Role of Cyclic AMP-Dependent Kinase Response Element-Binding Protein in Recombinant Adeno-Associated Virus-Mediated Transduction of Heart Muscle Cells

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Abstract

Recombinant adeno-associated virus (rAAV) vectors represent a promising approach to gene delivery for clinical use. Published data indicate that rAAV vector genomes persist *in vivo* as episomal chromatin in the skeletal muscle of nonhuman primates. In this study, we assessed the interconnection between the transcription factor cyclic AMP response element-binding protein (CREB) and recombinant AAV serotype 2 vector genomes after transduction *in vitro* and *in vivo*. rAAV-mediated myocyte transduction was potently blocked in the hearts of mice expressing CREB-S133A, which is a CREB-S133A dominant-negative mutant. Isoproterenol, a strong CREB activator, prominently increased rAAV transduction and the increase was abrogated by silencing the CREB gene with small interfering RNA. In addition, rAAV infection of muscle cells mildly but significantly induced CREB protein phosphorylation at serine-133, and was capable of stimulating CREB-dependent transcription from a reporter plasmid. Using chromatin immunoprecipitation and immunoblotting assays, both CREB and p300 were found to physically associate with two different rAAV genomes. Accordingly, CREB/p300 appears to have a role in rAAV transduction to establish active vector transcription in heart muscle cells.

Introduction

RECOMBINANT ADENO-ASSOCIATED VIRAL (rAAV) VECTORS are currently being developed for gene transfer to several tissues. Gene delivery to muscle by these vectors is an approach being used to treat myopathies (Xiao *et al.*, 1996, 2000; Asfour *et al.*, 2002; Goyenvalle *et al.*, 2004) and to produce secreted proteins (Flotte *et al.*, 2004; Wang *et al.*, 2005; Manno *et al.*, 2006). The small size and physical stability of rAAV make these vectors advantageous for *in vivo* use, and the resulting transgene expression can persist long term in a wide range of tissues including heart and skeletal muscle (Fisher *et al.*, 1997; Snyder *et al.*, 1997; Aikawa *et al.*, 2002; Palomeque *et al.*, 2007). rAAV vector genomes persist in skeletal muscle mainly in high molecular weight circular episomes (Schnepp *et al.*, 2005; Penaud-Budloo *et al.*, 2008). Data indicate that the rAAV genome persists *in vivo* in skeletal muscle of the nonhuman primate as episomal chromatin with a typical nucleosome structure (Penaud-Budloo *et al.*, 2008). We are interested in understanding why rAAV vector DNA is

able to establish episomal euchromatin that results in high-level gene expression that persists for several years. The cyclic AMP response element-binding protein (CREB) is a 43-kDa basic leucine zipper transcription factor that plays a critical role in regulating gene expression in response to a variety of extracellular signals (Hoeffler *et al.*, 1988; Mayr and Montminy, 2001). The CREB signaling pathway is also important for regulating gene expression and productive replication of several viruses, including wild-type AAV, adenovirus, and HIV (Lee *et al.*, 1987; Cole *et al.*, 1999; Di Pasquale and Chiorini, 2003). In the unphosphorylated state, CREB can bind to DNA but cannot activate transcription (Yamamoto *et al.*, 1988). Stimulation of the β -adrenergic receptor can activate protein kinase A (PKA) (Yamamoto *et al.*, 1988), which is capable of positively regulating the transcriptional activity of CREB by phosphorylation of serine-133 (Gonzalez and Montminy, 1989). Once phosphorylated, CREB-S133 promotes gene expression by recruiting the coactivator paralogs CREB-binding protein (CBP) and p300 to gene promoters regulated by cyclic AMP-responsive elements (CREs) (Lundblad

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et al., 1995; Gilchrist *et al.*, 1996) for subsequent chromatin remodeling. CBP and p300 possess histone acetyltransferase (HAT) activity that are involved in creating an open-chromatin architecture that promotes gene expression (Roth *et al.*, 2001). Overexpression of a CREB-S133A mutant, which cannot be phosphorylated but still occupies CRE sites, has been shown to inhibit CREB-dependent gene expression (Somers *et al.*, 1999; Kamiya *et al.*, 2007).

