Atrial natriuretic peptide inhibits cell cycle activity of embryonic cardiac progenitor cells via its NPRA receptor signaling axis

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Submitted 24 September 2014; accepted in final form 24 January 2015

Hotchkiss A, Feridooni T, Baguma-Nibasheka M, McNeil K, Chinni S, Pasumarthi KB. Atrial natriuretic peptide inhibits cell cycle activity of embryonic cardiac progenitor cells via its NPRA receptor signaling axis. Am J Physiol Cell Physiol 308: C557–C569, 2015. First published January 28, 2015; doi:10.1152/ajpcell.00323.2014.—The biological effects of atrial natriuretic peptide (ANP) are mediated by natriuretic peptide receptors (NPRs), which can either activate guanylyl cyclase (NPRA and NPRB) or inhibit adenylyl cyclase (NPRC) to modulate intracellular cGMP or cAMP, respectively. During cardiac development, ANP serves as an early maker of differentiating atrial and ventricular chamber myocardium. As development proceeds, expression of ANP persists in the atria but declines in the ventricles. Currently, it is not known whether ANP is secreted or the ANP-NPR signaling system plays any active role in the developing ventricles. Thus the primary aims of this study were to 1) examine biological activity of ANP signaling systems in embryonic ventricular myocardium, and 2) determine whether ANP signaling modulates proliferation/differentiation of undifferentiated cardiac progenitor cells (CPCs) and/or cardiomyocytes. Here, we provide evidence that ANP synthesized in embryonic day (E)11.5 ventricular myocytes is actively secreted and processed to its biologically active form. Notably, NPRA and NPRC were detected in E11.5 ventricles and exogenous ANP stimulated production of cGMP in ventricular cell cultures. Furthermore, we showed that exogenous ANP significantly decreased cell number and DNA synthesis of CPCs but not cardiomyocytes and this effect could be reversed by pretreatment with the NPRA receptor-specific inhibitor A71915. ANP treatment also led to a robust increase in nuclear p27 levels in CPCs compared with cardiomyocytes. Collectively, these data provide evidence that in the developing mammalian ventricles ANP plays a local paracrine role in regulating the balance between CPC proliferation and differentiation via NPRA/cGMP-mediated signaling pathways.

embryonic heart; ANP; natriuretic peptide receptors; gene expression; cardiac progenitor cells; cardiomyocytes; lineage tracking; knockin mice; cell proliferation and differentiation

ATRIAL NATRIURETIC PEPTIDE (ANP) is a 28 amino acid peptide that is synthesized and stored primarily in secretory granules of atrial cardiomyocytes in the adult heart. Ligand binding of ANP to its cognate natriuretic peptide receptors (NPRs) can either activate guanylyl cyclase (NPRA and NPRB) or inhibit adenylyl cyclase (NPRC) to modulate intracellular cGMP or cAMP, respectively. The primary stimulus for ANP secretion from atrial cardiomyocytes is mechanical stretch of the atrial wall (15). In addition to mechanical stimuli, several vasoconstrictor peptides including endothelin-1 (ET-1) (36) and angiotensin II (9), as well as a variety of neurohormones, growth factors, and cytokines, have been shown to modulate natriuretic peptide secretion (10). Once in the circulation, ANP acts in a true endocrine fashion by stimulating NPRA receptors in the kidneys, adrenal cortex, and vasculature to regulate fluid homeostasis and maintain blood pressure via diuretic, natriuretic and vasorelaxant effects (29). In the ventricles of the adult heart, the levels of ANP protein are normally ~1,000-fold lower than in the atria and secretory granules are rarely observed (31).

In contrast to the adult heart, developmental studies in rodents have documented higher levels of ANP mRNA (6, 46) and protein (43) in the embryonic ventricles compared with the atria. Spatial analysis of ANP mRNA in the murine heart during development revealed prominent labeling in the atria as well as the primitive ventricle at embryonic day (E)9 (46). Intriguingly, by E14 and neonatal stages, ANP labeling persisted in both atria but was gradually restricted to the trabecular myocardium of the left and, to a lesser extent, right ventricles (46). Based on this dynamic spatiotemporal expression pattern of ANP in the ventricles during cardiogenesis, we speculate that ANP may be an important paracrine regulator of cardiac growth in the ventricular compartment. In support of this notion, it has been shown that mice lacking NPRA display reduced survival, cardiac hypertrophy beginning at mid/late gestation, and morphological abnormalities including dextrocardia and mesocardia (7, 16, 26, 35). Currently, however, there is no direct evidence that ANP receptor-mediated signaling systems are biologically active in the embryonic heart.

Cardiac growth during embryonic development is achieved by proliferation of undifferentiated cardiac progenitor cells (CPCs) and cardiomyocytes containing myofibrillar proteins. Because the adult mammalian heart is considered a postmitotic organ, disturbances to the proliferation kinetics of CPCs or cardiomyocytes during development could have deleterious effects on heart formation and function in postnatal life. Several reports have pointed to the ability of ANP to modulate proliferation of various cardiovascular cell types including cardiac fibroblasts (8), vascular smooth muscle cells (37), mesangial cells (41), and cardiomyocytes from late embryonic/fetal stages of development (23, 32). Importantly, discrepancies regarding the effects of ANP on proliferation of cardiomyocytes have been reported. Specifically, exogenous addition of human ANP on embryonic chick cardiomyocytes was shown to increase proliferation (23), while addition of human/porcine ANP to fetal sheep cardiomyocytes was shown to inhibit angiotensin II-stimulated proliferation (32). Currently, it remains unknown whether ANP has any effect on the cell cycle kinetics of embryonic CPCs. Thus the primary aims of this study were to 1) examine biological activity of ANP signaling systems in embryonic ventricular myocardium, and 2) determine...
whether ANP signaling modulates proliferation/differentiation of undifferentiated CPCs and/or cardiomyocytes.

