DEVELOPMENT OF NOVEL MUSCLE-SPECIFIC ADENO-ASSOCIATED VIRAL VECTOR CONSTRUCTS FOR GENE THERAPY OF DUCHENNE MUSCULAR DYSTROPHY

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Abstract

To investigate efficacy of synthetic SPc5-12 promoter expression in skeletal and heart muscles and compare its activity with that of MYODI and MYLK2 muscle specific promoters. We hypothesized that we could achieve efficient, safe and global cardiac and muscle gene transfer in mice following a single intravenous injection of scAAV-SPc5-12-luc2 vector.

We reported relatively robust levels of luciferase expression in the heart and moderate levels in the skeletal muscles in particular the tibialis and gastrocnemius which are representative of fast twitch muscle fibers. No significant luciferase expression was observed in other tissues such as liver, lung, spleen and brain. The result of the luciferase expression quantified as (photons/sec/cm2/steridian) in the individual dissected organs were similar to the optical imaging results observed of the whole mice. This study report for the first time that the SPc5-12 promoter delivered by AAV9 serotype is robust and muscle specific.

Keywords: Gene therapy, Adeno-associated virus, Systematic gene transfer, SPc5-12 promoter, Luciferase

Introduction

Duchenne muscular dystrophy (DMD) is X-chromosome-linked recessive neuromuscular disorder found in 1 in 3500 live male births (Singh et al. 2010; Willmann., 2009). The disease is associated with continuous cycles of muscle cell regeneration and degeneration, ultimately resulting in a

failure of muscle regeneration. Symptoms include constant falling and out turned knees appear as early as age two and during extreme DMD, patients are unable to sit upright, move their arms or legs, or breathe on their own (Singh et al., 2010). Patient's life span rarely exceeds early mid-twenties due to cardiac or respiratory failure. This fatal muscle wasting diseases is caused by small deletions or point mutations that introduce a stop codon resulting in very little or no dystrophin production (Yue et al., 2008; Anderson et al., 2006; Dunant., 2003). The dystrophin gene spans 2.9 Mb, consisting of 79 exons and is expressed as a 14-kb transcript in muscle cells (Anderson et al., 2006). In normal skeletal muscle, dystrophin is localized to the cytoplasmic surface of the sarcolemma, where it is involved in a series of molecular interactions forming a mechanical or structural link between the actin based myofiber cytoskeleton and sarcolemma transmembrane glycoprotein complex (de León et al., 2005; singh et al., 2010) hence it stabilizes muscle cell membrane during cycles of contraction and relaxation (Willmann., 2009). The protein also plays a role in regulating the activity of membrane proteins as well as acting as a structural component of the membrane (Jeffrey S Chamberlain., 1993). The absence of dystrophin in DMD leads to a loss of function of the sarcolemma, causing the muscle fibers to be brittle and susceptible to membrane stress, this in turn leads to sarcolemmal fragility, muscle weakness and even muscle cell necrosis. In addition to its protective role, dystrophin has been proposed to act as a signaling molecule in the cell signaling pathways such as muscle cell growth, cytoskeleton organization, muscle homeostasis and atrophy/hypertrophy (Rando ., 2001)

Promising findings to treat DMD are emerging from gene therapy strategies in which the ultimate aim is to correct dystrophin disorder by stable transfer of a functioning gene into cells to ensure the permanence of the correction (Eldestein ., 2007; Rodino-Klapac et al., 2007) . To achieve this goal, appropriate regulatory elements that impart tissue-specific transgene expression in cardiac and skeletal muscle need to be identified (Anderson et al., 2006).

Materials and methods

Generation of constructs

scAAV expression plasmid designated pdsAAVscMONKEY (6199bp) was digested using appropriate enzymes ACC651 (GGTACC) and SphI (GCATGC) to remove unwanted Serp enhancer, TTRm promoter, MTTR 5'utr, MVM intron, FIX Co MT and bGH polyA fragments in order to replace a novel muscle specific expression cassette into this scAAV vector back bone (4001bp). Primers were designed to PCR out SPc5-12 promoter region from Human MDI plasmid. PCR was carried out using Finnzymes Phusion Hot start II High fidelity DNA polymerase Kit, the DNA

polymerase possess DNA polymerase and exonuclease activity. MVM intron to promote mRNA stability, sv40PA and luciferase Reporter gene sequences were synthesized and cloned into scAAV plasmid under the control of the synthetic muscle restrictive promoter Spc5-12.

