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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Construction of Stretch-Induced Reporter using Human Brain Natriuretic Peptide Promoter

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

by

Sung Min Kim

Committee in charge:

Professor Andrew McCulloch, Chair
Professor Barry Greenberg
Professor Jeffrey Omens

2014

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Chair

University of California, San Diego

2014

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ABSTRACT OF THE THESIS

Construction of Stretch-Induced Reporter

using Human Brain Natriuretic Peptide Promoter

by

Sung Min Kim

Master of Science in Bioegneering

University of California, San Diego, 2014

Professor Andrew McCulloch, Chair

Heart disease and the resultant heart failure is one of the leading causes of death in the world. Although advances in treating heart failure have improved patient outcomes, the morbidity and mortality of the disease still remains high. Based on the

finding that up-regulation of brain natriuretic peptide (BNP) is a biomarker of heart failure, which can be a result of abnormal stretch of cardiomyocytes, in this thesis, we attempted to make adenoviral reporter constructs using BNP promoter that is activated upon mechanical stretch to establish a basis for gene transfer therapy for heart failure. Using a mechanical device to mimic abnormal stretch of cardiomyocytes, we investigated the stretch-induced expression of several reporter constructs, which contained different combinations of regulatory sequences (GATA4, M-CAT and Nkx2-5) known to be involved in stretch-induced BNP expression. Our study concluded that among those reporter constructs we prepared, only GATA4-containing construct showed significant level of expression with up-regulation of 1.57 fold upon stretch. Although such result from this has much to be improved, the identification of stretch-inducible artificial gene construct will serve as an important framework to let us focus on increasing the degree of up-regulation for clinical applications.

Chapter 1

INTRODUCTION

One of the leading causes of death in the United States is heart disease, which in many cases results in heart failure if not treated properly [1]. Although advances in treating heart failure have improved patient outcomes, according to the survey conducted by Centers for Disease Control and Prevention, the morbidity and mortality of the disease still remains high [2, 3]. One potential novel therapeutic strategy for heart failure is gene transfer therapy (GTT), which works by introducing an artificial gene construct into the patient's cardiomyocytes via viral vector to confer an ability to express a specific protein or peptide. For example, the regulatory peptide angiotensin (1-7), a substance known to attenuate cardiac remodeling associated with heart failure [4, 5], could be over-expressed with this type of system to mediate the effects of hypertension caused by heart failure [6]. The key advantage of GTT is that, by employing an appropriate promoter, the regulatory peptide can be expressed only under a certain biological conditions caused by heart failure, as opposed to being constitutively expressed, thereby minimizing any potential side effects and making better use of metabolic resources.

Previous studies found that heart failure was accompanied by an increased wall stress of the heart, which could be the result of an increased degree of ventricular diastolic load [7]. It also was shown that increased stretch of ventricular myocytes leads to an up-regulation of brain natriuretic peptide (BNP) in cardiomyocytes [8]. This finding makes BNP an excellent biomarker of heart failure [9] and suggests that

a gene construct consisting of the BNP promoter followed by genes encoding appropriate regulatory peptides could be the basic layout for GTT for heart failure.

Previous studies have identified several important transcriptional regulatory sequences in stretch-induced BNP expression. It has been shown that GATA4 (5'-AGATAG-3') sequence is necessary and sufficient to confer hemodynamic stress-induced BNP expression *in vivo* [10], that muscle-CAT (M-CAT, 5'-CATTCC-3') promoter element mediates mechanical stretch-activated transcription of BNP [11], and that Nkx2-5 (5'-TTAAGTG-3') regulates transcription of cardiac fetal BNP [12]. However, the combined effects of these elements during BNP expression, especially when the gene construct is delivered into the host system via a viral vector, remain unclear.

As a preliminary step to develop a clinically potent GTT for heart failure, the aim of this study was to find a reporter construct with highest level of expression when induced by stretch. To do so, we used different combinations of above three regulatory sequences, GATA4, M-CAT and Nkx2-5, in addition to the BNP promoter to investigate their synergistic effect on stretch-induced expression *in vitro* (Figure 1). We also investigated whether the short version of the BNP promoter (-428 to +100) was sufficient for its transcriptional activity in our *in vitro* system as suggested by earlier *in vivo* study [10]. Such space optimization is important as viral vector for GTT has limited size capacity, especially when adeno-associated virus (AAV) is used as a vector (4.5 to 5 kb total) [13].

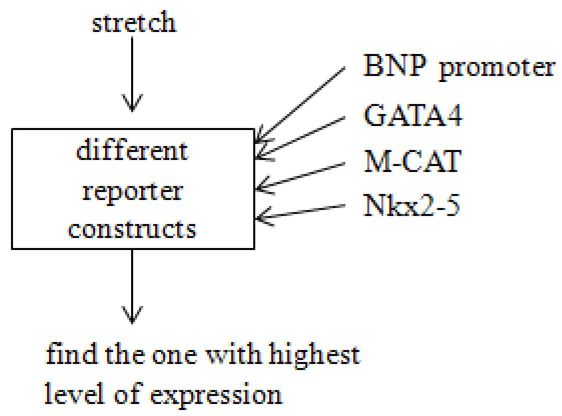

Figure 1. The key aim of this study was to find a reporter construct with highest level of expression when induced by stretch

Chapter 2

MATERIALS AND METHODS

The main goal of this study was to find a reporter construct with highest level of expression when induced by stretch. To do so, we used different combinations of three regulatory sequences, GATA4, M-CAT and Nkx2-5, in addition to the BNP promoter to build a total of five different reporter constructs (Figure 2, chapter 2.1). Then we infected neonatal mice cardiomyocytes with these constructs (chapter 2.2) and applied stretch using our stretchers or added endothelin-1 (ET-1) as a positive control for induction of BNP gene expression (chapter 2.3) since ET-1 has also been known to up-regulate endogenous BNP expression [14]. Then we fixed the cells (chapter 2.4) and measured their fluorescence (chapter 2.5). To confirm that *in-vitro* stretch up-regulated endogenous BNP, we used RT PCR (chapter 2.4).

2.1 Preparation of Adenoviral Vectors

A total of five different BNP constructs were made by repeated cutting and ligation with various restriction enzymes. The vector 1818hBNPluc was obtained from Dr. Margot C. LaPointe, and Dr. Pamela Harding (Henry Ford Hospital, Detroit, MI). The long version of BNP promoter (-1818 to +100) was taken out by cutting the vector 1818hBNPluc with restriction enzymes HindIII and BamHI [15]. The shorter version of BNP promoter (-428 to +100) was obtained by cutting the long version with SacII. To produce long BNP and short BNP plasmids (Figure 2A and B), we inserted long and short BNP promoter respectively into a plasmid backbone, ptdTomatoPA, which was modified from the plasmid, ptdTomato (Clontech

Laboratories, Inc.) to additionally contain a polyA site by Dr. Randy Cowling, UCSD School of Medicine.

For the preparation of GATA4 plasmid (Figure 2C), short BNP plasmid (Figure 2B) was cut with SacII and ligated with a GATA4 oligo (Figure 3A). GATA4 plasmid (Figure 2C) was then cut with SacII and ligated with a M-CAT oligo (Figure 3B) to make M-CAT plasmid (Figure 2D). M-CAT plasmid (Figure 2D) was then again cut with Sac II and ligated with a Nkx2-5 oligo (Figure 3C) to obtain Nkx2-5 plasmid (Figure 2E).

