su et al., 2013), TNF-related apoptosis inducing ligand (Holoch and Griffith, 2009) and Fas ligand (Modiano et al., 2012). The pros for selecting those upstream apoptosis genes is that those transgenes have very excellent efficacy because death signals would be amplified through the signal cascade. However, the cons is that the role of those upstream
apoptotic signals is “double-edged swords” (Aggarwal, 2003). The TNF family, was found that either they can induce tumor cell apoptosis to kill tumor cells, or they can also promote cancer progress through activating other genes to stimulate tumor cell proliferation, invasion and metastasis. It will depend on gene profile and mutations of host cells, whether TNF family plays a role of tumor suppressors or promoter. As a consequence, it has to be carefully considered the eligibility when TNF family is applied as the therapeutic gene.

Thus, it is a reasonable choice to select the downstream apoptosis gene, such as Caspase 3, Caspase 9, or Caspase 8, because they can directly induce cells into programmed death. Clearly, those downstream genes will be less affected by the complicity of cancer cells since they go through a shortcut. But there is a problem that Caspases are tight regulated within live cells. Without activation, expression of proto-Caspase is not able to induce cell death.

There are two ways to activate those apoptotic proteins after expression. The first way is developed by Rebecca and colleagues (MacCorkle et al., 1998). The modified fas ligand was fused with Caspase. After the recombinant protein is expressed, a chemical induced dimerization (CID) reagent is delivered into host cells. Then the fas ligand fused Caspase will start to dimerize and cleave themselves. Therefore the Caspase can be transformed to the activated form. The other way was developed by Srinivasula and
colleagues (Srinivasula et al., 1998), as our study shows. Simply changing the domain order, the constitutively active Caspase can be directly expressed.

The advantage of CID systems is that the safety can be improved since the delivered gene will not cause any damage without dimmerizing agent. The drawback is that the double administration increases the complicity and the cost of the system. Not only the gene complex, but also the safety and optimized dose of CID have to be considered. In our opinion, the safety of Caspase can be regulated by promoters and the system should be the simpler, the better. Thus we choose reverse Caspase 3 as our therapeutic gene.

3.4.8 The basal level activity of $^p$ran, $^p$brms1 and $^p$mcm5 and toxicity of $^p$ran-RevCasp3 for normal cells

According to our data, there still is the basal level of GFP expression driven by $^p$ran, $^p$brms1 and $^p$mcm5 and a week toxicity in some normal cells. It may cause potential safety concern for applying those promoter systematically. For targeted therapy, the ideal situation is that all targets are in cancer cells and “none” in the normal cells. However, due to the fact that the cancer cells evolve from normal cells, it is really hard to get the ideal situation. Thus, the alternative way is to find a promoter showing significantly different activity between normal cells and cancer cells. Then we can
optimize the delivery dose to achieve goals of killing cancer cells without harming normal tissue. Our data have shown that all those three promoter, \( p_{ran}, p_{brms1} \) and \( p_{mcm5} \) present an excellent selectivity and efficacy. Meanwhile, the \( p_{ran} \)-RevCasp3 can kill much more cancer cells than normal cells. Thus, they are eligible to be considered for further clinical practice.
3.5 Conclusion

According to our novel searching logic, three promoters, including \(^{p}\text{ran}, ^{p}\text{brms1}\) and \(^{p}\text{mcm5}\) were identified for their cancer-specific activity. Furthermore, the activity of those promoter within tested cancer cells is much higher than the golden standard cancer-specific promoter \(^{p}\text{hTERT}\). Reverse Caspase3 was shown as an excellent apoptosis inducing effect within tested cancer cells. Moreover, \(^{p}\text{ran-RevCAsp3}\) constructed plasmid has shown a significant cancer-specific killing effect \textit{in vitro}. 
Chapter 4

Verifying Minicells Therapeutic Effect in the Orthotopic Model of Murine Transitional Carcinoma
4.1 Background

Bladder cancer is one type of the most common urothelial carcinoma worldwide, the seventh leading cause of cancer death, and the fourth most common malignancy of men in United States (Siegel et al., 2014). An estimated 75% of all new bladder cancer patients present with Non muscle Invasive Bladder Cancer (NMIBC) (Sexton et al., 2010), which includes tumors confined to the uroepithelial mucosa (Ta), tumors invading the lamina propria but not yet the underlying muscle (T1), and carcinoma in situ (CIS). In some instances CIS may be concomitant with TaT1 disease. Currently, NMIBC patients are divided into low, intermediate and high-risk disease classifications, the designation of which is made based on tumor stage and grade. For TaT1 lesions, treatment calls for transurethral resection of bladder tumor(s) (TURBT), followed by administration of adjuvant intravesical therapy to aid in prevention of recurrence and progression of disease. In intermediate and high-risk disease, intravesical immunotherapy utilizing the live bacterial vaccine Bacillus Calmette-Guerin (BCG) is the most effective adjuvant therapy treatment option. Initially, 66.4% of patients with high-risk papillary disease (Akaza et al., 1995) and 70% of patients with CIS will have a complete response to BCG (Lamm et al., 1991). However, an estimated 27% will recur within 24 months and 54% will recur within 60 months (Herr et al., 2011). Those high-risk patients deemed to have failed BCG immunotherapy face radical cystectomy, a procedure that has a high impact on patient quality of life. Of equal importance, the instillation of live BCG is often associated with
serious local and systemic side effects, the latter of which includes fever, sepsis, and death in some cases. A study shown that 20.3% patients had to be ceased BCG treatment because of serious side effects (van der Meijden et al., 2003). Taken together, there remains great need for new and less toxic alternatives to BCG as well as for bladder-sparing second line salvage therapies for use in high-risk patients who have failed or are intolerant of BCG.

