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AAVPG: A vigilant vector where transgene expression is induced by p53

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ABSTRACT

Using p53 to drive transgene expression from viral vectors may provide on demand expression in response to physiologic stress, such as hypoxia or DNA damage. Here we introduce AAVPG, an adeno-associated viral (AAV) vector where a p53-responsive promoter, termed PG, is used to control transgene expression. *In vitro* assays show that expression from the AAVPG-luc vector was induced specifically in the presence of functional p53 (1038 ± 202 fold increase, $p < 0.001$). The AAVPG-luc vector was an effective biosensor of p53 activation in response to hypoxia (4.48 ± 0.6 fold increase in the presence of 250 μM $CoCl_2$, $p < 0.001$) and biomechanical stress (2.53 ± 0.4 fold increase with stretching, $p < 0.05$). *In vivo*, the vigilant nature of the AAVPG-luc vector was revealed after treatment of tumor-bearing mice with doxorubicin (pre-treatment, $3.4 \times 10^5 \pm 0.43 \times 10^5$ photons/s; post-treatment, $6.6 \times 10^5 \pm 2.1 \times 10^5$ photons/s, $p < 0.05$). These results indicate that the AAVPG vector is an interesting option for detecting p53 activity both *in vitro* and *in vivo*.

Introduction

Innumerous studies have shown that the tumor suppressor p53 plays a central role in the cellular response to a variety of physiological stimuli. p53 is typically thought of as a master regulator of the cell cycle arrest and cell death due its ability to activate or repress the expression of critical target genes upon DNA damage (Beckerman and Prives, 2010). However, p53 function is much more complex due to the large number of factors that can lead to p53 activation as well as the variety of physiological outcomes that it mediates.

Findings implicate p53 function extends to metabolism (Bensaad et al., 2006) autophagy (Crighton et al., 2006; Ravikumar et al., 2010) and regulation of microRNA expression (Hermeking, 2007; Junttila and Evan, 2009). In addition to ionizing radiation (Hildesheim et al., 2002; Klokov et al., 2012; Rashi-Elkeles et al., 2011), p53 activity can also be induced by oncogenic stress (Feng et al., 2006; Yan et al., 2005), oxidative stress (Achanta and Huang, 2004; Sinthupibulyakit et al., 2010), signaling cytokines (Doman et al., 1999; Kim et al., 2005), changes in temperature (Bajgelman and Strauss, 2006; Michalovitz et al., 1990; Zhang et al., 1994), hypoxia (Long et al., 1997; Sano et al., 2007; Sutton et al., 2008), chemotherapeutic agents (Chuang et al., 2012; el-Deiry et al., 1994; Lowe et al., 1993; Wang et al., 2005) and biomechanical stress (Leri et al., 1998, 2000; Liao et al., 2004). Molecular tools that reveal the activities of p53 would help deepen our understanding of many cellular processes.

The p53 protein typically functions as a transcription factor controlling the expression of a variety of target genes in order to bring about its pleiotropic effects. In general, these genes contain the canonical p53 binding site (5′–RRRCWWGYYY-3′ where R=G or A, W=T or A, Y=C or T) in their promoter region (el-Deiry et al., 1992). One of the first p53 responsive elements identified (referred to here as the PG element) was derived from the ribosomal gene cluster (Kern et al., 1991) and, when used to drive reporter gene expression, was shown to mediate transactivation by wild-type p53 (Kern et al., 1992). A few examples of p53 target genes include

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p21, GADD45, Mdm-2, and Bax, among others (Barak et al., 1993; el-Deiry et al., 1992, 1993; Funk et al., 1992; Juven et al., 1993; Kern et al., 1991, 1992). Since the transcriptional activities of p53 are central to its function, the expression of p53 target genes can reveal when and where p53 is called to duty.

We have developed biosensors that reveal p53 activity. These tools include recombinant viral vectors where transcription of the gene of interest is controlled by p53. In this way, the viral vector facilitates gene transfer and the observation of reporter gene activity can reveal the induction of p53 function. In our first generation vector, the PG element was used to modify LTR of the pCL retroviral vector (Strauss et al., 2005; Strauss and Costanzi-Strauss, 2004). The pCLPG retrovirus is especially well suited for *ex vivo* laboratory studies due to the biology of retroviral vectors derived from Moloney Murine Leukemia Virus.

