Chapter 16

Application of Synthetic Tumor-Specific Promoters Responsive to the Tumor Microenvironment

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Abstract

Activity of endogenous promoters can be altered by including additional responsive elements (REs). These elements can be responsive to features of the tumor environment or alternatively to signaling pathways specifically activated in cancer cells. These REs incorporated into tumor-specific promoters can improve cancer targeting, the replicative capacity, and lytic activity of conditionally replicative adenovirus. Here we outline an approach to incorporate hypoxia and inflammation REs into a specific fragment of the SPARC promoter and the steps to clone a nucleosome positioning sequence (NPS) identified in the osteocalcin promoter that contains a Wnt RE upstream of a heterologous synthetic promoter.

Key words Synthetic tumor-specific promoter, Hypoxia-responsive element, Nuclear factor kappa B-responsive element, Wnt-responsive element, Nucleosome positioning sequence, Adenovirus

1 Introduction

This chapter describes the construction and functional analysis of synthetic promoters designed for oncolytic viral applications. Tumor-specific promoters (TSP) were engineered with RE to the tumor microenvironmental features. Many TSPs have been identified, characterized, and incorporated in the design of different types of viruses to drive their replication and lytic capacity in experimental models and clinical trials in cancer therapy.

Examples of TSPs include the human telomerase reverse transcriptase (hTERT) promoter, active in multiple tumor types, and the prostate cancer-specific PSA promoter [1, 2]. Other examples of promoters driving viral replication are those obtained from the gene encoding the oncofetal carcinoembryonic antigen (CEA) and A33 antigen whose expression is reactivated in different types of adenocarcinomas [3–5]. Secreted protein acidic and rich in cysteine (SPARC) is a secreted protein involved in cell-extracellular matrix interactions [6]. Different groups have shown that SPARC

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overexpression is associated with increased aggressiveness and worse prognosis of malignant melanoma and other human cancers [6, 7]. An oncolytic adenovirus whose replication is driven by the SPARC promoter was able to inhibit the in vivo growth of established human melanomas and ovarian cancer tumors in nude mice [8].

A common feature of solid tumors is the presence of hypoxic regions, especially in more aggressive tumors, due to the proliferation of the tumor mass that rapidly exhausts nutrient availability in the absence of vascular supply. Due to its central role in tumor progression and resistance to various anticancer therapies, it has been suggested that hypoxia could be considered as a valuable target in the design of novel medicines in cancer treatment [9]. Under hypoxia, the labile HIF-1α subunit is stabilized, forms dimers with HIF-1β, and translocates to the nucleus, followed by binding to hypoxia response elements (HREs) [10]. Another paradigmatic characteristic of tumors is the generation of a pro-inflammatory microenvironment in which cytokines secreted by activated infiltrating immune cells activate transcription factors such as nuclear factor kappa B (NFκB), which translocate to the nucleus and activate the transcription of specific genes [11, 12].

The Wnt/β-catenin signaling pathway is involved in the development and progression of a significant proportion of human cancers [13]. Wnt stimulation leads to the nuclear translocation of β-catenin and transcriptional activation of target genes that function during both normal and malignant development. Constitutive activation of the Wnt pathway leads to inappropriate nuclear accumulation of β-catenin and gene transactivation, an important step in cancer progression, particularly in gastrointestinal cancer [14].

Eukaryotic DNA is packaged in nucleosome cores, with a 147 bp DNA wrapped around an octameric core of histones and with varying length of linkers between adjacent cores [15]. Nucleosomes have a major effect on gene transcription. Studies on DNA kinetics show that DNA wrapping of the nucleosomes is surprisingly dynamic with rapid uncoiling allowing the access of transcription factors (such as NFκB, HIF, or β-catenin) followed by rewrapping [15]. Genomic DNA sequences show considerable variability in their binding affinity to the histone octamer; these DNA regions or nucleosome positioning sequences determine the location and distribution of nucleosomes [16]. Therefore, the use of NPS in combination with DNA RE to cancer elements can be used to engineer TSPs and enhance their activity.

