Chapter 9

Synthetic Tumor-Specific Promoters for Transcriptional Regulation of Viral Replication

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Abstract

Here we describe a collection of methods that have been adapted to isolate and modify tumor-specific promoters (TSPs) to drive viral replication for cancer therapy and other uses. We will describe as examples the secreted protein acidic and rich in cysteine (SPARC) and the protease-activated receptor-1 (PAR-1) promoter. We outline strategies to select appropriate TSPs using bioinformatics resources and the methods utilized in their subsequent cloning, assessment of transcriptional activity, and their use in conditionally replicative oncolytic adenoviruses.

Key words Synthetic tumor-specific promoter, Adenovirus

1 Introduction

Regulation of viral replication is one of the milestones of successful oncolytic viral gene therapy in cancer. The clinical efficacy of conditionally replicative oncolytic adenoviruses (CRAds) is still limited by the inefficient viral replication in the tumor mass. The replication of second-generation CRAds is driven by TSPs selected from genes that exhibit differential expression in cancer versus nonmalignant tissue. Promoter length, activity, and specificity must be carefully assessed to increase viral efficacy. Furthermore, TSPs can be further engineered to harbor foreign sequences to obtain a final synthetic product that might greatly improve its expression characteristics [1–3].

Tumor growth is essentially the result of a continuous cross talk between malignant and cancer-associated stromal cells; therefore, targeting both cell compartments may profoundly influence cancer control. Based on this premise, we have developed the CRAd Ad-F512 whose replication is driven by a specific promoter fragment of the SPARC gene that is expressed both in malignant cells and in

cancer-associated stromal cells, fibroblasts, endothelial cells, and inflammatory cells such as macrophages and neutrophils [4–7].

A second CRAd, AdStroma2, was developed whose replication is driven by a TSP based on a specific fragment of the human *Par-1* gene promoter. PAR-1 plays a role in the malignant and physiological invasion processes [8] and in tumor angiogenesis [9, 10]. PAR-1 was the first identified member of the PAR gene family, currently including four seven transmembrane G-protein-coupled receptors activated by proteolytic cleavage. PAR-1 is overexpressed in several types of cancer [8, 11–15].

We outline all the steps that we have followed to obtain two synthetic TSPs based on the SPARC and PAR-1 genes.

2   Materials

2.1   In Silico Study of Promoters

1. Hardware: personal computer.

2. Software:
   For promoter searches:

   PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

   EPDnew (Eukaryotic Promoter Database - http://epd.vital-it.ch/).

   Neural Network Promoter Prediction (Berkeley Drosophila Genome Project, USA—http://www.fruitfly.org/seq_tools/promoter.html).

   Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/).

   PROMOSER (https://biowulf.bu.edu/zlab/PromoSer/).

   For promoter analysis and regulatory motifs search:

   GPMiner (http://gpminer.mbc.nctu.edu.tw/).

   Promoter and gene expression regulatory motifs search (http://linux1.softberry.com/berry.phtml?topic=index&group=programs&subgroup=promoter, Softberry, USA).

   MatInspector (https://www.genomatix.de/online_help/help_matinspector/matinspector_help.html).

   For primer design:

   Primer Premier (http://www.premierbiosoft.com/primerdesign/).

   qPCR Design Lab (http://www.qpcrdesign.com/qpcr-library).

   PerlPrimer and Beacon Designer (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1) [16].

2.2   Isolation of Promoter Fragments and Cloning

1. pGL3-Basic (Promega).

2. pShuttle vector (Agilent Technology).

3. pShuttle-I-Xp-luc, (See **Note 5** and [6, 7]).

4. DNeasy Blood & Tissue Kit (Qiagen).

5. MultiGene OptiMax Thermal Cycler.

6. Taq polymerase, $MgCl_2$ and polymerase buffer.

7. Primers.

8. 100 mM dNTPs set.

9. Agarose.

10. Ethidium bromide 10 mg/ml.

11. DNA gel extraction kit (such as QIAquick Gel Extraction Kit).

12. pCR4 TOPO vector (Invitrogen).

13. Saline solution 1.2 M NaCl; 0.06 M $MgCl_2$.

14. Chemically competent bacteria DH5α (Thermo Fisher Scientific).

15. Sterile LB broth and LB agar plates supplemented with 50 μg/ml ampicillin or kanamycin.

16. Qiagen Plasmid Midi and Maxi preparation kits.

17. Restriction enzymes.

18. Calf intestinal alkaline phosphatase (CIP).

19. pAdEasy-1 vector (Agilent Technologies) or pVK500C 5/3 [17].

20. Electrocompetent *E. coli* strain BJ5183 (Addgene).

21. DH5α ElectroMAX™ cells (Thermo Fisher Scientific).

22. Lipofectamine® LTX (Thermo Fisher Scientific).

23. 911 cells [18].

24. DMEM/F12 medium.

25. Fetal Bovine Serum (FBS).

26. 293HEK cells (Microbix).

2.3 Luciferase Analysis with Plasmid or Non-replicative Virus

1. Tumor cell lines.

2. Lipofectamine® 2000 (Invitrogen).

3. pRL-CMV plasmid (Promega Corp).

4. Dual Luciferase Reporter Assay System, (Promega Corp).

5. 24-well plates.