For the second generation of p53-responsive vectors, we turned to adenovirus (Ad) since these vectors offer high titer, efficient *in situ* gene transfer and remain episomal, thus avoiding problems associated with viral integration. To this end, we developed a chimeric promoter named PGTxβ, which is a fusion of the p53 responsive PG element (Kern et al., 1991), with the adenovirus E1B minimal promoter (Xavier-Neto et al., 1998) and a rabbit β-globin intron (Schambach et al., 2000). This chimeric promoter was inserted into a recombinant serotype 5 adenoviral vector (termed AdPG) and used to drive expression of the luciferase reporter gene. We observed both specific and high level transgene expression induced by p53 (Bajgelman and Strauss, 2008). Despite the wide use of adenovirus for *in vivo* applications, data from the literature suggest some drawbacks, such as its immunogenicity and short-term expression (Alemany et al., 2000; Olive et al., 2002; Worgall et al., 1997).

Recombinant vectors based on adeno-associated virus (AAV) offer many features that are desirable in a gene transfer vehicle. AAV vectors are much less immunogenic than Ad (Li et al., 2007) and provide long term expression in non-dividing cells (Leger et al., 2011; Rivera et al., 1999). In addition, it is possible to direct viral tropism to a specific tissue by manipulating the serotype or engineering the viral capsid (Asokan et al., 2012; Li et al., 2007; Palomeque et al., 2007). *In situ* gene transfer with AAV vectors is well tolerated and the virus may remain in the target cell for some time. With the use of a conditional promoter to drive expression, the vector can be applied even before the physiologic condition is manifested, thus providing vigilant, 'on-demand' expression of the transgene.

Here we present the development of AAVPG, our third generation vector where transgene expression is driven by p53. Using AAVPG encoding luciferase, we show induction of reporter gene activity 'on-demand' in response to several forms of cellular stress known to activate p53. The AAVPG vigilant vector offers tight control over transgene expression in response to p53 and, thus, may serve as a biosensor of p53 activity.

Results

AAVPG-luc expression is tightly regulated by p53

The three generations of p53-responsive vectors are represented in Fig. 1. The construction of the AAVPG vector began by inserting sequences to direct site-specific homologous recombination in a promoterless AAV2 transfer vector. In parallel, the chimeric promoter responsive to p53, called PGTxβ (Bajgelman and Strauss, 2008), and the gene of interest, luciferase, were inserted in a second plasmid already encoding sequences for site directed recombination that are compatible with the new AAV2 transfer vector. The final vector, AAVPG-luc, was derived by recombination between the two plasmids.

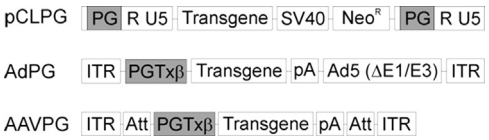

Fig. 1. Schematic representation of the three generations of p53-responsive viral vectors. First generation: pCLPG is a recombinant retroviral vector derived from Moloney murine leukemia virus. The p53-responsive element, PG, was fused with the viral LTR (substituting the U3 element), thus rendering transgene expression under the control of p53 (Strauss and Costanzi-Strauss, 2004). Second generation: AdPG is a serotype 5 adenoviral vector which has a chimeric p53-responsive promoter, PGTxβ, driving expression of the transgene (Bajgelman and Strauss, 2008). Third generation: AAVPG is a recombinant serotype 2 adeno-associated virus that contains the PGTxβ promoter driving expression of the transgene. Construction of AAVPG vectors is aided by site directed recombination at the Att sequence.