2 Materials

2.1 Cloning of RE in a Plasmid and Adenoviral Context

1. Sterile LB broth and LB agar plates supplemented with 50 μg/mL ampicillin or kanamycin.
2. Chloroform prechilled to 4 °C.
3. Ethanol (100%) prechilled to −20 °C.
4. Phenol/chloroform/isoamyl alcohol (25:24:1).
5. Restriction enzymes (New England Biolabs).
6. T4 DNA ligase, 10× T4 DNA ligase buffer.
7. Duplex buffer 100 mM potassium acetate; 30 mM Hepes, pH 7.5.
8. Taq polymerase, $MgCl_2$, and polymerase buffer (Invitrogen).
9. Plasmids:

   pGL3-F512-SPARC promoter.

   pGNL6 (containing NFκB-responsive elements).

   pH-Luc (containing HREs) was kindly provided by Dr. David Gould.

   pOC3.4 plasmid.

   pShuttle vector (Agilent Technologies).

   pShuttleI-Xp-Luc vector [3].

   pShuttle-XP-E1A vector [3].

   pAdEasy-1 vector (Agilent Technologies).

   pVK500C 5/3 [17].
10. QIAquick Gel Extraction Kit (Qiagen).
11. Qiagen Plasmid Midi Kit.
12. Bacterial strains: BJ5183, ElectroMAX™ DH5α cells, and DH5α chemically competent.
13. Electroporation cuvettes (BioRad).
14. Electroporator (BTX).
15. Complete medium: DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, 2 mM glutamine.
16. Temperature-regulated water bath.
17. MultiGene OptiMax thermal cycler (Labnet).
18. Lipofectamine® LTX (Thermo Fisher Scientific).
19. T4 DNA polymerase (Invitrogen).
20. 100 mM dNTPs set (Invitrogen).
21. Multi-block heater (Lab-Line).

22. Polyallomer centrifuge tubes (15 × 95 mm) (Beckman Coulter).
23. Cesium chloride solutions.

    D = 1.3 g/cc 55.00 g. CsCl add Tris-Cl 10 mM (pH 7.8) to **185 g**.

    D = 1.6 g/cc 58.33 g. CsCl add Tris-Cl 10 mM (pH 7.8) to **114 g**.

    D = 1.34 g/cc 106.59 g. CsCl add Tris-Cl 10 mM (pH 7.8) to **250 mL**.

    Sterilize by autoclaving.
24. Mineral oil (Sigma-Aldrich).
25. Spectrometer.

2.2 Cell Culture

1. A375N melanoma (ATCC, CRL-1619), low passage HEK 293 cells (ATCC CRL-1573), and HER 911 cells [18].
2. Complete medium: DMEM and DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin/streptomycin, and 2 mM glutamine.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and autoclave sterilized.
4. Ad WTs: Ad-5-WT (ATCC, VR-1516) and Ad-5/3-WT (was kindly provided by Dr. David T. Curiel).
5. Tumor necrosis factor-α (TNF-α) (Sigma-Aldrich).
6. Hypoxic chamber (Billups-Rothenberg Inc., Del Mar, CA).
7. Cell titer Promega/MTS solutions (Promega Corp).

2.3 Luciferase Assay

1. Luciferase reporter assay system (Promega Corp).
2. White 96 well-plates.
3. Plate-reading GENios luminometer (Tecan Group AG, Austria).
4. Pierce BCA Protein Assay.

3 Methods

3.1 Cloning of Responsive Motifs in Plasmid and Adenoviral Context

Since DNA RE can activate transcription at different distances from the transcription start, in our studies we have assessed different variants. Here we outline the methods to clone NFκB-REs upstream of the SPARC promoter (*see* **Note 1**) and HREs between bases −314 and −265. A similar approach could be used to introduce REs into other promoters.