6. Luminometer.

7. Protein assay.

2.4 Assessment of Promoter Activity: E1A Expression

1. Cancer cell line.

2. Lysis buffer: 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 0.05% SDS, 1% Triton X-100.

3. Sonicator.

4. SDS-polyacrylamide gel.

5. Nitrocellulose filter.

6. Mouse Anti-E1A (sc-430, Santa Cruz Biotechnology Inc).

7. ECL western blotting detection kit.

8. Anti-β-Tubulin III (Sigma).

2.5 Assessment of Promoter Activity: Viral Lytic Capacity

1. Cancer cell line.

2. MTT assay.

3 Methods

3.1 In Silico Study of Promoters

1. Download your promoter sequence from PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and save it as a word or TXT file. Genes and promoter sequences are often parts of full genomes or longer nucleotide sequences, and one needs to search manually for the start and end of the promoter you are interested in. As an example, PAR-1 promoter (from hereafter Stroma-2P TSP) was selected by an initial search in PubMed, using as keywords "stroma expression in cancer," "microarrays in cancer and normal tissue," and "promoter"; this initial search led us to select several promoters that were evaluated for their use in the adenovirus context (*see* **Note 1**). Make use of available bibliographies that might help you with the sequence you are dealing with.

2. In order to analyze the promoter sequence of interest, the presence of core promoter elements like TATA box, downstream promoter element (DPE), initiator element (INR), and transcription factor motifs [19–21] can be identified using a range of available programs. We present here some of the most useful online programs (see below), and, in Fig. 1, we show analysis of the SPARC promoter using the Eukaryotic Promoter Database (EPDnew).

   EPDnew (http://epd.vital-it.ch/) is a new collection of experimentally validated promoters in human, mouse, *Drosophila melanogaster*, and zebra fish genomes. Evidence comes from transcription start sites (TSS) mapping, high-throughput experiments such as cap analysis gene expression (CAGE) and Oligo-capping, and chromatin immunoprecipitation (ChIP)-sequencing experiments on H2AZ, H3K4me3, Pol-II, and DNA methylation. The program also includes promoter analysis tools [22]:
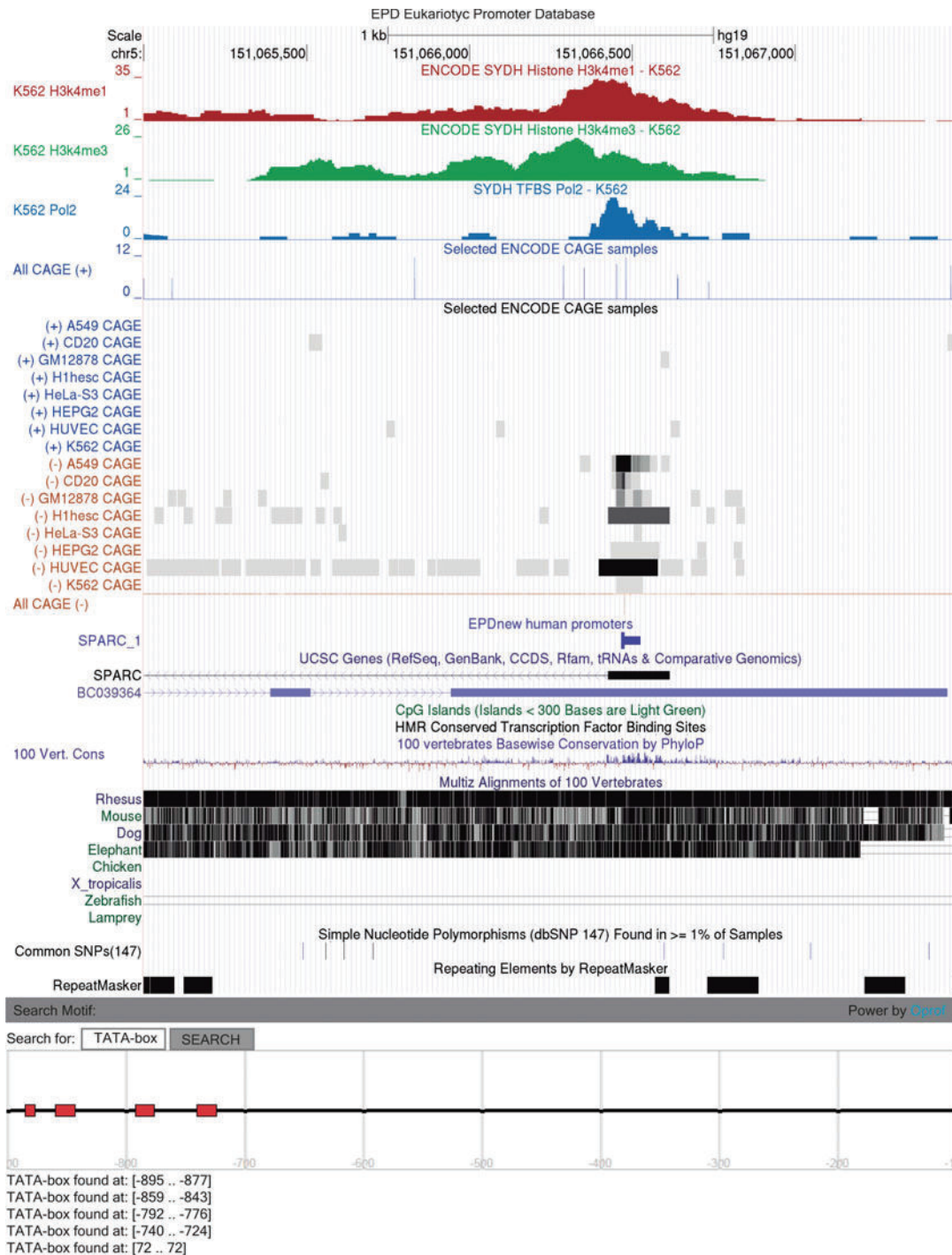
Fig. 1 SPARC promoter analysis using the Eukaryotic Promoter Database. The figure shows the results of a search by TATA box sequence. The SPARC promoter was defined as a TATA-less promoter; however, we demonstrated that it has an unconventional sequence active in the region of TATA box [2]

    (a) **Neural Network Promoter Prediction** (http://www.fruitfly.org/seq_tools/promoter.html, Berkeley Drosophila Genome Project, USA) [23].