Bacterial minicells are an emerging class of targeted molecular delivery vehicles for therapeutic use in oncology, with promising applications for tumor-specific targeted delivery of anti-neoplastic agents including small molecule drugs, nucleic acids, and protein payloads (Giacalone, et.al, 2006). Minicells are small, submicron-sized spherical particles best described as miniature versions of parental bacterial cells, complete with all of the bacterial components of parental cells with the exception of a chromosome. Because minicells lack a chromosome, they are inherently incapable of division and replication, and by definition, are non-infectious. What makes minicells unique is that they can be very easily designed to contain specific macromolecular constituents by applying standard recombinant techniques or by adding additional exogenous product components, such as small molecule drugs.

This work describes the development, characterization, and evaluation of VAX-IP, a bacterial minicell product candidate designed to selectively target and deliver the
cholesterol-dependent membrane pore-forming protein toxin, perfringolysin O (PFO) from *Clostridium perfringens* to bladder cancer cells. In studies using an aggressive variation of the syngeneic orthotopic murine MB49 bladder cancer model, VAX-IP was demonstrated to have significant dose dependent effects on the growth of well established tumors and confers a survival advantage at the optimal dose and regimen (Xuguang Chen and Shingo Tsuji, (In Preparation)).
4.2 Materials and Methods

4.2.1 Generation of VAX-IP.

Minicells were generated by Vaxion Therapeutics, Inc. (San Diego, CA) (Xuguang Chen and Shingo Tsuji, (In Preparation)). Full-length perfringolysin O (PFO) was synthesized via whole gene synthesis and cloned into a kanamycin resistant version of the L-rhamnose inducible bacterial expression plasmid, pRHA-67 as a transcriptional fusion with the Invasin gene (Inv) from Yersinia pseudotuberculosis. The resulting plasmid transformed into the minicell-producing strain and transformants selected for on MSB agar containing 50 µg/mL kanamycin, 10 µg/mL diaminopimelic acid (DAP), 111 µg/mL lysine, and 0.5% glucose at 30° C to make the VAX-IP minicell-producing strain, VAX14G8.

A streak plate of strain 14G8 on MSB agar containing 50 µg/mL kanamycin, 10 µg/mL diaminopimelic acid (DAP), 111 µg/mL lysine, and 0.5% glucose and grown at 30° C, was used to pick isolated colonies to inoculate overnight starter cultures in 50 mL of MSB liquid broth containing 50 µg/mL kanamycin, 10 µg/mL diaminopimelic acid (DAP), and 111 µg/mL lysine and grown at 30° C with vigorous shaking. The following day, 3 L of the same liquid media were inoculated by addition of the starter culture and grown at 30° C while shaking. Production cultures were monitored until reaching an optical density at 600 nm (OD<sub>600</sub>) of 0.1 at which time they were induced for
Invasin and PFO expression by the addition of L-rhamnose to a final concentration of 100 µM. At an OD$_{600}$ of 1.0, the minicell phenotype was induced by the addition of isopropyl thiogalactopyranoside (IPTG) to a final concentration of 100 µM and the culture allowed to incubate overnight. The following day, VAX-IP minicells were harvested by a combination of differential centrifugation steps and subsequent density gradient purification steps.

4.2.2 Cells.

MB49 cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA), 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen).

4.2.3 Animals.

Four-to-six-week-old female C57BL/6 mice were implanted with MB49 tumors as described below. Following verification of tumor implantation by palpation and hematuria, mice were randomized into several treatment groups. All animals were housed with normal bedding, with chow and water ad libitum for the duration of the study under an animal protocol approved by the IACUC at Tulane University.
4.2.4 MB49 tumor implantation.

Animals were lightly anesthetized with a mixture of isoflurane and oxygen. Once anesthetized, a small patch of hair was shaved from the back of each and a small amount of conductive gel applied. Following application of the gel, mice were placed on their backs onto a conductive surface and subsequently subjected to transurethral catheterization using a lubricated 24 gauge Teflon IV catheter. After successful catheterization and draining of urine from the bladder, the urothelium of each mouse was cauterized at two separate locations by inserting a platinum guide wire into the catheter until it reached the bladder wall upon which time a ~0.5 second pulse of low intensity (2.5W) monopolar high frequency was applied by touching a Bovie electrocautery unit cutting tool to the external portion of the platinum guidewire. This procedure was repeated a second time to generate the second cauterization site after repositioning of the guide wire in the bladder catheter and against the bladder wall. The platinum guide wire was removed and mice were implanted with 100,000 MB49 cells in 100 µL of sterile DMEM. The catheter was allowed to remain in place for 90 min to ensure optimal tumor take and tumor take rates were ~90% in sham treatment controls using this procedure. Unless indicated otherwise, tumors were allowed to grow for 6 days prior to the initiation of treatment.
4.2.5 Treatment of established MB49 tumors in tumor growth experiments.

Mice were anesthetized and transuretherally catheterized using a lubricated 24 gauge catheter in preparation for intravesical administration of each test agent. Unless indicated otherwise, an intravesical treatment dwell time of 120 min was used.