1. Firstly, determine restriction enzymes that cut upstream or within your promoter of interest. When we cloned NFκB-RE or HRE into the SPARC promoter construct, we firstly digested 10 μg of promoter plasmid (pGL3-F512-SPARC promoter) upstream of the promoter with 10 U of *MluI* or within the promoter with *StuI* in a final volume of 50 μL. Incubate the digest at 37 °C for 4 h.
2. Heat inactivate the enzyme for 15 min at 65 °C.
3. *StuI* produces a blunt cut but the 5′ protruding end of *MluI* sites are blunt by addition of an appropriate amount of 10× T4 polymerase buffer and fresh dNTPs to 100 μM. Incubate at room temperature for 30 min with 1 U T4 DNA polymerase/μg DNA (*see* **Note 2**).
4. Next, heat inactivate T4 polymerase for 10 min at 70 °C. Residual protein can be removed by one round of phenol/chloroform extraction and alcohol precipitation (*see* **Note 3**).
5. Chill on ice.
6. Prepare repeats of the required RE; this can be through the use of annealed oligonucleotides (*see* Subheading 3.3) or by removing repeats from another vector. We cut six repeats of the NFκB-RE from the vector pGNL6 [19] with *StuI* or repeats of the HRE with *StuI-SmaI* from the vector pH-Luc [20]. Then isolate released RE repeats by electrophoresis using 1% agarose gel, 100 V followed by DNA gel extraction using QIAquick Gel Extraction Kit.
7. T4 DNA ligase buffer should be thawed and mixed at room temperature. Combine in a molar ratio of 3:1 insert/vector in a ligation reaction and gently mix by pipetting up and down and microfuge briefly. For blunt ends or single base overhangs, incubate with 80 U of T4 DNA ligase at 16 °C overnight or room temperature for 2 h (alternatively, higher concentration T4 DNA ligase (400 U) can be used in a 10 min ligation (*see* **Note 4**)). Heat inactivate the ligase enzyme at 65 °C for 10 min (*see* **Note 5**).
8. Chill on ice and transform 1–5 μL of the reaction into 100 μL of chemically competent bacteria.
9. Then perform a shock of 1.5 min at 42 °C, incubate on ice for 1 min, and recover in 1 mL during 1 h at 37 °C without antibiotic in LB medium. Plate 100 μL of the cells in agar with antibiotic (ampicillin or kanamycin) and incubate overnight at 37 °C.
10. Correct clones of pGL3-kBF512HRE are determined by restriction analysis of mini-prep DNA and confirmed by sequencing. The scheme for the construction of pGL3-kBF512HRE is shown in Fig. 1a.

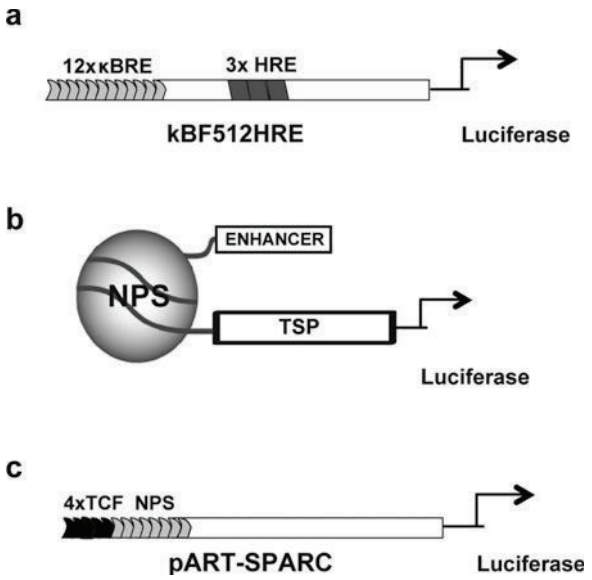

Fig. 1 Schematic representation of heterologous synthetic promoters. (**a**) kBF512HRE promoter. Incorporation of hypoxia and inflammation REs into a specific fragment of the SPARC promoter. (**b**) pART. Schematic representation of the potential spatial conformation of Enhancer-NPS-TSP sequences in the nuclear environment. (**c**) pART-SPARC. Incorporation of nucleosome positioning sequence (NPS) and Wnt RE upstream of specific fragment of the SPARC promoter

REs can also be combined with other DNA sequences in order to improve promoter activation. In the next two sections, we describe how to combine the REs (T-cell factor/lymphoid enhancer factor—TCF/LEF sites) for Wnt transcription factors and a nucleosome positioning sequence upstream of the SPARC gene promoter fragment. Figure 1b shows the schematic representation of the potential spatial conformation of Enhancer-NPS-TSP sequences in the nuclear environment.

3.2 Amplification of the NPS from the Rat Osteocalcin Gene

1. Firstly, amplify the 182 bp sequence containing the NPS and adjacent sequence from the osteocalcin (OC) gene; for this we use a pOC3.4 plasmid [21] containing the rat OC gene and the following specific primers:

   Sense: 5′-A*CTCGAG*GTCTCTAGGGCCAGCCAGT-3′

   Antisense: 5′-C*GAGCTC*AGGAGATGCTGCCAGGACTA-3′
2. The sense primer includes a 5′ *Xho*I site, and a *SacI* site is included in the antisense primer, and both are in italics.
3. Next, digest 5 μg of the amplified product with *XhoI/SacI*, and isolate the fragment by electrophoresis using 1% agarose gel, 100 V followed by QIAquick Gel Extraction Kit.
4. Store the product at −20 °C until use.