    (b) **Promoter 2.0 Prediction Server** (http://www.cbs.dtu.dk/services/Promoter/, S. Knudsen, Center for Biological Sequence Analysis, Technical University of Denmark) predicts TSS of vertebrate Pol II promoters in DNA sequences.

    (c) **PROMOSER** (https://biowulf.bu.edu/zlab/PromoSer/) Human, Mouse and Rat promoter extraction service (Boston University, USA), maps promoter sequences and TSS in mammalian genomes [24].

    (d) **Promoter and gene expression regulatory motifs search** (http://linux1.softberry.com/berry.phtml?topic=index&group=programs&subgroup=promoter, Softberry, USA) offers a variety of promoter-scanning programs.

    (e) **GPMiner** (http://gpminer.mbc.nctu.edu.tw/) identifies promoter regions and annotates regulatory features in user-input sequences.

    (f) **MatInspector** (https://www.genomatix.de) is a software tool that utilizes a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences. MatInspector is almost as fast as a search for International Union of Pure and Applied Chemistry (IUPAC) strings but has been shown to produce superior results. It assigns a quality rating to matches and thus allows quality-based filtering and selection of matches [25].

3. The selection of the most appropriate promoter fragment also depends upon the application and considers factors such as genome viral capacity, the presence of a singular core promoter element, or transcription factor binding sites that you would like to retain or avoid. We recommend you work with your file in your favorite word processor program. The original sequence can be subfragmented (always keeping the core promoter elements) in smaller fragments for further promoter activity analysis. We advise you highlight the most important motifs or promoter elements that are found in the in silico analysis, to help select the promoter fragments to test (Fig. 2).

3.2 Isolation of Promoter Fragments and Cloning

Once you decide the number of promoter fragments you would like to assess, you have to select the appropriate set of primers for the isolation of each promoter fragment by PCR. The primers should be designed for further subcloning in a reporter vector adding the appropriate restriction enzyme sites. As a reporter
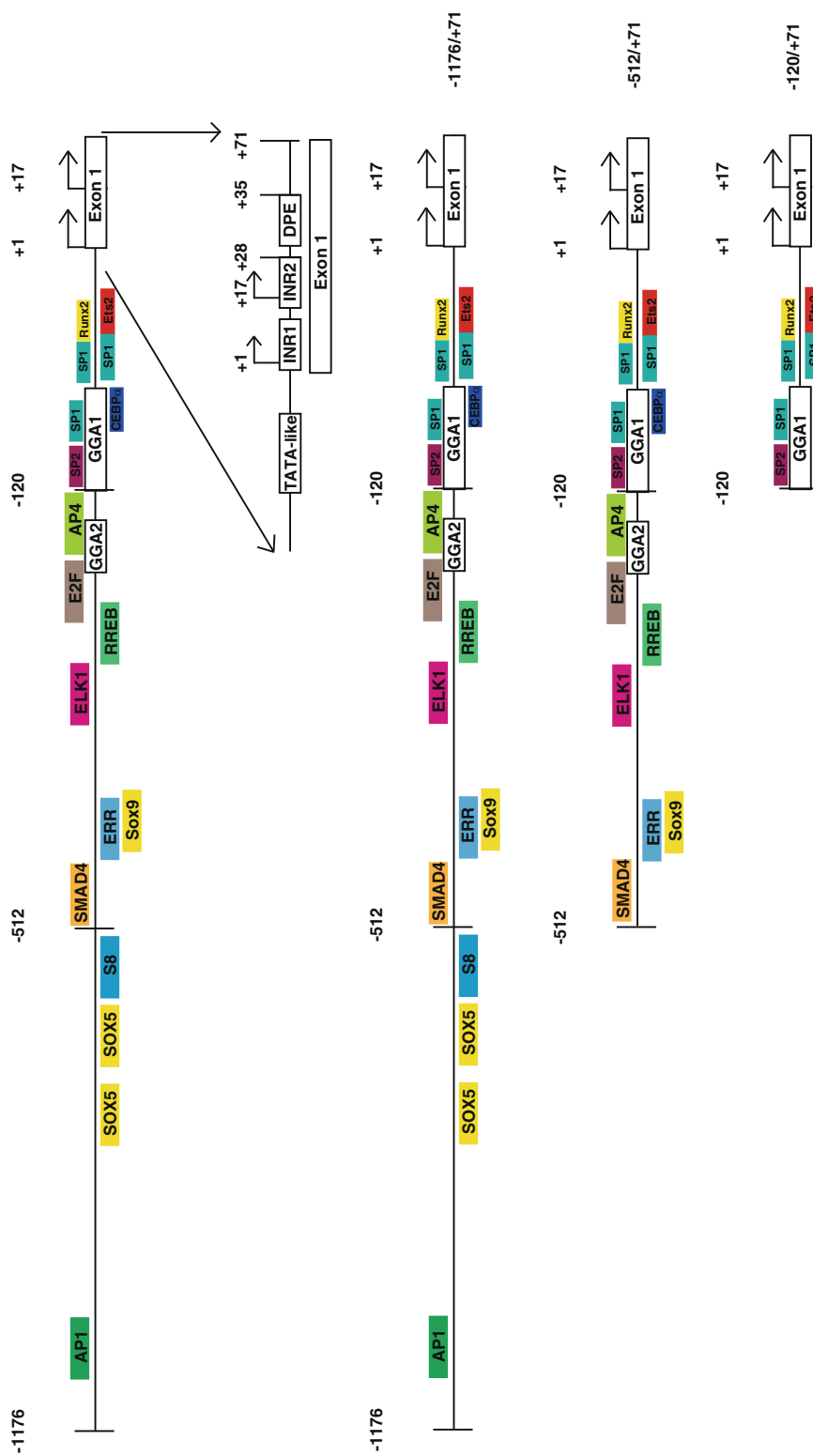
Fig. 2 Overview of the promoter fragment cloning process. We chose the SPARC promoter −1176/+71 for in silico *analysis*. We show the most relevant elements or transcription factor sequences. Then we fragmented the promoter in three smaller fragments as depicted

vector, we currently use the plasmid pGL3-Basic, while in the viral context, each promoter fragment is subcloned in a pShuttle vector (*see* **Note 2**).

