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JNMU
Journal of Nanjing Medical University, 2008, 22(4):205-210
www.elsevier.com/locate/jnmu
Research Paper

Construction of recombinant adeno-associated virus vector co-expressing hVEGF$_{165}$ and hBMP$_7$ gene☆

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Received 5 January, 2008

Abstract

Objective: To construct recombinant adeno-associated virus co-expressing human vascular epithelial growth factor 165(hVEGF$_{165}$) and bone morphogenetic protein 7(hBMP$_7$), measure the virus titer and verify the recombination. Methods:The AAV helper-free system was used as basis to generate recombinant AAV. The IRES sequence of plasmid pIRES was cut down and subcloned into ITR/MCS containing vector pAAV-MCS to construct recombinant plasmid pAAV-MCSa-IRES-MCSb. The hVEGF$_{165}$ and hBMP$_7$ gene was amplified by PCR and inserted into upstream MCSa and downstream MCSb respectively. Then, recombinant plasmid pAAV-hVEGF$_{165}$-IRES-hBMP$_7$, pAAV-RC and pHelper were co-transfected into AAV-293 cells to complete rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ packaging. The GFP labeled rAAV-IRES-GFP was simultaneously packaged by using the parallel plasmid pAAV-IRES-hrGFP. The efficiency of AAV packaging was monitored under fluorescent microscope and recombinant viral particles were harvested from infected AAV-293 cells. The virus titer was measured by infecting AAV-HT1080 cells, and the recombinant AAV-hVEGF$_{165}$-IRES-hBMP$_7$ was verified by PCR of the exogenous interest genes. Results:Recombinant pAAV-hVEGF$_{165}$-IRES-hBMP$_7$ was verified by double digestion. GFP expression in AAV-293 could be observed under fluorescent microscope 72 h after transfection and the system provided a high packing ratio of 95%. The recombinant adeno-associated virus has a high titer of 5.5×10^{11}vp/ml, and AAV-HT 1080 was infected at a ratio of 90%. The recombinant virus was confirmed by PCR of exogenous hBMP$_7$ and hVEGF$_{165}$ gene. Conclusion:Recombinant rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ was successfully constructed with a high virus titer, which may offer foundation for *in vitro* and *in vivo* experiments of hVEGF$_{165}$ and hBMP$_7$ co-expression and provide a new method for gene therapy of bone regeneration.

Key words: adeno-associated virus; human vascular endothelial factor; human bone morphogenetic protein; internal ribosome entry site

INTRODUCTION

Recent research shows that bone formation and renovation is a coordinated process involving various biological factors and their effects may be enhanced by each other. Among these factors VEGFs and BMPs play important roles and are surveyed extensively. Orchestrating the presentation of these two factors may greatly enhance the process of bone formation and regeneration. We have developed the recombinant AAV vector system capable of sustained co-expression of hVEGF$_{165}$ and hBMP$_7$, which may offer foundation for *in vitro* and *in vivo* experiments of hVEGF$_{165}$ and hBMP$_7$ co-expression and provide a new method for gene therapy of bone regeneration.

MATERIALS AND METHODS

Materials

AAV Helper Free System(pAAV-MCS vector, pAAV-IRES-GFP vector, pAAV-RC plasmid, and pHelper plasmid) and AAV-293 packaging cell line, AAV HT-1080 cells were purchased from Stratagene, USA. E.coli DH5α was a stocked strain from Shaanxi CDC. Plasmid pUC18-hHVEGF$_{165}$ and pBluescript KS-hBMP$_7$ were constructed previously by Dr. Zhibin Shi

☆ This work was supported by National Natural Science Foundation of China(No.30600624)

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and plasmid pIRES was kindly provided by Dr. Zhiming Hao(Department of Digestology, 1st Affiliated Hospital of School of Medicine, Xi'an Jiaotong University). Restriction enzyme and DNA marker were purchased from TAKARA, China. The primers were synthesized by Augtc, China. Plasmid Mini preps and Agarose Gel DNA Extraction Kit were purchased from Qiagen, Germany. DMEM-H Growth Medium and fetal bovine serum were purchased from GIBIC, USA. Calcium Phosphate Cell Transfection Kit was purchased from Invitrogen, USA.