Here we examine the relationship between rAAV transduction and CREB and show that rAAV transduction was strongly suppressed in the heart of CREB-S133A transgenic mice, which overexpress a dominant-negative mutant CREB protein. Both CREB and p300 physically associate with the rAAV genome, as shown in a chromatin immunoprecipitation (ChIP) assay. Isoproterenol, which is a strong CREB activator, prominently increased rAAV transduction and the increase was abrogated by silencing the CREB gene with small interfering RNA (siRNA).

Materials and Methods

rAAV vector production

Vectors rAAV-CMV-hGH/lacZ and rAAV-MHC-hGH/lacZ, with titers of 2×10^{11} to 1×10^{12} vector genomes (VG)/ml, were prepared as described previously (Aikawa *et al.*, 2002).

Protein analysis

Protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. Anti-CREB antibody (cat. no. sc-58; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-CREB antibody (cat. no. 9190; Cell Signaling Technology/New England BioLabs, Danvers, MA), and anti-p300 antibody (cat. no. sc-585; Santa Cruz Biotechnology) antibodies were used for Western blot analysis using an ECL Advance chemiluminescence system (RPN2135; GE Healthcare Life Sciences, Piscataway, NJ). Coimmunoprecipitation of p300 and CREB was performed 24 hr after rAAV infection by incubating cell lysates with anti-p300 antibody followed by precipitation with protein A beads (P1052; Sigma-Aldrich, St. Louis, MO), and the precipitate was analyzed with anti-CREB antibody.

Transfection

The CRE-luc reporter plasmid was constructed by cloning three copies of a somatostatin gene CRE element in front of the thymidine kinase promoter in the pGL2-basic plasmid (courtesy of J. Lepore, University of Pennsylvania, Philadelphia, PA). Transient transfection assays were performed in 12-well plates seeded at $2\text{--}3 \times 10^5$ cells per well with C2C12 (mouse skeletal myoblasts) and H9C2 (rat cardiomyocytes), using PolyFect reagent (cat. no. 301107; Qiagen, Hilden, Germany) with 50 ng of reporter plasmid. rAAV or $10 \mu\text{M}$ forskolin (Sigma-Aldrich) was added 5 hr after transfection. After 24 hr, the C2C12 and H9C2 cells were harvested and luciferase activity (luciferase assay system; Promega, Madison, WI) was measured with a luminometer (Luminoskan Ascent; Thermo Fisher Scientific, Waltham, MA). To control for transfection efficiency luminescence values were normalized to the total protein concentration measured by the bicinchoninic acid (BCA) protein assay (cat. no. 23223; Pierce Biotechnology/Thermo Fisher Scientific, Rockford, IL).

Cell culture and rAAV transduction assays

Twelve-well plates were seeded at $2\text{--}3 \times 10^5$ per well with H9C2 cells and C2C12 cells, and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 units/ml) in a humidified atmosphere at 37°C with 5% CO_2 . When cells were 70–80% confluent, the medium was replaced with serum-free fresh medium, and then rAAV-CMV-lacZ (2000 VG/cell) was added. After a 24-hr incubation, the cells were fixed and stained for β -galactosidase (β -Gal) activity (Aikawa *et al.*, 2002). For the siRNA experiments, both mouse CREB-1 siRNA (cat. no. sc-35111; Santa Cruz Biotechnology) and control siRNA (cat. no. sc-36869; Santa Cruz Biotechnology) were purchased, and rAAV-CMV-lacZ was infected 24 hr after transfection.

Animal studies

MHC-CREB-S133A mice (CREB mice) are courtesy of J. Leiden (Fentzke *et al.*, 1998). The Tufts University Institutional Animal Care and Use Committee approved all animal studies. Anesthetized 4- to 6-week-old wild-type or CREB mice received $50 \mu\text{l}$ containing 1×10^{10} VG of rAAV-hGH vector, injected into the anterior left ventricular wall under direct visualization through a left lateral thoracotomy, as previously described (Aikawa *et al.*, 2002). Blood samples were taken from the retro-orbital vein of anesthetized mice, and the plasma human growth factor (hGH) concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described (Aikawa *et al.*, 2002).

