In vivo natriuretic peptide reporter assay identifies chemical modifiers of hypertrophic cardiomyopathy signalling

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Aims

Despite increased understanding of the fundamental biology regulating cardiomyocyte hypertrophy and heart failure, it has been challenging to find novel chemical or genetic modifiers of these pathways. Traditional cell-based methods do not model the complexity of an intact cardiovascular system and mammalian models are not readily adaptable to chemical or genetic screens. Our objective was to create an in vivo model suitable for chemical and genetic screens for hypertrophy and heart failure modifiers.

Methods and results

Using the developing zebrafish, we established that the cardiac natriuretic peptide genes (nppa and nppb), known markers of cardiomyocyte hypertrophy and heart failure, were induced in the embryonic heart by pathological cardiac stimuli. This pathological induction was distinct from the developmental regulation of these genes. We created a luciferase-based transgenic reporter line that accurately modelled the pathological induction patterns of the zebrafish nppb gene. Utilizing this reporter line, we were able to show remarkable conservation of pharmacological responses between the larval zebrafish heart and adult mammalian models.

Conclusion

By performing a focused screen of chemical agents, we were able to show a distinct response of a genetic model of hypertrophic cardiomyopathy to the histone deacetylase inhibitor, Trichostatin A, and the mitogen-activated protein kinase kinase 1/2 inhibitor, U0126. We believe this in vivo reporter line will offer a unique approach to the identification of novel chemical or genetic regulators of myocardial hypertrophy and heart failure.

Keywords

Natriuretic peptides • Hypertrophy • Heart development • Heart failure • Hypertrophic cardiomyopathy

1. Introduction

The ability of cardiomyocytes to respond to stress is critical to the preservation of myocardial function in health and disease. If the heart is unable to adapt efficiently to haemodynamic changes, myocardial function declines and ultimately leads to the development of heart failure. Despite aggressive treatment with existing therapies, the prevalence of congestive heart failure (CHF) continues to increase. 1 A number of successful therapeutics have been developed using traditional target-driven screens in cell-based assays. These agents have proved effective in the later stages of the CHF syndrome, but the identification of effective agents operating earlier in the cascade, and thus capable of preventing CHF, has proved difficult. Even early in its pathogenesis, CHF is a multiorgan, multisystem disorder, and modelling this complexity is simply not feasible using existing in vitro techniques. Conversely, performing a large-scale screen for novel therapeutics using intact animal models is prohibitive. Recently, the emergence of the larval zebrafish as a tool for disease modelling and phenotype-driven small molecule screens has provided a viable in vivo alternative to traditional target-based screening techniques. 2

A signalling pathway that is consistently up-regulated in both myocardial hypertrophy and CHF in humans is the cardiac natriuretic peptide axis, with two major secreted ligands; atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP). The induction of these genes serves as a robust marker of cardiomyocyte stress in mammalian models of cardiovascular disease. However, these genes
are also highly expressed during cardiac development. Initial attempts at creating murine NPPA reporter lines using minimal promoter constructs were only able to recapitulate the developmental induction of these genes, but failed to mimic pathological responses. More recently, using BAC transgenesis, a mouse model was developed that captures both the developmental and pathological expression patterns of the NPPA gene. The zebrafish also exhibits dynamic expression of the cardiac natriuretic peptides during cardiogenesis, but it is unclear if the pathological responses are also conserved. We tested whether the regulation required for pathological natriuretic peptide responses is present during the early stages of cardiogenesis. We found that expression of the natriuretic peptides is increased in the embryonic zebrafish heart in response to traditional pathological stimuli and this response is distinct from the developmental induction of these genes. In an effort to create an animal model readily adaptable to screens for heart failure modifiers, we generated transgenic zebrafish reporter lines that model the pathological transcriptional responses of the cardiac natriuretic peptides. We provide evidence that this reporter line accurately reproduces the pathological expression patterns of the endogenous natriuretic peptides and their responses to a wide range of compounds that are either clinically utilized or in preclinical testing for CHF. In addition, using a zebrafish model of a human hypertrophic cardiomyopathy mutation, we demonstrate a distinct response to the histone deactylase (HDAC) inhibitor, Trichostatin A (TSA), and mitogen-activated protein kinase kinase (MEK) inhibitor U0126. These reporter lines will be useful to efficiently screen for both chemical and genetic modifiers of myocardial hypertrophy and heart failure.