pGL3-Basic vector is a reporter vector that provides a basis for the quantitative analysis of promoters and enhancers. The backbone of the pGL3-Basic vector contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. For pGL3-Basic vector, we design primers containing the restriction enzymes *Mlu*I/*Bgl*II (*see* **Note 3**). You should design the primers (*see* **Note 4**) or use software such as Primer Premier (http://www.premierbiosoft.com/primerdesign/), qPCR Design Lab (http://www.qpcrdesign.com/qpcr-library), PerlPrimer, and Beacon Designer (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1) [16].

pShuttle vector is an entry vector useful for cloning sequences inside the viral E1A region of the adenovirus genome that is usually deleted in adenoviruses backbones currently in use for gene therapy purposes (we engineered this vector by introducing an insulator sequence (named I) and a luciferase gene to obtain a new vector named pShuttle-I-Xp-luc; Xp means the MCS (*see* **Note 5** and [6, 7])). The insulator sequence helps to prevent any enhancer activity of the adenoviral inverted terminal repeats (ITR) on your TSP. This new vector is especially useful if you are planning to clone different promoters or engineer a promoter by adding additional sequences.

The source of DNA to clone your promoter could be a cell line of your lab; however, remember that sometimes cancer cells can contain DNA mutations. Another source of DNA is white blood cells from a healthy donor. DNA extraction is performed with an appropriate kit. The general protocol and in particular our work with SPARC and Stroma2 promoters are described in this section:

1. Perform PCR amplification in a reaction volume of 50 μl in a thermal cycler by using:

   300 ng of genomic DNA.

   1× polymerase buffer.

   1.5–2.5 mM of $MgCl_2$.

   0.1 mM of each dNTP.

   0.5 U Taq polymerase.

   10 μM each primer.

   The PCR cycle will differ depending on the target and primer sequences used. In our case the amplification conditions for the PCR are as follows: after an initial denaturation step at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at temperatures between 52 and 58 °C

Table 1
Primer sequences for SPARC promoter amplification and products generated

Primer	Sequence 5′–3′	PCR promoter fragment	Primers pair used
SPfse	CTAGCTAGCAGCTGGGTGTTGTGGCAT	–1175/+35	SPfse/R35
F120	GAACGCGTGGGAGAAGGAGGAG	–1175/+28	SPfse/R28
F513	CGACGCGTGCAGCTTGTCTTGTC	–513/+71	F513/R71
R35	CGAGATCTGCTCTCCGGGCAG	–513/+35	F513/R28
R28	CGAGATCTGGGCAGTCTGAAGGACC	–513/+24	F513/R24
R71	GCAGATCTCCTCAGTGGCAGGC	–120/+71	F120/SPas
R24	GCAGATCTAGTCTGAAGGACCGCG	–120/+35	F120/R35
SPas	ACGCGTCGACCTCAGTGGCAGGCA	–120/+28	F120/R28

The table shows the sequence of primers used for the different promoter fragments. Note: Only PCR fragments generated with the R35 and R71 primers contain the DPE [6]

(depending on your primers), and an extension reaction at 72 °C for 1 min (*see* **Note 6**)). A final extension step at 72 °C for 5 min terminates the reaction.

In the case of the SPARC promoter, we used pGEM-hSPPr plasmid containing the −1175/+71 SPARC promoter [5] as a template to obtain different SPARC promoter variants. Table 1 provides the list of primers and different promoter fragments. For the PAR-1 promoter, in silico analysis showed the presence of an SP1, AP-2, Egr-1, and androgen response element (ARE) transcription factor binding sequences. Three promoter variants were cloned, the largest fragment of 1900 bp (that included the ARE element), a fragment of 900 bp, and a fragment of 500 bp (named Stroma2). It is also important to include restriction sites into the primers to permit subsequent cloning.

2. Analyze PCR products by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide (*see* **Note 7**).

3. Gel purify products in the size range predicted, and clone them into pCR4 TOPO vector, and sequence the promoter fragment using T3 and T7 universal primers (*see* **Note 8**).

4. Next set up a ligation reaction as follows:

   4 μl of PCR fragment.

   1 μl of saline solution.

   1 μl of vector (TOPO).

   Total volume of ligation (6 μl).

   Incubate at room temperature for 30–60 min. If this mixture is going to be transformed in DH5α ElectroMAX™ bacteria, the

saline solution in the ligation reaction should be diluted in water at a proportion of 1/6.

5. Transform bacteria by incubating 100 μl of chemically competent bacteria with the ligation mixture for 10 min on ice, and then perform a heat shock of 1.5 min at 42 °C; again incubate on ice for 1 min, and then recover bacteria in 1 ml LB medium at 37 °C with shaking without antibiotic for 1 h. Plate 0.1 ml of the cells on agar with antibiotic (ampicillin or kanamycin), and incubate overnight at 37 °C.