Tissue culture

HEK 293 cells were seeded at 70% confluent and co-transfected with the expression plasmid (*pdAAVsc-SPc5-12-MVM-Luc2-sv40pA*), rAAV2/9 plasmid and Adenoviral helper plasmid (Stratagene) using calcium phosphate transfection kit supplied by Invitrogen. Cell pellets were harvested 48 hours post transfection, lysed by three freeze and thaw followed by sonication to release the viral particles. Cell suspension was purified by centrifugation at 3600rpm, 4^0c, 25 mins. The lysate were layered on a celsium chloride gradients and overnight centrifugation performed at 25,000rpm, 15^0c, 90Ti rotor, 18-20hrs and subsequently at 59,500rpm, 15^0c, 80Ti rotors, for 20-24hrs. The gradient fractions between 1.3670-1.3740 RI containing rAAV particles was determined using Abbe refractometer, isolated and dialyzed/concentrated against sterile 10ml PBS 1 mM $Mgcl_2$, through an Amicon ultracentrifugal filter Millipore.

Vector titration

The number of vector genome was determined relative to a plasmid DNA standard using quantitative PCR in ABI7500 FAST (Sequencer Detector Unit from Applied Biosystems, Foster, CA, USA) using SYBR Green mix and luciferase specific primers Forward-CCCACCGTCGTATTCGTGAG and Reverse-TCAGGGCGATGGTTTTGTCCC used to detect luciferase as transgene DNA target sequence.The sample (vector) was further diluted in the ratio of 1: 250 and a reaction mix containing ABI SYBR Green Mix, primers, and water to top up to required volume was put in each reaction well. After loading the samples, standard and the negative control (water) onto the 96 well Q-PCR plate, the Q-PCR was run from ABI7500 standard running: 50°C for 10minute, 95°C for 2 minutes and subsequent 40 cycles of 95°C for 15seconds and 60°C for 1 minute making each run last for about 1 hour and 35 minutes. Samples were loaded in triplicates.

Animal studies

Animal studies were approved by the institutional Animal Ethical Commission. $5x10^9$ vector genomes (vg) were injected (i.v) into the tail vein of Neonatal CB17-SCID mice purchased from Taconic Breeding Laboratories. Negative control mice were injected with 1X DPBS. The animals were held in the tail vein position and their tails were warmed before

the injection. The injections into the veins were carried out using 28 gauge needles. All the mice recovered from the injection quickly without loss of mobility or interruption of grooming activity.

***In vivo* imaging**

All images were acquired using a biospace *in vivo* photo Imaging System (IVIS). The CCD was cooled to -23^{0}C and the field of view (FOV) set at 25-cm height of the sample shelf. The charged coupled device (CCD) camera operates by converting photons that strike the CCD pixel into electrons at wavelengths between 400-100nm, allowing visible detection through infrared light signals. The mice were anesthetized with 2% isofluorane and oxygen. D-luciferin substrate was then injected intravenously, at a dose of 15 μg/g of body weight. The mice were then placed in a light-tight chamber and images generated using a cryogenically cooled charge-coupling device camera. For each mouse, the images were taken at a photo acquisition time limit of 10 minutes after the substrate injection and emitted light images of mice collected. The visual output represented the number of photons emitted/second/cm^2/steridian (sr) as a color image where the maximum was red and the minimum dark blue. Following imaging, the animals were kept warm with lamps and allowed to recover in the cages. All mice were imaged on a weekly schedule after AAV vector injection.

