Identification of Novel Muscle-Specific Promoters for AAV Gene Expression in Skeletal and Cardiac Muscles

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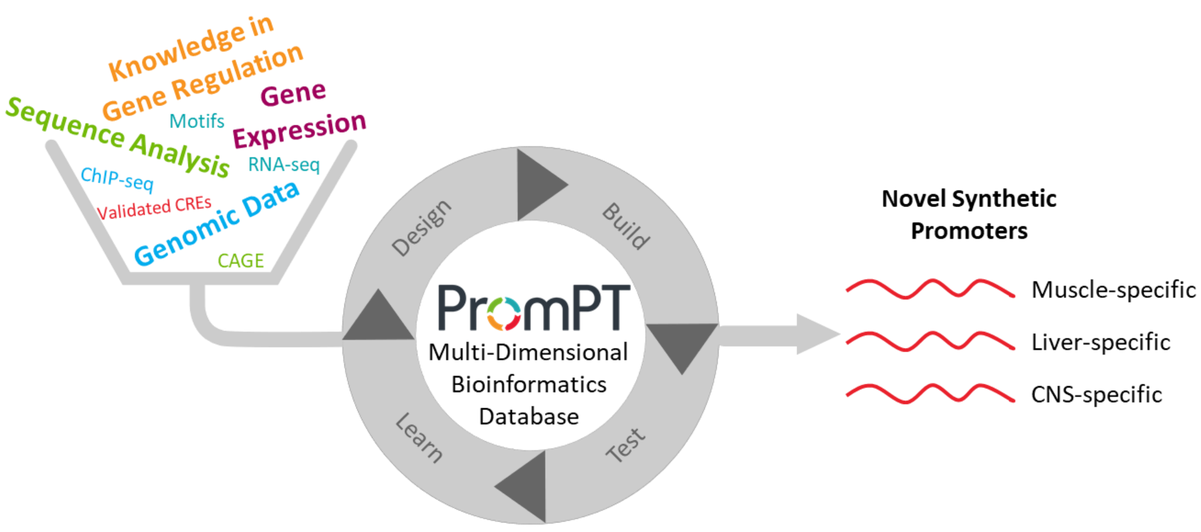
Abstract

Successful expression of an AAV transgene in a specific tissue relies on both the capsid tropism and the specificity of the promoter that drives the packaged expression cassette. We sought to engineer novel promoters for AAV-based gene transfer specifically in muscle tissue. Given that AAV has a limited packaging capacity, such promoters should not only drive strong muscle-specific expression but should also be as short as possible. A bioinformatics approach was employed to generate promoter sequences with predicted muscle specificity. These were first screened *in vitro* for specific expression in differentiated mouse myoblasts (C2C12 and H2K 2B4) and rat cardiac cells (H9C2). Candidates with the highest activity in comparison to a known muscle-specific promoter are additionally being characterized *in vivo* by both electroporation into tibialis anterior and systemic injection in mice, resulting in the comparison of several novel and potent muscle-specific promoters that can be used in AAV gene transfer to muscle. These findings are particularly relevant for treatment of diseases like Duchenne muscle dystrophy (DMD) with AAV gene transfer of microdystrophin (µDys), a smaller functional version of the missing or faulty dystrophin gene in DMD patients.