6. Pick ten colonies, culture the bacteria overnight in antibiotic-containing media, and then perform a mini-prep. Check the recovered plasmid by restriction fragment length polymorphism (RFLP) with the corresponding enzymes.

7. Prepare a midi-prep of the correct promoter clone.

8. Next subclone the promoter fragments into pGL3-Basic or pShuttle-I-Xp-luc. Firstly, release the promoter (insert) from pCR4-TOPO midi-prep DNA with the enzymes of choice. We dephosphorylate the digested vector using 1 μl of CIP during 60 min at 37 °C.

9. Purify the digested insert and vectors by gel electrophoresis and gel extract.

10. Perform the ligation reaction using 100 ng of vector, 80 units of T4 DNA ligase, and the following amount of insert:

$$\text{ng of insert} = \frac{1}{3} \times \frac{(100\,\text{ng of plasmid}) \times (\text{bp of insert})}{(\text{bp of vector})}.$$

11. Transform the ligation into bacteria, and plate in LB ampicillin (pGL3-Basic) or kanamycin (pShuttle vector), and after mini-prep DNA preparation, check the plasmid by RFLP with the specific restriction enzymes.

12. Once your promoter is cloned in pGL3-Basic, you can assess the activity using luciferase as the reporter gene (*see* Subheading 3.3). In the case of pShuttle vector, this can be used to generate adenoviral vector by recombination.

13. For this recombination reaction, digest the pShuttle vector with *Pme*I to linearize, and purify by gel extraction. Co-transfect the digested plasmid with pAdEasy-1 vector or pVK500C 5/3 [17] into *E. coli* BJ5183 by electroporation (1300 V, capacitance of 25 μF and the resistance of 200 Ω) to allow for homologous recombination [26]. Isolate one positive clone, sequence, and once the sequence is confirmed, amplify by transforming DH5α ElectroMAX™ cells followed by DNA

plasmid maxi-prep; the resulting plasmid is called pAd-TSP-luc.

3.3 Production of Adenoviral Vectors from Plasmids

1. Linearize the plasmid pAd-TSP-luc with *Pac*I, and purify the DNA by ethanol precipitation (*see* **Note 9**).

2. Transfect 911 cells with 10–20 μg of linearized plasmid plus 30 μl of Lipofectamine® LTX, mix, and incubate at room temperature in 1.5 ml of DMEM/F12 medium for 30 min (*see* **Note 10**). Then add the transfection mix to 911 cells plated in a 100 mm tissue culture dish (at 70–80% confluence) with 5 ml of DMEM/F12 containing 5% FBS. Incubate in a CO_2 incubator for 6 h, then remove the medium, and add 10 ml of DMEM/F12 containing 10% FBS.

3. Incubate the cells without medium change for 3 days, and then expand each 100 mm tissue culture dish in 2–3 150 mm tissue culture plates.

4. Keep the plates for 12 days without changing medium. During this period of time, the cytopathic effect (CPE) should be evident in the cells under the microscope. Scrape the monolayer into the medium, and transfer the suspension into a falcon tube and freeze (−80 °C). This is the bulk virus.

5. Use 100 μl of the suspension (bulk virus) to clone by limiting dilution the rescued adenovirus, and amplify the cloned virus (seed stock) in a 100 mm tissue culture dish and freeze (−80 °C) (*see* **Note 11**).

6. The rescued adenovirus can be amplified by using it to infect 293HEK cells and produce concentrated viral stocks [6].

3.4 Luciferase Analysis with Plasmid DNA

1. Plate $3–5 \times 10^4$ cells/well (seeded in 24-well plates) (*see* **Note 12**).

2. 24 h later transfect the cells using Lipofectamine® 2000. We follow this protocol, first mix in a 1.5 ml tube 0.8 μg of the luciferase plasmid (your plasmid), 0.1 μg of pRL-CMV plasmid in 50 μl of DMEM medium without antibiotic and in another tube mix 1 μl of Lipofectamine® 2000 in 50 μl of DMEM medium without antibiotic. After 5 min mix gently both tubes and incubate at room temperature for 20 min. Remove the medium from the cells and add 200 μl of DMEM medium without antibiotic and 100 μl of the transfection mix.

3. Incubate for 4 h in a cell culture 37 °C CO_2 incubator.

4. Add 500 μl of the specific medium of your cells with serum. Incubate for a further 48 h.

5. Lyse your cells and perform the dual luciferase activity quantification according to the assay manufacturer's recommendations using a luminometer (*see* **Note 13**).
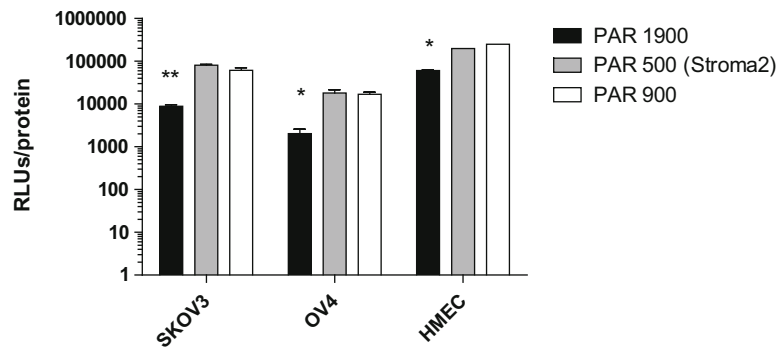
Fig. 3 Luciferase activity of the different promoter fragments of the PAR-1 promoter in two ovarian cancer cell lines (SKOV3 and OV4) and human micro-endothelial cells (HMEC). The bars represent mean $\pm$ SD ($n = 3$). **$p < 0.01$, *$p < 0.05$

6. Express the luciferase activity as RLU luciferase activity/Renilla activity $\times$ 100. This result is used to select the promoter fragment with highest activity in the cancer/stroma cells (in our case) and low activity in normal cells [6].