Quantifying luciferase expression in mice

For quantification of transmitted light signals, at 7 weeks post injection, the mice were killed and selected organs were dissected: brain, heart, liver, lung and skeletal muscles (biceps, triceps, quadriceps, tibialis and gastrocnemius). Region of interest (ROI) were drawn around each organ area and the bioluminescence signals emitted obtained as photons/sec/cm^2/sr.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was used to detect mRNA abundance for luciferase in all the twelve different tissues isolated. Messenger RNA (mRNA) was first extracted using an RNeasy kit (Qiagen) and then total mRNA concentration determined by Spectrophotometry using a Nanodrop. Superscript TM III First strand Synthesis system kit (Invitrogen) was used to convert mRNA from each sample into first strand cDNA. RNA primer mix in a final volume of 10ul/rxn (100ng total RNA, 50μM oligodT, 10mM dNTP mix, DEPC-treated water) was incubated at 65^{0}c for 5mins and placed in ice for 1 min. Added to CDNA synthesis mix in a final volume of 10ul per reaction (1 of 10x reverse transcriptase buffer, 4ul of 25Mm $Mgcl_2$, 2ul of 0.1M DTT, 1ul of 40U/ul RNaseOUT, 1ul of 200U/ul superscript ^{TM} III RT.

The reaction was incubated at 50^0c for 50 mins and the reaction terminated at 85^0c for 5 minutes then chilled on ice.

Real time PCR was performed with a reaction mixture for each sample of 2.5 ul forward primer (20 nmol/l), 2.5 ul reverse primer (20 nmol/l), 2.5 ul RNAse-free DNAse-free water, and 12.5 ul SYBR Green Master Mix (ABI). Specific luciferase primers CCCACCGTCGTATTCGTGAG (forward) and TCAGGGCGATGGTTTTGTCC (reverse) was used in the reaction.

For each experiment, a reaction mix was prepared; for each tube Aliquots (20 ul) of this mix were placed in the tubes and 5 ul cDNA solution was added to each reaction. All reactions were processed in triplicate. The experimental run protocol was denaturation (95 °C for 10 minutes) and 40 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds with a single fluorescence measurement. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA served as the internal control gene for normalization of sample loading.

Results

Kinetics of scAAV9-SPc5-12-luc2 vector mediated gene expression in mice

In this study we examined transduction efficiency and biodistribution of SPc5-12 muscle specific synthetic promoters in AAV9 following tail vein injection of newborn mice with $5x10^9$ vector genome per mouse. To visualize localization pattern of luciferase and vector biodistribution profile, mice were assessed by quantifying luciferase expression at a weekly time point interval using an *in vivo* bioluminescent photo imaging system. The heart exhibited the strongest level of luciferase signals (greenish red color) followed by skeletal muscles that had moderate luciferase signals (light blue color). No significant signals were detected in other organs like liver, lung, brain, diaphragm and spleen. This result also demonstrated that scAAV9–SPc5-12-luc2 vector efficiently transduced both the heart and skeletal muscles irrespective of the animal gender (Fig.1).