3.3 Construction of the 4× TCF/LEF Sites Responsive to Wnt/β-Catenin

1. Design primers with four copies of the TCF site. Below is the one we designed (with the first TCF site underlined) with *KpnI* (5′ italic sequence) and *XhoI* (3′ italic sequence) ends:

   *GGTACC*CTTACCCGCTACAAAGATTACCCGCTACAAA
   GATTACCCGCTACAAAGATTACCCGCTACAAAGAT
   TACCCC*CTCGAG*.

2. Anneal the sense sequence containing the 4× TCF sites with the corresponding antisense sequence generating a double strand DNA sequence to be inserted upstream to the NPS. Oligonucleotide annealing is performed in four steps:
3. Oligonucleotide resuspension: spin down lyophilized oligonucleotides and dissolve the pellet in 100 μL of duplex buffer at a concentration of 100 μM. Heat (around 94 °C) and mix (brief vortex) to facilitate resuspension.
4. Mix both oligonucleotides together at a concentration of 10 μM diluted in duplex buffer.
5. Anneal the oligonucleotides by heating to 94 °C for 2 min in a heat block. Then transfer to room temperature to permit a gradual cool down.
6. Store the annealed product at −20 °C until use.

3.4 Prepare the Cassette pART-SPARC

1. Clone into pCR4 Topo vector the product obtained from Subheading 3.3 (4× TCF fragment).
2. To clone this product, we use the following mix:

   4 μL of annealed fragment.

   1 μL of saline solution (1.2 M NaCl/0.06 M $MgCl_2$).

   1 μL of pCR4 Topo vector (Invitrogen).

   Total volume of ligation (6 μL).

   Incubate at room temperature for 30–60 min.

3. Transform bacteria by incubating 100 μL of chemically competent bacteria with the ligation mixture for 10 min on ice, then perform a heat shock for 1.5 min at 42 °C, again incubate on ice for 1 min, and then recover bacteria in 1 mL LB medium without antibiotic at 37 °C with shaking for 1 h. Plate 0.1 mL of the cells on agar with antibiotic (ampicillin or kanamycin) and incubate overnight at 37 °C.
4. 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.
5. Prepare a midi-prep of the correct promoter clone.

6. Next, digest 5 μg of the plasmid containing 4× TCF fragment with *XhoI/SacI* in order to clone downstream NPS fragment obtained in Subheading 3.2 for the generation of a pART (4× TCF + NPS) containing plasmid.
7. Digest 5 μg of plasmid containing pART with *KpnI/SacI* restriction enzymes during 4 h at 37 °C, and isolate the pART fragment by electrophoresis using 1% agarose gel, 100 V followed by QIAquick Gel Extraction.
8. Digest 5 μg of pGL3-F512-SPARC promoter with the same enzymes used in **step 7** during 4 h at 37 °C, and then inactivate the enzymes at 65 °C for 20 min.
9. Dephosphorylate the cut pGL3-F512-SPARC promoter vector with alkaline phosphatase CIP 1 h at 37 °C, and isolate the linearized pGL3-F512-SPARC fragment by electrophoresis using 1% agarose gel, 100 V followed by QIAquick Gel Extraction.
10. Ligate the purified pART fragment with pGL3-F512-SPARC promoter vector at a molar ratio of 1:3 of vector to insert using 1 μL of T4 DNA ligase at 16 °C overnight to obtain the pART-SPARC construction.
11. Correct cloning of pART-SPARC can be confirmed through restriction pattern with *KpnI/BglII* and by automatic sequencing using the following primers:

    pGL3FWD: 5′-CTAGCAAAATAGGCTGTC-3′

    LUC compl: 5′-CGCCGGGCCTTTCTTTATG-3′

Figure 1c shows the schematic representation of pART-SPARC construction.