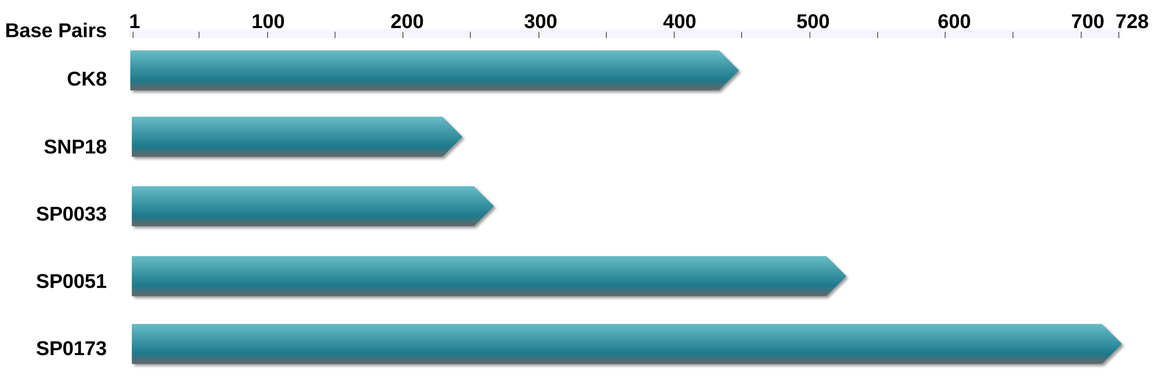
Background



PromPT®

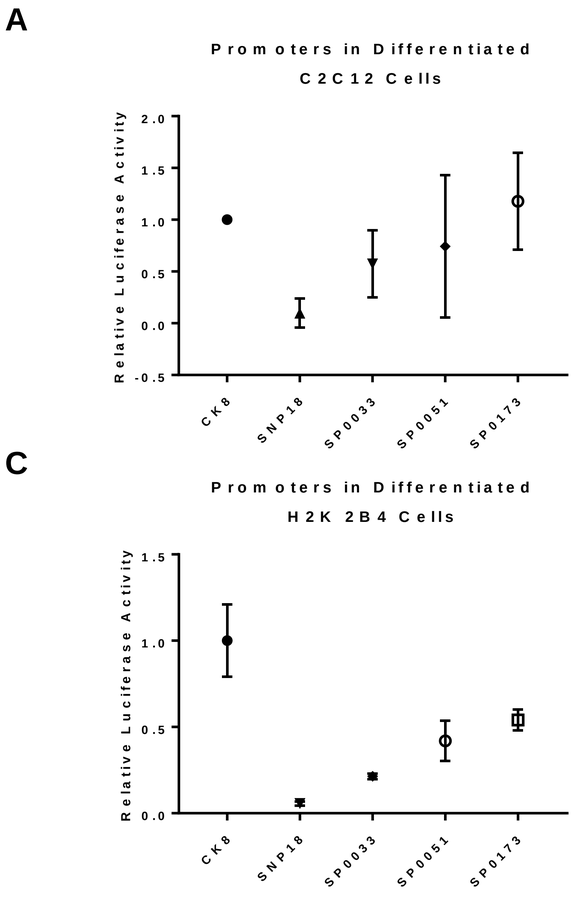
- **Synpromics' proprietary bioinformatics engine uses genomic data to design synthetic promoters. The designs are tested experimentally. The experimental results are fed into Machine Learning tools to help learn the regulatory code. The iterative process results in novel synthetic promoters matching the desired activity.**

Promoter Identification



- **Bioinformatics analysis was used to identify enhancer regions present in a set of genes differentially overexpressed in the muscle.**
- **Candidate enhancer elements were synthesized upstream of core promoters and transfected into a variety of muscle cell types.**
- **Enhancer elements were then combined to create novel muscle promoters .**
- **Novel promoters ranged in size from approximately 240 base pairs to 730 base pairs.**
- **CK8 is used as the benchmark for all novel promoter comparisons, as it is a potent muscle specific promoter that is ~450 base pairs in length.**

Results



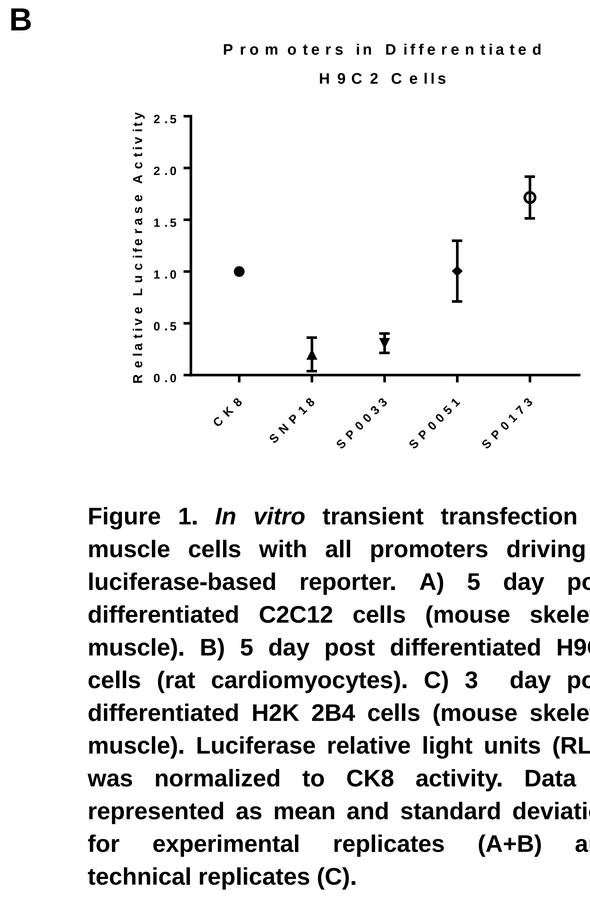


Figure 1. *In vitro* transient transfection of muscle cells with all promoters driving a luciferase-based reporter. A) 5 day post differentiated C2C12 cells (mouse skeletal muscle). B) 5 day post differentiated H9C2 cells (rat cardiomyocytes). C) 3 day post differentiated H2K 2B4 cells (mouse skeletal muscle). Luciferase relative light units (RLU) was normalized to CK8 activity. Data is represented as mean and standard deviation for experimental replicates (A+B) and technical replicates (C).