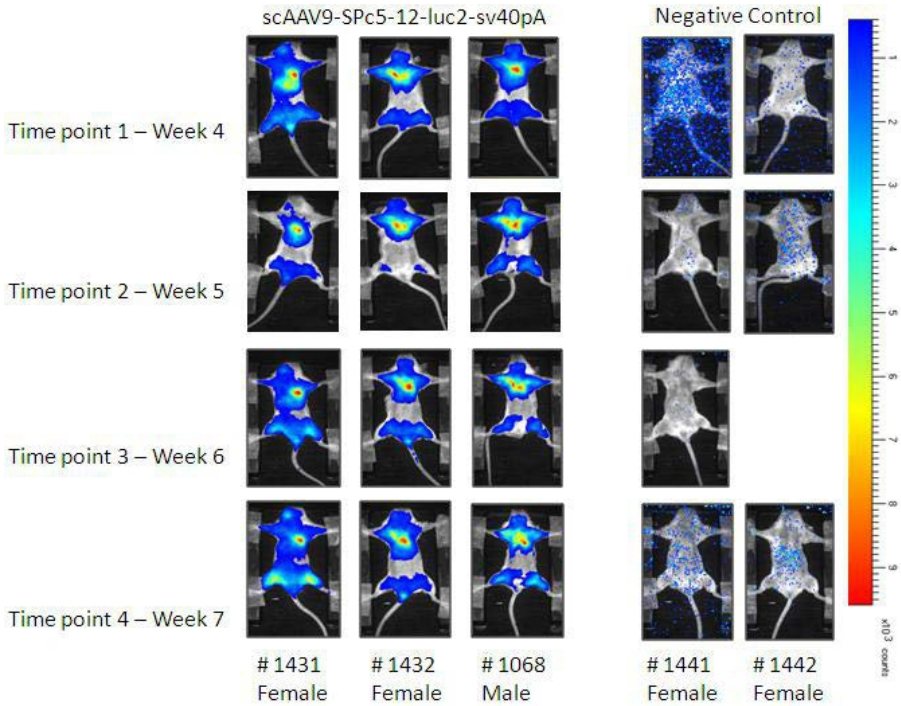

Figure 1: Optical imaging of luc2 reporter gene expression in vivo. Imaging was performed for firefly luciferase (luc2) expression after intravenous injection of D-luciferin substrate (15ug/g). Study mice transfected with AAV9-SPc5-12-luc2 ($5X10^9$) emitted significant cardiac luc2 activity (greenish red color) at 4, 5, 6 and 7 week post injection. Moderate luc2 activity (dark blue color) was observed in skeletal muscle. The control mice administered with PBS showed only background signals.

In vitro luciferase Assay in selected tissues

To observe differential expression driven by SPc5-12 promoter in specific tissues, seven weeks post injection the mice were euthanized and heart, spleen, liver, lung, kidney, diaphragm, brain and different skeletal muscle tissues from the leg and thigh (tibialis, gastrocnemius, quadriceps, triceps and biceps) were dissected out and observed under the imaging machine (Fig. 2). The results obtained correlated with the data observed during serial optical *in vivo* bioluminescence imaging. Gene transfer was more efficient in the heart than any other organ with an extensive region of the myocardium organ displaying intense luciferase signals (reddish-green light). Most of the skeletal, leg muscles (gastrocnemius and tibialis) and thigh muscles (quadriceps and biceps) yielded intermediate levels of luciferase signals. Moreover, there was no significant luciferase expression in other tissues such as lung, spleen, liver, kidney, brain and diaphragm.