Methods

Construction of the recombinant expression plasmid pAAV-hVEGF$_{165}$-IRES-hBMP$_7$

In order to construct AAV vector co-expressing two different genes, we firstly inserted a fragment of IRES sequence into the multiple clone sites of pAAV-MCS to get IRES linked bicistronic expression cassette. The plasmid pIRES was digested with *EcoR I* and *BamH I*, which respectively located on the upside and downside of IRES sequence. Then the IRES fragment was identified and extracted by 1% agarose gel electrophoresis. The purified IRES fragment was subcloned directly into the multiple cloning sites of pAAV-MCS which was also digested with *EcoR I* and *BamH I*. The positive recombinant clone was named pAAV-MCSa-IRES-MCSb. Secondly, hVEGF$_{165}$ gene was inserted into the upstream MCSa of pAAV-MCSa-IRES-MCSb. Primers were designed according to the sequence published at PubMed(NM_OD3376) to amplify hVEGF$_{165}$ cDNA. Primer F-vegf:5′-CCATCGATATGAACTTTCTGCTGTCTTG-3′, in which cleavage site of *Cla I* was added, primer R-vegf: 5′-CGGAATTCTCACCGCCTCGGCTTGTC-3′, in which cleavage site of *EcoR I* was added. The pUC18-hVEGF$_{165}$ was used as template to conduct PCR. The PCR products were separated and purified by 1.0% agarose gel electrophoresis. Both the PCR products and plasmid pAAV-MCSa-IRES-MCSb were digested with *Cla I* and *EcoR I*, then the PCR segments with adhesive ends were ligased into the upstream multiple cloning sites a(MCSa) of pAAV-MCSa-IRES-MCSb. After verifying with cleavage and PCR screening, the positive clone was named pAAV-hVEGF$_{165}$-IRES-MCSb. Finally, hBMP$_7$ gene was inserted into the downstream MCSb of pAAV-hVEGF$_{165}$-IRES-MCSb. Primers were designed according to the sequence published at PubMed(NM_001719). F-BMP was 5′-GGCCGGATCCATGCACGTGCGCTCACT GCG-3′, in which cleavage site of *BamH I* was added; R-BMP was 5′-GGCCGTCGACCTAGTGGCAGCC ACAG-3′, in which cleavage site of *Sal I* was added. The pBluescript KS-hBMP$_7$ was used as template to conduct PCR. Both the purified PCR products and plasmid pAAV-hVEGF$_{165}$-IRES-MCSb were digested with *BamH I* and *Sal I*, and with T4 DNA ligase the hBMP$_7$ segments were ligased into the downstream multiple cloning sites(MCSb) of pAAV-hVEGF$_{165}$-IRES-MCSb. The positive clone was named pAAV-hVEGF$_{165}$-IRES-hBMP$_7$. The whole procedure of recombination was detailed in Fig. 1. We further identified the positive clone by different combination of double digestion using *Cla I*, *BamH I* and *Sal I* for verifying of the reconstruction.

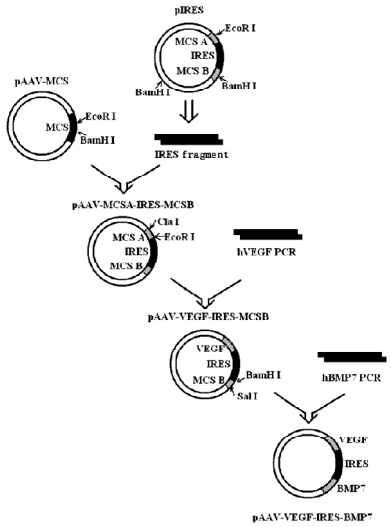


Fig. 1 Conceptual diagram of construction of pAAV-hVEGF$_{165}$-IRES-hBMP$_7$

Packaging of recombinant adeno-associated virus co-expressing hVEGF$_{165}$ and hBMP$_7$ gene