Results

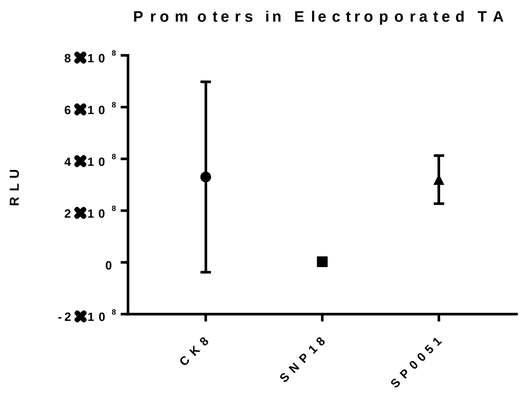


Figure 2. *In vivo* comparison of novel promoter activity versus CK8 by luciferase expression after electroporation of the tibialis anterior (TA) muscle of BALB/C male mice. The plasmids electroporated in this experiment are the same as those transfected into cells in Figure 1. Plasmids were injected into each leg of a total of 5 mice. Whole TA muscles were harvested 7 days post injection and lysed. Data represented as mean and standard deviation of technical replicates.

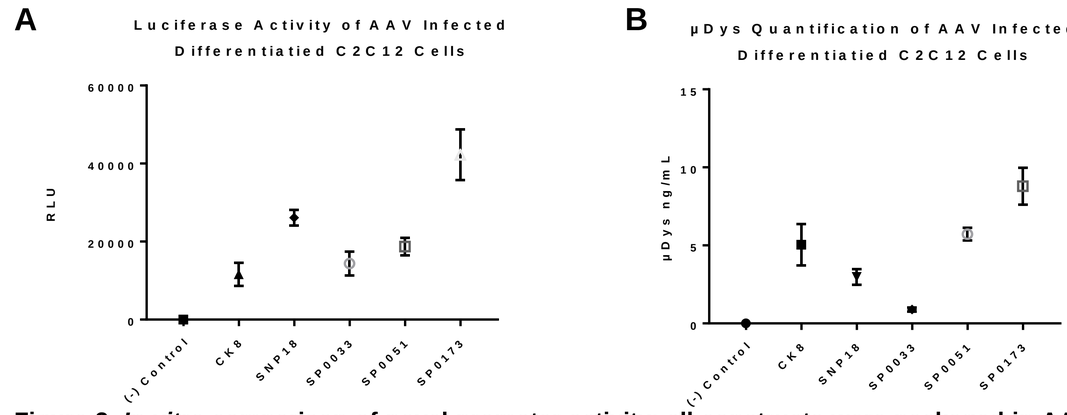


Figure 3. *In vitro* comparison of novel promoter activity; all constructs were packaged in AAV and transduced into differentiated C2C12s. Cells were harvested 48 hours post infection. A) Luciferase activity in C2C12 lysate following infection with AAVs containing the promoter and luciferase transgene measured in RLU. B) µDys levels in C2C12 lysate following infection with AAVs containing the promoter and µDys transgene quantified via MSD (meso-scale discovery electrochemiluminescent assay using a specific antibody for human dystrophin/µDys). Data is normalized to total protein content and is graphed as the mean and standard deviation of 2 experimental replicates.

In vivo Study Design

AAV9 vectors encoding for microdystrophin or luciferase

Promoter	Transgene	Dose (n=5)
CK8	Microdystrophin or luciferase	1E14 vg/kg
CMVIE		
SNP18		
SP0033		
SP0051		
SP0173		

Tissue collected
Quad (left and right)
Gastroc (left and right)
TA (left and right)
Diaphragm
Heart
Liver
Lung
Spleen
Kidney
Brain

Microdystrophin Readout (42 d)
Western
IF
Biodistribution (vg)

Luciferase Readout (weekly)
Activity
Biodistribution (vg)

- Deliver AAVs systemically (tail vein) in mdx mice
  - CK8 regulatory cassette will be compared head to head with known ubiquitous control and novel muscle-specific promoters
  - Regular imaging of luciferase mice to track kinetics over 6 weeks (42 d)
  - Priority analysis of select skeletal and cardiac muscles and liver

Conclusion

- **The *in silico* designed novel promoters are active in both skeletal and cardiac muscle cells.**
- **SP0173 is comparable to or better than CK8 *in vitro*. However, due to its large size, further optimization is desirable for use in AAV gene transfer with large transgenes such as µDys.**
- **SP0051 shows comparable activity to CK8 in the *in vivo* TA electroporation study.**
- **The size of these novel muscle specific promoters and the range of expression observed renders them amenable to viral mediated gene therapy for muscle diseases.**

Future

- **Evaluate and optimize various *in vitro* screening assays for muscle specific promoters to reduce variability.**
- **Continued pursuit of shorter and stronger promoters in additional rounds of bioinformatic analysis using PromPT®.**
- **Two ongoing *in vivo* studies:**
  - **AAV gene transfer in *DMD*^{mdx} mice, a relevant disease model for Duchenne, driven by promoters described here where expression is measured through weekly in-life luciferase imaging or quantification of µDys at 42 weeks.**
  - **TA electroporation of next generation promoters.**

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