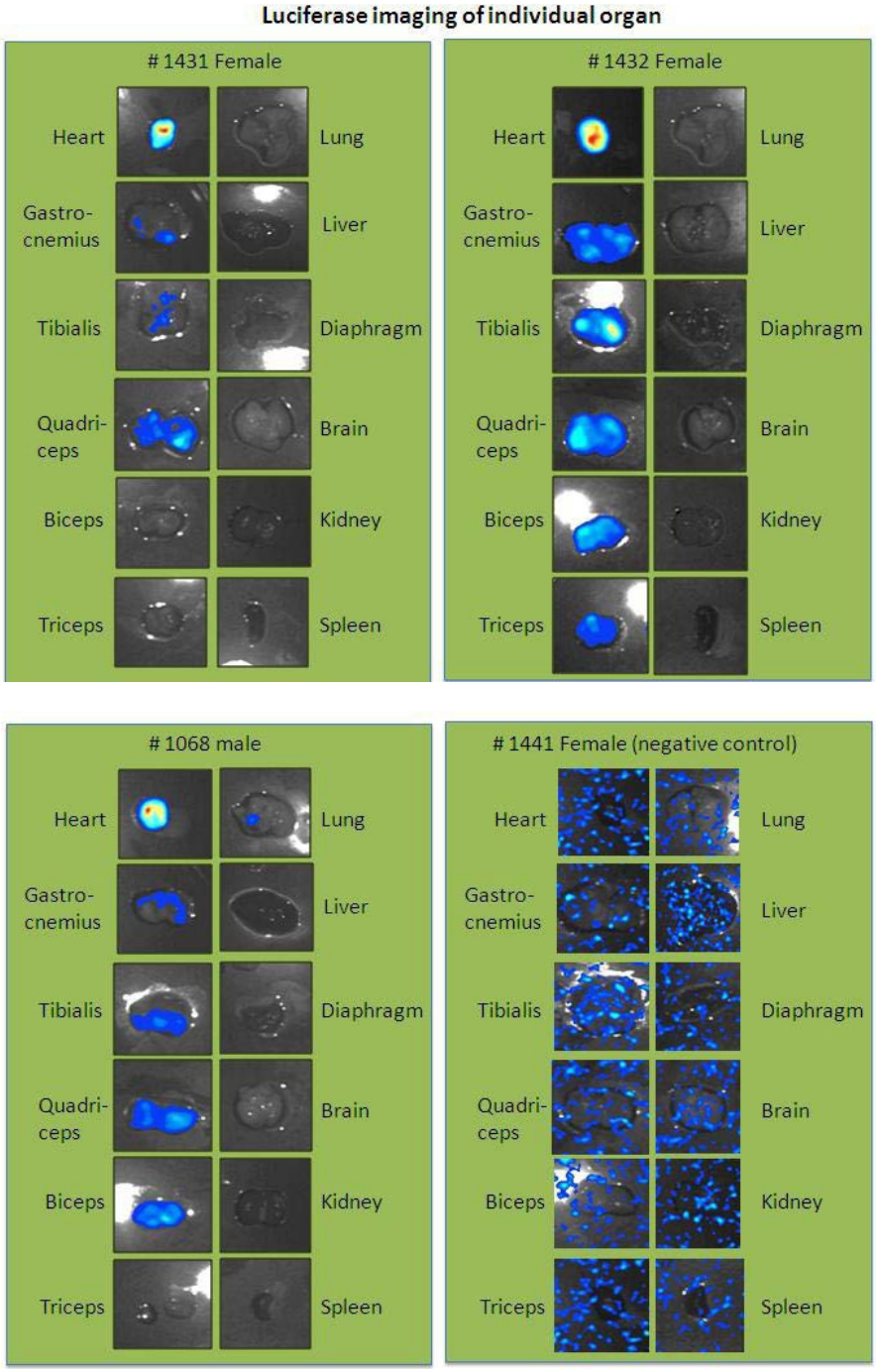

Figure 2: Representative bioluminescence images of luc2 expression in the different tissues of the neonatal mice. Luc2 expression could be observed in heart and skeletal muscles such as tibialis, quadriceps, biceps and gastrocnemius. The negative control mice injected only with PBS without the vector construct did not exhibit any luciferase expression.

To further verify whether the bioluminescence data obtained during non invasive optical imaging reflects actual luciferase activity observed *in vitro*, we plotted graphs of luciferase signals (photons/sec/cm2/sr) emitted against the different tissues of mice to effectively analyze the organs which were robustly transduced by scAAV9-SPc5-12 construct (Fig3). The luciferase expression signals obtained from the individual organs confirmed the biodistribution patterns observed during optical imaging. The main expression was in the heart which displayed the highest luciferase signals (1.30E+06) followed by quadriceps, gastrocnemius and tibialis of the skeletal muscle (fig.3)