Chromatin immunoprecipitation assay

Six-well plates seeded with H9C2 and C2C12 cells at 1×10^6 cells per well were grown to 80–90% confluence, and infected with rAAV-CMV-lacZ or rAAV-MHC-hGH vector at 500 VG/cell. Four hours after infection, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde at room temperature for 10 min. After being cross-linked, soluble chromatin was made as described (Braunstein *et al.*, 1993; Chen *et al.*, 1999). Anti-CREB or anti-p300 (cat. nos. sc-58 and sc-585, respectively; Santa Cruz Biotechnology) or control rabbit IgG (cat. no. sc-3871; Santa Cruz Biotechnology) was used for immunoprecipitation, using protein A beads (P1052; Sigma-Aldrich), and the precipitate was analyzed by polymerase chain reaction (PCR). The primers were used to amplify a 286-bp fragment of the rAAV-CMV-lacZ genome (Iwakura *et al.*, 2005). For the rAAV-MHC-lacZ genome, the MHC-344 primer and the MHC+ 19 primer were used (Aikawa *et al.*, 2002).

Statistical analysis

The mean and standard error (SE) were determined for replicate samples. For multiple treatment groups, a factorial analysis of variance was applied followed by the Fisher least significant difference test as computed by the StatView computer program. Values of p less than 0.05 were considered significant.

Results

Overexpression of CREB-S133A suppresses rAAV transduction in vivo

To analyze the role of CREB in rAAV transduction we used a previously published murine cardiomyopathy model

FIG. 1. Transgenic overexpression of dominant negative CREB in the heart suppresses rAAV transduction *in vivo*. (a) Western blot analysis demonstrates CREB mice have reduced phospho-CREB in the heart compared with skeletal muscle. (b) CREB mice (mean $\pm$ SE, $n = 4$) do not produce hGH after cardiac infection with rAAV.

induced by overexpression of the dominant-negative mutant CREB-S133A in the heart under the control of the tissue-specific α -myosin heavy chain (MHC) promoter (dnCREB mouse). Overexpression of nonphosphorylated CREB-S133A in the mouse heart inhibits CREB-mediated transcription activation (Fentzke *et al.*, 1998). We verified the CREB phosphorylation state in the dnCREB mouse in skeletal muscle compared with cardiac muscle. Phosphorylated CREB protein was detected in skeletal muscle of dnCREB mice, but not in heart (Fig. 1a). By comparison, phosphorylated CREB was detected in both heart and skeletal muscle of wild-type mice (data not shown). To examine the role of CREB in transduction of muscle by rAAV we used four rAAV2 vectors with two different promoters paired with two different transgenes: rAAV-MHC-hGH or lacZ and rAAV-CMV-hGH or lacZ (Aikawa *et al.*, 2002). We injected these vectors encoding human growth hormone (hGH) at a dose of 1×10^{10} VG per animal directly into the anterior left ventricular heart wall of wild-type and dnCREB mice. hGH was used as a secreted marker of rAAV expression for better quantitation of rAAV transduction. After injecting rAAV-MHC-hGH and rAAV-CMV-hGH, into the cardiac muscle of normal mice, the serum level of hGH remained stable for at least 12 weeks (Fig. 1b), similarly to previous results (Aikawa *et al.*, 2002). At the same time we found that neither rAAV-

CMV-hGH nor rAAV-MHC-hGH injection resulted in elevated serum hGH levels in hearts of dnCREB mice (Fig. 1b), indicating that overexpression of CREB-S133A in the heart tissue is capable of inhibiting rAAV gene expression.

Isoproterenol increases rAAV transduction through CREB

Because a β -adrenergic receptor stimulator, isoproterenol (ISO), can activate CREB protein (Yamamoto *et al.*, 1988), we examined whether upregulation of CREB by ISO can increase rAAV transduction. First, we observed that rAAV transduction was significantly inhibited after reducing CREB gene expression with siRNA in C2C12 cells (Fig. 2a). Next, pretreatment with ISO enhanced rAAV2 transduction in C2C12 cells more than 5-fold (Fig. 2b). When CREB expression is specifically downregulated by siRNA-CREB before rAAV2 infection and ISO treatment, lacZ transgene expression was downregulated to the baseline level (Fig. 2b).