All constructs in Figure. 2 were cut with HindIII and EcoRI, ligated with a linker oligo (SalI-HindIII) and transferred to kanamycine-resistant pENTR^{TM}1A plasmid backbone, which was cut with SalI and EcoRI to remove ccdB element. These pENTR^{TM}1A versions of each construct then went through a recombination with adenoviral backbone (pAd/PL-DEST^{TM}, 34864bp) by matching attR1 and attR2 elements in pAD/PL-DEST with attL1 and attL2 elements in pENTR^{TM}1A backbone to form their respective final adenoviral BNP constructs shown in Figure 4.

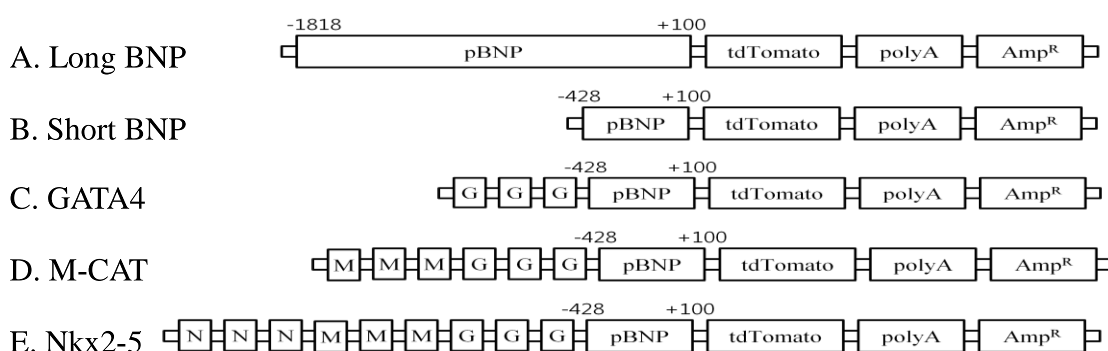

Figure 2. Initial Layout of Constructs on Amplicillin-Resistant Plasmid Backbone. In this study, A) was named as Long BNP, B) as Short BNP, C) as GATA4, D) as M-CAT, and E) as Nkx2-5.
Abbreviations in the diagram:
G: GATA4 sequence, 5’-AGATAG-3’
M: M-CAT sequence 5’-CATTCC-3’
N: Nkx2-5 sequence 5’-TTAAGTG-3’
pBNP: promoter of BNP

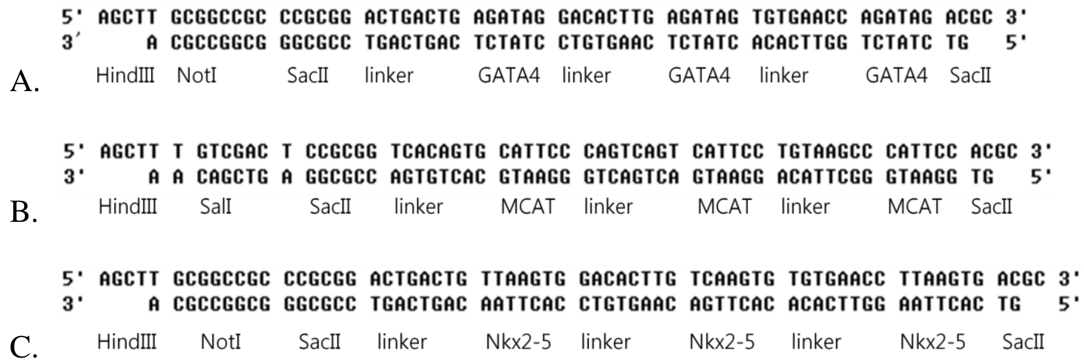

Figure 3. GATA4, M-CAT and Nkx2-5 Oligonucleotides. The 3’ end of each oligo has a modified SacII overhang, ACGC, instead of typical CCGC. This modified SacII overhang ligates with the other complimentary SacII overhang, but cannot be cut with SacII thereafter.

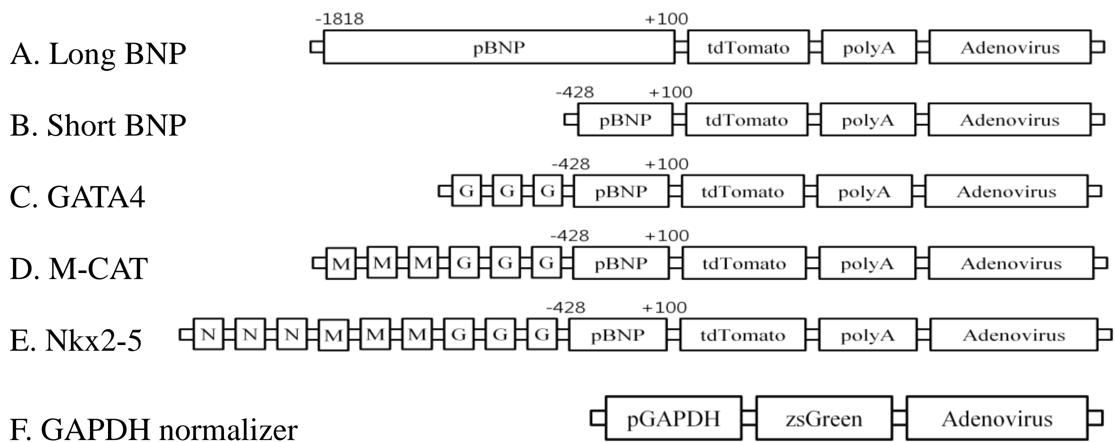

Figure 4. Final Adenoviral Vectors. All symbols followed the convention from Figure 2.

The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter was used as a normalizer since GAPDH is known to have relatively constant expression level regardless of experimental conditions [16]. This normalizing vector was constructed in two steps. First, zsGreen1 gene, which was cut from pIRES2-ZsGreen1 plasmid with KpnI and BtsI, was introduced along with a linker oligo (Not1-Pme1-EcoR1-BtsI) into pENTR^{TM}1A backbone, which was already cut with KpnI and Not1. Subsequently, this zsGreen1-containing pENTR^{TM}1A was cut with BamHI and NcoI, and was ligated with a linker (BamHI-Kpn-SpeI) along with a GAPDH promoter

fragment cut from pDRIVE-hGAPDH with SpeI and NcoI to form a pGAPDH-zsGreen-pENTR^{TM}1A vector. This again went through a recombination with pAD/PL-DEST^{TM} to form a final GAPDH normalizer (Figure 4F).

The correctness of all adenoviral constructs in Figure 4 was verified by restriction digestion with various enzymes to observe the characteristic pattern of the digested fragments in gel electrophoresis. To linearize and get rid of unnecessary plasmid sequences, these constructs were cut with PacI and were packaged into adenovirus 5 by transfection into 293A cells according to FuGene® 6 protocol, after which theses constructs were amplified and titered as plaque forming units.

