Differential activation of natriuretic peptide receptors modulates cardiomyocyte proliferation during development

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ABSTRACT

Organ development is a highly regulated process involving the coordinated proliferation and differentiation of diverse cellular populations. The pathways regulating cell proliferation and their effects on organ growth are complex and for many organs incompletely understood. In all vertebrate species, the cardiac natriuretic peptides (ANP and BNP) are produced by cardiomyocytes in the developing heart. However, their role during cardiogenesis is not defined. Using the embryonic zebrafish and neonatal mammalian cardiomyocytes we explored the natriuretic peptide signaling network during myocardial development. We observed that the cardiac natriuretic peptides ANP and BNP and the guanylate cyclase-linked natriuretic peptide receptors Npr1 and Npr2 are functionally redundant during early cardiovascular development. In addition, we demonstrate that low levels of the natriuretic peptides preferentially activate Npr3, a receptor with Gi activator sequences, and increase cardiomyocyte proliferation through inhibition of adenylate cyclase. Conversely, high concentrations of natriuretic peptides reduce cardiomyocyte proliferation through activation of the particulate guanylate cyclase-linked natriuretic peptide receptors Npr1 and Npr2, and activation of protein kinase G. These data link the cardiac natriuretic peptides in a complex hierarchy modulating cardiomyocyte numbers during development through opposing effects on cardiomyocyte proliferation mediated through distinct cyclic nucleotide signaling pathways.

KEY WORDS: ANP, BNP, Npr3, Cardiomyocyte proliferation, Heart development, Natriuretic peptides

INTRODUCTION

The cardiac natriuretic peptides, atrial natriuretic peptide (ANP) and brain (or B-type) natriuretic peptide (BNP), are dynamically regulated in the adult heart and in other tissues by changes in blood pressure and other pathological processes (de Bold, 1985; Sudoh et al., 1988). The active peptides exert paracrine effects on cardiomyocytes and other cell types, as well as endocrine effects on remote organs such as the kidneys, adrenal glands or vasculature. Although cardiac natriuretic peptides are produced at high levels during cardiogenesis, little is known of the fundamental roles of these peptides during this developmental time period (Bloch et al., 1986; Horsthuis et al., 2008; Tanaka et al., 1999; Zeller et al., 1987). Several murine genetic models have shed light on the role of the natriuretic peptides in the adult mammalian heart. Elimination of individual natriuretic peptide genes leads to significant effects on the post-natal heart, with myocardial hypertrophy in adult animals null for Nppa and myocardial fibrosis in older adults null for Nppb (John et al., 1995; Tamura et al., 2000). Investigators have attempted to dissect potential redundancies through elimination of Npr1, a particulate guanylate cyclase receptor that is activated by both ANP and BNP. A myocardial-restricted knockout of Npr1 confirmed that this receptor plays a direct role in blunting the hypertrophic response of adult myocardium (Holtwick et al., 2003), but it was also noted that early post-natal survival was decreased in Npr1 null mice (Oliver et al., 1997; Scott et al., 2009). These data suggest that the natriuretic peptide pathway is important for cardiac responses to specific stressors, but also infer that the exploration of potential redundancy in murine models may be limited by viability.

The complexity of the natriuretic peptide signaling pathway is further compounded by the interactions of the active peptides with two additional receptors, Npr2 (also known as guanylyl cyclase-B (GC-B)) and Npr3 (also known as Npr-C). Similar to Npr1, Npr2 is also a particulate guanylate cyclase-linked receptor. The role of Npr2 in cardiomyocyte development is poorly understood, but a transgenic rat that overexpressed a dominant-negative isoform of the Npr2 receptor developed cardiac hypertrophy despite a normal systemic blood pressure (Langenickel et al., 2006). Npr3 does not possess guanylate cyclase activity and it is thought to act as a clearance receptor by binding and internalizing circulating natriuretic peptides (Nussenzveig et al., 1990). However, the cytoplasmic domain of this receptor contains Gi activator sequences that cause inhibition of adenyl cyclase (Anand-Srivastava et al., 1996; Lelièvre et al., 2006; Murthy and Makhlof, 1999). Deletion of the Npr3 gene in mouse causes systemic hypotension and skeletal defects (Matsukawa et al., 1999).