In tumor growth experiments, mice were treated every 3 days for 4 total doses starting on Day 6 post-tumor instillation (fig. 4.1b). Intravesical treatments were given in 100 µL volumes of saline with either vehicle only (control), VAX-IP minicells ranging from $10^7$, $5.0 \times 10^7$, $10^8$, $5 \times 10^8$, and $1 \times 10^9$, or 500 µg gemcitabine (positive control for the MB49 model). On Day 18, mice were sacrificed. And then tumors were extracted and weighed.

4.2.6 Treatment of established MB49 tumors on survival.

In the first set of survival experiments, mice were treated every 3 days for 5 total doses starting on Day 6 post-tumor instillation (fig. 4.2b). Intravesical treatment was given in 100 µL volume of saline with either vehicle only (control), $10^8$ VAX-IP minicells, $5 \times 10^8$ VAX-IP minicells, or 500 µg gemcitabine. Animals were weighed and observed daily for up to 90 days.
In a second set of survival experiments designed to compare VAX-IP to what has been reported for BCG in the MB49 model, we initiated treatment 24 hr post tumor instillation and administered VAX-IP once weekly thereafter for 3 weeks (4 doses total) (fig. 4.2d). Again, intravesical treatment was given in 100 µL volume of saline with vehicle only (control), $10^8$ VAX-IP minicells, $5 \times 10^8$ VAX-IP minicells, or 500 µg gemcitabine. Animals were weighed and observed daily for 90 days where applicable.

### 4.2.7 Treatment for impeding tumor cell implantation

The tumor instillation procedure was the same as that used in the above set, except that the total number of tumor cells put into each bladder was 20,000 in 100 µl total volume (reduced from 100,000 in 100 µl total volume), and tumor cell incubation times will be 60 minutes (reduced from 90 minutes) (fig. 4.3b). In addition, bladders were irrigated with saline immediately following tumor cell exposure to mimic clinical routine postoperative bladder irrigation. Intravesical treatments were given after electrocautery burning in 100 µL volumes of saline with either vehicle only (Sham control), VAX-IP minicells ranging from $5 \times 10^7$, $5 \times 10^8$, and $1 \times 10^9$, or 500 µg gemcitabine (positive control for the MB49 model) for just one time. On Day 14, mice were sacrificed. And then tumors were extracted and weighed.
4.3 Results

4.3.1 Effect of VAX-IP treatment on well established MB49 tumor growth in vivo.

The ability of the intravesical administration of VAX-IP minicells to prevent tumor growth in mice with well-established orthotopic MB49 bladder tumors was evaluated in a dose-escalation study as compared to sham and gemcitabine (500 µg) control groups. This particular variation of the orthotopic MB49 model is considered to be very aggressive as 100,000 MB49 cells are used and treatments are not initiated until Day 6 post tumor cell instillation when tumors are very well established. Most experimental therapeutics tested in the MB49 model are evaluated when treatments are started 24 hours following tumor instillation. Such is the case with gemcitabine, apaziquone, and BCG. In contrast to results reported with the use of BCG in the standard early treatment model, it has been reported that BCG has no effect in this more aggressive late treatment model (Zhang and Godbey, 2011). To our knowledge, BCG is the only clinical stage therapeutic to have been evaluated in this late-treatment model. As shown in Figure 4.1, the median and average bladder weights, a surrogate for tumor burden, decreased with increasing doses of VAX-IP minicells when given by intravesical administration every 3 days for 4 treatments (fig.4.1, at the optimal dose of $5 \times 10^8$, respectively) until activity was lost at a high dose of $10^9$ VAX-IP minicells. No local or systemic adverse reactions were observed in any treatment group aside from the gemcitabine control group where
extremely low bladder weights were recorded in some animals. Some mice had stones in
their bladders probably resulting from catheterization with saline because the stones were
found in all groups, including sham treated, minicell treated and gemcitabine treated
control group.
Figure 4.1 a) Effect of VAX-IP treatment on well-established MB49 tumor growth *in vivo*. b) Scheme of treatment process. Mice were treated every 3 days for 4 total doses starting on Day 6 post-tumor instillation. Intravesical treatments were given in 100 µL volumes of saline with either vehicle only (Sham control), VAX-IP minicells.
ranging from $5 \times 10^6$, $5 \times 10^7$, $10^8$, $5 \times 10^8$, and $1 \times 10^9$, or 500 µg gemcitabine (positive control for the MB49 model). The bottom and top of each box shows the limits of the first and third quartiles, and the band within each box represents the median. The mean is shown by a single dot within each box. The highest whisker indicates the 1.5 interquartile range of the upper quartile and lowest whisker indicates the 1.5 interquartile range of the lower quartile. All data points are presented at the right side of box-whisker plot ($9 \leq n \leq 12$). The maximum and minimum data points have been removed from each individual group.
4.3.2 Effect of VAX-IP Treatment on Survival

The ability of VAX-IP to confer a survival advantage as compared to sham controls was conducted in both the early treatment MB49 orthotopic model as well as the late treatment MB49 orthotopic model. As demonstrated in Figure 4.2a, although not statistically significant, treatment with either $1 \times 10^8$ or $5 \times 10^8$ VAX-IP minicells every 3 days for 5 total doses starting on Day 6 post-MB49 tumor cell instillation resulted in extending the survival time as compared to sham controls (median survival days: sham=19days; $1 \times 10^8 = 32$days; $5 \times 10^8 = 29$days).