2.2 Cell Isolation, Culturing, and Viral Infection

1-2 day old CD-1 neonatal mice ordered from Charles River Laboratories were used to prepare cardiomyocytes according to the protocol in Appendix. The cells were plated ($3x10^5$~$5x10^5$ cells for fluorescence microscopy and microplate reader and $1.6x10^6$ cells for RNA extraction) on laminin-coated polydimethylsiloxane (PDMS) membrane (for stretching experiments) or 12-well plate (for ET-1 experiments) and were incubated with “Dark Media” (Appendix) for 24 hours at 37°C. Then viral constructs were added along with new culture media made of 4:1 ratio of Dulbecco’s Modified Eagle Medium (DMEM [+]4.5g/L D-Glucose [+]L-Glutamine [-]Sodium Pyruvate, Gibco®) and Medium 199 (M199, Gibco®) and the cells were incubated for another 24 hours at 37°C. For each sample, 50 MOI (multiplicity of infection) of BNP reporter constructs (Figure 4ABCDE) and 10 MOI of GAPDH normalizing constructs (Figure 4F) were added.

2.3 Stretching or Addition of Endothelin-1

We used a mechanical device depicted in Figure 5A as a stretcher according to the method described in [17, 18]. PDMS membrane was used as a basal membrane for cell plating and silica gel grease was used to build a wall to contain the culture media (Figure 5B). After 24 hours of virus infection, stretching was applied by rotating the top-screw by 720 degree which was known to result in 18% stretch along the short axis of the ellipse and 9% stretch along the long axis [18]. Such condition lasted for 24 hours at 37°C. For ET-1 experiment, ET-1 of 10^{-7} M per well was added to 12-well plates instead of stretchers and plates were incubated for 24 hours at 37°C.

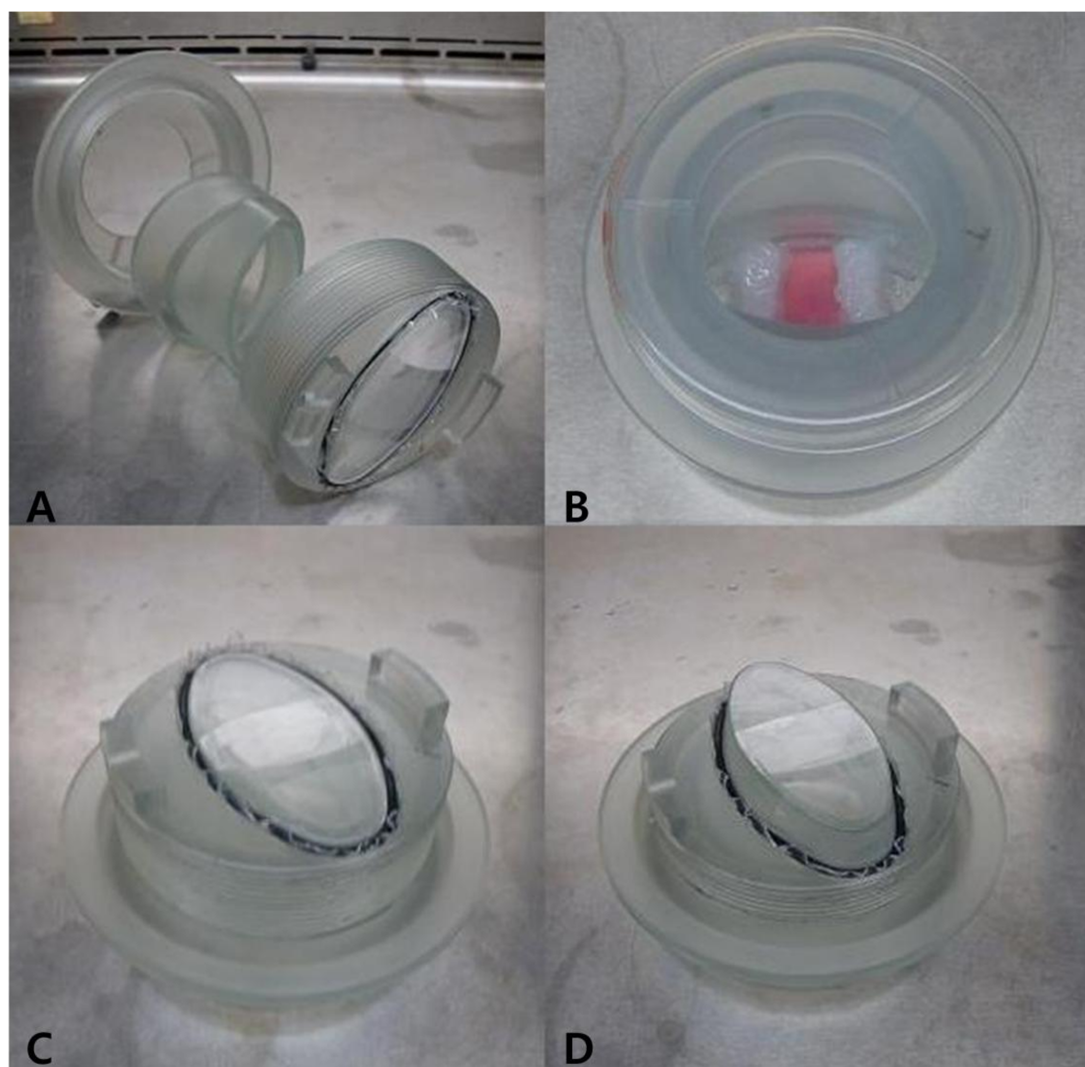

Figure 5. Stretcher. A) Components of the stretcher. B) Fully assembled stretcher with cell culture media. Silica gel grease was used on sides to build a wall to prevent leakage of the culture media. CD) PDMS membrane was stretched by rotating the top-screw.

2.4 Cell Fixation, RNA extraction, and RT PCR

To measure the up-regulation of endogenous BNP, we performed RT PCR (KAPA SYBR® FAST qPCR Kits by Kapa Biosystems) using Mastercycler® ep realplex machine after RNA extraction (RNeasy Kit by Qiagen) and cDNA synethesis (Transcriptor First Strand cDNA Synthesis Kit by Roche). GAPDH mRNA was used as a reference for calculating $\Delta Ct = Ct,_{BNP} - Ct,_{GAPDH}$. After obtaining ΔCt for both

with and without stretch (control), we calculated $\Delta(\Delta Ct) = (Ct,_{BNP} - Ct,_{GAPDH})_{control}$ - $(Ct,_{BNP} - Ct,_{GAPDH})_{stretch}$ to finally obtain the fold-increase = $2^{\Delta(\Delta Ct)}$. Table 1 shows the primers used in the RT PCR.

For fluorescence microscopy and microplate reader, cells were detached from the PDMS membrane with Trypsin-EDTA (Gibco®), and were fixed in suspension with 100% methanol.

Table 1. Primer Sequences for RT PCR to Measure Endogenous BNP Up-regulation upon Stretch.

BNP	Forward	5’ TGTTTCTGCTTTTCCTTTATCTGTC 3’
	Reverse	5’ CTCCGACTTTTCTCTTATCAGCTC 3’
GAPDH	Forward	5’ AGGTCGGTGTGAACGGATTTG 3’
	Reverse	5 TGTAGACCATGTAGTTGAGGTCA 3’

2.5 Fluorescence Measurement by Microscope and Microplate Reader

For fluorescence images, Olympus FV1000-IX81 confocal microscope was used with configurations specified in Table 2.

Table 2. Configuration of Olympus FV1000-IX81 Confocal Microscope.

	ZsGreen	TdTomato
excitation wave length (nm)	488	543
emission wave length (nm)	520	578
dye name	Alexa Fluor 488	TRITC
transmissivity (%)	5	50

For the quantification of fluorescence, Infinite® 200 PRO (TECAN) was used with configurations specified in Table 3. Fluorescence fold-increase was calculated as follows.