Previously, we showed that at the midgestational stage of E11.5 the murine ventricular myocardium is composed of both undifferentiated CPCs expressing the cardiac transcription factor Nkx2.5 and mature cardiomyocytes expressing sarcomeric myosin (48). At this developmental stage, we confirmed the presence of ANP secretory granules in cardiomyocytes but not in CPCs of the ventricular myocardium (48). In the present study we provide evidence that ANP is actively secreted from E11.5 ventricular myocytes and that ANP mediated activation of the NPRA/cGMP signaling pathway in CPCs is associated with reductions in cell number and DNA synthesis and a robust increase in nuclear p27 levels. These data suggest that ANP produced by cardiomyocytes within the embryonic ventricles may serve as a paracrine regulator of proliferation and differentiation of adjacent CPCs during cardiac development via the NPRA/cGMP signaling pathway.

**MATERIALS AND METHODS**

**Experimental animals.** All animal procedures were performed according to the Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animal Care (Protocol No. 10-008; 09-038, 12-013). CD1 and C57BL/6 (BL6) mice were obtained from Charles River Laboratories (Montreal, Canada). Generation of mice with Cre recombinase inserted into the Nkx2.5 allele was previously described (38). The R26R-lacZ reporter strain was obtained from the Jackson Laboratories, (Bar Harbor, ME). All knockin lines were maintained in BL6 background. For genotyping, genomic DNA was extracted from ear biopsies, and PCR amplification assay was performed using an RedExtract amplification kit (Sigma, St. Louis, MO) and appropriate primer sets for each knockin line (see Table 1). Male mice were mated with females, and noontime on the day when the copulation plug was found was designated as E0.5. Unless otherwise stated, CD1 mice were used for all experimental procedures.

**Total RNA extractions and quantitative PCR.** Total RNA was isolated from pooled ventricles at each developmental stage or from E11.5 ventricular cell cultures using Trizol reagent (Invitrogen, Burlington, Canada) and reverse transcribed into cDNA using Superscript II reverse transcriptase kit (Invitrogen). Quantitative (Q)PCR was performed on all samples in duplicate using EVOLution EvaGreen (Montreal Biotech, Quebec City, Canada) according to the manufacturer’s instructions. Primer sequences are provided in Table 1. Gene expression was normalized to a control reference gene (GAPDH) using the ΔΔCt method (25). All QPCR reactions were performed for 40 cycles: 15 s at 95°C and 60 s at 60°C using an ECO thermocycler (Illumina, San Diego, CA) except for GAPDH where 62°C was used as the annealing temperature.

**Enzyme linked immunosorbent assay.** Tissue lysates from pooled E11.5 or neonatal hearts were generated as previously described (40). Clear 96-well microtiter plates (Nunc, New York, NY; cat. no. 167008) were coated with tissue lysate samples in 100 μl/well of carbonate buffer (pH 9.6) overnight at 4°C. Subsequently, wells were washed with PBS-0.1% Tween (PBST), blocked for 1 h, and incubated with 8 ng of primary ANP antibodies (Chemicon; cat. no. CBL66) diluted in enzyme linked immunosorbent assay (ELISA) diluent (1% BSA in PBST) for 2 h at room temperature. After being extensively rinsed with PBST, secondary goat-anti-mouse antibodies (1:5,000; Bio-Rad, Mississauga, Canada; cat. no. 170–1019) conjugated to horseradish peroxidase (HRP) were added for 2 h at room temperature. Ultra TMB-ELISA (Thermo Scientific, Nepean, Canada) substrate was added to each well for 20 min at room temperature. The reaction was stopped by 2 M sulphuric acid, and absorbance was measured immediately at 450 nm using a BMG POLARStar Omega plate reader (BMG Labtech, Ortenberg, Germany). The concentration of ANP was deduced by extrapolating absorbance readings from a standard curve generated using synthetic ANP (Bachem; cat. no. H-2100.0500) and covered a range of 0.3125–40 ng/ml.

**Paraffin sections and immunohistochemistry.** Embryos were fixed in 10% neutral buffered formalin (Sigma) overnight. Embryos were embedded in paraffin, and 10-μM sections were generated using a microtome. Deparaffinized sections were subjected to antigen retrieval by boiling the samples in citrate buffer (10 mM sodium citrate dihydrate, 0.05% vol/vol, pH 6.0, and dH2O), blocked, and processed for immunofluorescence using antibodies for ANP (1:100; Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. sc-20158), phospho-histone-H3 (1:100; Cell Signaling, Danvers, MA; cat. no. 9701), and sarcomeric myosin (1:50; Developmental Studies Hybridoma Bank (DSHB), University of Iowa; no. MF20). Subsequently, slides were incubated with secondary goat anti-mouse antibodies, conjugated to Alexa Fluor 488 dye (1:200; Invitrogen; cat. no. A-12422) and goat anti-rabbit antibodies conjugated to Alexa Fluor 488 dye (1:200; Invitrogen; cat. no. A-11008). Nuclei were counterstained by immersing slides in a solution of 1 μg/ml Hoechst 33258 (Sigma) in PBS. Sections were examined using an epifluorescence microscope (Leica DM2500) or a confocal microscope (Zeiss LSM 510).