AAV-293 cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum at 37℃ and 5% CO_2. The cells were placed on 100 mm tissue culture plate 48 hours prior to transfection. With calcium phosphate transfection protocol, the triple transfection of pAAV-hVEGF$_{165}$-IRES-hBMP$_7$, pAAV-RC and pHelper was performed to package the recombinant AAV-hVEGF$_{165}$-IRES-hBMP$_7$(experimental group); the triple transfection of plasmid pAAV-IRES-hrGFP, pAAV-RC and pHelper was used to construct rAAV-IRES-GFP which was labeled with GFP and served as viral production parallel group; and a negative control group was performed for simultaneous observation by substituting the recombinant AAV expression plasmid with 10 μl TE buffer. The progress of AAV particle production was monitored under inverted microscope by observing phenotypic changes and cell-cytotoxic reaction of the AAV-293 cells, and the expression of green fluorescent protein in AAV-293 was detected under fluorescence microscope at 24 h, 48 h and 72 h after transfection. The ratio of cells labeled with GFP was calculated to ascertain the packaging efficiency.

After 72 h of incubation, the AAV-293 cells and culture medium was collected together to get viral particles. The cell suspension was subject to four rounds of freeze/thaw by alternating the tubes between dry ice-ethanol bath and 37℃ water bath. After being centrifuged at 10000 g for 10 minutes, the supernatant containing primary virus stock was collected. The virus stock was further concentrated and purified with chloroform/PEG8000/ chloroform protocols.

Viral titer measurement of recombinant AAV

AAV-HT1080 cells were cultured in high glucose DMEM as routine method for viral titer measurement. The viral stock was diluted with DMEM over a 10 fold dilution series, and each dilution was added to infect AAV-HT1080 cells cultured in 24-well plates. Each dilution was performed in triplicate and a no viral well was performed as negative control. The infection was incubated at 37℃ for 2 hours, during which the plates were swirled gently at 30-minute intervals. 48 hours after the infection, the expression of green fluorescent protein in AAV-HT1080 was detected under fluorescence microscope and fluorescing cells of each well was counted. Then the well of which the number of fluorescing cells between 10 and 100 was selected to calculate the viral titer(vp/ml=n × dilution multiple).

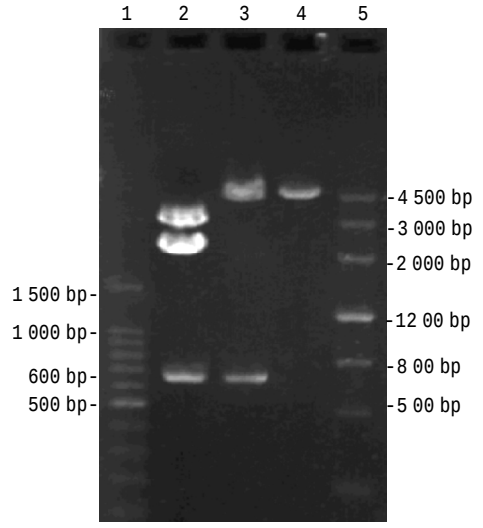
Verify of the recombinant virus rAAV-hVEGF$_{165}$-IRES-hBMP$_7$

To confirm that hVEGF$_{165}$ and hBMP$_7$ gene was successfully inserted into the recombinant AAV, we verified the recombinant virus by PCR of the exogenous interest genes. The virus rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ and rAAV-IRES-GFP were deliquated, DNase and RNase was added with final concentration of 1ug/ml and incubated for 30min to digest the residual DNA and RNA. Then protease K was added to split outer shell of the AAV and the virus genome was extracted using QIAamp VIRUS MINI KIT. 1 μl of virus genome was used as template to conduct PCR with hVEGF$_{165}$ primers or hBMP$_7$ primers. The PCR products were identified by 1% agarose gel electrophoresis.

RESULTS

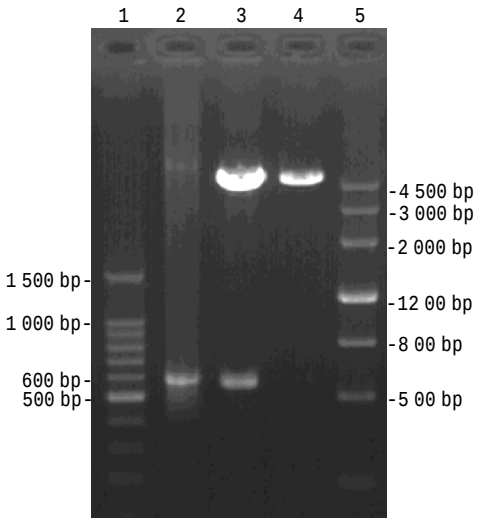
Construction of the recombinant expression plasmid pAAV-hVEGF$_{165}$-IRES-hBMP$_7$