Presence of CRE sites within rAAV vector genomes

Published studies have shown the importance of CRE sites within the cytomegalovirus (CMV) promoter for transcriptional activation of a transgene situated after this internal promoter in the context of an adenoviral vector (Wheeler and Cooper, 2001). Therefore we searched the rAAV genomes used in the present study for the presence of CREB-binding sites (CRE). Unlike the CMV promoter, there are no typical CRE sites (TGACGTCA) or CRE half-sites (TGACG) to which CREB can bind (Sands and Palmer, 2008) in the MHC promoter (Molkentin *et al.*, 1996; Gupta *et al.*, 1998). The inverted terminal repeat (ITR) of rAAV2 and the poly(A) sequences do not include a consensus CRE site (Table 1). We then looked for the presence of a noncanonical CRE site (TGTCGA) to which CREB is capable of binding to induce at levels up to 30% activity of a full CRE site (DiNardo *et al.*, 2001). As shown in Table 1, two noncanonical CRE sites exist in the MHC promoter and the poly(A) sequence. Both lacZ and hGH transgenes used in this study harbor CRE half-sites and noncanonical CRE sites (Table 1) that are capable of CREB binding. Thus multiple sites are available in these vectors for CREB binding.

rAAV mildly induces CREB phosphorylation and both CREB and p300 physically interact with the rAAV vector genome

To determine whether rAAV influences serine-133 CREB phosphorylation, CREB phosphorylation was analyzed by Western blot analysis after infection of H9C2 and C2C12 with rAAV2-CMV-lacZ at 2000 VG/cell. CREB phosphorylation was mildly but significantly increased at 15 and 30 min after rAAV infection. This effect required intact viral particles because boiled rAAV did not increase CREB phosphorylation (Fig. 3a). A study was carried out to determine whether rAAV-stimulated CREB phosphorylation could stimulate the CREB-dependent transcription of a CRE-regulated luciferase reporter plasmid (CRE-luc). After transfection with CRE-luc, H9C2 and C2C12 cells were infected with increasing amounts of rAAV vector or treated with the PKA activator forskolin. Infection with rAAV at 1000 and 2000 VG/cell significantly increased luciferase activity, and as expected,

FIG. 2. Isoproterenol (ISO) increases rAAV transduction through CREB. C2C12 cells were pretreated with control siRNA or CREB siRNA followed by infection with rAAV-CMV-lacZ at 1000 VG/cell, with or without preincubation with 10^{-6} M ISO. Twenty-four hours after infection, the cells were stained for β -Gal activity. Data represent means $\pm$ SE, * $p < 0.01$ ($n = 4$).

TABLE 1. NUMBER OF CRE SITES (TGACGTCA), CRE HALF-SITES (TGACG), AND NONCANONICAL CRE SITES (TGTCGA) IN EACH ELEMENT OF THE rAAV-CMV/MHC-lacZ/hGH GENOMES USED IN THE PRESENT STUDY^a

Element	TGACGTCA	TGACG	TGTCGA
ITR	0	0	0
Promoter			
CMV	4	4 ^b	0
MHC	0	0	2
Transgene			
lacZ	0	10	1
hGH	0	1	3
Poly(A)	0	0	2

Abbreviations: CMV, cytomegalovirus; CRE, cyclic AMP response element; hGH, human growth hormone; ITR, inverted terminal repeat; MHC, major histocompatibility complex; poly (A), polyadenylation site.

^aThe presence of the sites was determined for plus and minus DNA strands.

^bThese half-sites are not included in the CRE site count.

forskolin strongly activated the CRE-luc reporter plasmid (Fig. 3b).

To investigate further the possible role of CREB in mediating transgene regulation, we examined whether the CREB protein physically interacts with the rAAV-CMV or rAAV-MHC genome, using chromatin immunoprecipitation (ChIP). After infection of cultured H9C2 and C2C12 cells and formaldehyde-mediated cross-linking, the rAAV genome was recovered by immunoprecipitation with an anti-CREB antibody but not with a control immunoglobulin (Fig. 3c, left). CREB phosphorylation can enhance recruitment of CREB-binding protein and p300 cofactors to enhance transcription (Goodman and Smolik, 2000). To investigate the association of p300 with the rAAV genome, we performed the ChIP assay with an anti-p300 antibody. These studies confirmed p300 association with rAAV-CMV-lacZ or rAAV-MHC-lacZ (Fig. 3c, right), which could result via the interaction of the DNA-binding intermediary transcription factor CREB. Serine-133-phosphorylated CREB activates transcription through increased binding to coactivator proteins CBP and p300 (Goodman and Smolik, 2000). These results are consistent with CREB binding to CRE sites located in the rAAV genome and recruitment of p300.

To assess whether rAAV-induced CREB phosphorylation can increase the association of CREB with p300 we investigated the interaction of CREB with p300 after rAAV infection. Although the amount of p300 immunoprecipitated from H9C2 and C2C12 cells was not influenced by rAAV infection (Fig. 3d, top), a greater amount of CREB protein was found in the p300 immunoprecipitate 24 hr after rAAV infection (Fig. 3d, bottom).