**Cryosections and immunohistochemistry.** Embryos were harvested and placed in a cryoprotectant 30% sucrose solution overnight at 4°C. The following day, embryos were embedded in OCT medium (Sakura Finetek) and frozen at ~80°C. Cryosections (10 μm) were fixed with methanol for 15 min at 4°C, blocked, and processed for DAB staining. Sections were incubated with NPRA (1:50; Santa Cruz; cat. no. sc-25485) or NPRC (1:50; Santa Cruz; cat. no. sc-25487) antibodies overnight at 4°C. Subsequently, sections were incubated with secondary goat-anti-rabbit antibodies (Bio-Rad; cat. no. 722–1019) conjugated to HRP for 1 h at room temperature. DAB staining was performed using SIGMAFAST™ tablets (Sigma) according to the manufacturer’s instructions. For some experiments, 10-μM sections of adult murine ventricular myocardium were generated and processed for immunohistochemistry using anti-smooth muscle actinin IgM antibodies (undiluted; DSHB; no. 1E12) or anti-CD31 IgG antibodies (1:50; DSHB; no. 2H8-C). Subsequently, sections were incubated with secondary goat anti-mouse IgM antibodies conjugated to Alexa Fluor 555 (1:200; Invitrogen, cat. no. A-21426) or goat anti-mouse

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**Table 1. Primers used for genotyping and QPCR analysis**

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<th>Experiment/Primer Name</th>
<th>Sequence 5’-3’</th>
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<td>ROSA-lacZ genotyping</td>
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QPCR, quantitative PCR; ANP, atrial natriuretic peptide; NPRA and NPRC, natriuretic peptide receptor A and C; S, sense; AS, antisense; F, forward; R, reverse.
IgG antibodies conjugated to Alexa Fluor 488 (1:200; Invitrogen, cat. no. A-11001). Nuclei were counterstained by immersing slides in a solution of 1 μg/ml Hoechst 33258 (Sigma) in PBS.

**Western blot analyses.** Tissue lysates were prepared from pooled ventricles of different developmental stages, run on 12.5% polyacrylamide gels, and transferred to nitrocellulose membranes as previously described (40). For detecting the small 3-kDa ANP peptide, 16.5% gels were used and transfer was performed using Tris-tricine buffer (Bio-Rad) for shorter time periods. Blots were probed with antibodies for NPRA (1:200; Santa Cruz; cat. no. sc-25485), NPRC (1:200; Santa Cruz; cat. no. sc-25487), ANP (1:200; Chemicon; cat. no. CB66), and GAPDH (1:5,000; Santa Cruz; cat. no. sc-25778). Protein bands were detected by the enhanced chemiluminescence method using ECL Plus Western blotting detection system (GE Healthcare) according to manufacturer’s instructions. In some cases, imaging films were scanned and analyzed by National Institutes of Health ImageJ software, and the levels of target proteins (NPRA or NPRC) were normalized to those of GAPDH for each sample to correct for any variations in protein loads as described in our earlier studies (17).

**Second messenger assays:** cGMP and cAMP. Ventricles from E11.5 CD1 embryos were isolated, pooled, and digested in 0.2% type I collagenase (Worthington) for 30 min at 37°C. Human embryonic kidney epithelial cells (HEK293) were trypsinized for 3 min and resuspended in 10% FBS-DMEM. Cell pellets for E11.5 ventricular cells and HEK293 cells were obtained by centrifugation and resuspended in 10% FBS-DMEM. To measure cGMP, competitive immunoassays were performed using the two step protocol of the cGMP htrf assay kit (Cisbio; cat. no. 62GM2PEB) according to the manufacturer’s instructions. Briefly, in step 1 a volume of 5 μl of cells (64,000 cells/5 μl was optimal cell density determined empirically) in 10% FBS-DMEM was added to wells of white 38-well low volume plates (Greiner Bio-One; cat. no. 784075) with 5 μl of dilution buffer consisting of drug compounds, diluted in 10% FBS-DMEM. The broad substrate PDE inhibitor IBMX (500 μM; Sigma) was used to prevent cGMP degradation. The plate was sealed and incubated at room temperature for 30 min.

In step 2, 5 μl of the d2-cGMP analog and 5 μl mAb-cryptate was added to each well and the plate was sealed for 1 h at room temperature. The d2-cGMP fluorophore was excited at a wavelength of 357 nm and emission was detected at 665 and 620 nm using a POLARstar Omega plate reader (BMG Labtech). Results were calculated using the 665-nm to 620-nm ratio and expressed as delta F values using data reduction steps described in manufacturer’s instructions. A cGMP standard curve was generated by plotting the delta F values from standards with known cGMP concentrations and covered an average range of 0.49–500 nM (final concentration of cGMP/well).

To determine the level of cAMP in E11.5 cells, cAMP competitive immunoassays were performed using the two-step protocol of the cAMP dynamic 2 htrf assay kit (Cisbio; cat. no. 62AM4PEB) according to the manufacturer’s instructions. The two-step protocol of the cAMP competitive immunoassays was identical to that described above for the cGMP assay. The optimal cell density for cAMP assays was determined to be 4,000 cells/well. The cAMP standard curve covered an average range of 0.17–712 nM (final concentration of cAMP/well).