Fold increase =

$$\left[\frac{(\text{sample signal} - \text{blank})_{\text{TdTomato}}}{(\text{sample signal} - \text{blank}\ )_{\text{ZsGreen}}}\right]\text{stretch} \Bigg/ \left[\frac{(\text{sample signal} - \text{blank})_{\text{TdTomato}}}{(\text{sample signal} - \text{blank}\ )_{\text{ZsGreen}}}\right]\text{control}$$

Table 3. Configuration of Infinite® 200 PRO (TECAN) Microplate Reader.

	ZsGreen	TdTomato
excitation wave length (nm)	493	554
emission wave length (nm)	525	587
gain	112	177
excitation band width (nm)	9	
emission band width (nm)	20	
number of flashes	25	
integration time (µs)	20	
lag time (µs)	0	
settle time (µs)	0	

Chapter 3

RESULTS

3.1 Up-regulation of Endogenous BNP upon Stretch

In order to show that stretch of neonatal cardiomyocytes up-regulated endogenous BNP expression, we performed RT-PCR to measure BNP message with respect to GAPDH after stretch. Results showed that ΔCt decreased on average from 2.51 to 0.177 (= 2.33) with significance (p-value = 0.044) (Table 4, Figure 6). This result is equivalent to a BNP mRNA up-regulation of $2^{2.33}$ = 5.04 fold upon stretch, which suggests that our stretchers generated and transmitted stretch signal to the cultured cardiomyocytes.

Table 4. RT PCR Results for BNP Up-regulation.

	Replicates					Avg	Std. Dev
		#1	#2	#3	#4		
Control	$Ct_{,BNP}$	17.75	21.01	18.54	21.18		
	$Ct_{,GAPDH}$	16.54	17.22	16.82	17.86		
	ΔCt	1.21	3.79	1.72	3.32	2.51	1.24
Stretch	$Ct_{,BNP}$	18.2	16.16	16.39			
	$Ct_{,GAPDH}$	17.05	17.08	16.09			
	ΔCt	1.15	-0.92	0.3		0.18	1.04

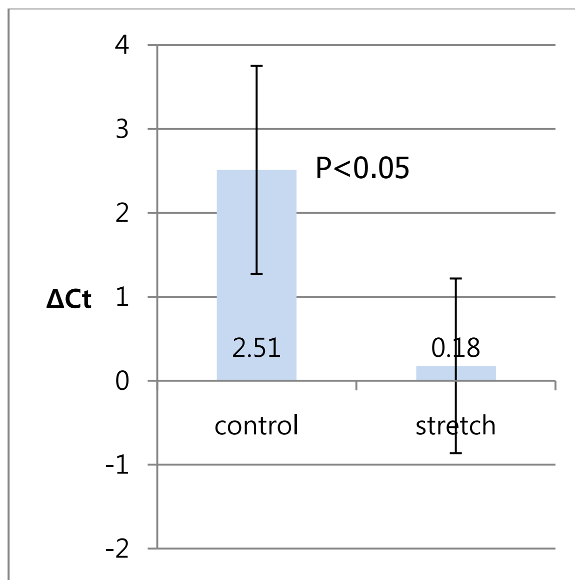

Figure 6. Up-regulation of Endogenous BNP upon Stretch. ΔCt decreased from 2.51 to 0.177 implying $2^{2.33}$ = 5.04 fold of up-regulation of endogenous BNP. P-value = 0.044.

3.2 Analysis of Long BNP and Nkx2-5 Constructs after Stretch

We first investigated the long BNP and Nkx2-5 constructs as long BNP had the longest promoter region and the Nkx2-5 construct contained all of our transcriptional regulatory sequences (Figure 4), increasing the chance of observing an up-regulation. However, fluorescence microscopy showed no significant signal from TdTomato meaning no significant level of transcriptional activity of BNP reporter construct (Figure 7). We quantified the fluorescence as shown in Table 5 and found that most of the signals from TdTomato were not well above the background signal obtained from uninfected cells. The calculated fold-increase was not consistent, ranging from 0.17 to 3.72 (Table 5).

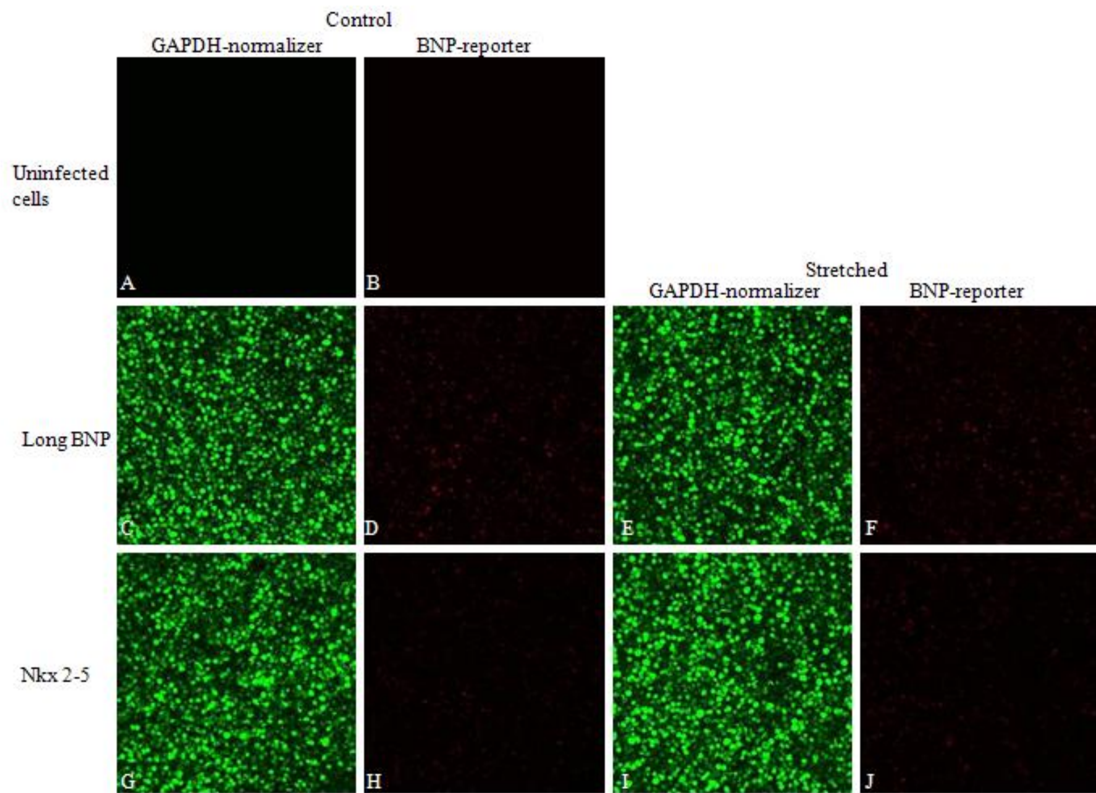

Figure 7. Fluorescence Microscopy of cells transfected with Long BNP and Nkx2-5 constructs After Stretching. AB) uninfected cells as a control. First and third columns are GAPDH normalizer and second and fourth columns are BNP-reporter. First and second columns are without stretch as a control and third and fourth columns are stretched. CDEF) Long BNP construct. GHIJ) Nkx2-5 construct.

Table 5. Fluorescence Quantification for Long BNP and Nkx2-5 after Stretching. All samples did not show a TdTomato signal well above background.