FIG. 3. Activation of CREB by rAAV and interaction of CREB and p300 with rAAV. (a) rAAV infection induces CREB serine-133 phosphorylation. (b) Activation of CRE-luc reporter plasmid in H9C2 cells (open columns) and C2C12 cells (solid columns) infected with the indicated number of rAAV vector genomes per cell or stimulated with 10μ M forskolin. Data represent means $\pm$ SE. * $p < 0.01$ ($n = 4$) compared with no-infection control. (c) CREB and p300 associate with the rAAV-CMV and rAAV-MHC genomes. After mock infection (–) or rAAV infection (+) of H9C2 or C2C12 cells, a chromatin immunoprecipitation assay was performed with nonspecific IgG antibody, anti-CREB (left), or anti-p300 (right). Final lane, plasmid control. Arrow shows 286- or 390-bp predicted PCR products. (d) Western blot analysis demonstrating immunoprecipitation of p300 (top), and CREB coimmunoprecipitation (bottom) 24 hr after mock infection (–) or rAAV infection (+).

Discussion

Molecular mechanisms important for wild-type AAV infection, latency, and viral gene expression are relevant to recombinant vector-mediated gene therapy. To explore the role of CREB in rAAV transduction we used CREB-S133A-expressing mice. Transduction using rAAV regulated by either a tissue-specific (MHC) or promiscuous promoter/enhancer (CMV) was blocked in the dnCREB mouse (Fig. 1b). Importantly, in dnCREB transgenic mice, the same MHC promoter is driving CREB-S133A expression, and only rAAV expression was suppressed after rAAV injection. Iso-proterenol (ISO) is a potent β -adrenergic receptor activator, which can activate CREB through the PKA pathway (Yamamoto *et al.*, 1988). Phosphorylated CREB can then recruit CBP and p300, which have HAT activity, and increase promoter accessibility by acting on chromatin structure (Gusterson *et al.*, 2002). Pretreatment with 10^{-6} M ISO significantly augmented rAAV transduction (Fig. 2a) and we observed that the ISO effect on rAAV transduction occurred in a dose-dependent manner between 10^{-7} and 10^{-5} M without an increase in vector genomes per cell (Nattanson *et al.*, unpublished data). ISO-augmented rAAV transduction was strongly inhibited by siRNA-CREB treatment (Fig. 2b), suggesting that CREB plays a role in CMV-driven transgene expression. Those experiments including loss of CREB function (Fig. 1) and gain of CREB function (Fig. 2) revealed that CREB is important for rAAV transduction. To address the possibility that, in addition to directly acting on the vector genome, CREB may be enhancing transduction by influencing viral uptake and trafficking, a pseudotype vector in which the rAAV2 genome was packaged in an rAAV1 capsid (rAAV1/2) was tested. Similar to the rAAV2 capsid vector, transduction with the rAAV1 capsid vector was significantly blocked by dnCREB overexpression *in vitro* (data not shown). By inhibiting CREB protein function (dnCREB), stimulating CREB function (with ISO), or knocking down CREB gene expression (with siRNA), we have shown that CREB plays an important role in rAAV transduction. Importantly, although the CMV promoter has abundant CRE sites and the MHC promoter has limited CRE sites (as shown in Table 1), both vectors were regulated by CREB. Using the ChIP assay we demonstrate an association of CREB with the rAAV-CMV vectors and rAAV-MHC vectors (Fig. 3c). CRE, CRE half-sites, and noncanonical CRE sites are present throughout the recombinant AAV vector genomes used here, and even though most of the sites are located outside of the actual promoter sequences, concatemerization and/or circularization of the rAAV genomes may enhance the effect of CREB/p300 by bringing distal CRE sites into closer proximity to the promoter region. In addition, 1 CRE half-site and 2 noncanonical CRE sites exist in the Rous sarcoma virus (RSV) promoter and 12 additional sites are present over the length of the rAAV-RSV-LEA29Y-WPRE vector used by Penaud-Budloo and colleagues (2008) (M. Penaud-Budloo, C. Le Guiner, and P. Moullier, unpublished data). Furthermore, 18 CRE half-sites or noncanonical CRE sites exist over the length of the wild-type AAV2 genome, 26 sites in AAV1, 29 sites in AAV4, 24 sites in AAV5, and 22 sites in AAV8, and some of these sites are located proximal to the P5 and P40 promoters (including a full CRE site at the AAV5 P40 promoter) (data not shown), indicating a role for CREB in the