3.5 Luciferase Analysis with Non-replicative Virus

1. Plate 5×10^4 cells/well (seeded in 24-well plates) (*see* **Note 12**).

2. 24 h later transduce the cells with Ad-TSP-luc at 500 MOI (*see* **Note 14**) in 200 μl of DMEM/F12 containing 2% FBS (or 2% FBS in the appropriate medium for your cells). Two hours later add 800 μl of fresh medium containing 10% FBS.

3. Harvest your cells 48 h later, and quantify luciferase activity following the assay manufacturer's recommendations. Luciferase activity is normalized by protein concentration in the cell lysate (see **Note 13**). In Fig. 3 we show this analysis for the PAR-1 promoter. Both promoter Stroma2 and PAR900 have almost the same activity in ovarian cancer lines (SKOV3 and OV4) and endothelial cells. We selected Stroma2 as our TSP since it has a shorter length that facilitates its cloning in the CRAd.

3.6 Construct the CRAd with the Selected Promoter

We have seen above how to assess TSP activity in a non-replicative virus expressing luciferase as a reporter gene. The next step is the construction of the CRAd. We construct the replicative virus by cloning the TSP into the pShuttle-I-Xp-E1A vector where the luciferase gene is replaced by the E1A gene [6, 7].

1. PCR amplify the viral E1A gene (560–1632) using specific primers:

   Sense: 5'- GAAGCTTATGAGACATATTATCTGCCA-3'

   Antisense: 5'- GTCGACTTATCTCACCCTTTATTAAACTT-3'

The primers include a 5′ *Hind*III site in the sense primer and a *Sal*I site in the antisense primer. Clean up the PCR product, digest it with the enzymes *Hind*III/*Sal*I, and purify by gel extraction.

2. Release the luciferase gene from pShuttle-I-Xp-luc with *Hind*III/*Sal*I, and purify the vector by gel extraction.

3. Ligate the vector and the PCR amplified and digested E1A gene (insert) using 100 ng of vector, 80 units of ligase (T4 DNA ligase), and the following amount of insert:

$$\text{ng of insert} = \frac{1}{3} \times \frac{(100 \text{ ng of plasmid}) \times (\text{bp of insert})}{(\text{bp of vector})}.$$

4. Transform the ligation into bacteria and plate on LB kanamycin plates, and after prepare mini-prep DNA of clones to check the plasmid by RFLP with the specific restriction enzymes.

5. Perform the recombination reaction by digesting the pShuttle vector with *Pme*I to linearize, and purify by gel extraction. Co-transfect the digested plasmid with pAdEasy-1 vector or pVK500C 5/3 [17] into *E. coli* BJ5183 by electroporation (1300 V, capacitance of 25 μF and the resistance of 200 Ω) to allow for homologous recombination [26]. Isolate one positive clone, sequence, and once the sequence is confirmed, amplify by transforming DH5α ElectroMAX™ cells followed by DNA plasmid maxi-prep; the resulting plasmid is called pAd-I-TSP-E1A.

6. Prepare the viral stock as described in Subheading 3.3, and perform two key experiments. The first is to check for E1A expression by western blot, and the second is the analysis of the lytic capacity of your CRAd.

3.7 Assess Expression of E1A by Western Blot

1. Plate 1×10^5 cells/well in a 6-well plate, and infect the cells with CRAd between 100 and 1000 MOI.

2. 24 h after adenoviral infection, scrape the cells into 200 μl lysis buffer, and sonicate on ice (*see* **Note 15**).

3. Electrophorese 10 μg of total protein in a reducing SDS-polyacrylamide gel, and transfer onto a nitrocellulose filter. Incubate the filter for 2 h with mouse anti-E1A (dilution 1/100), and visualize using enhanced chemiluminescence. We use anti-β-tubulin III as a housekeeping control.

3.8 Assess Viral Lytic Capacity

1. Seed cells in 24-well plates at a density of 1×10^4 cells/well.

2. The next day, cells are infected with the corresponding CRAd virus in 200 μl of DMEM/F12 containing 2% FBS.

3. After 4 h of infection, add 0.8 ml of fresh medium containing 10% FBS.

4. CPE can be monitored by staining viable cells with crystal violet, or the number of surviving cells can be determined by the MTT assay [27].

4 Notes

1. This initial bibliography searching is vital for your future work since you need to decide which promoter you are going to work with. The more information you get, the better.

2. We prefer to study our promoter directly in the viral context instead of using plasmid vectors as the first approach.

3. Be careful with the restriction enzymes you select before starting your work. Especially pay attention if the sites are present in other vector components before you start subcloning.

4. Remember the basics of primer design:

   (a) Primer length: Optimal length of PCR primers is 18–22 bp.

   (b) Primer annealing temperature: Primers with melting temperatures in the range of 52–58 °C generally produce the best results. Primers with melting temperatures above 65 °C have a tendency for secondary annealing.

   (c) GC content: The GC content of primers should be 40–60%.

   (d) GC clamp: The presence of G or C bases within the last five bases from the 3′ end of primers (GC clamp) helps promote specific binding at the 3′ end due to the stronger binding of G and C bases. More than three Gs or Cs should be avoided in the last five bases at the 3′ end of the primer.

   (e) Primer secondary structures: The presence of primer secondary structures (hairpins, self-dimers, cross dimer) produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. These interactions adversely affect primer template annealing and thus the amplification and greatly reduce the availability of primers in the reaction.