Lineage tracking in dispersed ventricular cell preparations and 3H-labeling assay. Ventricles from E11.5 embryos generated from crosses between Nkx2.5-Cre and Rosa-lacZ breeding pairs were dispersed in 0.2% type I collagenase and plated on fibronectin coated two-well chamber slides (Nunc) in 10% FBS-DMEM overnight in a CO2 incubator at 37°C. Fresh media supplemented with ANP or A71915 (NPRA-specific antagonist; Bachem; cat. no. H3048) + ANP were added to cultures for 24 h. Subsequently, fresh media supplemented with tritiated [3H]thymidine (GE Healthcare) were added to each well at a concentration of 1.0 μCi per 1 ml of medium for 6 h at 37°C. After being extensively washed with PBS, cells were fixed with methanol for 15 min at 4°C and processed for immunofluorescence and autoradiography as previously described (40, 42). Primary antibodies used to identify CPCs and cardiomyocytes were β-galactosidase (β-Gal; 1:50; Cappel, ICN; cat. no. 55976) and sarcromeric myosin (1:50; DSHB; no. MF20). Images were captured using a Leica DFC500 digital acquisition system.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.** Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed for detection of apoptosis in CPCs from E11.5 ventricular cell cultures using the TMR red in situ cell death detection kit (Roche Applied Science, Mannheim, Germany). E11.5 NCRL cells were fixed with FlowFix [2.7% (wt/vol) cacodylic acid, 2.5% (wt/vol) paraformaldehyde, 1.66% (wt/vol) NaCl, and ddH2O, pH 7.4] for 30 min at room temperature and incubated in X-gal (Goldbio) solution overnight at 37°C to visualize the insoluble blue reaction product in β-Gal+ cells. The following day, slides were rinsed in PBS, blocked, and processed for immunolabeling of sacromeric myosin (MF20; DSHB) to distinguish CPCs from cardiomyocytes. Nuclei were counterstained by immersion in Hoechst solution. Subsequently, slides were rinsed in PBS and processed for TUNEL assay as described earlier (17). Slides were mounted with propyl gallate solution, and images were captured using a Leica DFC500 digital acquisition system.

**Immunocytochemistry.** After methanol fixation (15 min at 4°C), primary E11.5 CD1 cultures were permeabilized in 0.1% Triton X-100 (Sigma) and then covered in blocking buffer solution [10% goat serum and 1% bovine serum albumin (BSA) in PBS] for 1 h at room temperature. Subsequently, primary antibodies diluted in blocking buffer were added at the following concentrations: anti-p27 (1:100; Santa Cruz; cat. no. sc-528), anti-sarcromeric myosin (1:50; DSHB), anti-smooth muscle actinin (undiluted; DSHB), and anti-CD31 (1:50; DSHB). Sections were subsequently incubated with appropriate goat anti-rabbit or anti-mouse secondary antibodies conjugated to Alexa Fluor 555 or Alexa Fluor 488 (Invitrogen). Nuclei were stained with 1 μg/ml of Hoechst 33342 for 5 min.

**Statistical analyses.** Data are presented as means ± SE. A two-tailed unpaired t-test was used to compare between two groups only. The dose-response data for second messenger levels and DNA synthesis were analyzed by nonlinear regression with a sigmoidal dose-response (variable slope) curve fit method (GraphPad Prism 4.0 software; San Diego, CA). The runs test and goodness of fit parameters were used to confirm that the curves did not significantly deviate from the data tested. Between groups comparisons were analyzed by ANOVA and Tukey multiple comparisons post hoc test. Significance for all analyses was assigned at P < 0.05. All statistical analyses were performed using Graphpad Prism software.

**RESULTS**

**ANP mRNA and immunoreactive protein are present in the E11.5 ventricles.** The developmental profile of ANP gene expression in the murine ventricular myocardium was determined by quantifying the relative abundance of mRNA transcripts at E11.5 and several later developmental and postnatal stages by QPCR (Fig. 1, A and B). Because we have previously shown that expression levels of GAPDH remain unchanged across all developmental and postnatal stages (20), this gene was used to normalize data by correcting for variations in quantities of cDNA used as template. The highest level of ANP mRNA expression was observed at E11.5 and gradually declined at later developmental and postnatal stages (Fig. 1, A and B). Compared with the E11.5 stage there was an approximately twofold decrease in ANP mRNA at E14.5 (0.51 ± 0.03 vs. E11.5). Gene expression did not vary significantly among E14.5,
E16.5, and neonatal (day 1) stages but decreased significantly at adult stages (~10-fold decrease from E11.5 to adult stage). To determine whether ANP mRNA was also translated into immunoreactive protein, an ELISA was used to quantify ANP protein from ventricular tissue lysates at both E11.5 and neonatal stages. Consistent with mRNA expression data, the concentration of ANP protein in the ventricles at E11.5 was approximately twofold higher compared with the neonatal stage (E11.5: 4.3 ± 0.7 ng ANP/µg of lysate vs. neonatal: 2.3 ng ± 0.5 ng ANP/µg of lysate; Fig. 1C). The presence of ANP protein in the E11.5 ventricles was also confirmed by analyzing the spatial expression pattern of ANP in paraffin sections by immunofluorescence. Strong ANP labeling was observed primarily in the trabecular myocardium of the left ventricle and also in specific regions of the inner atrial wall (Fig. 1D). ANP expression was also detectable, albeit at lower levels, in the trabecular myocardium of the right ventricle in some sections but was essentially absent from the developing compact myocardium of both ventricles (Fig. 1D). Higher magnification images of the left ventricle at E11.5 revealed the broad expression of sarcomeric myosin (cardiomyocyte-specific marker) in the compact and trabecular myocardium (Fig. 1E), while ANP expression remained primarily localized within cells of the trabecular layer (Fig. 1, F and G).