wild-type AAV life cycle. However, other transcription factors that are capable of binding CRE elements may have a role too, including CREM (cAMP response element modulator) and ATF-1 (activating transcription factor-1), and CREB can bind to CRE as a homodimer or heterodimer with other transcription factors belonging to the CREB/ATF or Jun/Fos families (Hai and Curran, 1991). CREB phosphorylation can enhance recruitment of CBP and p300 cofactors to establish transcriptionally active chromatin (Roth *et al.*, 2001). It is also possible that CREB influences rAAV transduction via an as yet unknown indirect mechanism. As shown in Fig. 3a, the enhanced phosphorylation of CREB starts as early as 15 min post-AAV infection, which is a time point after infection when AAV particles are detected in the nucleus of about 50% of cells grown *in vitro* (Seisenberger *et al.*, 2001). The rapid appearance of AAV particles in the nucleus may trigger CREB phosphorylation by activating signal transduction pathways in addition to DNA binding.

The ChIP assay carried out with a p300 antibody revealed an association with both the rAAV-CMV and rAAV-MHC genomes (Fig. 3c). Because p300 is able to interact with the rAAV genome via CREB (Fig. 3d), CREB may be functioning as a transcription regulator either by itself or in association with as yet unidentified factors. It is possible that p300 acetylates rAAV-associated histones to establish a transcriptionally active chromatin structure, which may relate to findings demonstrating that the rAAV genome is converted to circular episomal chromatin with a typical nucleosomal pattern *in vivo* in the skeletal muscle of the nonhuman primate (Penaud-Budloo *et al.*, 2008). Accordingly, CREB involvement may be a general feature of rAAV vector expression in muscle cells.

HATs may be required to establish rAAV open chromatin, which is the basis of long-term gene expression in muscle. In agreement with our observations, Okada and colleagues demonstrated an association of acetylated histone H3 with the rAAV vector genome in cancer cells *in vitro* (Okada *et al.*, 2006). *In vitro*, the vector may exist in a circular episomal state (Duan *et al.*, 1999) or integrated into the host chromosome where it can be repressed, but reactivated with histone deacetylase inhibitors (Chen *et al.*, 1997; Chen and Townes, 2000). Previous reports show that p300 and CBP have similar tissue patterns of HAT activity, with the highest level in the brain; a relatively high level in the lung, spleen, and heart; an intermediate level in testes and skeletal muscle; and a lower level in liver and kidney (Li *et al.*, 2002). The data presented in this report support a role for CREB and HATs in the regulation of rAAV vector chromatin in muscle cells.

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Author Disclosure Statement

R.O.S. is an inventor on patents related to recombinant AAV technology and owns equity in a gene therapy company

that is commercializing AAV for gene therapy applications. To the extent that the work in this manuscript increases the value of these commercial holdings R.O.S. has a conflict of interest.