The hVEGF$_{165}$ and hBMP$_7$ gene was successfully amplified and verified from plasmid PUC18-VEGF$_{165}$ and pBluescript KS-hBMP$_7$. The IRES sequence was successfully cleaved and purified from plasmid pIRES and finally subcloned into pAAV-MCS. The recombinant bicistronic expression plasmid pAAV-MCSa-IRES-MCSb was confirmed by double digestion. The amplified hVEGF$_{165}$ and hBMP$_7$ gene was successfully inserted into upstream MCSa and downstream MCSb by turns. With several combinations of double digestion, the size of each fragment was exactly in accordance with expectation(*Fig.* 2,3,4). The pAAV-hVEGF$_{165}$-IRES-hBMP$_7$ was successfully constructed.



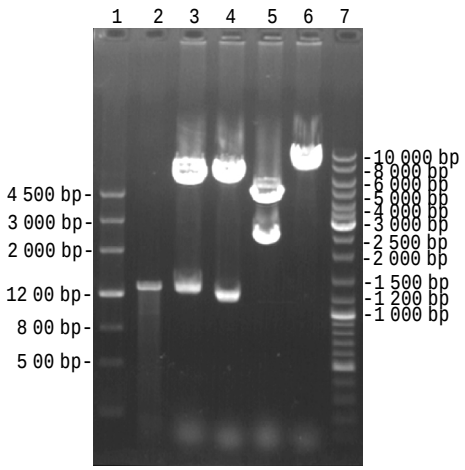
1:100 bp DNA Ladder; 2:pIRES double digested with *EcoR* Ⅰ and *BamH* Ⅰ;3:recombinant pAAV-MCSA-IRES-MCSB double digested with *EcoR* Ⅰ and *BamH* Ⅰ; 4:pAAV-MCS digested with *EcoR* Ⅰ; 5. DNA Marker Ⅲ

Fig. 2 Identification of the recombinant pAAV-MCSA-IRES-MCSB



1:100 bp DNA Ladder; 2:VEGF$_{165}$ PCR; 3: recombinant pAAV-VEGF$_{165}$-IRES-MCSB double digested with *Cla* Ⅰ and *EcoR* Ⅰ; 4: pAAV-MCSA-IRES-MCSB digested with *EcoR* Ⅰ;5:DNA Marker Ⅲ

Fig. 3 Identification of the recombinant pAAV-hVEGF$_{165}$-IRES-MCSB



1:DNA Marker Ⅲ; 2:Bmp_7 PCR; 3:recombinant pAAV-hVEGF$_{165}$-IRES-hBMP$_7$ double digested with *BamH* Ⅰ and *Sal* Ⅰ (BMP$_7$ 1.3 kb); 4:recombinant pAAV-hVEGF$_{165}$-IRES-hBMP$_7$ double digested with *Cla* Ⅰ and *BamH* Ⅰ (hVEGF$_{165}$+IRES 1.2 kb); 5: recombinant pAAV-hVEGF$_{165}$-IRES-hBMP$_7$ double digested with *Cla* Ⅰ and *Sal* Ⅰ (hVEGF$_{165}$+IRES+hBMP$_7$ 2.5 kb) 6: recombinant pAAV-VEGF$_{165}$-IRES-hBMP$_7$ digested with *BamH* Ⅰ; 7:GeneRuler DNA Ladder Mix

Fig 4 Identification of the recombinant pAAV-hVEGF$_{165}$-IRES-hBMP$_7$

Packaging efficiency of recombinant adeno-associated virus

24 h after the transfection, the color of the medium obviously changed from red to orange or yellow in the experimental group and parallel group compared to the negative control group. Some of the AAV-293 cells rounded up and detached from the plate, and were seen floating in the medium. The expression of green fluorescent protein in AAV-293 was detected 24 h post transfection in the parallel group, and the proportion of cells expressing GFP increased rapidly as time passed. The intensity of fluorescence also strengthened gradually. The ratio of cell labeled with GFP reached a climax of 95% at 72 h after the transfection(*Fig.* 5). There was no phenotypic change and cell-cytotoxic reaction in the negative control group and GFP expression in AAV-293 was negative.