2. Methods

The investigation conforms to the ‘Guide for the Care and Use of Laboratory Animals’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1 Whole-mount in situ analysis

nppa and nppb CDNA constructs were subcloned into pDONR221 or pCR8 (Invitrogen) and then subcloned into pCSDest (kindly provided by Dr N. Lawson, University of Massachusetts). A pCS2+ eGFP plasmid (kindly provided by Dr W. Chen, Vanderbilt University) was utilized to create the YFP antisense probe. For additional details, please see Supplementary material online, Methods.

2.2 Morpholino injections

Morpholinos (Gene Tools, LLC, Philomath, OR, USA) were resuspended in 1x Danieu’s solution and 500 μM (2 nL drop size, 7.6 ng) of morpholino was injected into fertilized eggs from TuAB fish at the single cell stage. Tntt2 splice targeting morpholino (tntt2sp M0) and tntt2 translation targeting morpholino (tntt2 M0) were both utilized.

2.3 Quantitative RT–PCR

Quantitative RT–PCR was performed using RNA extracted from whole embryos using Trizol reagent (Invitrogen) and reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). Gene-specific primers and Sybr Green reagent (Applied Biosystems) were utilized to perform the PCR portion of the experiment. The 2−ΔΔCt method was used to normalize the gene of interest to the endogenous housekeeping gene rpl13a and determine the fold change relative to control. For additional details, please see Supplementary material online, Methods.

2.4 Reporter construction

Natriuretic peptide reporter constructs were created using the zebrafish nppa and nppb promoters placed upstream of either YFP or Firefly luciferase. Stable transgenics were created using Tol2 transposase-mediated transgenesis. These lines were outbred for at least two generations. For additional details, please see Supplementary material online, Methods.

2.5 Whole-mount immunocytochemistry

Control or tntt2 morpholino-injected embryos were fixed in phosphate buffered saline (PBS) with 4% paraformaldehyde overnight at 4°C. The embryos were permeabilized with 0.5% Triton-X 100 in PBS (PBS-T) and then blocked with 2 mg/mL BSA, 2% goat serum, 1% dimethyl sulfoxide (DMSO) in PBS-T. Embryos were then incubated overnight at 4°C with a monoclonal antibody directed against Firefly luciferase protein (L2164, Sigma). The embryos were then washed with PBS-T and incubated with the secondary antibody (A10667, Invitrogen) for 3 h at 25°C. They were washed again in PBS-T and then imaged with a fluorescent dissecting microscope equipped with a digital camera.

2.6 Luciferase assay

Morpholino injected or uninjected nppa:F-Luc or nppb:F-Luc embryos were placed into a 96-well microtiter plate with buffered embryo water (E3) and the chemical that was being tested. The total volume of the embryos and the E3 was 100 μL. These plates were then incubated at 28°C. At a specified time point (72 hpf, 96 hpf, etc.), the plate was removed from the incubator and 50 μL of long half-life firefly luciferase reagent (Perkin Elmer, Steady-Glo) was added to the well. The plate was then incubated in the dark for 60 min. After 60 min, the plate was read in a high-sensitivity luminoscope plate reader (PE Elmer Victor X, or Biotek Synergy HL-1). The luminescence values obtained between different readers were calibrated to use the same scale.

2.7 Drug exposure

All chemicals were stored in DMSO stocks and diluted into working concentrations for specific experiments. The final DMSO concentration was kept below 1% for all experiments. All chemicals were applied at 24 hpf unless otherwise noted. The following concentrations were utilized: Isoproterenol (ISO) 250 μM, Carvedilol 25 μM, TSA 25 nM, Milrinone (MIL) 10 μM, PD98059 10 μM, U0126 10 μM, SB203580 10 μM, SB202190 10 μM, SP600125 500 nM, LY294002 10 μM. If additional drug concentrations are utilized, it is noted from the figure.

2.8 Statistics

To compare two continuous variables, a Student’s t-test was used. Data are expressed as mean ± SEM, unless a box plot is utilized. The box plot data show sample minimum (lowest point), lower quartile (bottom of shaded box), median (central black square), upper quartile (top of shaded box), and largest observation (highest point).