To more closely mimic a clinical treatment situation, trials were run where tumor cells were only allowed to incubate for 24 hours post-surgery (implantation), and treatments were administered once every seven days. It was found that groups receiving a total of 4 doses of $1 \times 10^8$ VAX-IP minicells had a significant survival advantage ($P=0.03$) (figure 4.2.b).
Survival Analysis
3days Group

Survival Rate vs. Time (days)

- Sham
- 3Days 1*108
- 3Days 5*108
- 3Days GEM

Tumor Implantation
First Treatment
Second Treatment
Third Treatment
Fourth Treatment
Fifth Treatment

6 Days 3 Days 3 Days 3 Days 3 Days 72 Days
Figure 4.2 Effect of VAX-IP Treatment on Survival.  a) Survival analysis of every three days treatment group b) Treatments schedule of every 3 days group  c) Survival analysis of every seven day treatment group. d) Treatments schedule of every 3 days group.  Figure a shows the survival graph. in which mice were treated every 3 days for 5 total doses starting on Day 6 post-tumor instillation.  Intravesical treatment was given in 100 µL volume of saline with either vehicle only (control), $10^8$ VAX-IP minicells, $5 \times 10^8$ VAX-IP minicells, or 500 µg gemcitabine. Animals were weighed and observed daily for up to 90 days. Figure c shows that the survival graph of treatment 24 hr post tumor...
instillation and administered VAX-IP once weekly thereafter for 3 weeks (4 doses total).

Again, intravesical treatment was given in 100 µL volume of saline with vehicle only (control), $10^8$ VAX-IP minicells, $5 \times 10^8$ VAX-IP minicells, or 500 µg gemcitabine.

Animals were weighed and observed daily for 90 days where applicable. “*” stands for that there is a significant difference between the drug treated group with the sham group.

Survival data were analyzed for statistical significance using the log-rank test.
4.3.2 Effect of VAX-IP Treatment on impeding tumor cell implantation

Minicells treatment also presents dose-dependent inhibition effect on tumor cell implantation. As shown in Figure 4.3, the median and average bladder weights also decreased with increasing doses of VAX-IP minicells. When given by intravesical administration at the optimal dose of $5 \times 10^8$, the tumor burden became the lowest. Then therapeutic effect was lost at an increasing high dose of $10^9$ VAX-IP minicells.
Figure 4.3  a) Effect of VAX-IP Treatment on impeding tumor cell implantation. b) Scheme of treatments. The tumor instillation procedure was the same as that used in the
above set, except that the total number of tumor cells put into each bladder was 20,000 in 100 µl total volume (reduced from 100,000 in 100 µl total volume), and tumor cell incubation times was 60 minutes (reduced from 90 minutes). In addition, bladders was irrigated with saline immediately following tumor cell exposure to mimic clinical routine postoperative bladder irrigation. Intravesical treatments were given in 100 µL volumes of saline with either vehicle only (Sham control), VAX-IP minicells ranging from $5 \times 10^7$, $5 \times 10^8$, and $1 \times 10^9$, or 500 µg gemcitabine (positive control for the MB49 model). The bottom and top of each box shows the limits of the first and third quartiles, and the band within each box represents the median. The mean is shown by a single dot within each box. The highest whisker indicates the 1.5 interquartile range of the upper quartile and lowest whisker indicates the 1.5 interquartile range of the lower quartile. All data points are presented at the right side of box-whisker plot ($8 \leq n \leq 10$). The maximum and minimum data points have been removed from each individual group.
4.4 Discussion

The most effective and widely used agent utilized in the treatment of intermediate and high risk papillary disease and CIS in NMIBC patients is BCG adjuvant immunotherapy. While effective, BCG is a live pathogen, and as such is associated with a high incidence of adverse side effects ranging from dysuria to systemic toxicity, sepsis, and death (Lukacs et al., 2013). The use of BCG can be difficult from a practical standpoint. For example, administrating personnel must be vaccinated against BCG, dedicated BCG treatment rooms must be established and maintained, and dedicated toilets must be sanitized following each patient micturition cycle, the latter of which continues with the patient at home where protocol stringency is difficult to monitor and control. Of distinct clinical importance, it is estimated that up to 20.3% of NMIBC patients treated with BCG voluntarily halt therapy due to toxicity (van der Meijden et al., 2003). It is also estimated that about 54% of those NMIBC patients that complete treatment are either BCG refractory or will become BCG resistant and recur (Herr et al., 2011). In the high risk disease setting, patients having failed one course of BCG are typically given a second course in an attempt to restore immunotherapeutic responses. Cystectomy is the most common treatment for those patients that fail two courses of BCG and comes with major changes in patient lifestyle, quality of life, and can be complicated by its high incidence of acute and chronic morbidity (Zlotta et al., 2009). Many patients are deemed unfit for cystectomy due to age and other co-morbidities. The only
marketed product labeled for use in these patients is valrubicin (Valstar®, Endo Pharmaceuticals, Inc.) (FDA Application No.:20-892) and it is limited to use in BCG failure CIS patients that are unfit for cystectomy. Moreover, Valstar only works in 21% of patients treated with a durable response of 30 months (Steinberg et al., 2000).

Relative to other cancer types, there are few clinical trials with novel or existing agents being conducted in NMIBC patients and many hopeful candidates have recently failed to meet primary clinical endpoints in their respective disease populations. Taken together, there is a clear need for novel therapies in the treatment of NMIBC.

In this report, the development of VAX-IP, a novel, recombinant, non-living bacterial minicell product candidate and its ability to generate significant anti-tumor responses in the widely used MB49 syngeneic orthotopic model of superficial bladder cancer is described. Results show that VAX-IP has significant, dose dependent anti-tumor effects and confers a survival advantage over the saline control group, even in a late treatment variation of the MB49 model in which BCG has been reported to have no effect (Zhang and Godbey, 2011).