3.5 Clone Promoters into an Ad Shuttle Vector

pShuttle vector is an entry vector to clone sequences in the viral E1A region that is usually deleted in non-replicative adenoviral vectors. In pShuttle-I-Xp, we have modified this vector so that the RSV promoter is replaced with a multiple cloning site so that synthetic promoters can be readily cloned. Upstream from the MCS, we have introduced an insulator element so that the ITR does not alter the activity of inserted promoters. Downstream from the gene, we have positioned the termination signal from the bovine growth hormone gene. The activity of inserted promoters is monitored with the pShuttle-I-Xp-luc vector containing luciferase as a reporter gene [17].

1. Clone the synthetic promoter of interest in the plasmid vector pShuttle-I-Xp-luc or a similar vector; we restrict the vector pShuttle-I-Xp-luc at the *SpeI/SalI* sites for this purpose.

2. Prepare synthetic promoters either as restricted fragments released from vectors or as digested PCR products with compatible sites *SpeI/SalI*.
3. Isolate the fragments by electrophoresis using 1% agarose gel at 100 V followed by QIAquick Gel Extraction.
4. Ligate promoter inserts and the pShuttle-I-Xp-luc vector, and use correct clones to assess promoter activity in transfected cells.

3.6 Construction of Non-replicative Adenoviral Vectors

1. In order to prepare the non-replicative adenoviral vector, linearize the shuttle vector with *PmeI*.
2. Inactivate the enzyme by heating at 65 °C for 20 min.
3. For homologous recombination, co-transform the digested plasmid with pAdEasy-1 vector or pVK500C 5/3 into *E. coli* BJ5183 by electroporation.
4. Prechill two DNase-free microcentrifuge tubes and two electroporation cuvettes (0.2 cm gap) on ice.
5. Remove three aliquots of BJ5183 electroporation competent cells from −80 °C storage and thaw on ice.
6. Gently pipet 40 μL of the competent cells into each of the chilled microcentrifuge tubes.
7. Pipet 1 μL (1 μg) of linearized, dephosphorylated shuttle vector and 1 μL of pAdEasy-1 or pVK500C 5/3 supercoiled vectors (100 ng/μL) into one of the tubes. Mix by tapping the tube gently and keep on ice.
8. Pipet (1 μL) 1 μg of linearized, dephosphorylated shuttle vector into the second tube. Mix by tapping the tube gently and keep on ice. This controls for background contributed by the shuttle vector.
9. Set the electroporator to the following settings by referring to the instructions provided with the instrument: 200 Ω, 2.5 kV, 25 μF.
10. Transfer the contents of one microcentrifuge tube from **step 4** into one of the chilled electroporation cuvettes, and tap the cuvette gently to settle the mixture to the bottom.
11. Slide the cuvette into the electroporation chamber until the cuvette connects with the electrical contacts.
12. Pulse the sample once, and then quickly remove the cuvette. Immediately add 1 mL of sterile LB broth, and pipet up and down to resuspend the cells [22].
13. Transfer the cell suspension to a sterile 1.8 mL Eppendorf tube.

14. Repeat the electroporation for the other transformation reaction. Incubate all of the transformations at 37 °C for 1 h while shaking at 500–550 rpm.
15. For the recombination reaction and associated control, plate the entire volume of recovered cells onto three LB-kanamycin plates (e.g., three plates containing 100, 250, 650 μL, respectively).
16. Use a sterile spreader to spread the mixture.
17. Incubate the plates overnight at 37 °C.
18. Pick 10–20 of the smallest colonies, and grow each in 2 mL LB medium containing 50 μg/mL kanamycin for 10–15 h in a 37 °C orbital shaker.
19. Perform mini-preps. We recommend you use the conventional alkaline lysis method [23].
20. Sequence and amplify the selected positive clones by transforming ElectroMAX™ DH5α cells followed by DNA midi-prep using Qiagen Plasmid Midi Kit.