	Control		Stretched		Fold-increase
	GAPDH-normalizer	BNP-reporter	GAPDH-normalizer	BNP-reporter	
Uninfected cells	39	57			
Long BNP	9689	53	8234	71	ND
Nkx2-5	13680	75	4118	77	3.72
	14217	78	13197	79	1.13
	9661	112	12389	69	0.17

3.3 Analysis of All Constructs after ET-1 Addition

Since the addition of ET-1 is known also to up-regulate endogenous BNP [14], we stimulated with ET-1 for all constructs as a positive control. Only GATA4 construct (Figure 4C) showed distinct TdTomato signals both from microscopy and microplate reader (Figure 8 L and N, Table 6).

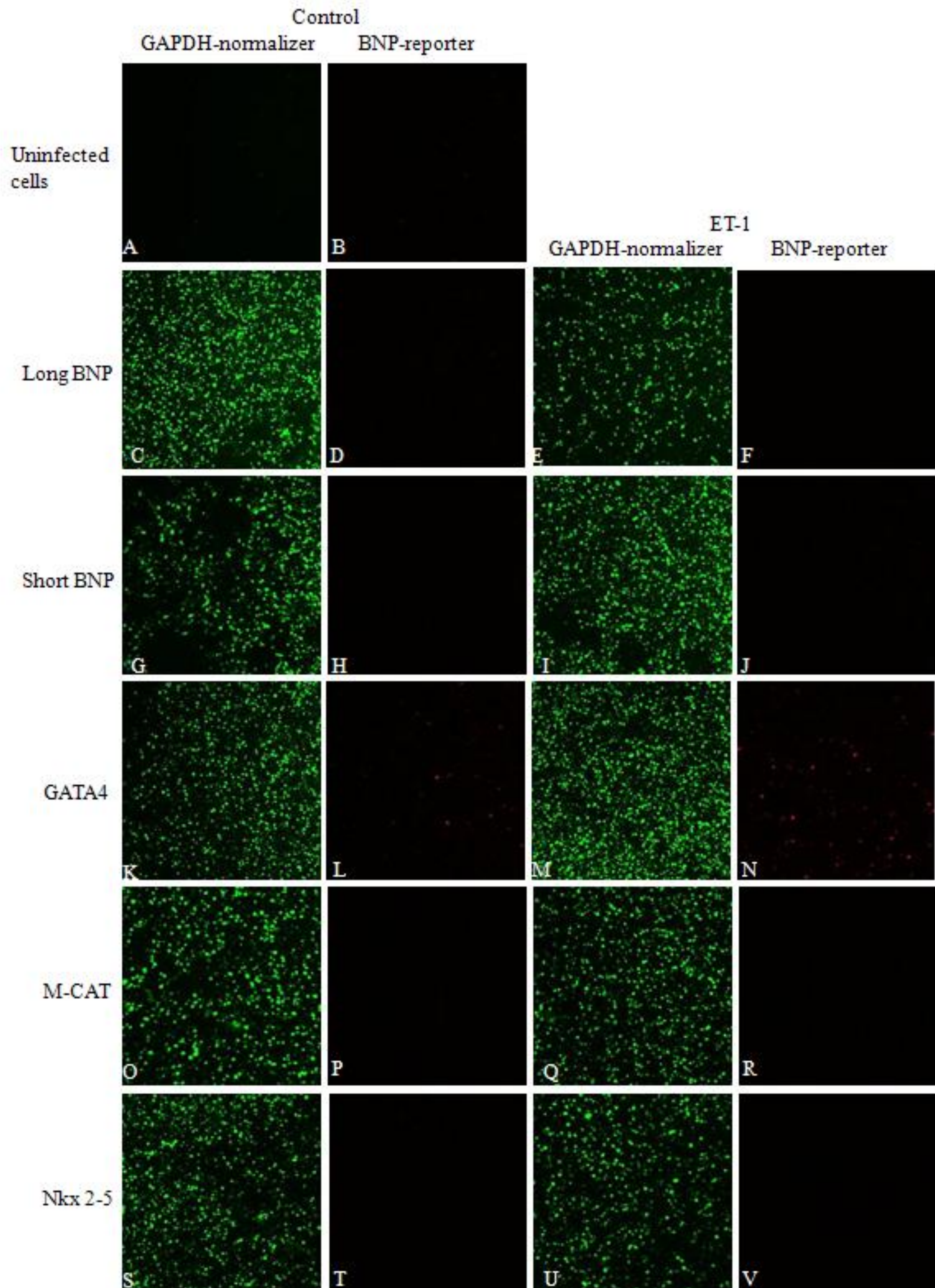

Figure 8. Fluorescence Microscopy of Myocytes Expressing Constructs After ET-1 Addition. AB) uninfected cells as a control. First and third columns are for GAPDH normalizer and second and fourth columns are for BNP-reporter. First and second columns are without ET-1 as a control. Third and fourth columns are when ET-1 was added. CDEF) Long BNP construct. GHIJ) Short BNP construct. KLMN) GATA4 construct. OPQR) M-CAT construct. STUV) Nkx2-5 construct.

Table 6. Fluorescence Quantification for All Constructs after ET-1 Addition. Only GATA4 construct showed a distinct signal well above background.

	Without ET-1		ET-1		Fold-increase
	GAPDH-normalizer	BNP-reporter	GAPDH-normalizer	BNP-reporter	
Uninfected cells	27	138			
Long BNP	30347	235	37258	261	1.03
Short BNP	31248	193	39503	217	1.14
GATA4	11590	**1181**	14183	**2193**	**1.61**
M-CAT	25130	163	34724	178	1.16
Nkx2-5	42553	170	37541	235	3.44

3.4 Analysis of GATA4 Construct after ET-1 Addition

We further investigated GATA4 construct with ET-1 to obtain statistical significance of its up-regulation upon ET-1 addition. Fluorescent microscopy showed a noticeable increase of TdTomato signal when ET-1 was added, while ZsGreen signal remained about the same, suggesting GATA4 construct was indeed up-regulated (Figure 9 D and F). Quantification of fluorescence with the microplate reader confirmed the significance ($p\text{-value} < 0.01$) of up-regulation of normalized TdTomato fluorescence, with a 1.40 fold-increase (Table 7, Figure 10).

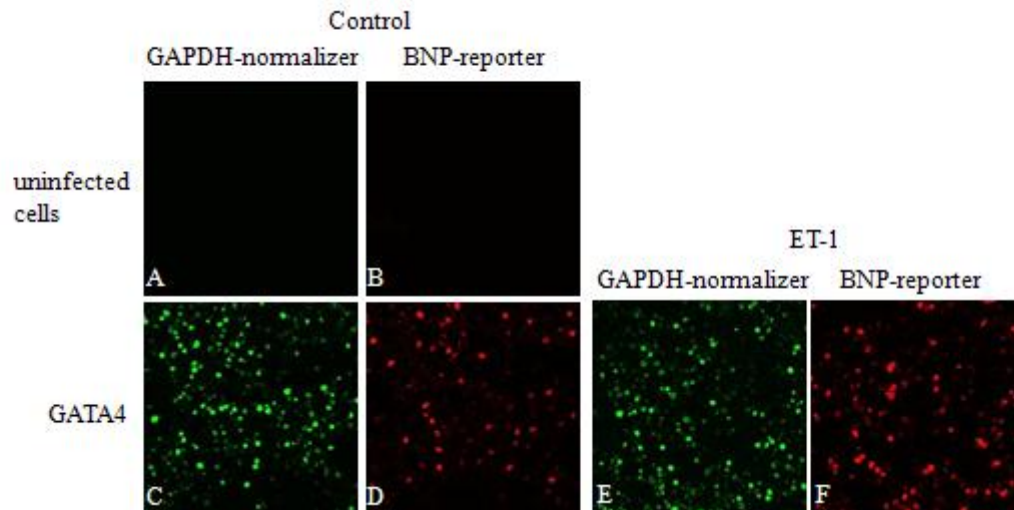

Figure 9. Fluorescence Microscopy for GATA4 Construct after Addition of ET-1. AB) Uninfected cells as a control. CD) Cells without ET-1. EF) Cell with ET-1. First and third columns are for GAPDH normalizer and second and fourth columns are for BNP-reporter

Table 7. Fluorescence Quantification for GATA4 Construct after ET-1 Addition.. Average fold increase was 1.4.