By applying knockdown and transgenic techniques in the zebrafish and in mammalian cardiomyocyte cultures, we show a novel role for the cardiac natriuretic peptides in dynamically regulating embryonic and neonatal cardiomyocyte proliferation in a concentration-dependent manner. Low concentrations of natriuretic peptides enhanced proliferation of embryonic zebrafish and neonatal rodent cardiomyocytes through Npr3-dependent modulation of cAMP signaling. By contrast, elevated concentrations of natriuretic peptides inhibit cardiomyocyte proliferation through protein kinase G (PKG)-mediated signaling that is dependent on Npr1 and Npr2. These results demonstrate a novel role for the natriuretic peptides in regulating developmental cardiomyocyte proliferation via the distinctive coupling of the natriuretic peptide receptors to discrete cyclic nucleotide signaling pathways.

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RESULTS

Perturbation of natriuretic peptide levels during embryogenesis reveals a role for these peptides in cardiac development

The full-length sequence of the zebrafish nppa was available (NM_198800.2) and we identified and characterized the zebrafish ortholog of the nppb gene (supplementary material Fig. S1). Whole-mount in situ hybridization analysis of nppa and nppb was undertaken at various developmental stages (Fig. 1A). The expression of zebrafish nppb is similar to that of nppa, with more robust expression in the ventricle compared with the atrium. The earliest expression was detected at 24 hours post-fertilization (hpf). We performed quantitative RT-PCR of nppa and nppb to determine the changes in expression levels during early heart development. Using the 24 hpf measurement as the reference point, nppa and nppb expression increase 50- and 22-fold, respectively, by 48 hpf. Expression of both genes decreases substantially from 72 hpf to 96 hpf but remain above the 24 hpf levels (Fig. 1B).

We then designed morpholino antisense oligonucleotides to eliminate the translation of the cardiac natriuretic proteins. Morpholino specificity was confirmed using nppa:YFP and nppb:YFP constructs incorporating the respective oligonucleotide target sequences (supplementary material Fig. S2A,B). The knockdown of either Nppa or Nppb in isolation did not cause any discernible changes in early cardiac development or function. However, when both genes were knocked down simultaneously, we observed a substantial enlargement of the zebrafish heart (Fig. 2A). In addition, we generated a transgenic zebrafish model to overexpress nppb using the Gal4:UAS transactivator system (Fig. 2B,D). The overexpression of nppb caused the overall zebrafish heart size to decrease substantially (Fig. 2C,D).

Cardiomyocyte proliferation is modulated by changes in natriuretic peptide levels

To see if changes in cell number might account for the divergent phenotypes seen with reduction or overexpression of the cardiac natriuretic peptides in the embryonic heart, we next counted total cardiomyocytes. The Nppa/Nppb double knockdown embryos exhibited a hyperplastic response with an ~15% increase in total cardiomyocytes at 48 hpf, whereas in the nppb-overexpressing embryos there was a 20% reduction in total cardiomyocytes at 48 hpf compared with control embryos (Fig. 3A,B). The overall body length at 48 hpf of both Nppa/Nppb double knockdown and nppb overexpression embryos was ~5% different from control embryos (control 3146±20, nppb overexpression (HS/nppb) 3016±26, Nppa/Nppb MO 3109±39 μm; mean ± s.e.m.,) suggesting that these effects were not a result of generalized effects on growth. The reduction in total cardiomyocyte numbers was not a result of increased cardiomyocyte apoptosis (Fig. 3C), but was a consequence of significant reduction in cardiomyocyte proliferation (Fig. 3D,E).

Cardiomyocyte cell size was not increased in the Nppa/Nppb double knockout embryos, which suggests cell hypertrophy was not the primary cause of the larger hearts in the Nppa/Nppb double knockdown embryos (supplementary material Fig. S2D,E). However, the cell size was decreased in the nppb overexpression embryos compared with controls, suggesting that both reduced cardiomyocyte proliferation and size are contributing to the reduced overall heart size in HS/nppb.

To confirm that the effects of the cardiac natriuretic peptides were independent from any extra-cardiac or systemic actions of the natriuretic peptides, we measured proliferation in the H9C2 cardiomyocyte cell line and neonatal rat cardiomyocyte primary cell cultures. Rat and mouse ventricular cardiomyocytes retain the capacity to proliferate for ~96 hours post-natally if cultured in high serum conditions (Fig. 4A,B; supplementary material Fig. S3A,B) (Hammoud et al., 2009; Kerkela et al., 2008; Li et al., 1996; McKoy et al., 2007). This allowed us to assess accurately the effects of natriuretic peptide concentrations on mammalian neonatal cardiomyocyte proliferation. We found that low concentration natriuretic peptide supplementation increased the baseline rates of proliferation of H9C2 cells by 11% and neonatal cardiomyocytes by up to 27%, whereas high concentration natriuretic peptide supplementation decreased proliferation by up to 18% for H9C2 cells (supplementary material Fig. S3C) and 24% for neonatal cardiomyocytes (Fig. 4C). Fluorescence-activated cell sorting analysis of annexin V- terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)- and propidium iodide-stained neonatal rat ventricular myocytes (NRVMs) confirmed that the changes in total cell populations were not a result of changes in cell viability (Fig. 4D,E).