ANP is present in conditioned media from E11.5 ventricular cell cultures. To achieve a paracrine effect, embryonic ventricular cells would be required to actively secrete ANP into their surrounding environment. To gain insight into this issue, ANP protein levels were measured by Western blot analyses in conditioned media samples collected from primary cultured E11.5 ventricular cells. Following a 24-h incubation period, conditioned media samples were separated by electrophoresis, transferred to a nitrocellulose membrane, and probed using ANP-specific antibodies. Results from these analyses revealed immunoreactive bands at 17 and 3 kDa in conditioned media collected from ventricular cell cultures but not in control media samples that were not exposed to cells (Fig. 2). The 17-kDa band corresponds to the expected molecular weight of proANP, while the 3-kDa band corresponds to the proteolytically processed and biologically active form of ANP. These data provide evidence that proANP is actively secreted from
ANP inhibits embryonic CPC cell cycle activity

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Determination of the effects of ANP on cGMP and cAMP production in E11.5 ventricular cells. To determine whether NPR-linked signal transduction pathways are biologically active in the ventricular myocardium at midgestation, levels of the second messenger molecules cGMP and cAMP were measured in acutely isolated E11.5 ventricular cells in response to ANP stimulation. The dose-response curves were plotted using nonlinear regression with sigmoidal dose-response (variable slope) curve fit method, and further statistical analysis confirmed that the curves did not significantly deviate from the data tested (Fig. 5A). The level of cGMP measured under basal conditions was 25.7 ± 3.7 nM/64,000 cells. Due to considerable variability in basal levels of cGMP between independent experiments, basal levels from each experiment were set to a value of 1.0 and data were represented as fold changes in cGMP concentration in response to different doses of ANP (Fig. 5B). At the two lowest concentrations of ANP tested (1 and 10 ng/ml), there was no significant increase in cGMP observed compared with control (Fig. 5B). By contrast, 100 ng/ml ANP was able to induce a significant, 1.5-fold increase in cGMP production (1.5 ± 0.1 vs. basal). A 10-fold higher dose of ANP (1,000 ng/ml) resulted in an apparent further increase in cGMP production, but this result was not statistically significant compared with 100 ng/ml (100 ng/ml: 1.5 ± 0.1 vs. 1,000 ng/ml: 1.7 ± 0.1; Fig. 5B). Because the induction of cGMP was relatively modest in E11.5 ventricular cells, cGMP experiments were also conducted in HEK293 cells as a positive control since these cells are known to express endogenous NPRA. The level of cGMP measured under basal conditions in HEK cells was 20.7 ± 2.0 nM/32,000 cells. In response to either 100 or 1,000 ng/ml of ANP, an ~2.5-fold increase in cGMP was observed (100 ng/ml: 47.2 ± 2.8 vs. 1,000 ng/ml: 53.0 ± 4.1; Fig. 5C).

Under basal conditions, the level of cAMP in E11.5 cells was 3.2 ± 0.9 nM/4,000 cells. Compared with baseline, ANP (1–100 ng/ml) had no effect on levels of cAMP production (Fig. 5D). By contrast, stimulation with isoproterenol (ISO; 100 nM) alone was able to induce an approximately fourfold increase in cAMP levels compared with basal controls (basal: 3.2 ± 0.9 nM vs. ISO: 14.8 ± 5.2 nM). In parallel experiments, ANP (100 or 1,000 ng/ml) was unable to blunt the ISO-induced increase in cAMP production (Fig. 5D). Collectively, data from cGMP and cAMP second messenger assays provide evidence that ANP-sensitive guanylyl cyclase receptors (NPRA and/or NPRB) are biologically active in the ventricles at E11.5, while ANP signal transduction pathways coupled to adenylyl cyclase activity could not be confirmed under our experimental conditions.

ANP high expression zones are associated with a low mitotic index in the E11.5 ventricles. Previous studies showed that ANP/cGMP signaling could either increase proliferation or decrease proliferation of embryonic/fetal cardiomyocytes from the avian or ovine heart, respectively (23, 32). The effects of ANP on proliferation of either CPCs or cardiomyocytes from earlier developmental stages have not yet been determined. As a first approximation toward determining whether ANP is involved in regulating proliferation of E11.5 ventricular cells, paraffin sections were immunolabeled with antibodies against ANP and the mitotic marker phosphohistone H3 (PH3). In the ANP-rich trabecular region of the left ventricle, there was an observably lower number of PH3+ nuclei compared to areas with low ANP expression.

E11.5 ventricular cells and gets cleaved into its biologically active form.

Natriuretic peptide receptors are expressed in E11.5 ventricles. The developmental profiles for ANP high affinity receptors (NPRA and NPRC) were determined at the mRNA level by QPCR. Both receptor subtypes were present at E11.5 and at later developmental stages and displayed differential expression patterns (Fig. 3, A and B). Specifically, NPRA gene expression was lowest at E11.5 and increased significantly during the gestational period achieving a peak in expression at the neonatal stage (neonatal ~3-fold higher vs. E11.5). By contrast, NPRC gene expression was highest at E11.5 and decreased significantly by E16.5 and remained low at subsequent postnatal stages (E11.5 expression was ~2-fold higher vs. E16.5). Side by side comparison of NPRA and NPRC expression at each developmental stage more clearly illustrates the receptor subtype switch that occurred from early to late developmental stages (Fig. 3C). Western blot analyses confirmed the presence of NPRA and NPRC at the protein level at all developmental/postnatal stages using NPRA- and NPRC-specific antibodies (Fig. 3D). Distinct bands were detected at 120 and 66 kDa, corresponding to the expected molecular weights of NPRA and NPRC, respectively. Quantification of the developmental changes in immunoreactive NPRA revealed some consistencies with gene expression data obtained by QPCR analysis. Specifically, both of these analyses revealed an apparent increase in NPRA levels from E11.5 to E14.5 as well as a peak in expression at the neonatal stage (Fig. 3, D and E). Also consistent with QPCR analyses of NPRC, levels of immunoreactive protein appeared to be higher at the embryonic stages (E11.5 and E14.5) compared with postnatal stages (Fig. 3, D and F). To gain insight into the spatial distribution patterns of NPR subtypes in the embryonic heart, E11.5 cryosections were processed for immunohistochemical staining using NPRA- and NPRC-specific antibodies (Fig. 4, A and B). Immunoreactive NPRA and NPRC were both broadly expressed in the ANP-rich trabecular myocardium as well as the ANP negative compact myocardium, with minimal expression observed in adjacent endocardial cushion tissue (Fig. 4, A and B).