   (f) Repeats: A repeat is a dinucleotide occurring many times consecutively and should be avoided because they can misprime, for example, ATATATAT. A maximum number of dinucleotide repeats acceptable in an oligo are four dinucleotides.

(g) Runs: Primers with long runs of a single base should generally be avoided as they can also misprime. For example, AGCGGGGGATGGGG has runs of base 'G' of values 5 and 4. A maximum number of runs accepted are 4 bp.

(h) 3′ end stability: It is the maximum ΔG value of the five bases from the 3′ end. An unstable 3′ end (less negative ΔG) will result in less false priming, since high 3′ stability means there are many C/G pairs at the 3′ end of your primer that may cause nonspecific binding of only the 3′ portion.

5. The vector pShuttle-I-Xp-luc was constructed as follows: First, we replaced the RSV promoter in the vector-pADPSY [28] with a multiple cloning site (MCS: *Spe*I, *Bcl*I, *Kpn*I, *Nhe*I, *Mlu*I, *Bgl*II, *Eco*RV, *Cla*I, *Sna*BI, *Sal*I) to create the pAd-Xp shuttle vector. Next, a fragment of 234 bp corresponding to the polyadenylation signal of the bovine growth hormone gene [29] was PCR cloned in the *Spe*I/*Kpn*I sites downstream of the ITR in the MCS to create a new shuttle vector, pAd-I-Xp (using primers INSU-F-SpeI (CCactagtGCTAGAGCTCGCT-GATCAGC) and INSU-R-KpnI (CGGTACCATCCCCAG-CATGCCTGC)) [6]. pAd-I-Xp-luc was constructed by adding the luciferase gene obtained from pGL3–513/+35 with *Bgl*II/*Bam*HI and cloned in the *Bgl*II site of pAd-I-Xp vector to produce pAd-I-Xp-luc. Then, the fragment I-Xp-luc was extracted with *Spe*I/*Sal*I and cloned in the sites *Xba*I/*Sal*I of the pShuttle vector.

6. Remember: 1 min for each 1000 bp of PCR product

7. If you have more than one band, try changing the $MgCl_2$ concentration, or modifying the annealing temperature, or if the problem persists, try purifying the correct band, and use this to re-amplify.

8. Even when you add restriction sites in the primers, we suggest you use a T vector to do the first cloning since you have more source of the promoter fragment and also you can easily sequence with this type of vector. We also recommend you use the PCR product fresh (otherwise add A-tailing) and use at a high concentration. We have successfully used 7 μl of purified PCR fragment in A-tailing reactions, and then add 1 μl of *Taq* DNA polymerase 10× reaction buffer with $MgCl_2$, dATP to a final concentration of 0.2 mM, 5 U of *Taq* DNA polymerase, and PCR water to 10 μl of volume. Incubate at 70 °C for 30 min, and use 4 μl to ligation with TOPO vector.

9. For each 100 μl of digestion reaction, add 10 μl of sodium acetate 3 N pH 5.2 and 200 μl of ethanol 100%. Incubate at −20 °C for 30 min, and centrifuge during 10 min at

$21,000 \times g$. Then remove the supernatant, and wash the pellet with 70% ethanol. Remove the supernatant, and dry the pellet 5 min at room temperature. Overdrying can make DNA hard to redissolve. Remember that DNA should be resuspended drunk but not drowned. Resuspend the pellet in 50 μl of water DNase-free.

10. The procedures that involve use of cells should be done in a laminar flow hood.

11. A day before, plate 911 cells (5×10^3 cells/well) in a 96-well plate. Then infect the cells with 100 μl of serial dilution of the adenovirus bulk virus suspension. Between 5 and 10 days, you can see virus clones by CPE. Choose the well seeded with the maximum dilution that produces CPE. Then scale the virus cloned, first adding the infected cells plus supernatant (make three cycles of freeze and thaw) in a 60 mm and then in 100 mm tissue culture dishes.

12. Selection of the suitable cells is another issue. Here are some tips; the cells should be easily transfected or susceptible to adenoviral infection. The cells also, at least for the first assays, should have your promoter active, and finally cells should be compatible with common culture techniques.

13. Alternatively, you can use only a luciferase assay and refer the RLUs to total protein of the samples. In that case to solve the problem of measured total protein of your sample which is dissolved in luciferase buffer, you must use DC Protein Assay (Bio-Rad).

14. MOI (multiplicity of infection) is the ratio of virus to target cells. For example, for MOI of 500 of 5×10^4 cells, you must add 2.5×10^7 viral particles (vp) to each well. Each stock virus has a titer, for example, 10^{12} vp/ml, so in this case you should add 0.025 μl of stock or 2.5 μl of a dilution 1/100 of the same stock.

15. To sonicate in Branson Digital Sonifier, set at 50% amplitude three times for 2 s each with at least 1 min rest on ice between each two-second pulse. If lysate is still viscous, repeat sonication.

References

1. Xiong J, Sun WJ, Wang WF, Liao ZK, Zhou FX, Kong HY, Xu Y, Xie CH, Zhou YF (2012) Novel, chimeric, cancer-specific, and radiation-inducible gene promoters for suicide gene therapy of cancer. Cancer 118:536–548

2. Viale DL, Cafferata EG, Gould D, Rotondaro C, Chernajovsky Y, Curiel DT, Podhajcer OL, Veronica Lopez M (2013) Therapeutic improvement of a stroma-targeted CRAd by incorporating motives responsive to melanoma microenvironment. J Invest Dermatol 133 (11):2576–2584

3. Hogg RT, Garcia JA, Gerard RD (2010) Adenoviral targeting of gene expression to tumors. Cancer Gene Ther 17:375–386

4. Podhajcer OL, Benedetti L, Girotti MR, Prada F, Salvatierra E, Llera AS (2008) The role of the matricellular protein SPARC in the dynamic interaction between the tumor and the host. Cancer Metastasis Rev 27 (3):523–537