Although H9C2 cardiomyocytes are a homogenous cell population, primary cultures of neonatal cardiomyocytes can be contaminated by non-myocytes. Therefore, to confirm our results we directly measured the number of cardiac troponin T (Tnnt2)-positive cells that progressed through the S phase of the cell cycle by
measuring the incorporation of the thymidine analog 5-ethynyl-2′-deoxyuridine (EdU) (supplementary material Fig. S3D). This enabled us to confirm that the changes in cell numbers seen in the cardiomyocyte cultures resulted from changes in cardiomyocyte proliferation. We found an increased number of EdU-positive cardiomyocytes in the low concentration (10 nM ANP exposure) group and a lower number of EdU-positive cardiomyocytes in the high concentration (10 μM ANP exposure) group (Fig. 4F). In addition, the proportion of EdU-positive cells that were binucleate was <6% in all three treatment groups, supporting the conclusion that the primary effect measured was cell division and not just karyokinesis.

Natriuretic peptide effects on cardiomyocyte number are mediated via the three natriuretic peptide receptors

In order to define the mechanisms through which the natriuretic peptides exert their effects in vivo, we identified the zebrafish orthologs of Npr1, Npr2 and Npr3 and found that all three receptors are expressed in zebrafish cardiac tissue (supplementary material Fig. S4A,B). In contrast to nppa and nppb, the expression of the natriuretic peptide receptors did not increase as dramatically through the early stages of heart development (supplementary material Fig. S4B). However, npr3 expression did surge at 48 hpf coinciding with peak expression of nppa and nppb in early zebrafish heart development.

As npr1 and npr2 both exhibit guanylate cyclase activity, we investigated if the knockdown of these receptors could phenocopy the Nppa/Nppb double knockdown morphants. We designed morpholinos to disrupt the normal splicing of these genes to generate transient loss of function (supplementary material Fig. S4C,D). To compare the different groups directly, we used cmlc2:CFP labeling of the embryonic heart and estimated heart dimensions in all of the different experimental groups at the same time point during development (48 hpf) (Fig. 5A,B). When npr1 or npr2 alone were targeted, there were minimal changes in early cardiac morphogenesis or physiology. However, simultaneous disruption of both npr1 and npr2 resulted in perturbation of cardiac chamber formation comparable to that observed in the Nppa/Nppb double knockdown embryos.

Next, we injected the npr1 and npr2 morpholinos into HS:nppb transgenic embryos. The decreased heart size observed in HS:nppb embryos was prevented by simultaneous knockdown of npr1 and npr2, suggesting that the effects of nppb overexpression are mediated via Npr1 and Npr2 activation (Fig. 5A,B). Importantly, it was also possible to block the effects of nppb overexpression using the cognate nppb translation-blocking morpholino.

After selectively targeting the particulate guanylate cyclase-linked natriuretic peptide receptors npr1 and npr2, we then performed a morpholino knockdown of npr3 (supplementary material Fig. S4E). We found that embryos injected with the npr3 morpholino...
developed at a normal rate except that their overall heart sizes were smaller than control embryos at 48 hpf (Fig. 3A,B). The Npr3 knockdown embryos had very similar heart size and morphology to HS/nppb embryos. We then measured cardiomyocyte proliferation in embryos lacking the natriuretic peptide receptors. We found that embryos lacking the particulate guanylate cyclase natriuretic peptide receptors npr1 and npr2 displayed increased levels of cardiomyocyte proliferation. However, embryos lacking npr3 exhibited reduced embryonic cardiomyocyte proliferation (Fig. 3C).

We also evaluated the addition of cardiomyocytes to the arterial pole of the zebrafish ventricle from 30 to 48 hpf to determine if disruption of natriuretic peptide signaling in the embryonic heart was perturbing differentiation of cardiomyocyte progenitors. In all three groups, new ventricular cardiomyocytes were added to the arterial pole of the zebrafish ventricle from 30 to 48 hpf to determine if disruption of natriuretic peptide signaling in the embryonic heart was perturbing differentiation of cardiomyocyte progenitors. In all three groups, new ventricular cardiomyocytes were added to the arterial pole of the zebrafish ventricle (identified by green cells indicating non-photoactivated kaede), which suggests that our observations are not a result of altered proliferation in progenitor compartments outside the primary heart field, but rather of differentiated ventricular cardiomyocytes (Fig. 3D).