	Replicates					Normalized avg	Std. Dev
		#1	#2	#3	#4		
Control	BNP-reporter	5479	5165	5430	2817	0.16	0.026
	GAPDH-normalizer	29998	27983	35510	21708		
ET-1	BNP-reporter	5726	7285	9425	7876	0.23	0.013
	GAPDH-normalizer	23916	31913	45154	33768		

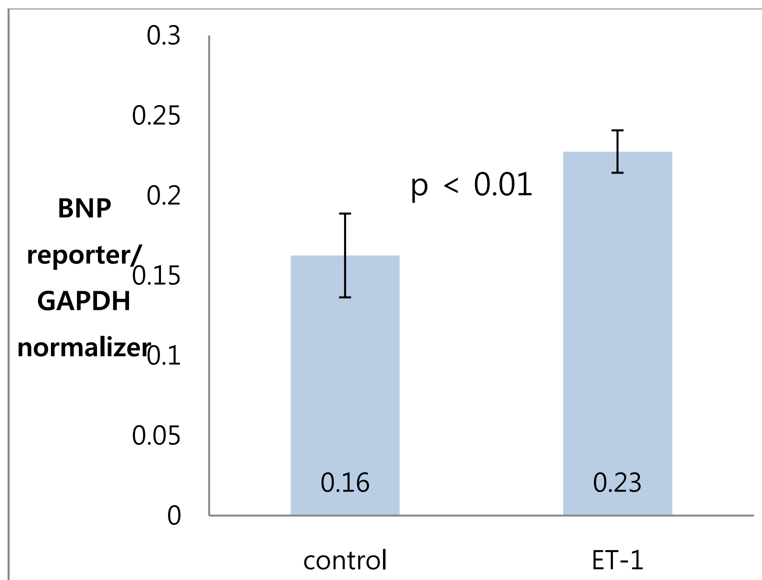

Figure 10. T-test for GATA4 construct after addition of ET-1. P-value = 0.009 and average fold increase = 1.4

3.5 Analysis of GATA4 Construct after Stretch

Finally, we investigated the GATA4 construct with stretch. Again, the results were similar to those of ET-1 experiment. The fluorescence of TdTomato noticeably increased after stretch while that of ZsGreen remained about the same (Figure 11 D and F). Quantification of fluorescence confirmed the significance (p-value < 0.05) of up-regulation with 1.57 fold-increase (Table 8, Figure 12).

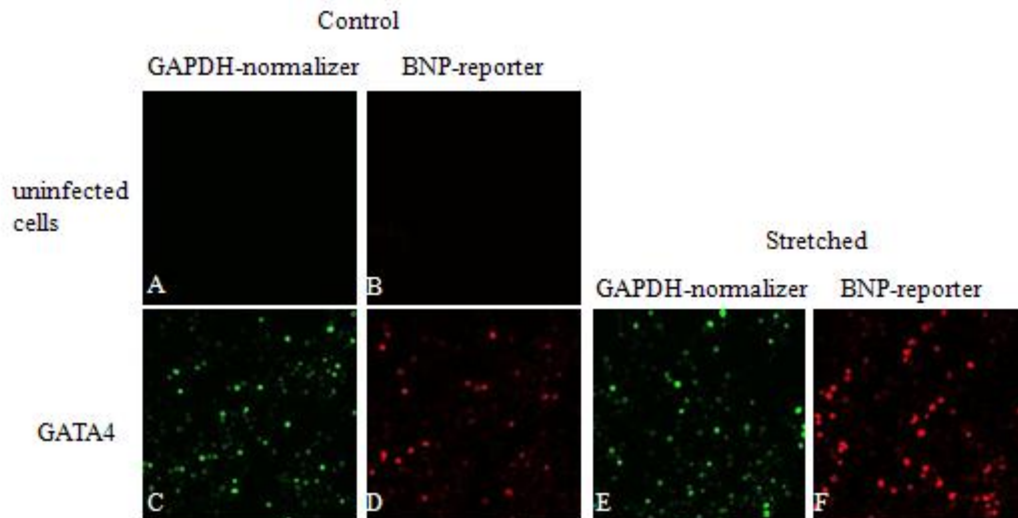

Figure 11. Fluorescence Microscopy for GATA4 Construct after Stretching. AB) Uninfected cells as a control. CD) Cells without stretch. EF) Cells with stretch. First and third columns are for GAPDH normalizer and second and fourth columns are for BNP-reporter

Table 8. Fluorescence Quantification for GATA4 Construct after Stretching. Average fold increase was 1.57.

	Replicates				Normalized Avg	Std. Dev
		#1	#2	#3		
Control	BNP-reporter	1967	2986	2176	0.19	0.050
	GAPDH-normalizer	14699	13393	10135		
Stretched	BNP-reporter	4875	2359	4899	0.30	0.040
	GAPDH-normalizer	17276	8753	14304		

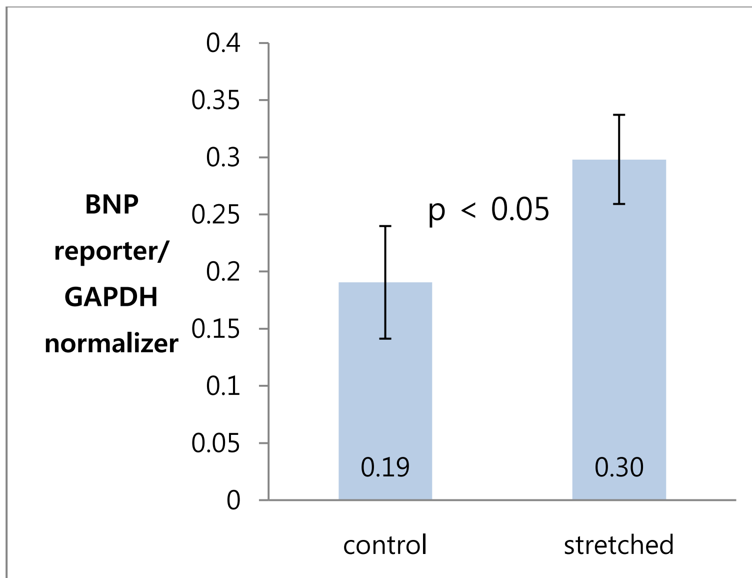

Figure 12. T-test for GATA4 Construct after Stretching. P-value = 0.044 and average fold increase = 1.57