The variance of tumor burden is large in our *in vivo* assay. Even though we tried our best to reduce the system error, some factors are hard to control. For example, during tumor implantation process, we use a platinum guidewire through a catheter to generate a cauterization site (mimics the TURBT procedure while providing a tumor
attachment site). Because the thickness of bladder in each individual mouse is not same and there are hand tremors during the process of controlling catheter, the size of burning wound is potentially different. Therefore, the initial cancer cell number planted in this wound site would be different. Because of cell replication in an exponential profile, there would be no surprise that the tumor weight will have a huge difference after 18 days. However, the variation of tumor size after VAX-IP minicell treated (dose: $5 \times 10^8$ (figure 4.1, 4.2)) is highly restricted, which indicates that minicell do have an anti-tumor growth effect in the orthotopic murine bladder tumor model regardless of the variability in tumor size at treatment initiation.

It is much more common within chemotherapy treatment that there is a linear correlation between drug dose and antitumor efficacy. Due to the direct toxicity, the higher dose produces more cell death. Usually, the constraint for applying a higher dose of chemotherapy drug is the toxicity to normal, healthy cells and accompanying side-effects. However, according to our data, the minicell treatment efficacy does not linearly correlate with drug dose. There is a bell-shaped structure in the dose response curve. High dose of minicell treatment will lose tumor inhibition effect. Interestingly, the similar phenomenon also exists in BCG treatment (Lamm et al., 1982). There is also a bell-shaped curve between BCG dose and tumor progression. High dose of BCG administration will decrease the anti-tumor efficacy and potentially promote the tumor growth.
As is known for BCG, the bell-shaped curve of mincell treatment may indicate the
tumor inhibition is in part because of an immunotherapeutic response (in addition to
VAX-IP’s direct cytotoxic effect). It is very common to observe the bell-shaped
response in adaptive immune reaction (Bocharov et al., 2004). At the start stage, small
amount of antigen cannot activate the immune system until the concentration of antigen
reaches an appropriate level. However, antigen tolerance starts to build up with the
antigen concentration increase. Finally, the excessive amount of antigen will disable the
immune system. Another mechanism contributing to BCG bell-shaped response curve
is that there is a shift from immunotherapeutic TH1 response at the right dose to a
non-immunotherapeutic TH2 response at higher dose. The minicell treatment curve did
not follow the linear toxicity correlation curve. In contrast, it complies with the
bell-shaped curve similar with adaptive immune reaction (Bocharov et al., 2004) and
immunotherapeutic shift. Thus, it is indicated that minicell present strong anti-tumor
effect as an immunonomodulatory therapeutic in addition to direct PFO-mediated
tumoricidal activity.
4.5 Conclusion

The therapeutic effects of VAX-IP minicells were tested within our orthotopic model of murine transitional cell carcinoma. Whether administered in the established tumor model or the anti-implantation model, there was a bell-shaped curve in the relation between drug dosage concentration and tumor burden. The median and average bladder weights, which are surrogates for tumor burden, decreased with increasing doses of VAX-IP minicells when given by intravesical administration to a point, after which the benefit of VAX-IP minicells administration was decreased with higher dosages. Furthermore, compared with the sham-treated group, a total of 4 doses of $1 \times 10^8$ VAX-IP minicells treated 24-hours post-surgery with follow-up treatments every 7 days had a significant survival advantage ($P=0.03$).
Chapter 5

Future-work:

Cancer-specific and Stress-inducible Promoters for Expression-targeted Gene Therapy: $^{p}txnll$, $^{p}grp94$, $^{p}polh$ and $^{p}znf394$
5.1 **Introduction to Future Work**

Expression-targeted gene delivery is aimed at delivering a gene of interest (GOI) into cells of a targeted population to elicit a desired response. Inducible gene expression also belongs to the expression-targeted gene delivery, which take advantage of the inducible promoters, impart the capability to turn the expression of the functional gene on or off in response to a stimulus. For example, the tetracycline-inducible expression system (Tet-on) is a powerful tool that has been used to achieve inducible transgene expression. Driven by $^{\beta}TRE3G$, a gene of interest will be expressed when tetracycline or its derivative doxycycline is administrated. By adjusting the concentration of tetracycline or doxycycline, the expression of the GOI can be controlled by artificial stimulation.

Stressors can alter gene expression patterns within the cell. Examples include hypoxia, low glucose availability, or changes in pH. At the molecular level, the cell adapts to the stressors by expressing specific genes to handle the altered environment. This is accomplished through the binding of transcription factors, often specialized, to the promoters/enhancers of stress response genes. Transformed cells occasionally turn on their stress response genes constitutively. For example, the heightened metabolism of many cancer cells may generate a greater need for glucose because of the extra energy required for replication. In response to their own generated need, they may turn on
certain glucose responsive genes. We have isolated four very interesting promoters that are typically associated with specific stresses, but are quite active in cancer cells. This presents a unique opportunity for adding to the expression-targeting library as it applies to cancer treatment.

The glucose-regulated protein Grp94, also named Endoplasm, is a heat shock protein which is expressed in correlation with unexpected temperature stresses. The Grp94 protein is a chaperone whose major function is to fold proteins into an appropriate conformation. Although Grp94 is a heat shock protein, grp94 expression occurs not only in response to temperature changes, but also to stresses such as hypoxia (Paris et al., 2005), starvation (Morita et al., 2000), and oxidative stress (Chen et al., 2002). Grp94 also plays an important role in cancer progression, being overexpressed in multiple types of cancer such as colon (Liu et al., 2010b), lung (Wang et al., 2002), and esophageal (Langer et al., 2008).