We also measured expression of nppa, nppb, npr1, npr2 and npr3 when there was knockdown or overexpression of different components of the cardiac natriuretic peptide signaling pathway (supplementary material Fig. S5). When there was knockdown of Nppa/Nppb or Npr1/Npr2 there was a robust increase in nppa/nppb expression (greater than fourfold increase). By contrast, Npr3 knockdown caused a reduction in nppa and nppb expression (60% and 23% reduction, respectively). Overexpression of nppb caused a modest but significant reduction of npr1. Lastly, the efficacy of our splice-targeting morpholinos (npr1, npr2, npr3) was also confirmed with reduction of the target transcripts of >95%.

The inhibitory effects of natriuretic peptides on cardiomyocyte proliferation are mediated via activation of PKG

We then utilized mammalian cardiomyocyte primary culture to explore further the downstream signaling pathways involved in natriuretic peptide suppression of cardiomyocyte proliferation. First, we verified that all three natriuretic peptide receptors are expressed in mammalian cardiomyocytes at different stages of heart development (supplementary material Fig. S6A). This was performed by analyzing publicly available microarray datasets collected in the Gene Expression Omnibus (National Library of Medicine) (n=3-10 biological replicates/group). We found that Npr1, Npr2 and Npr3 were all expressed in embryonic, neonatal and adult murine cardiomyocytes at levels similar to the beta-1 adrenergic receptor (Adrb1) (a receptor highly expressed in cardiomyocytes). Of note, Npr2 was expressed at higher levels in neonatal and adult cardiomyocytes than Npr1 (P<0.001). The cardiomyocyte sarcomeric protein, cardiac troponin T (Tnnt2), was used to verify the accuracy of the cardiomyocyte expression data.

We then tested to see if Npr1 and Npr2 were biologically active in primary cardiomyocyte cell culture using selective peptide agonists. Dendroaspis natriuretic peptide (DNP, an Npr1-selective agonist) and C-type natriuretic peptide (CNP, an Npr2-selective agonist) were each able to inhibit neonatal cardiomyocyte proliferation at elevated concentrations (10 μM CNP and 1 μM DNP) in a manner similar to ANP 10 μM (supplementary material Fig. S6B,C). This confirmed that Npr1 and Npr2 were functionally
active in mammalian cardiomyocytes and contributed to the inhibition of cardiomyocyte proliferation seen with high concentrations of natriuretic peptides.

Activation of Npr1 and Npr2 leads to increased cGMP levels, which in turn result in the activation of PKG. We targeted PKG with selective inhibitors to determine if this kinase contributed to the natriuretic peptide effect on cardiomyocyte proliferation (Fig. 6A). We first used Rp-cGMP and were able to almost eliminate the inhibitory effects of high concentration ANP on cardiomyocyte proliferation. We then employed a second selective PKG inhibitor, KT5823, and prevented the reduced cardiomyocyte proliferation seen with high concentration ANP exposure. In fact, cardiomyocyte proliferation rates were similar to those observed with low concentration ANP, suggesting that complete blockade of PKG allowed the proliferative effects of Npr3 activation by ANP to be unmasked even in the setting of high concentration ANP. Of note, both selective PKG inhibitors were used at a concentration that did not alter baseline cardiomyocyte proliferation (supplementary material Fig. S6D).

Finally, the direct application of 8-pCPT-cGMP (a cell-permeable analog of cGMP that activates PKG) mimicked the inhibitory effect of high concentrations of ANP on NRVM proliferation (Fig. 6B). These data suggest that cGMP activation of PKG mediates the anti-proliferative effects of high concentration ANP signaling through Npr1 and Npr2.

Enhanced proliferation of cardiomyocytes at low concentrations of natriuretic peptides is mediated by activation of Npr3 and cAMP pathways

To verify that Npr3 was involved in the induction of cardiomyocyte proliferation observed with low concentrations of ANP, we used the selective Npr3 antagonist AP-811 (Veale et al., 2000; William et al., 2008). Although AP-811 did not alter baseline cardiomyocyte proliferation across a wide range of concentrations (supplementary material Fig. S7A), when we exposed proliferating cardiomyocytes to low concentration ANP (10 nM) in the setting of an Npr3 antagonist, the increased cell proliferation was blocked (Fig. 6C). In contrast to PKG inhibition, blocking Npr3 had minimal effects on the anti-proliferative actions of high concentration ANP. We then combined Npr3 antagonism with AP-811 and PKG inhibition with KT5823 and found that we could completely block the effects of ANP on cardiomyocyte proliferation (supplementary material Fig. S7A).