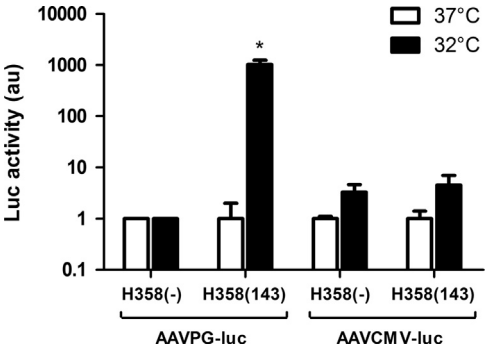

Fig. 2. Robust expression from AAVPG-luc driven by a temperature-sensitive allele of p53. H358(−) cells (that lack endogenous expression of p53) and H358(148) cells (that express a temperature sensitive allele of p53) were transduced with a preparation of AAVPG-luc or AAVCMV-luc, using MOI of 100, and incubated at 37 °C or 32 °C, as indicated. Luciferase activity, presented in arbitrary units (au), was normalized by protein concentration, considering the activity obtained at 37 °C as 1, for the parental lineage H358(−). The graph represents the mean and standard deviation of three independent experiments wherein each condition was performed in duplicate. *$P < 0.001$, two-way ANOVA followed by the Bonferroni post hoc test.

To explore regulation of expression directed by p53, the H358 cell line (human lung carcinoma, p53-null) was modified with the introduction of a retrovirus encoding the temperature sensitive allele of human p53 mutant at codon 143 (Strauss et al., 2005). P53(143) assumes a mutant phenotype when the cells harboring it are cultivated at 37 °C, yet switches to a transcriptionally active conformation at 32 °C (Zhang et al., 1994). As seen in Fig. 2, H358(143) cells transduced with AAVPG-luc (MOI = 100) and cultivated at 32 °C show a 1000 fold induction of reporter activity over cells cultivated at 37 °C. This temperature sensitive induction was not observed in parental H358 cells, which do not have expression of p53. A slight increase in reporter activity was observed when AAVCMV-luc was used to transduce either parental or H358(143) cells which were then cultivated at 32 °C. This apparent induction is likely non-specific and is not strong enough to alter the interpretation of the performance of the AAVPG-luc vector. When induced, the AAVPG vector provides transgene activity that is superior to that seen with the AAVCMV vector, a point that is made clearer when the data is presented with normalization only for protein content (Fig. S1). This assay shows that expression of the transgene encoded by the AAVPG vector is specifically controlled by p53 and that high levels of induction can be achieved.

AAVPG-luc as a biosensor of chemotherapeutic activity

The use of chemotherapeutic agents such as doxorubicin can induce the activity of endogenous p53 (el-Deiry et al., 1994). In this experiment, B16 cells (mouse melanoma, wild-type. p53) were

co-transduced with a mixture of viral preparations AAVPG-luc and AAVCMV-LacZ (serving as an internal control), using a MOI of 30,000, subjected to treatment with increasing concentrations of doxorubicin. As seen in Fig. 3, luciferase reporter activity was correlated with the drug dose. In contrast, we did not find a significant alteration in LacZ expression. Treatment of B16 cells with doxorubicin was associated with the induction of p53-responsive genes p21 and Mdm2 as well as an altered cell cycle profile (Fig. S2). In this way, the AAVPG-luc vector served as a biosensor of the induction of p53 by doxorubicin.

The vector AAVPG-luc can be used as a sensor of hypoxia

We next sought to confirm the utility of AAVPG-luc as a reporter of hypoxia, a condition known to activate p53 (Long et al., 1997; Sano et al., 2007). In this assay, NIH3T3 cells (immortalized mouse fibroblast, wild-type p53) were co-transduced with AAVPG-luc along with AAVCMV-LacZ and treated with cobalt chloride, a hypoxia mimetic. We found that the addition of cobalt chloride induced a dose-dependent increase in the reporter gene activity provided by AAVPG-luc (Fig. 4). In contrast, we found that LacZ activity diminished in response to the increasing concentrations of cobalt chloride, in agreement with reports from the literature (Vordermark et al., 2001). The treatment of NIH3T3 cells with $CoCl_2$ was associated with the induction of p53-target genes p21 and Mdm2 (Fig. S3). These findings suggest a dose-dependent modulation of expression of AAVPG-luc which we attribute to activation of p53.