5. Lopez MV, Blanco P, Viale DL, Cafferata EG, Carbone C, Gould D, Chernajovsky Y, Podhajcer OL (2006) Expression of a suicidal gene under control of the human secreted protein acidic and rich in cysteine (SPARC) promoter in tumor or stromal cells led to the inhibition of tumor cell growth. Mol Cancer Ther 5:2503–2511

6. Lopez MV, Viale DL, Cafferata EG, Bravo AI, Carbone C, Gould D, Chernajovsky Y, Podhajcer OL (2009) Tumor associated stromal cells play a critical role on the outcome of the onco-lytic efficacy of conditionally replicative adeno-viruses. PLoS One 4:e5119

7. Lopez MV, Rivera AA, Viale DL, Benedetti L, Cuneo N, Kimball KJ, Wang M, Douglas JT, Zhu ZB, Bravo AI et al (2012) A tumor-stroma targeted oncolytic adenovirus replicated in human ovary cancer samples and inhibited growth of disseminated solid tumors in mice. Mol Ther 20(12):2222–2233

8. Tellez C, Bar-Eli M (2003) Role and regulation of the thrombin receptor (PAR-1) in human melanoma. Oncogene 22:3130–3137

9. Arisato T, Sarker KP, Kawahara K, Nakata M, Hashiguchi T, Osame M, Kitajima I, Mar-uyama I (2003) The agonist of the protease-activated receptor-1 (PAR) but not PAR3 mimics thrombin-induced vascular endothelial growth factor release in human smooth muscle cells. Cell Mol Life Sci 60:1716–1724

10. Yin YJ, Salah Z, Maoz M, Even Ram SC, Ocha-yon S, Neufeld G, Katzav S, Bar-Shavit R (2003) Oncogenic transformation induces tumor angiogenesis: a role for PAR1 activation. FASEB J 17:163–174

11. Even-Ram S, Uziely B, Cohen P, Grisaru-Granovsky S, Maoz M, Ginzburg Y, Reich R, Vlodavsky I, Bar-Shavit R (1998) Thrombin receptor overexpression in malignant and phys-iological invasion processes. Nat Med 4:909–914

12. Henrikson KP, Salazar SL, Fenton JW 2nd, Pentecost BT (1999) Role of thrombin recep-tor in breast cancer invasiveness. Br J Cancer 79:401–406

13. Rudroff C, Schafberg H, Nowak G, Weinel R, Scheele J, Kaufmann R (1998) Characteriza-tion of functional thrombin receptors in human pancreatic tumor cells (MIA PACA-2). Pancreas 16:189–194

14. Wojtukiewicz MZ, Tang DG, Ben-Josef E, Renaud C, Walz DA, Honn KV (1995) Solid tumor cells express functional "tethered ligand" thrombin receptor. Cancer Res 55:698–704

15. Kaushal V, Kohli M, Dennis RA, Siegel ER, Chiles WW, Mukunyadzi P (2006) Thrombin receptor expression is upregulated in prostate cancer. Prostate 66:273–282

16. Marshall O (2007) Graphical design of primers with PerlPrimer. Methods Mol Biol 402:403–414

17. Dmitriev I, Krasnykh V, Miller CR, Wang M, Kashentseva E, Mikheeva G, Belousova N, Curiel DT (1998) An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsack-ievirus and adenovirus receptor-independent cell entry mechanism. J Virol 72:9706–9713

18. Fallaux FJ, Kranenburg O, Cramer SJ, Hou-weling A, Van Ormondt H, Hoeben RC, Van Der Eb AJ (1996) Characterization of 911: a new helper cell line for the titration and propa-gation of early region 1-deleted adenoviral vec-tors. Hum Gene Ther 7:215–222

19. Decker KB, Hinton DM (2013) Transcription regulation at the core: similarities among bac-terial, archaeal, and eukaryotic RNA poly-merases. Annu Rev Microbiol 67:113–139

20. Arimbasseri AG, Rijal K, Maraia RJ (2014) Comparative overview of RNA polymerase II and III transcription cycles, with focus on RNA polymerase III termination and reinitiation. Transcription 5:e27639

21. Juven-Gershon T, Kadonaga JT (2010) Regu-lation of gene expression via the core promoter and the basal transcriptional machinery. Dev Biol 339:225–229

22. Dreos R, Ambrosini G, Perier RC, Bucher P (2015) The eukaryotic promoter database: expansion of EPDnew and new promoter anal-ysis tools. Nucleic Acids Res 43:D92–D96

23. Reese MG (2001) Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. Comput Chem 26:51–56

24. Halees AS, Leyfer D, Weng Z (2003) Promo-Ser: a large-scale mammalian promoter and transcription start site identification service. Nucleic Acids Res 31:3554–3559

25. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayer-lein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcrip-tion factor binding sites. Bioinformatics 21:2933–2942

26. Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M (1996) Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. J Virol 70:4805–4810

27. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 89:271–277

28. Jakubczak JL, Ryan P, Gorziglia M, Clarke L, Hawkins LK, Hay C, Huang Y, Kaloss M, Marinov A, Phipps S et al (2003) An oncolytic adenovirus selective for retinoblastoma tumor suppressor protein pathway-defective tumors: dependence on E1A, the E2F-1 promoter, and viral replication for selectivity and efficacy. Cancer Res 63:1490–1499

29. Martin-Duque P, Jezzard S, Kaftansis L, Vassaux G (2004) Direct comparison of the insulating properties of two genetic elements in an adenoviral vector containing two different expression cassettes. Hum Gene Ther 15:995–1002