References

- Aikawa, R., Huggins, G.S., and Snyder, R.O. (2002). Cardiomycocyte-specific gene expression following recombinant adeno-associated viral vector transduction. *J. Biol. Chem.* 277, 18979–18985.
- Asfour, B., Baba, H.A., Scheld, H.H., Hruban, R.H., Hammel, D., and Byrne, B.J. (2002). Uniform long-term gene expression using adenoassociated virus (AAV) by *ex vivo* recirculation in rat-cardiac isografts. *Thorac. Cardiovasc. Surg.* 50, 347–350.
- Braunstein, M., Rose, A.B., Holmes, S.G., Allis, C.D., and Broach, J.R. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* 7, 592–604.
- Chen, H., Lin, R.J., Xie, W., Wilpitz, D., and Evans, R.M. (1999). Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* 98, 675–686.
- Chen, W.Y., and Townes, T.M. (2000). Molecular mechanism for silencing virally transduced genes involves histone deacetylation and chromatin condensation. *Proc. Natl. Acad. Sci. U.S.A.* 97, 377–382.
- Chen, W.Y., Bailey, E.C., McCune, S.L., Dong, J.Y., and Townes, T.M. (1997). Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5798–5803.
- Cole, S.W., Jamieson, B.D., and Zack, J.A. (1999). cAMP up-regulates cell surface expression of lymphocyte CXCR4: implications for chemotaxis and HIV-1 infection. *J. Immunol.* 162, 1392–1400.
- DiNardo, D.N., Butcher, D.T., Robinson, D.P., Archer, T.K., and Rodenhiser, D.I. (2001). Functional analysis of CpG methylation in the *BRCA1* promoter region. *Oncogene* 20, 5331–5340.
- Di Pasquale, G., and Chiorini, J.A. (2003). PKA/PrKX activity is a modulator of AAV/adenovirus interaction. *EMBO J.* 22, 1716–1724.
- Duan, D., Yan, Z., Yue, Y., and Engelhardt, J.F. (1999). Structural analysis of adeno-associated virus transduction circular intermediates. *Virology* 261, 8–14.
- Fentzke, R.C., Korcarz, C.E., Lang, R.M., Lin, H., and Leiden, J.M. (1998). Dilated cardiomyopathy in transgenic mice expressing a dominant negative CREB transcription factor in the heart. *J. Clin. Invest.* 101, 2415–2426.
- Fisher, K.J., Jooss, K., Alston, J., Yang, Y., Haecker, S.E., High, K., Pathak, R., Raper, S.E., and Wilson, J.M. (1997). Recombinant adenoassociated virus for muscle directed gene therapy. *Nat. Med.* 3, 306–312.
- Flotte, T.R., Brantly, M.L., Spencer, L.T., Byrne, B.J., Spencer, C.T., Baker, D.J., and Humphries, M. (2004). Phase I trial of intramuscular injection of a recombinant adeno-associated virus α_1 -antitrypsin (rAAV2-CB-hAAT) gene vector to AAT-deficient adults. *Hum. Gene Ther.* 15, 93–128.
- Gilchrist, C.A., Orten, D.J., and Hinrichs, S.H. (1996). Evidence for the role of cyclic AMP-responsive elements in human virus replication and disease. *J. Biomed. Sci.* 3, 293–306.
- Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 59, 675–680.
- Goodman, R.H., and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. *Genes Dev.* 14, 1553–1577.
- Goyenvall, A., Vulin, A., Fougerousse, F., Leturcq, F., Kaplan, J.C., Garcia, L., and Danos, O. (2004). Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. *Science* 306, 1796–1799.
- Gupta, M., Zak, R., Libermann, T.A., and Gupta, M.P. (1998). Tissue restricted expression of the cardiac α -myosin heavy chain gene is controlled by a downstream repressor element containing a palindrome of two ETS-binding sites. *Mol. Cell. Biol.* 18, 7243–7258.
- Gusterson, R.J., Jazrawi, E., Adcock, I.M., and Latchman, D.S. (2002). The transcriptional co-activators CREB-binding protein (CBP) and p300 play a critical role in cardiac hypertrophy that is dependent on their histone acetyltransferase activity. *J. Biol. Chem.* 278, 6838–6847.
- Hai, T., and Curran, T. (1991). Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3720–3724.
- Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L., and Habener, J.F. (1988). Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. *Science* 242, 1430–1433.
- Iwakura, A., Dean, J., Hamada, H., Eaton, E., Qin, G., Losordo, D.W., and Aikawa, R. (2005). Use of recombinant adeno-associated viral vectors as a tool for labeling bone marrow cells. *J. Mol. Cell. Cardiol.* 38, 799–802.
- Kamiya, K., Sakakibara, K., Ryer, E.J., Hom, R.P., Leof, E.B., Kent, K.C., and Liu, B. (2007). Phosphorylation of the cyclic AMP response element binding protein mediates transforming growth factor β -induced downregulation of cyclin A in vascular smooth muscle cells. *Mol. Cell. Biol.* 27, 3489–3498.
- Lee, K.A., Hai, T.Y., Sivaraman, L., Thimmappaya, B., Hurst, H.C., Jones, N.C., and Green, M.R. (1987). A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proc. Natl. Acad. Sci. U.S.A.* 84, 8355–8359.
- Li, Q., Xiao, H., and Isobe, K. (2002). Histone acetyltransferase activities of cAMP-regulated enhancer-binding protein and p300 in tissues of fetal, young, and old mice. *J. Gerontol. A Biol. Sci. Med. Sci.* 57, B93–B98.
- Lundblad, J.R., Kwok, R.P., Lurance, M.E., Harter, M.L., and Goodman, R.H. (1995). Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. *Nature* 374, 85–88.
- Manno, C.S., Pierce, G.F., Arruda, V.R., Glader, B., Ragni, M., Rasko, J.E., Ozelo, M.C., Hoots, K., Blatt, P., Konkle, B., Dake, M., Kaye, R., Razavi, M., Zajko, A., Zehnder, J., Rustagi, P.K., Nakai, H., Chew, A., Leonard, D., Wright, J.F., Lessard, R.R., Sommer, J.M., Tigges, M., Sabatino, D., Luk, A., Jiang, H., Mingozzi, F., Couto, L., Ertl, H.C., High, K.A., and Kay, M.A. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat. Med.* 12, 342–347.
- Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat. Rev. Mol. Cell. Biol.* 2, 599–609.
- Molkentin, J.D., Jobe, S.M., and Markham, B.E. (1996). α -Myosin heavy chain gene regulation: Delineation and characterization of the cardiac muscle-specific enhancer and muscle-specific promoter. *J. Mol. Cell. Cardiol.* 28, 1211–1225.
- Okada, T., Uchibori, R., Iwata-Okada, M., Takahashi, M., Nomoto, T., Nonaka-Sarukawa, M., Ito, T., Liu, Y., Mizukami, H., Kume, A., Kobayashi, E., and Ozawa, K. (2006). A histone deacetylase inhibitor enhances recombinant adeno-associated virus-mediated gene expression in tumor cells. *Mol. Ther.* 13, 738–746.