3. Results

3.1 The cardiac natriuretic peptides are induced secondary to pathological stimuli during zebrafish development

There is a robust expression of both natriuretic peptides during zebrafish cardiogenesis, but the pathological responses of the natriuretic peptide system during cardiogenesis are unknown. We performed whole mount in situ analysis of nppa and nppb expression in the intact embryo and found that there was a qualitative increase in the expression of both nppa and nppb in response to two distinct genetic stressors of cardiomyocyte physiology: abnormal splicing of cardiac...
troponin T or homozygous mutation of the gene gridlock (grl, hey2) (Figure 1A). The tnnt2sp MO embryos are injected with a morpholino that causes missplicing in the zebrafish cardiac troponin T (tnnt2) gene directly paralleling a known human mutation that causes hypertrophic cardiomyopathy. The gridlock2/2 embryos are genetic mutant embryos that are homozygous, a point mutation in the hey2 gene that causes luminal obstruction of the proximal aorta in the developing embryo. Notably, these embryos did not exhibit any extra-cardiac expression of the natriuretic peptides (Figure 1B).

We performed quantitative RT–PCR to confirm the whole mount in situ results (Figure 1C). We found that at 96 hpf, the tnnt2sp MO embryos exhibited a 2.6-fold induction of nppa and a 5.2-fold induction of nppb when compared with control embryos. The grl homozygote mutants exhibited a 2.3-fold induction of nppa and a 12.32-fold induction of nppb compared with grl heterozygote mutants (no phenotype). Since the cardiac natriuretic peptides are also induced during cardiogenesis, we sought to determine whether the pathological induction was an exaggeration of the developmental expression pattern or if unique regulators were controlling the pathological induction. Therefore, we measured the induction of nppb from 24 hpf until 96 hpf in both control and mutant embryos and we found that both groups had an early induction of nppb expression at 48 hpf, with the morphant embryos exhibiting a higher level of expression (Figure 1D) (control 25 ± 7.8 vs. TNNT2sp 35 ± 4.3-fold induction, P = N.S.). However, by 96 hpf, the control embryos had a dramatic decrease of nppb expression. In contrast, the tnnt2sp MO embryos revealed persistent elevation of nppb expression (control 4 ± 1.5 vs. tnnt2sp 39 ± 12-fold induction, P < 0.01). Thus, both control and mutant embryos responded to developmental stimuli at 48 hpf, but by 96 hpf, elevated nppb expression was maintained only in the context of the pathological stimuli in the tnnt2sp morphant embryos. These data suggest that cardiac hypertrophic stimuli do not appear to perturb the initial developmental induction of the natriuretic peptides, but markedly dysregulate subsequent control of natriuretic peptide expression and implicate discrete regulation in the control of developmental and pathological induction.

3.2 Transgenic natriuretic peptide reporter lines phenocopy endogenous gene expression patterns

The small size and translucency of the zebrafish embryo make it well suited for the real-time observation of fluorescent protein expression. We developed stable transgenic zebrafish lines that expressed fluorescent proteins under the control of the nppa and nppb minimal
promoters to serve as tools in the investigation of natriuretic peptide regulation during normal development and in the face of pathological stimuli. We first created transgenic lines using minimal promoters for the \(nppa\) or \(nppb\) gene controlling the expression of yellow fluorescent protein (\(Figure 2\)). For the \(nppa\) gene, we tested multiple different upstream genomic fragments before identifying fragments that recapitulated endogenous gene expression. We found that both 1 and 2 kb upstream genomic fragments had similar but not identical expression patterns compared with endogenous \(nppa\) expression (\(Figure 2A\); Supplementary material online, \(Figure S1\)). Specifically, there was less atrial expression of YFP in the transgenic reporter lines compared with the endogenous \(nppa\) expression. Attempts at using larger genomic fragments (\(>2\) kb) for \(nppa\) were not successful. This may reflect the location of the zebrafish \(nppb\) gene 2.8 kb upstream of the \(nppa\) gene with inclusion of this gene in the promoter fragment preventing efficient translation of the YFP.