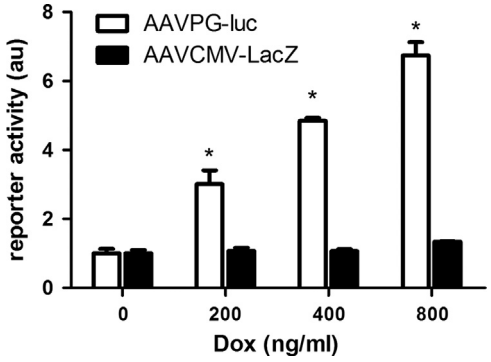

Fig. 3. Expression from AAVPG-luc is regulated by endogenous p53 in B16 cells. Cells were co-transduced with AAVPG-luc and AAVCMV-LacZ, using MOI of 30,000, incubated 24 h at 37 °C before the media was changed to include the indicated amounts of doxorubicin (Dox). Cells were harvested 24 h later for analysis of luciferase activity and β-gal activity. The reporter activities were normalized considering the sample with transduction, but without drug treatment, as 1 and presented in arbitrary units (au). *$P < 0.001$, two-way ANOVA followed by the Bonferroni post hoc test. The graph represents the mean and standard deviation of three independent experiments wherein each condition was performed in duplicate.

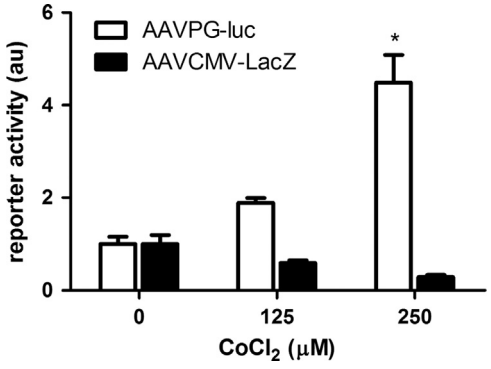

Fig. 4. Hypoxia activates p53 and induces AAVPG-luc expression. NIH3T3 cells were co-transduced with a mixture of AAVPG-luc and AAVCMV-LacZ (MOI=500) and incubated 24 h. Media was changed, adding the indicated concentration of cobalt chloride ($CoCl_2$). Cells were harvested after 24 h when luciferase and β-gal activities were determined. The condition without cobalt chloride was called 1. *$P < 0.001$, two-way ANOVA followed by the Bonferroni post hoc test. The graph represents the mean and standard deviation of three independent experiments wherein each condition was performed in duplicate.

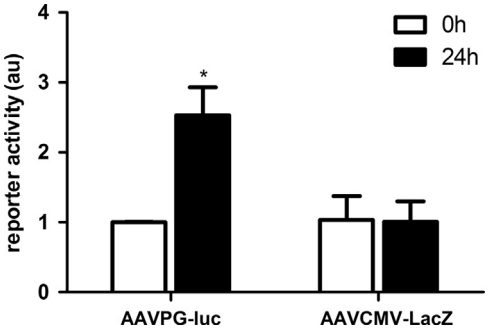

Fig. 5. Biomechanical stress activates p53 and induces AAVPG-luc expression. NIH3T3 cells were seeded on Bioflex® culture plates and co-transduced with AAVPG-luc and AAVCMV-LacZ, using MOI of 500. After 24 h of 10% stretching, cells were harvested to quantify luciferase and β-gal expression. The reporter activity in the absence of stretching was called 1. *$P < 0.05$, two-way ANOVA followed by the Bonferroni post hoc test. The graph represents the mean and standard deviation of three independent experiments wherein each condition was performed in duplicate.

Biomechanical stress activates expression from the AAVPG-luc vector

Data from the literature (Leri et al., 1998; Liao et al., 2004) have suggested that stretching can lead to p53 activation. Here NIH3T3 cells were again co-transduced with AAVPG-luc and AAVCMV-LacZ, but cultivated in the presence of biomechanical stress for 24 h. We observed an increase in luciferase activity when compared with resting (non-stressed) cells, indicating that the expression from the AAVPG-luc vector was modulated by the biomechanical stress (Fig. 5). In contrast, there was no change in LacZ reporter gene activity between resting and stretched cells. In this final example, the AAVPG-luc vector again showed its utility as a biosensor of p53 activity.