To determine if Npr3 inhibition of adenylate cyclase was playing a role in cardiac proliferation, we tested whether direct inhibition of adenylate cyclase could mimic the proliferative effects of low concentration ANP. We exposed neonatal rat cardiomyocytes to the selective adenylate cyclase inhibitor SQ 22536 and found that proliferation could be enhanced by adenylate cyclase inhibition in a concentration-dependent manner (Fig. 6D; supplementary material Fig. S7B), mimicking the low concentration ANP activation of cAMP.
Npr3. Conversely, the addition of a cAMP analog (8-Br-cAMP) could block the enhanced proliferation of cardiomyocytes seen with low concentrations of ANP in a concentration-dependent manner (Fig. 6D; supplementary material Fig. S7B). We then explored the effects of activation of adenylyl cyclase on cardiomyocyte proliferation. We used the β-adrenergic receptor agonist isoproterenol to activate adenylyl cyclase and found that cardiomyocyte proliferation was decreased (supplementary material Fig. S8). We found that we could block the effect of adenylyl cyclase activation by simultaneous addition of low concentration ANP (10 nM). Conversely, the reduced cardiomyocyte proliferation seen with high concentration ANP (10 μM) was enhanced in the setting of adenylyl cyclase activation by isoproterenol.

Finally, we performed siRNA knockdown of Npr3 in NRVMs. When Npr3 was eliminated using siRNA we were able to completely block the enhanced proliferation and DNA synthesis seen with low concentration ANP (Fig. 7A,B). In addition, we performed direct measurement of EdU-positive cardiomyocytes using fluorescence microscopy to confirm that DNA synthesis was indeed reduced compared with control cells stimulated with 10 nM ANP (Fig. 7C,D).

**DISCUSSION**

In the work we have outlined, we establish a novel role for the cardiac natriuretic peptides in modulating cardiomyocyte proliferation during cardiogenesis by differentially activating the guanylate cyclase-linked natriuretic peptide receptors (Npr1 and Npr2) and Npr3 (a clearance receptor and activator of Gi subunit). Our data establish in both a zebrafish model and in proliferating mammalian cardiomyocytes, that the natriuretic peptides modulate cardiomyocyte division in a concentration-dependent manner. Low concentrations of natriuretic peptide increase cardiomyocyte proliferation whereas high concentrations of these same peptides inhibit cardiomyocyte proliferation. The enhanced proliferation seen with low levels of cardiac natriuretic peptides is primarily mediated through Npr3, whereas the inhibition of proliferation seen with high concentrations of natriuretic peptides is mediated through Npr1 and Npr2 (Fig. 8).

Natriuretic peptides are dynamically expressed during heart development in both zebrafish and mammals (Bloch et al., 1986; Zeller et al., 1987). In our zebrafish model, the experimental results suggest that the surge of natriuretic peptides that occurs at 48 hpf reduces the proliferative capacity of differentiated cardiomyocytes.
at that time point. When the translation of \textit{nppa} and \textit{nppb} was reduced during that time period, cardiomyocyte proliferation was increased. Conversely, if \textit{nppb} expression was increased during that time period there was a further reduction of cardiomyocyte proliferation below baseline proliferation rates. The production of cardiac natriuretic peptides decreases substantially by 96 hpf in the developing zebrafish (Fig. 1B), which also coincides with an increase of cardiomyocyte proliferation in the zebrafish heart (Choi et al., 2013). The \textit{in vivo} role of natriuretic peptides during later stages of cardiac development cannot be readily studied in the embryonic zebrafish; however, murine models lacking or overproducing natriuretic peptides will enable further exploration of the \textit{in vivo} role of these peptides at later developmental time points.

Both Npr1 and Npr2 are expressed in cardiomyocytes during mammalian heart development (supplementary material Fig. S6A) and cardiac natriuretic peptides can activate these receptors at low nanomolar concentrations or less (Johns et al., 2007; Nir et al., 2001; Suga et al., 1992). By contrast, Npr3, which binds all natriuretic peptides, does not exhibit guanylate cyclase activity, but can act as a clearance receptor and can activate Gi, which inhibits adenyl...
heterogeneous mixture of different cell types it will be necessary to (Khambata et al., 2011). As the mammalian heart is composed of a smooth muscle cell proliferation was inhibited by Npr3 activation whereas cell proliferation was augmented by activation of Npr3 whereas been explored in other cell types and it was found that endothelial effects of this receptor (adenylyl cyclase inhibition) from its role as a modulator of systemic natriuretic peptide levels (clearance role). 

restricted elimination of Npr3 will help further define its role in a cGMP-mediated pathway is supported by previous work in fetal cardiomyocyte proliferation through regulating multiple cyclic nucleotide signaling pathways in a concentration-dependent fashion. A further understanding of how these endogenous cardiac hormones regulate the growth characteristics of cardiomyocytes will enhance our understanding of the mechanisms regulating cardiac growth during development and beyond, in health and disease.