Chapter 4

CONCLUSION

4.1 Experiment Summary

This study was conducted as part of developing a GTT for heart failure, which can be accomplished by building an artificial gene construct that can be inserted into patients' cardiomyocytes via viral vector and express therapeutic peptide whenever heart failure condition occurs. As a preliminary step to accomplish this goal, based on the finding that up-regulation of BNP is a biomarker of heart failure [8, 9] and that this up-regulation can be triggered by mechanical stretch of cardiomyocytes [11], we attempted to build a reporter construct with highest stretch-inducibility. To do so, we built a total of five reporter constructs using the BNP promoter along with different combinations of its regulatory sequences, GATA4, M-CAT and Nkx2-5 (Figure 4), and compared each of their expression level before and after stretch by measuring the fluorescence. The result suggests that only GATA4-containing construct (Figure 4C) showed significant level of basal expression with about 50% of up-regulation when induced by stretch, which was also confirmed by stimulation with ET-1. This sufficiency of GATA4 element in stretch-induced BNP expression agrees with the result from the previous *in vivo* study [10]. All the other constructs showed weaker levels of expression after stretch. Possible reasons for such weaker levels of expression could be that just a subtle difference in sequence around the promoter region might have caused drastic effect on the promoter's affinity with other transcription factors involved in BNP expression. Or it could be that M-CAT and Nkx2-5 might have had stronger affinity to the transcriptional machinery than GATA4,

masking the effect of GATA4, but their location was not ideal for transcription to be initiated. Additional experiments may need to be conducted for more comprehensive understanding.

Our results also suggest that the short BNP promoter (-428 to +100) is sufficient to be transcriptionally active when the GATA4 element was present around the promoter, which is in accord with the result from the previous *in vivo* study [10]. The experimental result showing that the M-CAT-containing constructs (Figure 4D and E) had low transcriptional activity even with GATA4 element suggests that M-CAT somehow represses transcriptional activity, the details of which still remain unclear.

4.2 Suggestions for Future Work

In order to obtain clinical significance to develop GTT for heart failure, we need to increase the inducibility — defined in this study as a ratio of the expression level of BNP reporter with versus without stretch. Here are two suggestions. The first idea is that we can put another gene encoding a transcriptional activator at the end of the gene of our interest, along with inserting its regulatory sequence around the promoter. These two genes can be linked via IRES (internal ribosome entry site) linker, making the construct bicistronic (Figure 13). A possible candidate for such transcriptional activator for the purpose of targeted gene expression can be GAL4-UAS system as demonstrated in [19].

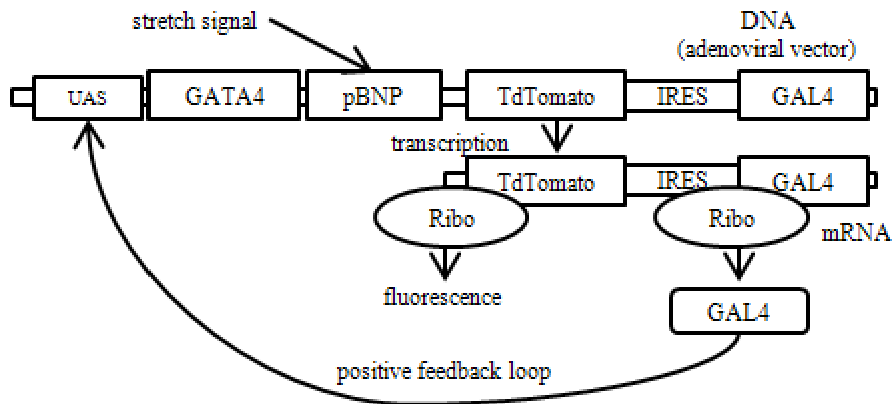

Figure 13. Suggested Positive Feedback Loop by Transcription Activator Leading to an Amplification of Gene Expression

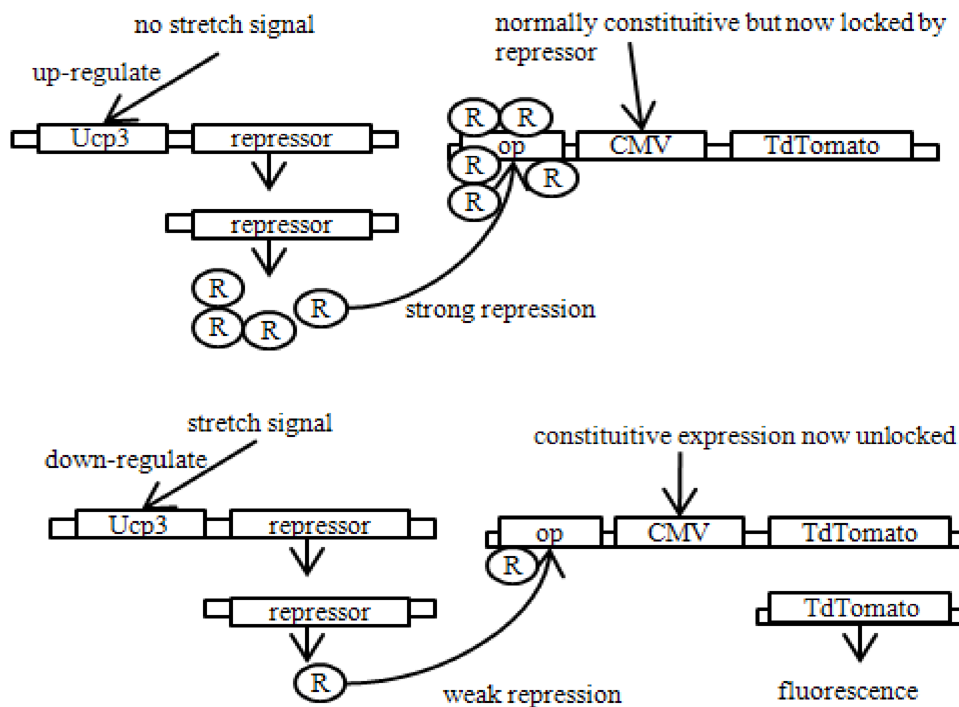

Figure 14. Suggested Repression System using Down-regulated Promoter upon Stretch.

The second idea, as depicted in Figure 14, is that we can use cytomegalovirus(CMV) promoter, which is constitutively expressed regardless of stretch. This can give us overall increase of expression level, but we need to provide a stretch-inducibility by employing another construct. This second construct can contain a promoter that expresses repressors and become down-regulated upon stretch.

One possible example is uncoupling protein 3 (Ucp3) promoter, which is down-regulated to 20% of its original expression level when stretch is applied. Thus, in the absence of stretch, this second construct will produce high level of repressors which will inhibit CMV promoter. In the presence of stretch, repressors are produced in low level and CMV promoter can become active, up-regulating the reporter or a gene of our interest. However, the type of repressor/operator pair that would work best for this purpose still needs to be determined. Together with targeted gene amplification using GAL4-UAS system, this repression-based modulation may provide a solution to increase the inducibility as well as absolute level of expression. However, the outcome of this type of competition between activators and repressors heavily depends on the actual expression level of respective components, which cannot be predicted before an experiment is conducted.

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APPENDIX

Neonatal Mouse Cardiomyocyte Isolation Protocol

CMRG 10.10.11

Derived from Valeria Mezzano by Jen Stowe, Emily Pfeiffer.