In vivo bioluminescent imaging of AAVPG-luc reveals p53 activity

We next evaluated the potential application of AAVPG as an *in vivo* sensor of p53 activity upon induction by doxorubicin. For this, melanoma-derived B16 cells were transduced *ex vivo* with AAV vectors and implanted subcutaneously in syngeneic C57BL/6 mice. In Fig. 6 we show that luciferase activity was increased after doxorubicin treatment only in the AAVPG-luc group. In contrast, there was a reduction in luciferase activity in the AAVCMV-luc group upon drug treatment. This assay demonstrates the vigilant, bio-sensing role of the AAVPG vector *in vivo*.

Discussion

In this work we show that our third generation p53-responsive viral vector can be used as a biosensor of p53 activity in response to temperature, chemotherapy, hypoxia and mechanical stress. The PGTxβ promoter, previously described by our group (Bajgelman and Strauss, 2008), proved to be reliable in the context of an AAV vector. The AAV vector developed here also offers compatibility with the Gateway cloning technology, a feature that may facilitate the construction of vectors containing varied promoters and/or genes of interest.

As compared to our first and second generation vectors (retroviral and adenoviral, respectively), the use of an AAV vector offers

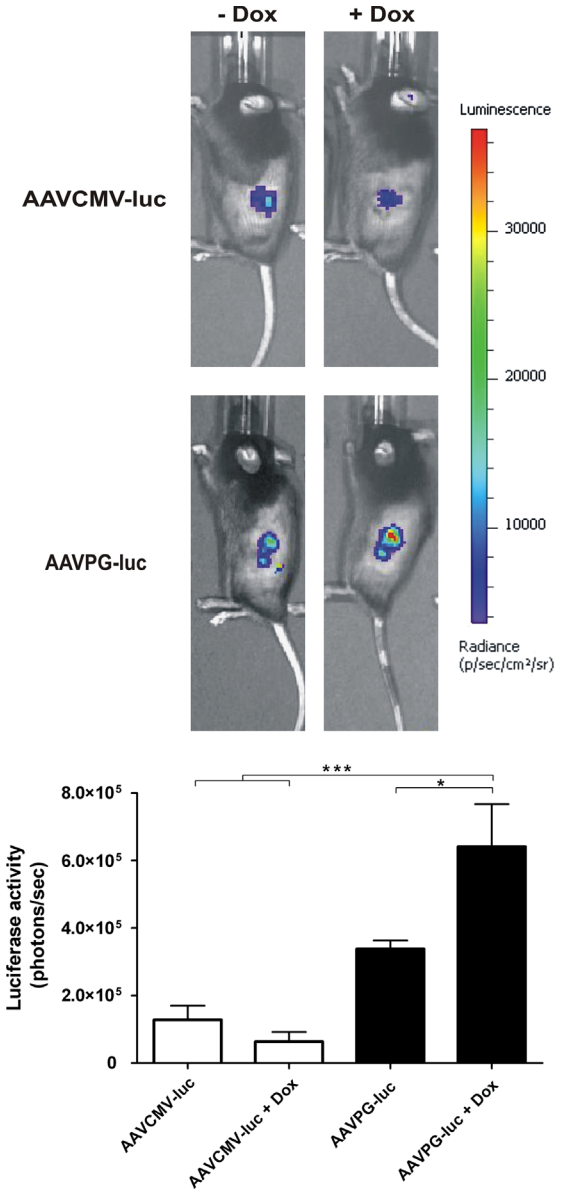

Fig. 6. Demonstration of the vigilant, bio-sensing role of the AAVPG vector *in vivo*. B16 cells were transduced *ex vivo* with either AAVCMV-luc or AAVPG-luc and expanded before 5×10^5 cells were implanted subcutaneously in C57BL/6 mice ($n=5$/group). One week later, the animals were subjected to bioluminescence imaging before i.p. injection of doxorubicin (Dox, 1.5 mg/kg). The animals were then maintained for 24 h before a second image was captured. * $p < 0.05$, *** $p < 0.001$, one-way ANOVA followed by Tukey's Multiple Comparison test.

several improvements, especially with respect to vector biology. For example, AAV remains predominantly episomal, yet maintains long term expression, thus avoiding the pitfalls of retroviral integration and short term adenoviral expression. AAV is also much less immunogenic than adenovirus, a feature that contributes to the permanence of AAV-transduced cells *in vivo* and, as a result, prolongs transgene expression (Li et al., 2007). In addition, the tropism of AAV vectors can be easily modified by pseudotyping with naturally occurring or engineered viral capsids (Asokan et al., 2012).