Fig. 2. ANP is present in conditioned media samples from E11.5 ventricular cell cultures. Western blot experiments were performed to detect ANP in conditioned media samples. Lane 1 and 2 are positive controls, using either 50 ng (lane 1) or 100 ng (lane 2) of synthetic ANP peptide. Lanes 3 and 4 correspond to conditioned media samples collected from E11.5 ventricular and atrial cell cultures, respectively, following a 24-h culture period. Lane 5 is a negative control (10% FBS-DMEM; equal amount of protein). Bands were detected at 17 kDa, corresponding to proANP (1–126), as well as at 3 kDa, corresponding to the proteolytically processed/biologically active form of ANP (99–126). Positions of molecular weight markers are indicated using solid lines, and position of the synthetic ANP is shown using a dashed line; n = 4 independent experiments.
with the adjacent compact layer (Fig. 6A). Similarly, in the right ventricle, PH3+/nuclei appeared to be less abundant in the trabecular myocardium despite having lower levels of immunoreactive ANP (Fig. 6B).

To determine the mitotic indices in the left and right ventricles, the percentage of PH3+/nuclei out of the total number of nuclei was determined in regions of both high (designated ANP+/) and low ANP (designated ANP−) expression that were defined based on immunolabeling with ANP-specific antibodies. Results from these analyses confirmed that, in the left ventricle, the ANP+/region had a significantly lower mitotic index compared with ANP−regions (ANP+/: 0.6 ± 0.07% vs. ANP−: 1.5 ± 0.1%; Fig. 6C). Because it was difficult to reliably distinguish between ANP+/ and ANP−regions in the

Fig. 4. Spatial expression pattern of immunoreactive NPRA and NPRC in the embryonic heart at E11.5. Immunolabeling using DAB staining method on serial sections of the E11.5 heart revealed a broad expression pattern of both NPRA (A) and NPRC (B) that was strong in the trabecular and compact layers of the ventricles. EC, endocardial cushion. C: DAB-negative control; primary antibodies were omitted. Scale bar = 100 μm.
right ventricle, the mitotic index was determined in the trabecular vs. the compact myocardium. From these analyses, it was determined that the mitotic index was significantly lower in the trabecular myocardium compared with the surrounding compact layer (Fig. 6D; trabecular: 0.8 ± 0.1% vs. compact: 1.5 ± 0.06%).

Exogenous ANP decreases cell number and DNA synthesis of E11.5 cardiac progenitor cells but not cardiomyocytes. To distinguish CPCs from cardiomyocytes in culture, we used a combination of Cre/LoxP-based cell labeling for the early cardiac transcription factor Nkx2.5, and immunostaining for the differentiation marker sarcomeric myosin (using MF20 antibodies). In the Cre/LoxP system, mice from the Nkx2.5-Cre line (designated NC) (38) were crossed with a Cre-dependent β-Gal (lacZ) reporter mouse strain (R26R-lacZ; designated RL). In the resulting double knockin offspring (designated NCRL), expression of Nkx2.5 resulted in Cre-mediated excision of the floxed stop cassette located in the S′-region of lacZ sequence and resulted in reporter gene expression in cells of the Nkx2.5+ lineage thereafter (Fig. 7A). Cells positive for β-Gal, but not MF20 (β-Gal+/MF20−), were considered to be CPCs (Fig. 7, A–C) since previously we demonstrated that Nkx2.5+/MF20− cells differentiate into MF20+ expressing cardiomyocytes in vitro (30). Conversely, cells positive for both β-Gal and MF20 (β-Gal+/MF20+) were considered to be cardiomyocytes (Fig. 7, A–C).

Because ANP (100 ng/ml) was shown to stimulate guanylyl cyclase activity (Fig. 5A), we investigated the effects of ANP on proliferation and differentiation kinetics of cultured E11.5 ventricular cells. Cell counts following a 24-h incubation with exogenous ANP (100 ng/ml) revealed a significant reduction in cell number and DNA synthesis in E11.5 CPC and cardiomyocyte populations was assessed. Primary cultures were subjected to a [3H]thymidine incorporation assay following 24 h of incubation with various