Protocol for up to 90 pups

Day 1:

1. **Prepare Reagents:** Weigh out 35mg Trypsin and mix with 75ml cold HBSS (0.5mg/ml). Store at 4°C.
2. Make Base Media Mix: Mix together and vacuum filter. Keep sterile.
    - 375ml DMEM
    - 125ml M-199
    - 5ml 100x Pen/Strep/Glut solution
    - 5ml HEPES (1M)

3. Make Light and Dark Media: Store media at 4°C for no more than 2 weeks.
    - Light Media: 191.25ml Base Media Mix (from above)
    - Dark Media: mix together in a sterile container.
        - 318.75ml Base Media Mix (from above)
        - 37.5ml Horse Serum
        - 18.75ml FBS

4. **Prepare BSC work area**: Obtain bucket and/or deep tray of ice. Clean BSC with 70% ethanol. Place ice, a large beaker of 95% ethanol, a stack of paper towels, large weigh boats, a body bag, and clean surgical instruments into hood.
5. Place dish(es) of cold HBSS on ice in hood. This is for collecting and rinsing hearts after removal. Tissue must be kept on ice after removal from animal. Dish can be 6-well, 60mm Petri, or other similar, sterile dish. Use a new dish for each litter.
6. Transfer the pups to the BSC in a box lined with paper. Place pups in a weigh boat.
7. **Extract Tissue**: In one motion, cut off the head with the large scissors.
8. Pick up pup by pinching it's back. This will pull the front feet apart and make the chest bulge out. Dunk body in 95% ethanol and blot on clean paper towel. Make a shallow incision between the front feet with the small scissors. Cut deeply enough to penetrate the rib cage, but not so deep as to cut into the heart. The heart should pop out of this incision. Lungs and other tissue may also be exposed. Carefully excise the entire heart from the pup leaving any other tissue behind. Immediately place heart in dish of cold HBSS. Repeat with remaining pups. Remove any excess non-cardiac tissue from hearts.
   Note: if intestines rupture during the process, discard the pup and heart and sterilize all surgical equipment in 95% ethanol to prevent bacterial contamination of the culture.
9. Trim off atria and discard, but do not mince ventricular tissue.

10. Swirl hearts gently in dish to rinse. Transfer hearts to the HBSS/Trypsin container.
11. Predigest overnight at 4°C on an orbital shaker at 80rpm (24 hours max).

Day 2:

1. **Prepare:** Pre-heat shaking water bath, and pre-warm the Light Media and a 50 ml aliquot of Dark Media and/or CMRG Plating Media to 37°C in a water bath.
2. Weigh 80mg collagenase type 2 (330U/ml) and mix with 100ml HBSS (0.8mg/ml).
    a. For 2 litters: 24 mg and 30 ml. For more litters, can prepare multiple batches of two litters.
    b. Filter-sterilize and store in hood at RT. Make solution fresh for each isolation.
3. Place ice bucket in hood and pre-chill 1-2 x 50ml conical tubes (per batch of 2 litters) with 10ml Dark Media in each. Keep in ice bucket.
4. Remove the Trypsin & tissue bottle from 4°C and place in hood. Carefully remove half the volume (~35-40ml) of the Trypsin/HBSS ensuring all hearts are left behind. Add same volume (~35-40ml) warm Light Media to hearts. **Shake at 37°C for 3-4min at 100-150rpm.** Do not leave any longer or tissue will be over-digested and cells will die.
5. Carefully aspirate off all solution from hearts (or, pick up hearts with a 25 ml pipette and pour off solution). Add 15ml collagenase/HBSS to tissue. Cap tightly and shake in 37°C water bath for **2min only!**
6. Allow hearts to settle (or pick up with pipette) and discard all supernatant. This initial digest is mostly RBCs and dead cells.
7. **Digest:** Add 15ml collagenase/HBSS to tissue, triturate (pipette up and down) briefly, then shake at 37°C for 7mins at 150rpm. Use a clamp to stabilize bottle in shaking water bath. After digest, complete by triturating thoroughly.
8. **Remove tissue chunks and RBCs:** Pass contents of conical tube thru a cell strainer into a 50 ml conical tube containing 10ml of ice cold Dark Media.
9. Spin at 150rcf (xg) for 4min at RT. (This pellets the CMs and leaves dead cells and some fibroblasts in suspension).
10. Carefully aspirate off supernatant (pellet is loose!) without disturbing pellet. Add 15ml/flask of Dark Media to pellet and resuspend by gently pipetting up and down.
11. **Pre-plate**: Plate each pellet (in 15ml Dark Media) in a T-75 flask and incubate for **1hr 30min at 37°C and 5% CO_2.** Note: Use one T-75 flask when working with less than 30 hearts. Fibroblasts will adhere and CMs will remain in suspension.
12. Place adequate Dark Media in 37°C water bath for next steps.
13. Remove the suspension from the T-75 flask(s).
14. After the second round of plating, remove all suspension media from the flask and add to 50ml conical. Rinse flask with 10ml warm Dark Media and add to conical. Spin at 150rcf for 4min to pellet cells.
15. Aspirate off the supernatant without disturbing the pellet. Resuspend in 10ml warm Dark Media (decrease volume if fewer than 10 hearts).

16. **Count cells:** Mix 40µl Dark Media + 40µl cell suspension + 20µl Trypan Blue. Load 10µl into both chambers of a hemocytometer and count 2 squares on each side (4 total). Record cell counts and average. Multiply by 2.5 (dilution factor), then multiply by 10,000 to get # million cells/ml. Multiply by 10mls to get total # of cells in isolation. Divide by # of hearts to get cells/heart.
17. Proceed to downstream experiments.

Required Reagents and Equipment:

Product	Function	Vendor	Catalog #
Small Scissors	Tissue excision	Any	----
Large Scissors	Pup beheading	Any	----
Blunt Forceps	Holding Pups	Any	----
Fine Forceps	Tissue Handling	Any	----
Hank's Balance Salt Solution (HBSS)	Washing and enzyme dilutions	Invitrogen (Gibco) buy in 100ml aliquots	14170-120
Trypsin Powder	Heart predigestion	Affymetrix (USB)	US 22715
Collagenase 2	Heart Digestion	Worthington BioChem	MOE 3983
Vacuum Filter Flasks (250ml)	Filtering media/enzyme	Millipore	SCGVU02RE
DMEM High Glucose w/out pyruvate	Cell Culture	Invitrogen (Gibco)	11965-092
Media 199	Cell Culture	Invitrogen (Gibco)	11043023
HEPES	Cell Culture	Invitrogen (Gibco)	15630080
Horse Serum	Cell Culture	Invitrogen (Gibco)	16050130
Fetal Bovine Serum (FBS)	Cell Culture	Invitrogen (Gibco)	16000036
100X Pen/Strep/Glutamine	Cell Culture	Invitrogen (Gibco)	10378016

Also Needed:

Consumables: 60mm tissue culture dishes (does not need to be TC treated), 50ml conical tubes, T-75 flasks, 6-well TC dishes, plastic pasture pipettes, other plates/vessles for culturing cells, large weigh boats, paper towels, plastic bag, serological pipettes and pipette aid

Equipment: Shaking water bath at 37°C, Orbital shaker at 4°C, TC incubator at 37°C & 10% CO_2.

Other: Ice bucket and ice

Mice: Litter of mouse pups with lactating mother (pups should be 0.5-1.5 days old), paper-lined box for transporting pups

Culture substrates: coat substrates with ECM, such as laminin, gelatin, fibronectin or collagen to promote cell adhesion.