MATERIALS AND METHODS

Cloning of zebrafish nppb, npr1a and npr2 genes
Utilizing existing databases (Ensembl, NCBI) we identified the zebrafish orthologs of Nppb, Npr1 and Npr2 genes. We performed RACE experiments (Ambion) to determine the coding sequence of each gene.

Whole-mount in situ hybridization analysis
nppa and nppb cDNA constructs were cloned into pDONR221 or pCR8 (Invitrogen) and then subcloned into pCSDest (kindly provided by Nathaniel Lawson, University of Massachusetts). Digoxigenin (dig)-labeled sense and antisense probes were generated and purified (Roche). Control and experimental embryos were fixed in 4% paraformaldehyde (PFA) at 25°C for 2 hours, and then stored in 100% methanol at −20°C until beginning of rehybridization protocol. Embryos were rehydrated in PBST (0.1% Tween in PBS), and briefly incubated with proteinase K (4-8 minutes depending on age) and then re-fixed. Hybridization with dig-labeled probe was performed for 12 hours at 65°C. After washes and re-equilibration in PBST, embryos were incubated with anti-Dig antibody (1:5000; Roche, 11093274910) for 12 hours at 4°C, washed, and then staining was performed. Full protocol is outlined in Thiss and Thissè (Thissè and Thissè, 2008).

Gene knockdown in vivo
Morpholinos (Gene Tools) were resuspended in 1× Danieu’s solution and 125-500 μM (2 nl drop size, 1.9-7.6 ng) of morpholino was injected into fertilized eggs from TuAB fish at the single-cell stage. The nppa and nppb translation-inhibiting morpholino specificity was evaluated using modified nppa:YFP and nppb:YFP constructs as described in supplementary material Fig. S2. The npr1a, npr2 and npr3 morpholinos were splice-targeting morpholinos and their effectiveness is detailed in supplementary material Figs S4 and S5. As an additional measure of morpholino specificity, we tested all of the morpholinos listed below in a p53 (tp53) null zebrafish line to verify that the phenotypes seen in wild-type embryos were not secondary to non-specific activation of p53 (Robu et al., 2007). See supplementary material Table S1 for morpholino sequences.

Gene knockdown in vitro
Control siRNA (Invitrogen, 4390843) and Npr3 siRNA (Invitrogen, s129528) were resuspended in siRNA buffer (Dharmacon). Neonatal cardiomyocytes
were transfected with 5 nM of siRNA using DharmaFECT reagent (Thermo Scientific) and the media was changed daily. After 48 hours of siRNA exposure, measurement of the target RNA was performed to verify knockdown efficiency. For all proliferation experiments, an additional 48 hours of siRNA exposure was performed with or without the addition of ANP and cell numbers were measured using the methods described below.

**Quantitative RT-PCR**
Quantitative RT-PCR was performed using RNA extracted from whole embryos or heart tissue 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 to interest to the endogenous housekeeping gene eef1a1l1. See supplementary material Table S1 for primer sequences.

**Overexpression of nppb in vivo**
The pCH:Gtwy:G4VP16 and pBH:UAS:Gtwy:YFP vectors (kindly provided by Michael Nonet, Washington University St Louis, MO, USA) were utilized to make stable transgenic zebrafish lines. The pBH-UAS-Gtwy-YFP vector was modified by removal of the YFP sequence and replacement of the cmlc2 (msl7 – Zebrafish Information Network) promoter with a Xenopus crystallin promoter to drive mCherry expression in the lens of the eye; this new vector is referred to as pBE-UAS-Gtwy. The zebrafish nppb cDNA and 1.5-kb heat shock (HS) 70 promoter were first recombined into pDonor221 (Invitrogen) using BP clonase (Invitrogen). Then LR clonase-mediated recombination was used to place the HS promoter upstream of the Ga4VP16 site in pCH:Gtwy:G4VP16, creating pCH:HS:G4VP16. Likewise, the pDonor:nppb construct was recombined using LR clonase to create pBE:UAS:nppb. Single-cell embryos were injected with 25 ng/ml vector DNA and 25 ng/ml of Tol2 transposase RNA. Stable transgenics were selected for and outbred for two generations. Adult pCH:HS:G4VP16 and pBE:UAS:nppb were then crossed and embryos were collected. The embryos were stored in a 28°C incubator. At 24 hpf, the embryos were placed in a 37°C incubator for 60 minutes, and then returned to a 28°C incubator. At 48 hpf, the embryos were analyzed. Embryos positive for both the cmlc2:CFP (Gal4+) and crystallin:mCherry (UAS:nppb+) fluorescent markers were shown to be positive for ectopic nppb expression using whole-mount in situ analysis for nppb. Embryos containing only cmlc2:CFP or crystallin:MCherry did not have ectopic nppb expression (supplementary material Fig. S2C).