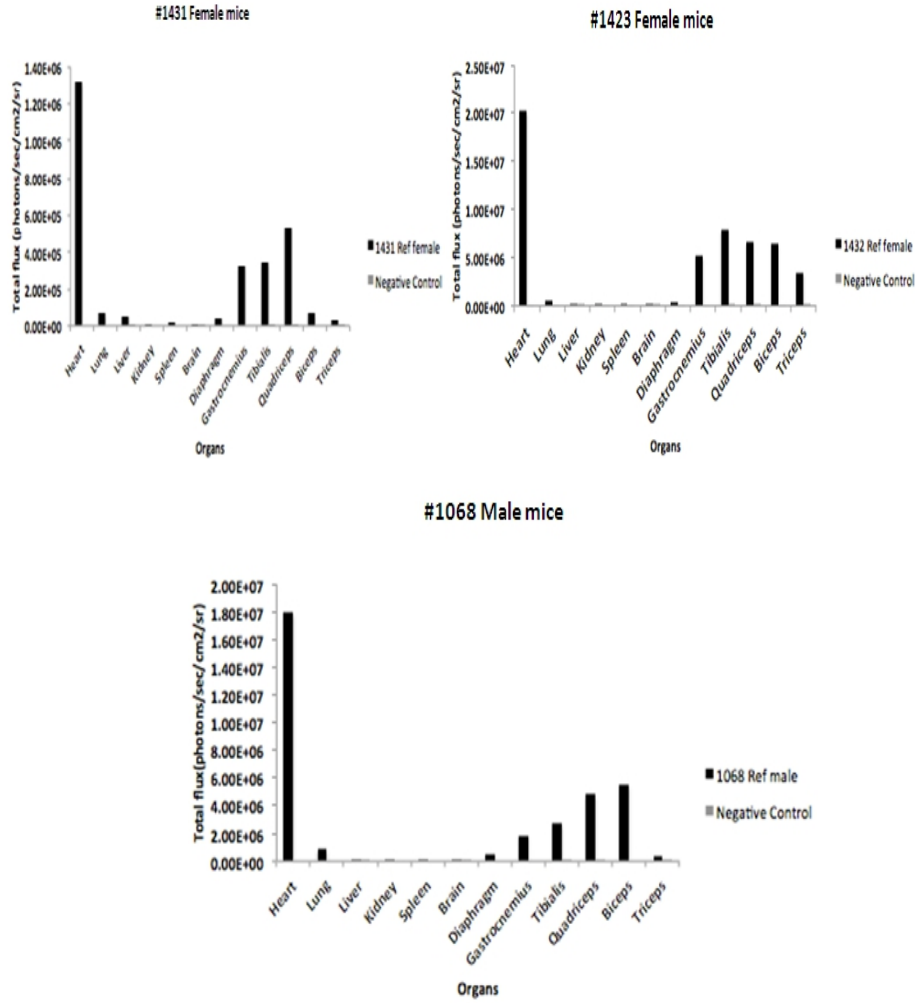

Figure 3: **Plot of luciferase activity in different tissues of mice**. *Signal intensities represented as photons/sec/cm2/s***r** *plotted against the different tissues. The heart muscle still emerged as the best transduced organ, followed by the skeletal muscles such as quadriceps, gastrocnemius and tibialis. Signal intensities in the lung, liver, kidney, brain and spleen are insignificant. No luc2 signals were obtained in the negative control mice.*

RT-PCR and genomic copy number assays are currently ongoing to further confirm if the levels of luciferase mRNA and remaining viral genome copy numbers in the heart, skeletal tissues and all the other different organs mimics the results observed during luciferase localization pattern and the analyzed levels of luciferase signals (photons/cm2/radian) using the BLI machine .

This imaging data shows that the SPc5-12 promoter is robust and specific for cardiac and moderate for skeletal tropism. The non targeted tissues such as liver normally efficiently transduced by AAV9 serotype did not express luciferase despite the presence of substantial amount of vector genomes injected. A reasonable explanation for this is that the SPc5-12 promoter incorporated in our expression cassette was not active in these other tissues rather was specific for heart and skeletal muscle. It has been shown that gene expression in the skeletal muscles following systemic delivery can be increased by using muscle specific promoters such as SPc5-12 element that has been reported to restrict transgene expression to the skeletal muscle cells (Li et al 1999).

Discussion

Gene therapy aims to target the various underlying cellular and molecular abnormalities causing a disease and it has a great potential for the treatment of several diseases which no definite pharmacological agents exists i.e. heart failure, muscular dystrophies and cystic fibrosis (Zincarelli et al., 2008). Many genetic diseases affect multiple organs/tissues and the challenge for gene therapy for these diseases lies in delivery of the therapeutic gene to all the affiliated tissues. More than 50% of the gene therapy clinical trials are currently using viral vectors for efficient gene delivery (Daya & Berns., 2008) and recombinant adeno-associated virus vectors (rAAVs) has emerged as one of the most promising vectors for systemic and local long term delivery of gene therapy for clinical diseases (Akache et al., 2006; Aikawa et al., 2002). There persistence in a wide range of tissues, lack of pathogenicity, physical stability and divergence of the capsid sequence in the new serotypes has resulted in striking differences in their transduction profile (Ghosh et al., 2007; Akache., 2006, Aikawa.,2002). The promiscuous tropism of AAV may lead to undesirable expression of therapeutic genes in non-targeted cells and this limitation can be circumvented by use of tissue specific promoter i.e. SPc5-12 (X. Li et al.,1999) to specifically drive the gene of interest to target tissues as cardiac and skeletal using rAAV. Body wide dissemination has particularly been reported for AAV 6, 8 and 9 hence this vectors are of major interest to cure many genetic diseases that requires global transduction of all the affected tissues i.e. in the case of gene therapy of DMD where mortality results from