Fig. 5. ANP stimulates cGMP production in E11.5 ventricular cells. A: cellular cGMP levels after treatment with various doses of ANP were shown as a dose-response (DR) curve. Log (agonist) values were plotted against cGMP levels as a percent maximal response using a nonlinear curve fit method. The runs test indicated that the curve did not deviate significantly from the data analyzed. The zero-concentration on x-axis indicates cGMP levels in control or unstimulated (basal) cultures. B: with the use of a cGMP competitive immunoassay, basal levels of cGMP were determined to be 25.7 ± 3.7 nM/64,000 cells. Basal cGMP levels were set to a value of 1.0 and data represented fold change in cGMP in response to ANP. Compared with basal levels, a significant increase in cGMP production was observed in response to either 100 ng/ml (1.5 ± 0.1-fold) or 1,000 ng/ml (1.7 ± 0.1-fold) ANP. A and B: n = 8 independent experiments, performed in duplicate wells. *P < 0.05 vs. basal. C: basal levels of cGMP in HEK293 cells were determined to be 20.7 ± 2.0 nM/32,000 cells. Compared with basal levels, a significant increase in cGMP production was observed in response to either 100 ng/ml (2.3 ± 0.1 fold) or 1,000 ng/ml (2.5 ± 0.1-fold) ANP; n = 5 independent experiments, performed in duplicate wells; *P < 0.05 vs. basal. D: with the use of a cAMP competitive immunoassay, basal levels of cAMP were determined to be 3.2 ± 0.9 nM/4,000 cells. Basal cAMP levels were set to a value of 1.0 and data represent fold change in cAMP in response to ANP, isoproterenol (ISO), or ANP + ISO. Compared with basal, ANP (1–100 ng/ml) had no effect on cAMP levels. By contrast, stimulation with ISO (100 nM) alone was able to induce a ~4-fold increase in cAMP levels. ANP (100 or 1,000 ng/ml) was unable to blunt the ISO induced increase in cAMP production; n = 8–10 independent experiments/treatment group, performed in duplicate wells. *P < 0.05 vs. basal.
concentrations of ANP (1–100 ng/ml), and the labeling index (LI) was assessed as the percentage of cells displaying [3H]thymidine silver grains (Fig. 7, D and E) out of the defined cell population. In vehicle (H2O)-treated control cultures, the CPC population had an average LI of 53.8 \pm 5.6\% which was significantly higher compared with the LI observed in the cardiomyocyte population (15.2 \pm 3.2\%; Fig. 8, B and C). Treatment of cultures with ANP (1–100 ng/ml) was associated with a dose-dependent decrease in DNA synthesis of CPCs that reached statistical significance at the concentration of 100 ng/ml (30.5 \pm 0.5\%; Fig. 8B) but had no effect on the cardiomyocyte population (Fig. 8C). In parallel experiments, E11.5 cultures were pretreated for 30 min with 1 \mu M A71915, which is an NPRA receptor-specific antagonist (12), before 24-h ANP treatment. Results from these experiments revealed that the reduction in CPC DNA synthesis associated with a dose-dependent decrease in DNA synthesis of CPCs that reached statistical significance at the concentration of 100 ng/ml (30.5 \pm 0.5\%; Fig. 8B) but had no effect on the cardiomyocyte population (Fig. 8C). In parallel experiments, E11.5 cultures were pretreated for 30 min with 1 \mu M A71915, which is an NPRA receptor-specific antagonist (12), before 24-h ANP treatment. Results from these experiments revealed that the reduction in CPC DNA synthesis associated
with 100 ng/ml ANP could be abolished by preincubating cultures with A71915 (49.9 ± 3.1%; Fig. 8D), supporting a role for ANP and its high-affinity NPRA receptor signaling axis in modulating CPC cell cycle activity. To examine whether ANP plays a proapoptotic effect on CPCs, we monitored levels of apoptosis in E11.5 ventricular cell cultures treated with or without 100 ng/ml ANP. Levels of apoptosis were not significantly different in cultures treated with ANP (11.4 ± 1.5% for CPCs and 12.2 ± 3.2% for cardiomyocytes).

To gain insight into potential mechanisms whereby ANP modulates CPC DNA synthesis, gene expression levels of the positive cell cycle regulator cyclin D1 and negative cell cycle regulator p21 were examined following a 24-h incubation with 100 ng/ml ANP, but no significant differences were observed compared with controls (data not shown). Because the effects of cell cycle regulatory proteins also depend on subcellular localization, nuclear translocation of the cell cycle inhibitor p27 was also measured by immunocytochemistry in ANP treated E11.5 ventricular cell cultures. Interestingly, ANP (100 ng/ml) treatment was associated with a significantly higher percentage of CPCs in response to exogenous ANP (100 ng/ml) (3.4-fold increase vs. control, *P < 0.05, ANP vs. respective controls). The percentage of CMs displaying nuclear localization of p27 was also significantly increased vs. control, albeit to a lesser extent compared with CPCs (1.6-fold vs. control, #P < 0.05, ANP vs. respective controls); n = 3 independent experiments/treatment group, ~500 cells counted/experiment.