3.7 Production of Non-replicative Adenovirus

1. To produce a non-replicative adenovirus, the plasmid produced in Subheading 3.6 above should be linearized with *PacI* and purified by ethanol precipitation (*see* **Note 3**).
2. Mix 10–20 μg of linearized plasmid with LTX Lipofectamine (30 μL), and then incubate at room temperature in 1.5 mL of DMEM/F12 medium. Then add the mix dropwise to 911 cells (at 70–80% confluence) in 10 cm tissue culture plate with 5 mL of DMEM/F12 containing 5% FBS.
3. Incubate in a CO_2 incubator for 6 h, and then remove the medium and add 10 mL of DMEM/F12 containing 10% FBS. Incubate for 15 days.
4. Keep the plates without media change for the three first days (*see* **Note 6**).
5. When over 95% of the HEK 293 cells have detached from the plates due to cytopathic effect (CPE), scrape the monolayer into the medium and transfer the suspension into a falcon tube and freeze (−80 °C).
6. Then perform adenovirus purification by standard cesium chloride banding (*see* **step 11**).
7. Or for the large-scale amplification, plate HEK-293 cells in 30 × 15cm dishes (approximately 10^7 cells per dish) so that they are 90–100% confluent at the time of infection 6–15 h later.
8. Infect HEK-293 cells with viral supernatant at a multiplicity of infection of approximately ten infectious viral particles per cell. When all infected cells round up (usually at 1–2 days after

infection), collect the infected cells from all dishes. Centrifuge for 10 min at approximately 1250 × *g* in a benchtop centrifuge and remove supernatant. *See* **Note 7**.

9. Combine cell pellets and resuspend the pellet in 8.0 mL sterile PBS. Perform four freeze-thaw cycles to release the viruses from cells.
10. Centrifuge viral lysate for 10 min in a Sorvall centrifuge at 5000 × *g* at room temperature.
11. Add 2.4 mL of CsCl density of 1.6 g/cc to each polyallomer tube. Gently overlay with 2.4 mL of CsCl density of 1.3 g/cc.
12. Apply 4 mL of cleared virus supernatant to the top of each gradient.
13. Top off tubes with mineral oil.
14. Spin at 200,000 × *g* in a SW40 rotor at 4 °C, for 2 h.
15. Collect virus band with a needle and syringe by piercing the side of the tube.
16. Apply the collected virus on the top of 5 mL of CsCl density of 1.34 g/cc.
17. Top off tubes with mineral oil.
18. Spin at 200,000 × *g* in a SW40 rotor at 4 °C, for 16–20 h.
19. Collect virus band with a needle and syringe by piercing the side of the tube (*see* **Note 8**).
20. Purify the virus using PD-10 desalting columns containing Sephadex G-25 medium.

3.8 Determination of Viral Titer

1. Determine viral titer by optical density, and the results are less reliable than the functional tests. One OD unit (A260) contains approximately 10^{12} viral particles/mL.
2. The 50% tissue culture infectious dose (TCID50) method is based on the development of CPE in HEK-293 cells using endpoint dilutions to estimate the titer. This is the standard titration method used (AdEasy vector system).
3. Store the purified virus in small aliquots at −70 °C with glycerol to a final concentration of 10%.

3.9 Assessment of the Activity of the DNA Responsive Motifs in Non-replicative Adenoviruses Expressing Luciferase

1. Plate 4×10^4 A375N cells per well in 24-well plates (*see* **Note 9**.)
2. After 24 h transduce the cells with Ad-Luc [multiplicity of infection (MOI), 100] in 200 μL of DMEM/F12 containing 2% FBS (*see* **Note 10**).
3. 4 h later add 800 μL of fresh medium containing serum.
4. Place transduced cells in normoxic (21% O_2) or hypoxic (0.1% O_2) conditions or stimulate with TNF-α (5 ng/mL). TNF-α-stimulated cells placed in hypoxia will demonstrate the effect of combined stimulation.

5. 48 h later, harvest cells and perform a luciferase assay on cell lysates by following the manufacturer's recommendations, and determine activity using an automated luminometer.
6. The luciferase activity can be normalized by total protein content (*see* **Note 11**) of the cells lysates.

3.10 Construction of the Conditionally Replicative Adenovirus

To prepare the oncolytic vector, the luciferase gene should be replaced by the adenoviral E1A gene.

1. Amplify the viral E1A gene (560–1632) from Ad 5 WT adenoviral genome using specific primers:

   Sense: 5′- GAAGCTTATGAGACATATTATCTGCCA-3′.

   Antisense: 5′-GTCGACTTATCTCACCCTTTATTAAACTT-3′.