Viral titer measurement of recombinant AAV

The expression of green fluorescent protein in AAV-HT1080 was detected under fluorescence microscope 48 hours after the AAV infection, and the number of fluorescing cells of each well was correlated with the concentration of virus administered. The efficiency of infection was 95%(*Fig.* 6). The viral titer was calculated as 5.5×10^{11} vg/ml.

Verify of rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ by PCR of the exogenous interest gene

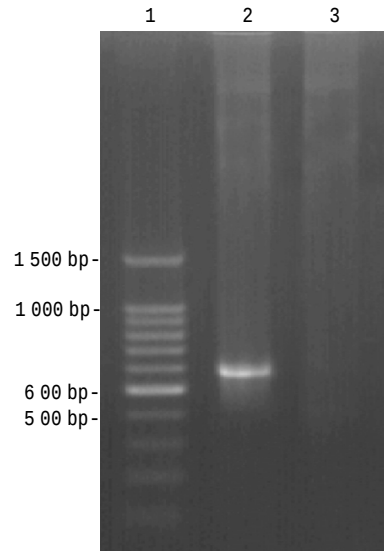
The 600 bp and 1 300 bp target band were separated by 1% agarose gel electrophoresis in experimental group, respectively in accordance with the length of exogenous hVEGF$_{165}$ gene and hBMP$_7$ gene fragment. There was no target band in rAAV-IRES-GFP parallel group(*Fig.* 7,8). The recombination of hVEGF$_{165}$ and hBMP$_7$ gene into rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ genome was confirmed.

A:fluorescence microscope, the GFP expression at 510 nm; B:inverted microscope, white light source.

Fig. 5 Packaging efficiency of recombinant adeno-associated virus 72 h after transfection(× 30)

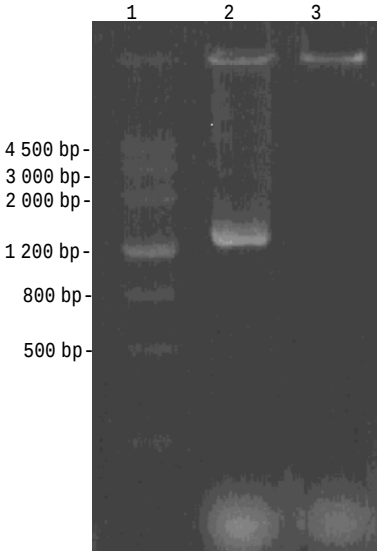
A:fluorescence microscope, the GFP expression at 510 nm; B:inverted microscope, white light source

Fig. 6 Infection efficiency of recombinant AAV for HT1080 cells 48 h after infection(× 30)



1: 100 bp DNA Ladder; 2:rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ group 3: rAAV-IRES-GFP group.

Fig. 7 Identification of VEGF$_{165}$ PCR



1:DNA Marker; 2: rAAV-hVEGF$_{165}$-IRES-hBMP$_7$ group; 3:rAAV-IRES-GFP group.

Fig. 8 Identification of BMP$_7$ PCR

DISCUSSION

Bone renovation is a complicated process involved with many kinds of cytokines, of which VEGFs and BMPs play important roles and are surveyed extensively. Vascular endothelial growth factor is one of the most important cytokines of angiogenesis. VEGF can specifically promote the division and growth of the vascular endothelial cells and finally induce angiopoiesis. VEGF is essential for bone formation and reparation as the whole process of osteogenesis is closely related with VEGF-mediated angiogenesis. Moreover, endogenous or exogenously added VEGF directly attracts endothelial cells and osteoclasts and enhances the differentiation of osteoblasts, as a result regulates bone formation and remodeling[1,2]. On the other hand, osteoblasts can be induced by most osteoinductive growth factors to express VEGF[3]. Inhibition of VEGF blocks FGF$_2$ or BMP$_2$ induced angiogenesis, BMP$_7$ induced primary osteoblast differentiation, and BMP$_4$ induced bone formation[4-6]. BMPs are multifunctional cytokines which are members of TGF-beta superfamily. BMPs have definite effects of stimulation of mesenchymal and osteoprogenitor cell proliferation and differentiation[7,8]. They are the only signaling molecules which can singly induce de novo bone formation at orthotopic and heterotopic sites. Extensive studies demonstrate that BMPs are important factors regulating chondrogenesis and skeletogenesis during normal embryonic development[9]. In bone formation, BMP$_7$ mainly acts by inducing the expression of the critical transcription factors Runx2 and Osterix in mesenchymal stem cells, thereby committing and directing them in osteoblast differentiation. It is reported recently that bone morphogenetic proteins also stimulate osteoblastogenesis and angiogenesis through the production of VEGF-A. In the presence of a VEGF-A antibody, both unstimulated and BMP-stimulated angiogenesis were arrested[3]. These results strongly suggested that angiogenesis and bone induction is a correlated and coordinated process during bone formation and their effects may be enhanced by each other. VEGF participates in BMP induced osteogenesis by taking part in bone cell differentiation and by promoting angiogenesis at the site of bone formation, and BMP regulating osteogenesis and bone renovation by increasing VEGF expression of osteoblast.