Next, we created a transgenic line with the \(nppb\) promoter controlling the expression of YFP. Again, we tested numerous genomic fragments and found that a 4 kb fragment most closely recapitulated the expression of the endogenous \(nppb\) gene (\(Figure 2B\); Supplementary material online, \(Figure S1\)). The \(nppb\):YFP transgenic appeared to have stronger YFP fluorescence in the atria compared with the endogenous \(nppb\) expression. However, YFP RNA expression more closely matched the endogenous gene expression. This could reflect the \(\approx 12\) h half-life of YFP protein.

We compared the expression of these fluorescent reporters over the course of cardiac development in the zebrafish to fully characterize the expression of the reporters and to detect any non-cardiac expression (\(Figure 3\)). Both reporters were initially visible at 24 hpf paralleling the onset of endogenous gene expression. Over the next 48 hpf as the embryonic heart underwent looping and further growth, the expression of the reporters remained quite high, although the intensity qualitatively decreased at 72 hpf in the \(nppb\):YFP transgenics (\(Figure 3A\)). Importantly, there was no evidence of expression of the fluorescent reporters in non-cardiac regions of the embryo (\(Figure 3B\)).

### 3.3 Development of a luciferase-based whole embryo heart failure model

Fluorescent reporters are useful to observe the developmental expression patterns of the natriuretic peptides and to determine the optimal promoters for our reporter constructs. However, one of our primary goals in studying the natriuretic peptide expression pattern in the embryonic zebrafish was to develop an \(in vivo\) heart failure model that could be readily adapted for small molecule and genetic screens. Semi-quantitative measurement of fluorescent expression in the zebrafish embryo does not offer the rigorous assay characteristics that are necessary for high-throughput screens.
Therefore, we coupled the validated promoters for both nppa and nppb with Firefly luciferase.

Once we had created stable transgenic zebrafish lines expressing Firefly luciferase downstream of the nppa or nppb promoter, we then exposed these embryos to either a chemical (ISO) or genetic morpholino stimulus (tnnt2 morpholino). We found that fish transgenic for the nppa promoter driving luciferase did not recapitulate the pathological induction pattern of the native nppa gene (Figure 4B) (nppc:F-Luc, DMSO: 89 ± 7, Iso: 114 ± 10, tnnt2 MO: 66 ± 7 average luciferase units/embryo, P < 0.04). In contrast, the nppb promoter luciferase lines increased luciferase expression in a manner parallel to that of the endogenous nppb gene (tnnt2 MO stimuli—nppb mRNA 5.2-fold increase, nppb:F-Luc 5.8-fold increase; ISO stimuli—nppb mRNA 3.1-fold increase, nppb:F-Luc 2.6-fold increase, Supplementary material online, Figure S2). This reporter line appeared to accurately model the pathological induction pattern of the native gene (nppb:F-Luc, DMSO: 207 ± 9, Iso: 546 ± 45, tnnt2 MO: 1197 ± 245 average luciferase units/embryo, P < 0.001).

In order to verify that the luciferase protein expression in the nppb:F-Luc transgenic was restricted to the heart, we performed immunocytochemistry using a monoclonal antibody specific for Firefly luciferase protein (Figure 4C). At baseline, we could barely detect expression of the Firefly luciferase protein. However, with the tnnt2 morpholino stimulus, there was a large increase of Firefly luciferase protein. V, ventricle; A, atria. Scale bar = 100 μm.

In order to verify that the luciferase protein expression in the nppb:F-Luc transgenic was restricted to the heart, we performed immunocytochemistry using a monoclonal antibody specific for Firefly luciferase protein (Figure 4C). At baseline, we could barely detect expression of the Firefly luciferase protein. However, with the tnnt2 morpholino stimulus, there was a large increase of Firefly luciferase protein expression that was restricted to the heart.

Next, we performed a time course experiment comparing the control (DMSO) and ISO-treated nppb:F-Luc embryos exposed from 24 to 120 hpf (Supplementary material online, Figure S3A). Similar to the endogenous nppb gene expression, we found a clear divergence between control embryos and embryos exposed to ISO (48 hpf DMSO 4 ± 0.6 vs. ISO 5 ± 1, P = N.S., 72 hpf DMSO 6 ± 0.5 vs. ISO 19 ± 1.5, P < 0.001, 96 hpf DMSO 7 ± 0.5 vs. ISO 19 ± 1.7, P < 0.001, 120 hpf DMSO 3 ± 0.3 vs. ISO 16 ± 1.0-fold units, P < 0.001). However, the nppb:F-Luc embryos reached peak luciferase expression at a later time point (96 hpf) compared with the endogenous nppb gene expression (48 hpf).