**Cardiac chamber cross-sectional area**
All embryos carried a cmlc2:CFP or cmlc2:GFP cardiomyocyte marker to delineate the atrial and ventricular chambers. At 48 hpf, a lethal dose of tricaine was applied to the embryos causing cessation of myocardial contraction and uniform myocardial relaxation. The embryos were then positioned in the left lateral decubitus position and imaged using a fluorescence dissecting microscope (Zeiss Stereo Discovery v8, AxiocamMRm camera). Once the images were acquired they were analyzed using ImageJ software (NIH) to determine the chamber cross-sectional area. The nuclei of the cardiomyocytes were then placed in acetone for 10 minutes, washed and bleached. A primary antibody directed against BrdU (G3G4, DSHB) was then used to detect BrdU incorporation. Secondary antibody was Alexa 568-conjugated goat anti-mouse (A11004, Invitrogen). To detect CFP-positive cardiomyocytes an Alexa 488-conjugated anti-GFP antibody was utilized (A21311, Invitrogen).

**Cardiomyocyte quantification and proliferation in vivo**
Zebrafish embryos carrying a cmlc2:GFP transgene were injected with control or nppa/nppb morpholino at the one-cell stage. The HS:nppb dual transgenic embryos also have a cmlc2:CFP fluorescent marker. At 48 hpf, the embryos were euthanized, their hearts were excised and placed at 4°C. DAPI stain was applied, and the hearts were imaged using a Zeiss LSM5 Pascal confocal microscope. The nuclei of the CFP-positive cells were then counted using ImageJ software. Cardiomyocyte proliferation in zebrafish was assessed using a modified BrdU (5-bromo-2'-deoxyuridine) protocol (Lazic and Scott, 2011). Briefly, 48 hpf cmlc2:GFP zebrafish embryos were bathed in 10 mM BrdU for 30 minutes with 15% DMSO on an ice bath, then placed in a 28°C incubator for 3.5 hours. Embryos were then fixed in 4% PFA for 60 minutes, and washed in PBST. The embryos were then placed in acetrone for 10 minutes, washed and bleached. A primary antibody directed against BrdU (G3G4, DSHB) was then used to detect BrdU incorporation. Secondary antibody was Alexa 568-conjugated goat anti-mouse (A11004, Invitrogen). To detect CFP-positive cardiomyocytes an Alexa 488-conjugated anti-GFP antibody was utilized (A21311, Invitrogen).

**Assessment of zebrafish ventricular cardiomyocyte differentiation between 30 to 48 hpf**
Dual transgenic embryos [cmlc2:GAL4 x UAS:kaede (Scott et al., 2007)] (30 hpf) were anesthetized and mounted in 1% methylcellulose on a glass slide, and exposed to 330-385 nm light on a Olympus FV1000 confocal microscope. Fluorescence in the cells was measured using a microtiter plate reader (Spectramax M5) at 485-nm excitation and 530-nm emission settings. The remaining wells on the plate were then exposed to peptides or small molecules at varying concentrations for the next 48 hours. After 48 hours of exposure, the exposed wells were washed with PBS and the DNA-binding reagent (Cyanquant NF, Invitrogen) was added to a subset of the wells to obtain a baseline (0 hour) measurement. Fluorescence in the wells was measured using a microtiter plate reader (Spectramax M5) at 485-nm excitation and 530-nm emission settings. The remaining wells on the plate were then exposed to peptides or small molecules at varying concentrations for the next 48 hours. After 48 hours of exposure, the exposed wells were washed with PBS and the DNA-binding reagent was added and measured on a fluorescent microtiter plate reader. In order to normalize fluorescence values to cell number, we did triple replicate cell counts manually using a hemocytometer and plated a range of 500 to 6000 cells/well. After cell adherence, the plates were measured and a formula [350 nm emission=0.6065(cell count) + 1.4532] was derived using linear regression (R²=0.9998) to convert raw fluorescence values to cell number in subsequent experiments.