severe pathology not only in the skeletal muscles but also heart, successful gene therapy may require lifelong expression of therapeutic gene in all the affected muscles and this vectors have been found to have the ability to transduce both cardiac and skeletal muscles (Foster et al. 2008; Rodino-Klapac et al. 2007).

When developing gene therapy constructs, it is important to minimize adverse responses to protein expression in unnecessary sites by restricting transgene expression to areas where most suitable. An approach to confine and or to increase specificity is to use a gene delivery vehicle with high tropism for the tissue of interest and select a promoter that drives the expression of a particular gene in the tissue(s) of interest (Pacak et al., 2008). The synthetic SPc5-12 promoter used in our study has been reported to have *in vivo* specificity in cardiac and skeletal muscle tissues and that their transcriptional potency greatly exceeds transcriptional levels obtained from natural myogenic and viral gene promoters (Li et al., 1999). Studies have also shown that robust expression system requires interplay between Transcription factor complexes binding to the multiple TFBS of a promoter and enhancer regulatory regions (Li et al., 1999). This study explored the potential of systemic gene transfer with scAAV9 and SPc5-12 muscle specific promoter to direct expression of luciferase gene to targeted skeletal and heart tissues. Comparative studies are still currently underway with MYOD1 and MYLK2 promoters to determine the most robust and specific promoter conferring long-term and tissue-specific transcriptional activity in the muscles. In this study, rAAV- Spc5-12-luc2-MVM-sv40 construct was successfully cloned and all the clones were confirmed using multiple restriction enzymes analysis. The corresponding AAV9 vector was produced and 95% transfection efficiency was achieved 48 hr after transfection. This step was crucial to achieve good AAV vector titer after production. We used tail vein injection route of administration which best allows for investigation of tissue tropism and allows tissue-specific expression if tissue specific promoters/enhancers are used in conjunction with the desired serotype. Immunodeficient CB17SC mice were chosen in order to assess efficiency without the complication of antibody response to the transgene product (Y. Liu et al., 2004). A dose of 5x109vg was injected into each mouse and analysis done using a bioluminescence imaging system. AAV9 has been reported to stand out as an attractive vehicle for high transduction levels in cardiac and skeletal tissues (VandenDriessche et al. 2007; Bostick et al. 2007; Pacak et al. 2008; Inagaki et al. 2006). Consistent with these reports, we also observed efficient transduction and robust luciferase levels of expression in the skeletal and cardiac tissue following intravenous administration. In a study Wang et al., 2008 also showed that neonatal mice respond more efficiently following intravenous administration of AAV

vector in heart and muscles gene transfer compared to adult mice that also showed expression but was less efficient. Our results concurred with these findings since we obtained significant levels of expression in cardiac and skeletal after intravenous administration of vector to newborn mice 48hrs old. Further exploration of age associated difference in AAV9 transduction may have significant clinical applications.