We then shifted the interval of exposure of the nppb:F-Luc embryos to a later time point in order to further separate the developmental induction pattern from the pathological induction pattern (Supplementary material online, Figure S3B). We found that older embryos had a lower absolute level of luminescence when treated with either DMSO or ISO. However, since the control embryo luminescence levels were lower, the ISO-treated embryos had a greater fold induction when treated from 96 to 168 hpf (6.0-fold induction) compared with embryos treated from 24 to 96 hpf (2.8-fold induction) (24–96 hpf exposure: DMSO 205 ± 16, ISO 582 ± 54, 96–168 hpf: DMSO 62 ± 4, ISO 377 ± 40 average luciferase units/embryo, P < 0.001 for both). These data suggest that this transgenic line accurately models multiple aspects of native nppb gene expression and may be useful as a model to study pathways regulating cardiac hypertrophy and heart failure.

### 3.4 Development of a method to rapidly measure luminescence in the intact zebrafish embryo

In order to maximize the utility of our transgenic luciferase line, we needed to develop an assay that would be readily adaptable to
large-scale screening. Luciferase measurement in zebrafish after manual homogenization is feasible for small numbers of embryos, but is not readily adaptable to a 96 or 384 well microtiter plate format. Therefore, we incorporated the use of a long half-life luciferase transgene. This induction could be completely blocked by administration of carvedilol, \( p < 0.0001 \) compared with DMSO, \( **p < 0.0001 \) compared with ISO alone. The combination of MIL, a phosphodiesterase type 3 inhibitor, with isoproterenol showed a significantly larger induction of the luciferase transgene. This induction could be completely blocked by administration of carvedilol, \( p < 0.0001 \) compared with ISO alone, \( **p < 0.0001 \) compared with MIL/ISO. (B) A targeted kinase inhibitor screen shows inhibition of reporter induction, \( **p < 0.04 \) compared with ISO alone.

3.5 Validation of pathological natriuretic peptide responses using known agents

After we developed methods to consistently measure luciferase expression in high-throughput, we began to test our nppb:F-Luc reporter line responses to agents commonly used in human heart failure (Figure 5A). We first tested the non-selective beta-receptor agonist ISO, which mimics the elevated catecholaminergic state seen in late stage human heart failure patients. The application of this agent from 24 to 96 hpf consistently induced the reporter gene expression. Next, we co-administered the ISO with carvedilol, a non-selective beta and alpha-adrenergic blocker. This agent is one of the most effective agents in the treatment of human heart failure. When the agents were co-administered, the induction of the reporter expression was blocked. These data suggest that even at this stage of development, the embryonic zebrafish heart has an intact adrenergic signalling pathway. Next, we utilized the class I and II histone deacetylase (HDAC) inhibitor TSA, which has been shown to be effective in reducing or preventing myocardial hypertrophy in pre-clinical animal models. TSA has not been shown to interfere with the stimulation of the beta-adrenergic receptors, but instead modulates the expression of numerous genes by inhibition of HDACs. We found that TSA could also block the ISO induction of the nppb reporter gene (control 198 ± 6, ISO 421 ± 20, ISO/Carvedilol 213 ± 8, ISO/TSA 250 ± 12 average luciferase units/embryo, Ctl vs. ISO, \( p < 0.0001 \), ISO vs. ISO/Carvedilol, or ISO/TSA, \( p < 0.0001, n = 20–33 \) embryos/group). The final agent that we tested was milrinone (MIL), a type 3 phosphodiesterase inhibitor that increases cardiomycyte cyclic adenosine monophosphate (cAMP) levels and has a positive inotropic response in mammals. When this agent is administered alone to the zebrafish embryo, there is no significant change in nppb reporter expression. However, when ISO, an agent that increases intracellular cAMP levels is co-administered, there is a synergistic effect between both agents in the induction of the reporter gene. Notably, the addition of carbedilol can block this synergistic induction (MIL 219 ± 8, MIL/ISO 640 ± 52, MIL/ISO/Carvedilol 193 ± 13 average luciferase units/embryo, MIL vs. MIL/ISO, \( p < 0.0001 \), MIL/ISO vs. MIL/ISO/Carvedilol, \( p < 0.0001, n = 11–12 \) embryos/group). These data suggest that a broad selection of adult mammalian pharmacological responses is conserved in the larval zebrafish.