- Palomeque, J., Chemaly, E.R., Colosi, P., Wellman, J.A., Zhou, S., Del Monte, F., and Hajjar, R.J. (2007). Efficiency of eight different AAV serotypes in transducing rat myocardium *in vivo*. *Gene Ther.* 14, 989–997.
- Penaud-Budloo, M., Le Guiner, C., Nowrouzi, A., Toromanoff, A., Chérel, Y., Chenuaud, P., Schmidt, M., Von Kalle, C., Rolling, F., Moullier, P., and Snyder, R.O. (2008). Adeno-associated viral vector genomes persist as episomal chromatin in primate muscle. *J. Virol.* 82, 7875–7885.
- Roth, S.Y., Denu, J.M., and Allis, C.D. (2001). Histone acetyltransferases. *Annu. Rev. Biochem.* 70, 81–120.
- Sands, W.A., and Palmer, T.M. (2008). Regulating gene transcription in response to cyclic AMP elevation. *Cell. Signal.* 20, 460–466.
- Schnepp, B.C., Jensen, R.L., Chen, C.L., Johnson, P.R., and Clark, K.R. (2005). Characterization of adeno-associated virus genomes isolated from human tissues. *J. Virol.* 79, 14793–14803.
- Seisenberger, G., Ried, M.U., Endress, T., Büning, H., Hallek, M., and Bräuchle, C. (2001). Real-time single-molecule imaging of the infection pathway of an adeno-associated virus. *Science* 294, 1929–1932.
- Snyder, R.O., Spratt, S.K., Lagarde, C., Bohl, D., Kaspar, B., Sloan, B., Cohen, L.K., and Danos, O. (1997). Efficient and stable adenoassociated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum. Gene Ther.* 8, 1891–1900.
- Somers, J.P., Deloia, J.A., and Zeleznik, A.J. (1999). Adenovirus-directed expression of a nonphosphorylatable mutant of CREB (cAMP response element-binding protein) adversely affects the survival, but not the differentiation, of rat granulosa cells. *Mol. Endocrinol.* 13, 1364–1372.
- Wang, L., Dobrzynski, E., Schlachterman, A., Cao, O., and Herzog, R.W. (2005). Systemic protein delivery by muscle-gene transfer is limited by a local immune response. *Blood* 105, 4226–4234.
- Wheeler, D.G., and Cooper, E. (2001). Depolarization strongly induces human cytomegalovirus major immediate-early promoter/enhancer activity in neurons. *J. Biol. Chem.* 276, 31978–31985.
- Xiao, X., Li, J., and Samulski, R.J. (1996). Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J. Virol.* 70, 8098–8108.
- Xiao, X., Li, J., Tsao, Y.P., Dressman, D., Hoffman, E.P., and Watchko, J.F. (2000). Full functional rescue of a complete muscle (TA) in dystrophic hamsters by adeno-associated virus vector-directed gene therapy. *J. Virol.* 74, 1436–1442.
- Yamamoto, K.K., Gonzalez, G.A., Biggs, W.H., III, and Montminy, M.R. (1988). Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* 334, 494–498.

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