The thioredoxin-like protein 1TXNL-1 has a redox potential which is associated with oxidative stress (Lu et al., 2012) and glucose deprivation (Jimenez et al., 2006). Oxidative stress is sensed when there is an imbalance between reactive oxygen species (free radicals) production and antioxidant reduction. The extra free radicals will adversely react with DNA, proteins and lipids. The thioredoxin family of proteins
typically has a highly conserved active motif (Cys-x-x-Cys), which has redox activity due to the reversible oxidation of thiol groups in cysteine pairs to form disulfide bonds

\[(\text{R–SH} + \text{HS–R'} \rightarrow \text{R–S–S–R'})\].  A hydride ion is transferred to NAD$^+$ in the process, and a proton is also generated. The thioredoxin family is one of the major systems responsible for maintaining the cell in a reduced state. TXNL-1 also assists the cell in surviving glucose-deprived conditions (Jimenez et al., 2006). (Note the similarities to GRP94.)

Polymerase η (POLH) is a specialized DNA polymerase that is in charge of replicating UV-damaged DNA (Ohkumo et al., 2006). The high energy of UV radiation can cause thymine dimers to form within the DNA bases of the genome. Unlike the typical polymerase-blocking action that results from the abnormal thymine dimers, POLH is able to synthesize new strands of DNA by tolerating and skipping the damaged segment. This type of DNA replication is named “translesion synthesis”. Thus, there is a strong connection between POLH and UV radiation stress.

Zinc finger protein 394 (ZNF394) is a transcription factor. There is no direct evidence to link its correlation with cellular stress, but there is one report that indicates that it can inhibit the transcriptional activities of c-Jun and Ap-1 (Huang et al., 2004), which are well known as stress-responsive transcription factors. They are regulated by multiple extracellular stressors, including hypoxia (Laderoute et al., 2002), oxidative
stress (Karin and Shaulian, 2001), and ultraviolet radiation (Devary et al., 1991). There is at least an indirect relationship between ZNF394 and environmental stresses.

We have found that the promoters for the four genes just described – $^{p}grp94$, $^{p}txnl1$, $^{p}polh$, and $^{p}znf394$ – do indeed serve as markers of certain cancer cells. Future work will further characterize the activity of these promoters and their suitability for expression-targeted gene therapy of tumor cells. In the process, a set of engineered genes that may be turned on in response to certain stressors will be produced.
5.2 Material and Methods

5.2.1 Cell culture

All cells were maintained in a humid incubator maintained at 37 °C and a 5% (v/v) CO₂ atmosphere. A total of 10 cell types were included in the investigations, in which six originated from normal tissue and four types from cancer tissue. The selected cell lines spanned human and murine species. The details of each line with its growth media were presented in Table 5.1

Table 5.1 Cell lines and culture media.

<table>
<thead>
<tr>
<th>Cell Name</th>
<th>Species</th>
<th>Description</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF-1</td>
<td>Homo sapiens</td>
<td>Normal foreskin fibroblast cells</td>
<td>DMEM with 15% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>HASMC</td>
<td>Homo sapiens</td>
<td>Normal aortic smooth muscle cells</td>
<td>SmGM-2 BulletKit(Lonza)</td>
</tr>
<tr>
<td>HAEC</td>
<td>Homo sapiens</td>
<td>Normal aortic endothelial cells</td>
<td>EGM-2 BulletKit(Lonza)</td>
</tr>
<tr>
<td>PrEC</td>
<td>Homo sapiens</td>
<td>Normal prostate epithelial cells</td>
<td>PrEGM BulletKit(lonza)</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>Homo sapiens</td>
<td>Normal prostate epithelial cells</td>
<td>K-SFM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Homo sapiens</td>
<td>Mammary gland/breast adenocarcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>PC-3</td>
<td>Homo sapiens</td>
<td>Prostate adenocarcinoma cells</td>
<td>RPMI 1640 with 10% FBS, and 100 U/ml P/S</td>
</tr>
<tr>
<td>Bg-1</td>
<td>Homo sapiens</td>
<td>Ovarian carcinoma cells</td>
<td>DMEM with 10% FBS, and 100 U/ml P/S</td>
</tr>
</tbody>
</table>

PS: DMEM - Dulbecco’s Modified Eagle’s Medium
RPMI 1640 - Roswell Park Memorial Institute (RPMI) 1640 Medium
FBS - Fetal Bovine Serum
P/S - Penicillin/Streptomycin
K-SFM - Keratinocyte Serum Free Medium Kit (Invitrogen)
5.2.2 Promoter Cloning

All tested promoters were obtained via PCR amplification (Table 2). The templates came from whole-genome extracts of human foreskin fibroblasts (HFF-1).

The identification and verification of the amplified promoters were carried out by DNA sequencing.

### Table 2 Promoter names and cloning sites

<table>
<thead>
<tr>
<th>mRNA ID</th>
<th>Gene Name</th>
<th>Start*</th>
<th>End*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_004786</td>
<td>Homo sapiens thioredoxin-like 1 (TXNL1)</td>
<td>52456706</td>
<td>52457762</td>
</tr>
<tr>
<td>NM_003299</td>
<td>Homo sapiens heat shock protein 90kDa beta (Grp94)</td>
<td>102847507</td>
<td>102848411</td>
</tr>
<tr>
<td>NM_006502</td>
<td>Homo sapiens polymerase (DNA directed), eta (POLH)</td>
<td>43651064</td>
<td>43652030</td>
</tr>
<tr>
<td>NM_032164</td>
<td>Homo sapiens zinc finger protein 394 (ZNF394)</td>
<td>98935675</td>
<td>98936598</td>
</tr>
</tbody>
</table>

*Start and End designations refer to positions obtained from the Human Genome Browser – HG-18 assembly.