To further validate our reporter line, we performed a targeted screen with a variety of kinase inhibitors (Figure 5B). We found significant reductions in reporter activity with U0126 (Mek1/2 inhibitor), SB202190 (p38 MAPK inhibitor), and SP600125 (JNK inhibitor) (Control 206 ± 9, ISO 581 ± 32, ISO/U0126 237 ± 38, ISO/SB202190 368 ± 43, and ISO/SP600125 439 ± 25 average luciferase units/embryo, ISO vs. U0126, \( p < 0.0001 \), ISO vs. ISO/SB202190, \( p < 0.0001, n = 6–15 \) embryos/group). Upon further testing, we found that U0126 was the most effective agent at reducing ISO-induced activation of the nppb:F-Luc reporter (Figure 6A). At higher doses, SB202190 and SP600125 did not show any increased efficacy in reducing reporter activation (Supplementary material online, Figure S5).

Finally, we tested a genetic form of hypertrophic signalling, tnt2sp morpholino (Figure 6B). We found that the tnt2sp morpholino caused a significant induction of the reporter gene, and co-administration of MIL showed no ability to block this induction. Interestingly, we also found that carvedilol was ineffective in reducing the induction of the reporter gene in the tnt2sp morphant embryos. In contrast, TSA and U0126 were both effective in reducing the induction of the reporter by at least 60% (control 230 ± 10, tnt2sp MO 1366 ± 110, tnt2sp MO/MIL 1473 ± 235, tnt2sp MO/Carvedilol 1705 ± 508, tnt2sp MO/TSA 528 ± 61, and tnt2sp MO/U0126 462 ± 78 average luciferase units/embryo, tnt2sp MO vs. tnt2sp MO/MIL, \( p < 0.01 \) or tnt2sp MO/U0126, \( p < 0.01, n = 7–15 \) embryos/group).

4. Discussion

We have established that the genetic pathways controlling the pathological induction of the cardiac natriuretic peptides are intact during
In vivo natriuretic peptide reporter assay

We have validated this developed a rapid and reliable method to screen for genetic and pharmacological modifiers of cardiac natriuretic peptides promoters. Utilizing these reporter lines, we were able to peptides and firefly luciferase under the control of cardiac natriuretic pathways that regulate the developmental induction of these peptides. We have generated transgenic reporter lines that express fluorescent cardiogenesis in the zebrafish embryo and are distinct from the pathways that regulate the developmental induction of these peptides. We have generated transgenic reporter lines that express fluorescent peptides and firefly luciferase under the control of cardiac natriuretic peptides promoters. Utilizing these reporter lines, we were able to develop a rapid and reliable method to screen for genetic and pharmacological modifiers of cardiac natriuretic peptide expression, a highly conserved biomarker of cardiomyocyte dysfunction and hypertrophy. We have validated this in vivo system across a range of pathological and genetic stimuli. Finally, a targeted screen identified the HDAC inhibitor, TSA, and the MEK1/2 inhibitor, U0126, as potent inhibitors of the pathological reporter induction in a zebrafish model of hypertrophic cardiomyopathy.

There are two distinct settings in which cardiac natriuretic peptide production is induced, in cardiac development and in pathological states where myocardial function is impaired, such as CHF or myocardial hypertrophy. Many of the upstream transcriptional regulators of natriuretic peptide expression during heart development have been identified, and most of these transcription factors (Nkx2.5, Gata4, SRF, Tbx5) are also important for cardiomyocyte differentiation. These transcription factors not only control the developmental induction of the cardiac natriuretic peptides, but also are important in restricting the expression of NPPA and NPPB to the embryonic atria and ventricle.