AAVPG-luc was tested in different experimental conditions that are known to activate p53, such as hypoxia and stretching. While the tumor suppressor functions of p53 are well known, p53 may also play a critical role mediating the response to physiologic conditions that are also important for other pathologies. For example, hypoxia is a common trait of ischemia, such as occurs in the myocardium upon infarct or uncompensated hypertrophy (Sano et al., 2007). In another example, biomechanical stress, such as stretching, is associated with cardiovascular remodeling and is known to involve the activation of p53 (Leri et al., 1998, 2000). Even though p53's typical role is related to tumor biology, it also plays a part in other disease processes. Hence the need for molecular tools that reveal p53 activity, and thus physiologic stress, extends to fields of study beyond cancer.

Interestingly, we were able to use co-transduction of the AAVPG and AAVCMV vectors and reveal two distinct responses of viral expression upon induction of stress. Our data show that there was no crosstalk between the vectors since the measurement of the activity of each reporter gene was reliable and, more importantly, their responses were distinct. This is an important point since intermolecular cis activation (where an enhancer encoded by one AAV vector can influence gene expression in a second vector that includes a minimal promoter, but not an enhancer) has been described (Duan et al., 2000). However, this was not observed in our studies. Each of our vectors contains a complete expression cassette and appears not to have been influenced by the other vector (*i.e.*, the AAVCMV vector did not promote expression from the AAVPG vector in the absence of p53 function). Experimental evidence for this will be shown in a separate study by our group. Therefore, we have shown that two AAV vectors can be applied together and provide distinct, yet complimentary, results.

As shown here, the AAVPG vector is an asset for *in vivo* whole animal imaging and may even prove to be an interesting vector for gene therapy. A few reports in the literature have used a similar approach to measure the role of p53 in the response of tumors to treatment (Wang and El-Deiry, 2003; Wang et al., 2006). Since p53 is important for other pathologies, the application of AAVPG may be well suited for *in situ* gene transfer at the time experimental surgeries, such as induction of ischemia. Such studies have been initiated by our group and will be described elsewhere. In this way, the AAVPG vector may reveal p53 function, but may also harness p53 activity in order to bring about expression of a therapeutic transgene.

The AAVPG platform offers several characteristics that are desirable in a gene transfer vector, such as safety, stability and expression controlled by p53, a critical sensor and mediator of the response to physiologic cell stress. We propose that the AAVPG vector is beneficial for functional assays and represents the first steps in developing a vigilant vector that can provide the expression of therapeutic transgenes on demand in response to the activation of p53.

Material and methods

Cell culture and lines

The cell lines 293T, NIH3T3 and B16 were maintained in DMEM (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FBS (HyClone, Logan, UT, USA), 100 mg/ml gentamycin, 50 mg/ml ampicillin, and 2.5 mg/ml fungizone, at 37 °C, in a humidified atmosphere of 5% CO_2. The cell line H358 was maintained in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) supplemented as described above and maintained in a humidified atmosphere of 5% CO_2, at 37 °C or 32 °C, as indicated in the experiments.

Vector construction

The AAV Helper-Free System (Stratagene, USA) was modified to become compatible with the Gateway site specific recombination system and pEntr vectors (Invitrogen Life Technologies). Initially, we constructed pAAVdest by cloning the recombination site

excised from pCDNA/V5-DEST (Invitrogen) into pAAVCMV (Stratagene) after removing the CMV promoter/enhancer sequences. To generate the pEntr vector, we used PCR to amplify the expression cassette PGTxβ-luc from pAdPG-luc (Bajgelman and Strauss, 2008), with primers PG-Topo D Forward (5′CACCTCTTTCCCACCCTTAACCAC3′) e PG-Topo D Reverse (5′TAGGCTCAAGCAGTGATCCT3′) and inserted this into pEntr-D-topo (Invitrogen). Recombination with pAAV-dest yielded the AAVPG-luc vector. Cloning details can be provided upon request. AAVCMV-LacZ is a constitutive β-galactosidase reporter vector and was supplied in the AAV Helper-Free System (Stratagene, USA). AAVCMV-luc was cloned in two steps. Firstly a 1.4 kb fragment containing a partial 3′ ires element followed by an eGFP cDNA was cut from p-Ires2-eGFP (Clontech, USA) with *Not*I and *Hin*dIII, and ligated to AAVCMV-MCS (Stratagene, USA). Next, we digested the plasmid vector FE2FLIGfW (Strauss et al., 2006) with *Hin*dIII, to isolate a 2 kb fragment containing the firefly luciferase reporter gene, followed by a partial 5′ ires element, generating the final construct, encoding the luc-ires-eGFP cassette driven by a CMV promoter.