When the skeletal muscles were examined by measuring photons/sec/cm2/sr there was expression of the transgene product but it was not as strong and impressive as in the heart muscles. The levels were much lower than expected despite the fact that the design of our expression cassette incorporated SPc5-12 promoter intended to maximize transgene expression to skeletal muscles (li et al., 1999). SPc5-12 promoter contains a combination of muscle specific transcriptional regulatory elements SREs, E-boxes, MEF -2, MEF-1 and SP1 that has been reported to be responsible for long-term transgene expression and up regulation of expression predominantly in the cardiac and skeletal muscles both in neonatal and adult mice *in vivo* (Li et al., 1999). Pacak et al.,2008 and Aikawa et al.,2002 demonstrated that efficient viral transduction of muscles require direct injections into a specified tissue or at least injection into the arterial supply of target muscle to help concentrate transduction to that exact location . Furthermore, Mary Mc Menamin also observed expression of micro-dystrophin in AAV1 at high levels was via intramuscular and intraperitoneal administration but not via intravenous route of administration. Gregorevic et al., 2008 demonstrated that the use of AAV6 in conjunction with 10ug VEGF also allowed a widespread transduction of most skeletal and cardiac muscles. Moreover Intravenous technique using pressure has also demonstrated its easiness and efficiency to delivery in skeletal muscles in several species though skeletal muscle in general, may prove difficult to transduce upon systemic administration of AAV due to blood vessel barriers (Z. Wang, T. Zhu, et al., 2005). However, most studies including our own have utilized the intravenous pathway for AAV9 delivery since it has the advantage of reaching most organs in the body when gene therapy vector is introduced into the bloodstream compared to localized transduction. The utilization of AAV9 with the use of a robust and muscle specific promoter allows for efficient systematic gene delivery specifically to the skeletal and heart muscles. Our finding is therefore important for the potential use of AAV9 for gene therapy of muscle related diseases such as Duchene Muscular Dystrophy and Myotonic dystrophy. Since superior cardiac transduction may have been recognized as a feature of AAV9 following intravenous injection with a dose of 5x109, dose response experiment using different doses is ongoing in the lab to examine the potency and kinetics of gene expression at lower and higher doses of viral particles. This is to assess

whether expression is dose depedent and determine if high efficiency can still be conferred in the heart and skeletal when the SPc5-12 muscle specific restrictive promoter is maintained at a higher or lower dose. Skeletal muscles are composed of fast twitch muscle fibers that depends mainly on glycolytic energy and slow twitch myofiber that depends on oxidation energy for metabolism in the muscles (Dunant et al., 2003: Giger et al.,2005). In our study we used SPc5-12 promoter construct to evaluate whether there was any selectivity for expression within fast twitch glycolytic muscles because DMD involves preferential loss and or degeneration of large glycolytic fast muscle fibers compared to the slow twitch oxidative muscle fibers (Dunant et al., 2003). The use of fast twitch muscle promoters can lead to sufficient transgene expression which might be of particular clinical relevance. It has also been shown that AAV2 preferentially transduces slow twitch fibers while AAV6 transduces both fiber types though it still wasn't clear whether AAV9 displays a fiber type preference (Gosh et al., 2006). In our experiment detailed analysis of tissue biodistribution of the luciferase revealed that luc2 expression driven by SPc5-12 promoter was predominant in the gastrocnemius, tibialis and quadriceps which are fast twitch muscles. The transgene was not expressed in other tissues like spleen, brain, kidney, liver, lung and diaphragm. This observation clearly showed that the combination of AAV9 and use of SPc5-12 promoter led to a significant gene transfer particularly in fast twitch muscles. This is an important observation and has important implication for gene therapy for DMD.

Conclusion and Prospects

This study confirmed that the SPc5-12 promoter delivered by AAV9 serotype is robust and muscle specific. Experiments are in progress to determine if the SPc5-12 promoter is truly muscle specific when a provocative AAV9 vector dose is used. Other potentially novel muscle specific promoters such as MYOD1 and MYLK2 identified using the DDM algorithm are to be compared side by side with the SPc5-12 promoter in future experiments to identify the most robust and specific promoter. Since glycolytic muscle fibers are more vulnerable to the dystrophic process in DMD, comprehensive studies should further be conducted to examine in detail and compare the activity/ expression of muscle specific promoters in the main representative fast twitch (tibialis anterior, quadriceps, gastrocnemius) and slow twitch skeletal muscles tissues i.e. Soleus and Extensor Digitorum Longus (EDL). All these findings could later on be used to improve constructs of gene therapy for DMD and explored as experimental tools particularly to validate therapeutic approaches in animal disease models.

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