In contrast, the transcriptional control of NPPA under pathological conditions is not well defined. This is highlighted by the fact that multiple transgenic reporter mouse lines with a range of NPPA promoters failed to respond to pathological stimuli (aortic banding) despite accurate recapitulation of the endogenous developmental expression patterns. The only mouse transgenic NPPA reporter line that has a preserved response to pathological stimuli was constructed using BAC transgenesis incorporating a segment of chromosomal DNA that spans from ~141 kb upstream to +58 kb downstream of the NPPA gene. In contrast, the developmental and pathological regulation of NPPB expression can be accurately reproduced with much less extensive promoter constructs. Importantly, we were able to demonstrate robust developmental and pathological induction of nppb in the zebrafish using relatively limited promoter fragments. In order to build a truly quantitative reporter system, we created luciferase-based reporter lines, which are able to rapidly measure single embryo luciferase expression at a scale similar to cell-based luciferase assays. Our method, unlike those previously published, does not require the manual homogenization of zebrafish embryos. This modification allows this assay to be readily adapted to primary genetic or chemical screens.

Modelling complex disease states such as CHF is challenging using in vitro systems, which are limited in their representation. Further, currently available cardiomyocyte cell lines either share properties of both cardiac and skeletal muscle (H9C2) or are derived from a cardiac tumour (HL-1). Using the intact zebrafish embryo with a functioning multi-chambered heart, intact endothelium, and active circulation is a better model of complex disease states such as CHF. The incorporation of a quantitative natriuretic peptide luciferase reporter enables the rapid assessment of a gene network that is conserved in most forms of myocardial hypertrophy and heart failure across multiple vertebrate species.

We were able to validate our reporter lines against several classes of cardiotoxic agents. Those agents that increased luciferase expression in the nppb reporter line (Beta-receptor agonists and Phosphodiesterase Type 3 inhibitors) have also been shown to increase mortality in human heart failure patients. In contrast, the agents that decreased expression of NPPB luciferase reporter line were the beta-receptor antagonist, Carvedilol, the class I and II HDAC inhibitor TSA, and the MEK1/2 inhibitor U0126. Carvedilol is a well-established agent administered to human heart failure patients and has been shown to significantly improve mortality in these patients. However, TSA has only recently emerged as a promising agent to inhibit and reverse the development of left ventricular hypertrophy in murine animal models. Likewise, U0126 has been shown in cell culture to be effective in blunting the induction of hypertrophic signal pathways, but has limited in vivo efficacy data. Of note, we also tested PD98059 a selective inhibitor of MEK1. This compound was ineffective in reducing pathological induction of the reporter gene. These results suggest that both MEK1 and MEK2 may be contributing to the pathological signalling cascade, or that MEK2 may be the primary regulator. Mouse models genetically targeting the Raf/MEK/ERK signalling cascade have shown divergent results with regards to the role of this signalling cascade in the pathological hypertrophic response. However, recent data from a mouse model with a Raf1 (L613V) mutation that causes Noonan syndrome in humans suggest that pharmacological inhibition of MEK may be a viable strategy in preventing myocardial hypertrophy.
Finally, to further illustrate the utility of our reporter line, we injected a morpholino that causes missplicing in cardiac Troponin T paralleling a human mutation that causes hypertrophic cardiomyopathy. The reporter embryos showed a robust induction of the luciferase transgene, but in contrast to the ISO-treated embryos, the TNNT2sp morphants did not uniformly respond to carvedilol. However, there was a significant inhibitory response to the HDAC inhibitor TSA and MEK 1/2 inhibitor U0126. This is the first experimental evidence from an in vivo model that sarcomeric gene mutations associated with hypertrophic cardiomyopathy may respond to HDAC inhibition or MEK1/2 inhibition. Currently, the pharmacological agents available to treat this condition are limited and are unable to induce regression of established ventricular hypertrophy.

The cardiac natriuretic peptide axis is a well-established marker of pathological cardiac signalling. Our zebrafish natriuretic peptide reporter lines enable the in vivo identification of genetic and chemical modifiers of this signalling pathway. We used this line to identify two compounds, TSA and U0126, which could potentially be useful in modulating this signalling pathway and could be used to rapidly assess the in vivo effectiveness of agents to modulate heart failure and myocardial hypertrophic signalling pathways.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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