As bone formation is a coordinated process involving BMPs and VEGFs, orchestrating the presentation of these two factors may greatly enhance this process. It is reported that combined delivery of recombinant BMP and VEGF in scaffolds resulted in a significant increase in the quantity of regenerated bone compared with delivery of single factor[10]. And in rabbit models with femoral head bone defects, combined administration of BMP and VEGF lead to a elevation of microvessel count at 2 weeks and lead to a elevation of ALP activity, calcium content for 12 weeks[11]. In gene therapy, introduction of multiple synergistic genes into cells can also provide a better effect than single gene alone[12]. Among the several different strategies to coexpress multiple genes, the incorporation of IRES (internal ribosome entry site) into gene therapy vector represents one of the more promising strategies. IRES functions as a ribosome-landing pad for the efficient internal initiation of translation ensuring coordinate expression of several genes[13]. IRES initiate ribosome

binding and translation in the absence of a 5' CAP, which overcome the main disadvantage of traditional strategy which express two different genes under two different promoters, two transcripts and two polypeptides[14]. This character is especially useful for AAV production due to packaging size limitation imposed by AAV vectors. In this study, IRES sequence was incorporated into pAAV-MCS to construct a bicistronic vector, then $hVEGF_{165}$ and $hBMP_7$ gene was respectively inserted into upstream MCS and downstream MCS located on either side of IRES, and the length of the bicistronic frame is 2.5 kb which within the capacity of the vector. We finally confirmed that exogenous gene of $hVEGF_{165}$ and $hBMP_7$ has been successfully inserted and incorporated with genome of the recombinant AAV.

Choosing of a safe and effective vector system to transfer and correctly express the target gene is also important issue to consider in gene therapy. Adeno-assnciated virus has many natural features that make it attractive for a human viral vector. It is a non-pathogenic defective human parvovirus that requires the presence of a helper virus such as adenovirus or herpes virus for productive infection[15]. Other advantages of this vector system includes the low immunogenicity, the ability to transduce both dividing and non-dividing cells, the potential to integrate site specifically and to achieve long-term gene expression even *in vivo*, and its broad tropism allowing the efficient transduction of diverse organs[16]. All these features make AAV attractive and efficient for gene transfer *in vitro* and local injection *in vivo*. Using the AAV helper-free system in combination with IRES sequence, we successfully constructed adeno-associated virus co-expressing $hVEGF_{165}$ and $hBMP_7$. The viral titer was measured by infecting AAV-HT1080 optimized by Stratagene, which precluded the disturbance due to empty viral particle and difference of cell liability. The recombinant adeno-associated virus has a high titer of 5.5×10^{11}vp/ml, meeting the requirements of experiments *in vitro* and *in vivo*. GFP fluorescence in the system may be used to monitor the process of AAV production and measure the titer of the recombinant virus directly. In addition, since both proteins are translated from the same transcript, GFP expression may be used to ascertain the infection efficiency for the desired target cells and also serves as a useful expression marker for the inserted gene of interest. The construction of rAAV-$hVEGF_{165}$-IRES-$hBMP_7$ lays a basement for later study on the synergistic action of $hVEGF_{165}$ and $hBMP_7$ and may provide a new method for bone regeneration therapeutics.

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