Preparation of AAV

Viral preparations were produced transiently by co-transfection of 293T cells with the viral plasmid and serotype 2 packaging plasmids from the AAV Helper-Free System (Stratagene-USA). The supernatants and cell lysates were harvested and virus was purified using the AAV Purification Kit (Cell Biolabs, USA). Titration was done by qPCR, where a standard curve was generated using the plasmid form of the AAV vector.

In vitro luciferase and β-gal assay

Cells were treated as indicated in the figures before being harvested and lysed in 0.1% Triton X-100, 0.1 M Tris/HCl, pH 8.0 by a freeze/thaw treatment. The lysate was then clarified by centrifugation, 5 min, 10,000 rpm. The supernatant was transferred to a fresh tube and stored at −20 °C. For luciferase assay, 10 μl of lysate was added to 50 μl of LAR (Promega, USA), and activity was measured using a luminometer. Luc activity was normalized by total protein, measured using Bradford reagent (Bio-Rad, USA). For the β-galactosidase assay, 30 μl of lysate was added to 100 μl of ONPG solution (77 ml of 0.1 M Na_2HPO_4, pH 7.5, 22 ml of 4 mg/ml ONPG, 1 ml of 0.1 M $MgCl_2$, 4.5 M 2-mercaptoethanol) in a 96 well plate. The reactions were allowed to proceed for 5 to 15 min, 37 °C, and stopped using a 1 M sodium carbonate solution. Absorbance measurements were made at 405 nm using a microtiter plate reader. Beta gal activity was normalized by total protein, measured using Bradford reagent (Bio-Rad, USA).

Biomechanical stress

Briefly, 10^5 NIH3T3 cells/well were seeded on a 6 well Bioflex® culture plate. Cells were incubated at 37 °C for 24 h, transduced with virus and incubated at 37 °C for additional 24 h. Media was changed and cells were stretched 10% for 24 h.

In vivo imaging

All experimental procedures followed institutional guidelines for care and use of laboratory animals, and the protocols were approved by the Institutional Review Board of the University of São Paulo School of Medicine (FMUSP), Brazil. Female C57BL/6 mice, 7-weeks old, were obtained from the Centro de Bioterismo, FMUSP, and housed in the Heart Institute animal facility. Water and food were supplied *ad libitum* and the animals' well-being was monitored closely. All procedures for each experimental group were performed in parallel such that images and drug treatments were performed in the same experimental session and in the same manner, thus minimizing differences in handling.

B16 cells were transduced (MOI=30,000) *ex vivo* with either AAVCMV-luc or AAVPG-luc and expanded before being trypsinized, counted and implanted subcutaneously, 5×10^5 cells in each C57BL/6 mouse ($n=5$/group). After 7 days, when tumors were palpable, the animals were subjected to bioluminescence imaging (IVIS Spectrum, Caliper Life Science). For this, the animals received 15 mg/kg of luciferin (Promega) via i.p. injection and, 10 min later, were anesthetized with isoflurane (Cristalia) using the Xenogen anesthesia system before imaging (performed by Mara de Souza Junqueira, Centro de Bioterismo, FM-USP). Periodic images were captured until the luciferin signal dissipated and only the strongest signal was included in the analysis. The animals then received an i.p. injection of doxorubicin, 1.5 mg/kg, and were maintained for 24-h before repeating the imaging process. Data was analyzed using Living Imaging 4.3 software (Caliper Life Science). As a negative control, animals were implanted with B16 cells transduced with the AAVCMV-LacZ vector, resulting in no emission of photons (data not shown).

Acknowledgments

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.09.004.

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