Promoters were amplified via PCR using a “Matryoshka doll” method. In this method, a longer fragment spanning the desired range is first amplified based on the whole genomic template. Then, using the newly amplified PCR fragments as a template, the promoters within the specific range (table 2) (with added restriction sites) are amplified during the second PCR run. The primer details are as follows:
\$^p\text{txnl1}\$

First round:

Forward: GGG TCA GGC TCA GGC AAG

Reverse: TCA CTG CCT TCC ATC CTC CT

Second round:

Forward: (EcoR I) GTG TGT \underline{GAA TTC} AAG AGT ACA CTG GCG TTA GC

Reverse: (Age I) ATA TAT \underline{ACC GGT} TGG CTT TGA AAC TGA AGG AG

\$^p\text{grp94}\$

First round

Forward: GAG CCA GAG CCT CAG AGA G

Reverse: GAG CCA GAG CCT CAG AGA G

Second round

Forward: (EcoR I) GTG TGT \underline{GAA TTC} GCT GTG TAC AAT GGA GAC T

Reverse: (Age I) ATA TAT \underline{ACC GGT} GTG AGT CTC AAG TCC CCT TC

\$^p\text{polh}\$

First round

Forward: GAG CAG AGC ATT CGG GTC TC

Reverse: CCC AAT CCG CAC AAA ACA AGT
Second round
Forward- (EcoR I) GTG TGT GAA TTC GCA GGA GAG ACC ATA AAG AAC
Reverse- (Age I) ATA TAT ACC GGT AGT TTC GAG CGA GAT TTA TTC

\[ \text{pznf394} \]

First round
Forward- GAC GAC AGA GTT CTC GGA GC
Reverse- TGA GCC TCA ATT TCC TCA TCT TTT T

Second round
Forward- (EcoR I) GTG TGT GAA TTC TGA GCC TCA ATT TCC TCA TCT
Reverse- (Age I) ATA TAT ACC GGT TCC TGG AGT CTT CTT TTC AGG T

5.2.3 EGFP Reporter Indication Assay

All plasmids with the EGFP reporter gene were constructed using the PGL3 vector (Promega, Madison, WI, USA) as a backbone. The luciferase reporter exon was replaced with the enhanced green fluorescent protein exon (EGFP) from the plasmid pEGFP-N1 (Clontech, Palo Alto, CA, USA). Promoters to drive EGFP expression were inserted into the MCS of the vector. The promoter and EGFP elements of each constructed plasmid were verified by DNA sequencing.
Branched poly(ethyleniminie) (PEI) with a weight-average molecular weight of 25 kDa (Sigma-Aldrich, St. Louis, MO, USA) was used to deliver plasmids into cells. PEI/DNA complexes were prepared at a 7.5:1 N: P ratio, using 3.6 µg of DNA per dose (Zhang et al., 2008, Dobek et al., 2011). The transfection procedure consisted of plating 100,000 cells into wells of 6-well plates 16 hours prior to the administration of gene delivery complexes. At the time of transfection, the growth medium in each well was replaced by 2 ml of serum-free medium plus 100 µl of gene delivery complexes in normal saline, prepared as just specified. Cells were incubated in the transfection medium for 2 hours at 37°C, after which the medium was replaced by the appropriate growth medium for that cell type. Analysis of EGFP expression took place at the 48-hour time point.

EGFP expression was measured by flow cytometry (FACSCantoII, BD Biosciences, San Jose, CA, US). Cells were trypsinized with 0.3ml trypsin and then flushed from the plate with an additional 0.7ml of FBS-free medium, and the entire 1 ml was immediately used for flow cytometry. A value for population intensity was recorded, being defined as the mean fluorescence intensity of the strongest 10% cells within the population. Intensity values were normalized to the positive control: \[^{\text{P}}_{\text{cmv}}\text{-driven EGFP expression.}\]
5.3 Preliminary data

The four selected promoters – $p_{tnxl1}$, $p_{grp94}$, $p_{polh}$, and $p_{znf394}$ – all yielded excellent cancer-cell selectivity and levels of reporter expression. To demonstrate the strength of activity, the selected promoters were compared with the activity of the human telomerase reverse transcriptase gene promoter ($p_{hTERT}$), which has been used in clinical trials as a cancer treatment (Nemunaitis et al., 2010). The data in figure 5.1 show that the activities of $p_{hTERT}$ in all three cancer cell lines, including MCF-7 (breast), PC-3 (prostate), and Bg-1 (ovary), are comparatively versus the results obtained with our four gene constructs. The activity of $p_{txnl1}$ in particular was very strong, inducing reporter transcription (and expression) to levels comparable to the strong $p_{cmv}$ in the cancer cells tested (fig. 5.1a). The results indicate that $p_{txnl1}$, $p_{grp94}$, $p_{polh}$ and $p_{znf394}$ possess strong cancer-targeting specificity.