   The primers include a 5′ *HindIII* site in the sense primer and a *SalI* site in the antisense primer.
2. Release the luciferase gene by digesting 20 μg of pShuttle-I-Xp-Luc with *HindIII/SalI* in a final volume of 50 μL. Incubate at 37 °C for 4 h and isolate the vector by electrophoresis using 1% agarose gel at 100 V followed by DNA gel purification.
3. Clone the E1A fragment in *HindIII/SalI* sites to obtain the final pShuttle-I-Xp-E1A.
4. Confirm correct cloning through restriction patterns and by automatic sequencing.
5. Conditionally replicative virus is then obtained by *PmeI* linearization of the E1A containing shuttle vector and following the steps in Subheadings 3.6 and 3.7.

In order to analyze the transcriptional activity of promoters previously constructed, we try to replicate features of the tumor microenvironment. In the case of plasmids containing HREs, we use a hypoxic chamber. To mimic inflammatory conditions present in tumors, we stimulate cells with TNF-α which activates NFκB.

3.11 Assessment of the Activity of the DNA Responsive Motifs in an Oncolytic Adenovirus

1. Plate 4×10^4 A375N cells per well in 24-well plates.
2. After 24 h infect the cells with CRAds (multiplicity of infection (MOI), 0–100) and Ad-WTs as a control of maximal lytic activity (MOI, 0–100) in 200 μL of DMEM/F12 containing 2% FBS.
3. 4 h later add fresh medium containing serum (800 μL).
4. Place the cells in normoxic (21% O_2) or hypoxic (0.1% O_2) conditions or stimulate with TNF-α (5 ng/mL). Stimulated cells placed in hypoxia will demonstrate the effect of combined stimulation.

5. Six days later wash cells with 500 μL of PBS, and add 100 μL of MTS solution following manufacturer's recommendations.
6. Measure absorbance at 490 nm using a microplate reader.
7. The lytic activity is determined as % of cellular survival, referred to control without infection or specific treatments such as TNF-α stimulation or hypoxic conditions.

4 Notes

1. The SPARC promoter vector pGL3-F512 (containing the −513/+35 region of the human SPARC promoter driven luciferase gene) was described in previous work [17].
2. Do not heat T4 polymerase over 70 °C, or most of your DNA will get caught in the complex and trapped in the gel well. T4 poly can be used in NEB Buffers 1.1, 2.1, and 3.1 and CutSmart® Buffer as well as NEB Buffers 1–4 and T4 DNA ligase reaction buffer.
3. Alcohol precipitation procedure:

   Add 1/10 volume of sodium acetate (3 M, pH 5.2), and then add 2.4× volume (calculated after addition of sodium acetate) of 100% ethanol.

   Incubate on ice for 15 min. In case of small DNA fragments or high dilutions, overnight incubation gives best results, and incubation below 0 °C does not significantly improve efficiency.

   Centrifuge at 14,000 × *g* for 30 min at 4 °C.

   Discard supernatant being careful not to throw out DNA pellet which may or may not be visible.

   Rinse with 70% ethanol.

   Centrifuge again for 15 min at room temperature.

   Discard supernatant and dissolve pellet in desired buffer. Make sure the buffer comes into contact with the whole surface of the tube since a significant portion of DNA may be deposited on the walls instead of in the pellet.

   This last step should be done with sterile water in the hood if you plan to use the plasmid for transfection into animal cell.
4. For cohesive (sticky) ends, incubate at 16 °C overnight or room temperature for 10 min.
5. T4 DNA ligase should be added last. Note that the ligation reaction below shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA.

20 μL of reaction.

2 μL of 10× T4 DNA ligase buffer.

0.020 pmol of DNA vector.

0.006 pmol of DNA insert.

1 μL of T4 DNA ligase.

Nuclease-free water to 20 μL.

6. You can split the cells and expand the 10 cm plates into two or three 15 cm plates. Keep the plates for 15 days without change of medium. You can add 1–2 mL fresh complete DMEM every 5–7 days after transfection.
7. The virus-containing waste should be disinfected with chlorine bleach.
8. To collect virus band, puncture the side of the tube just below the virus band with a needle and syringe. Collect the virus band in the smallest volume possible.
9. All the procedures should be performed in a laminar flow hood.
10. Ad-Luc refers to adenoviruses whose activity is driven by different synthetic promoters.
11. We use Pierce BCA Protein Assay (green-to-purple ($A_{562\text{nm}}$)), a two-component, detergent-compatible assay reagent to measure total protein concentration vs. protein standard.

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