To measure the number of NRVMs in S phase of the cell cycle, we incubated the cells with 5-ethyl-2'-deoxyuridine (EdU; Invitrogen) for 5 hours after the initial 48-hour exposure to the specified concentrations of ANP peptide. The cells were then fixed with 4% PFA and permeabilized with 0.5% Triton X-100. EdU detection was performed using Alexa Fluor 594-conjugated azide (A10270, Invitrogen). To selectively label cardiomyocytes, we incubated the fixed NRVMs in either a monoclonal antibody specific for cardiac Troponin T (ab10214, Abcam) or sarcomeric alpha actinin (ab9465, Abcam). Detection of the primary mouse monoclonal antibodies was performed using Alexa Fluor 488-labeled goat-anti mouse.
secondary antibodies (A11029, Invitrogen). Imaging was performed on either confocal microscope (Zeiss LSM510) or upright fluorescence microscope (Zeiss Axioplan 2). The number of EdU-positive and cardiac troponin T dual-positive cells were then manually counted and divided by the total number of cardiac troponin T-positive cells.

Apoposis assessment
Apoposis assessment in vitro was performed using Annexin V and TUNEL assessment on proliferating NRVMs exposed to ANP. Primary NRVMs were cultured and exposed to ANP for 48 hours. The cells were then washed and exposed to Annexin V–Alexa Fluor 488 conjugate (Invitrogen, A13201) and propidium iodide. The cells were sorted using a BD FACSaria II with an average of 10,000 counts/biological replicate with three biological replicates for each treatment group. For the TUNEL assessment, NRVMs were cultured and exposed to ANP for 48 hours and then fixed with 1% PFA on ice for 15 minutes and stored in 70% (v/v) ethanol at −20°C for 48 hours. The cells were then washed, labeled with BrdU labeling solution and then stained with Alexa Fluor 488-labeled anti-BrdU antibody (Invitrogen, A23210). The labeled cells were then analyzed by flow cytometry as described above. Acrinide Orange staining was performed to determine the number of apoptotic cells in the zebrafish heart. Zebrafish embryos with normal or increased expression of Nppb were exposed to 2 μg/ml of Acrinide Orange for 30 minutes and anesthetized with tricaine. The embryos were then imaged using a fluorescence dissecting microscope (Olympus SXZ12). The number of Acrinide Orange-positive heart cells was then manually counted.

Microarray dataset analysis
Repository gene expression data were obtained from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; for accession numbers, see supplementary material Table S1). Data sets that analyzed purified cardiomyocyte samples were included for analysis. Each data set was separately normalized using robust multi-array average (RMA) for Affymetrix data or quantile normalization followed by log2 transformation for the non-Affymetrix data sets. Only the experimental channel was used for the Agilent two-color array data. To compare receptor expression across studies, normalized log2 hybridization signal value ratios were calculated using two different internal housekeeping genes (GAPDH and Actb), which generated similar values. Only cardiomyocyte data sets that expressed high levels of cardiac troponin T (Tnt2) were considered acceptable for further analysis. Likewise, we used endothelial cell expression data to serve as a negative control to verify that Tnt2 expression could adequately differentiate between cardiomyocyte and non-cardiomyocyte populations.

Peptides and small molecules
The following peptides and small molecules were used: atrial natriuretic peptide (ANP) (0.5-10,000 nM; AnaSpec, 20652); C-type natriuretic peptide (CNP) (1-10,000 nM; Phoenix Pharmaceuticals, 012-03); dendraosip fatal atriat tripletid epine (DNP) (0.1-1000 nM; Phoenix Pharmaceuticals, 013-01); AP-811 (10-500 nM; California Peptide Research); 8-Br-CAMP (1-100 μM; Santa Cruz Biotech, sc-201564); Rp-8-pCPT-cGMP (1 μM; Enzo, BML-CN206-0001); 8-pCPT-cGMP (100-800 μM; Sigma, C5438); SQ 22536 (250 nM-5 μM; Santa Cruz Biotech, sc-201572); isoproterenol (1 μM; Sigma, I6504); and KT 5823 (1 nM; Tocris, 1289).

Statistics
All data displayed as mean ± s.e.m. unless otherwise noted. Data were analyzed using Student’s t-test for comparisons between two groups or one-way ANOVA for comparisons between three or more groups. Significance was defined as P<0.05.

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Competing interests
The authors declare no competing financial interests.

Author contributions

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