The tests of inducible activity for those promoter are part of the planned future work of the laboratory. The activities of these promoters will be tested in cells exposed to various stresses, including temperature, hypoxia, oxidative, glucose deprivation, and ultra-violet radiation.
a

![Graph comparing relative intensity of Promoter-less, hTERT, and TXNLI in Normal and Cancer Cells.]

b

![Graph comparing relative intensity of Promoter-less, hTERT, and GRP94 in Normal and Cancer Cells.]*
Figure 5.1 – Promoters activities between normal and cancer cell lines. a) ptnxl1 b) pgrp94 c) ppolh d) pznf394. Eight cell types, including five kinds of normal cells and three kinds of cancer cells, were transfected to verify the cancer-specific activity of each promoter. Plasmids with GFP driven by ptnxl1, pgrp94, ppolh and pznf394 were transfected into cells. Furthermore, the promoter-less plasmid was also used to be the
negative control. To demonstrate the efficacy, the $^p hTERT$ driven GFP plasmid was also used to be a comparable control. GFP fluorescence intensity was measured by flow cytometry. The data shown have been normalized to CMV driven positive controls. One-way ANOVA was first applied to analyze the differences between groups, then the Holm-Sidak Post Hoc Test was applied to compare the differences between selected promoter and promoter-less plasmid, $^p hTERT$ control. "*" indicates significant differences between selected promoter and promoter less control (P<0.05). "#" indicates significant differences between selected promoter and $^p hTERT$ (P<0.05) Error bars indicate standard deviations (n≥3).
5.4 Discussion

There are too many common in gene profile when cells suffer from stress or become cancerous. When cells suffer some stress, such as temperature suddenly changes, hypoxia, lack of nutrition, in order to survive those conditions, cells have to respond to those kinds of stress by changing their gene expression profile (Baluchamy et al., 2010). They may release some signal to ask for more nutrition supply from the body. Or they may express some proteins to change the metabolic mode to keep the minimal function to survive. Similarly, transformed cancer cells also usually express the similar protein to trick the body as they are suffering stress, by those tricky signals they can get more competitive advantages to replicate infinitely, such as inquiring more nutrition supply, more space to replicate, angiogenesis for metastasis.

Cyclooxygenase-2 (Cox2) expression is a good example of carcinoma cells tricking the body as they are under stress. Both carcinoma cells and cells experiencing inflammation over-produce Cox2. Cox2 in normal cells is an inducibly expressed protein by many other cytokines, mitogens and pro-inflammatory factors. The major function of Cox2 is to convert arachidonic acid (AA) to prostaglandin endoperoxide H2, which is an essential step for inflammatory signal cascade. Nonsteroidal anti-inflammatory drug (NSAIDS), such as celecoxib, are designed to inhibit the Cox2 function to treat pain and inflammation. However, in multiple types of carcinoma, the
Cox2 is a constitutively expressed protein. Cox2 contribute to multiple hallmarks of cancer, including tumorigenesis (Telliez et al., 2006), angiogenesis (Gately and Li, 2004), metastasis (Singh et al., 2007) and resistance to apoptosis (Kern et al., 2006). Thus, those anti-inflammatory drugs targeting Cox2 have been transferred into the tumor treatments. It is not hard to build a connection between carcinogenesis and inflammation through the Cox2 involvement. Carcinoma cells potentially trick the human body to construct new blood vessels and deliver more oxygen and nutrients to their site by mimicking inflammation signal cascade. By this connection, the promoter of cox2 has a dual function as a cancer-specific promoter (Godbey and Atala, 2003) and inflammatory inducible promoter (Rachakonda et al., 2008).

Thus, we start to consider a new logic think about how to look for stress inducible promoters. Firstly, we start to investigate whether a promoter is cancer-specific. Thorough the connection between stress and carcinogenesis, finally we hope to find a set of stress-inducible promoters.

The promoter activity in the (Landolin et al., 2010) from strong to week were ranked at first and a sublist of top-strong promoter was concluded. Then from the most strong promoter list, we start to filter the potential cancer-specific promoter. Then, we get four promising inducible promoter candidates, including txnl1, grp94 and polh and znf394,
by a large amount of clues of those gene function and expression involving in responding to the extracellular stress.

The expression and function of *txnl1*, *grp94*, *polh*, and *znf394* are all related to cellular responses to multiple types of stresses, such as glucose deprivation, hypoxia, oxidative stress, or UV radiation. Our data have shown that the activity of these promoters is high in cancer cells, but not in normal, unstressed cells. The promoters are also very promising for use in the production of functional, inducible transgenes. Our future work will focus on the use of these promoters in driving suicide genes for expression-targeted gene therapy of transitional cell carcinomas. The production of engineered genes that can be activated in response to non-chemical signals is a closely related application to be pursued in future work as well.
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BIOGRAPHY

Xuguang Chen was born in 1985, Baotou, which is an industrial city in the Inner Mongolia Autonomous Region of the People's Republic of China. Xuguang obtained his bachelor degree of Chemical Engineering and Technology in Wuhan Polytechnic University in 2006. After that, Xuguang was accepted by Dalian University of Technology to pursue the master degree. Xuguang was conducting research about developing high-efficient and long-life nanosized catalyst for proton exchange membrane fuel cells, under the guidance of Dr. Gaohong He and Dr. Zhigang Shao (Dalian Institute of Chemical Physics, Chinese Academy of Sciences). After successfully obtaining the master degree in Chemical Engineering in 2009, Xuguang was accepted by Tulane University and joined in Dr. W T. Godbey’s laboratory to pursue the degree of Doctor of Philosophy in developing advanced treatments for transitional cell carcinoma. In his spare time, Xuguang really enjoys chasing the modern technology development, no matter in Electronic, Information technology, or Biotechnology. His dream is that someday he can change the world by translating cutting edge science into technology for serving people and society.