**Fig. 8.** ANP decreases cardiac progenitor cell DNA synthesis via guanylyl cyclase receptor signaling pathway. A: quantification of the percentage of cardiac progenitor cells (CPC) and cardiomyocytes (CM) in E11.5 ventricular cell cultures treated with or without exogenous ANP (100 ng/ml). The relative percentage of CPCs (β-gal−/MF20−) decreased, while the percentage of CMs (β-gal+/MF20+) increased in response to ANP; n = 4 independent experiments/treatment group, ~500 cells counted/experiment. *P < 0.05 vs. control (CPC); #P < 0.05 vs. control (CM). B and C: with the use of a [3H]thymidine incorporation assay, the labeling index (LI; % of cells in S phase out of the total number of cells in the defined cell population) was determined in the CPC and CM populations from E11.5 primary ventricular cell cultures. B: in the CPC population, ANP treatment (100 ng/ml) was associated with a decrease in LI compared with control cultures. C: in the CM population, ANP had no effect on LI compared with control cultures. D: preincubation with the NPRA receptor-specific antagonist A71915 (1 μM) abolished the decrease in LI associated with 100 ng/ml ANP in the CPC population. E: quantification of the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive CPCs and CMs in E11.5 ventricular cell cultures treated with or without exogenous ANP (100 ng/ml). Inset represents a CPC (X-gal− and MF20−) that shows positive TUNEL signal (red) in Hoechst stained nucleus. Scale bar = 20 μm. B–E: n = 3 independent experiments/treatment group, ~500 cells counted/experiment, *P < 0.05. F: nuclear localization of the cell cycle inhibitor p27 was observed in a significantly higher percentage of CPCs in response to exogenous ANP (100 ng/ml) (3.4-fold increase vs. control, *P < 0.05, ANP vs. respective controls).
pronounced (control: 10.9 ± 1.6% vs. ANP: 17.9 ± 1.5%) compared with that seen with CPCs (Fig. 8 F). To examine the possibility that ANP treatment may be associated with increased differentiation of CPCs toward noncardiomyocyte cell lineages, E11.5 ventricular cell cultures were immunolabeled with the smooth muscle cell marker (smooth muscle actinin; Fig. 9, A and D) and the endothelial cell marker (CD31; Fig. 9, B and E) following a 24-h incubation period in the presence or absence of 100 ng/ml ANP. Results showed that there was no apparent labeling of cells with smooth muscle or endothelial cell-specific markers in either control or ANP-treated cultures.

**DISCUSSION**

In the present study, we sought to determine whether the ANP/NPR signaling systems play any role in the proliferation of embryonic CPCs or cardiomyocytes in midgestation (E11.5) ventricular myocardium. To our knowledge, this is the first report that shows that 1) ANP is actively secreted from E11.5 ventricular cells and processed to its biologically active (3 kDa) form and 2) NPRA/cGMP signaling system serves as an inhibitor of CPC cell cycle activity.

Previous studies with neonatal rat ventricular cardiomyocytes have demonstrated that ANP is secreted from these cells in a constitutive manner as well as in response to stimuli such as ET-1 (3, 21). While we have previously shown that secretory granules containing ANP exist in mouse ventricular cardiomyocytes at E11.5 stage (48), it is not known whether ANP is actively secreted from the embryonic cells. In the present study, we have discovered that proANP (17 kDa) synthesized by E11.5 ventricular cells can be actively secreted into the extracellular compartment and proteolytically processed into its active form (3 kDa). These data are further supported by the fact that corin, the type II transmembrane protease that cleaves proANP into its active form (45), was shown to be expressed in the embryonic heart as early as E9.5 (44). In addition, corin was shown to be most abundantly expressed in the trabecular myocardium between E11.5 and E13.5 stages (44). These observations support the view that a high local concentration of biologically active ANP could be achieved in the embryonic trabecular myocardium.

Specific binding of radiolabeled ANP has been detected in various tissues of fetal and neonatal rats, including the heart, kidney, adrenal gland, and brain (4, 19). A recent study has conducted an extensive analysis of NPR gene expression patterns in the developing murine nervous system (E10.5-E14.5) and revealed predominant expression of NPRB and NPRC subtypes in various regions of the central and peripheral nervous systems (13). However, expression profiles of NPR subtypes during cardiac development have not been determined. In this study, we have found that transcripts coding for ANP high affinity receptors (NPRA and NPRC) are present throughout cardiac development and the transcription profiles display a subtype switch such that NPRC expression is highest at midgestational stage (E11.5), while NPRA is predominant later in development. Although NPRA mRNA expression is the lowest at E11.5 stage, we have chosen to study the effects of ANP on ventricular cells from this stage. This is primarily due to the fact that CPCs are abundant in E11.5 ventricular myocardium and their numbers decline precipitously at later developmental stages (30, 47, 48). While NPRA mRNA levels are lowest at E11.5 stage, Western blot analyses indicate that NPRB protein levels at E11.5 are comparable to later developmental stages.

Exogenous addition of ANP has been shown to stimulate or inhibit cell proliferation via NPRA- or NPRC-mediated signaling pathways in numerous noncardiovascular cell types (2, 13, 18). Similar reports on the ability of ANP to stimulate or inhibit proliferation have also been reported in fetal/embryonic cardiomyocyte cultures. Specifically, exogenous addition of human ANP to embryonic chick cardiomyocytes was shown to

**Fig. 9.** ANP treatment is not associated with induction of smooth muscle or endothelial cell markers. A–C: in control E11.5 ventricular cell cultures, there was no apparent expression of the smooth muscle marker, smooth muscle actinin (red), or the endothelial cell marker CD31 (green). Scale bar = 50 μm. D–F: no induction of either marker was observed in response to ANP treatment (100 ng/ml). Scale bar = 50 μm. G: positive control for smooth muscle actinin antibody performed on a thin section of adult murine myocardium reveals strong labeling of vascular smooth muscle cells (red) in a blood vessel. Scale bar = 50 μm. H: positive control for CD31 antibody performed on a thin section of adult murine myocardium reveals strong labeling of the luminal surface of endothelial cells in a blood vessel. Scale bar = 50 μm.
stimulate proliferation (23). In contrast, addition of human/porcine ANP to fetal sheep cardiomyocytes was shown to inhibit angiotensin II-induced proliferation (32). These studies indicate that the biological effects of ANP are cell type specific and may depend on the NPR subtypes expressed in a given cell type. Notably, heterologous systems (i.e., cells treated with ANP from different species) were employed in studies of cardiomyocyte proliferation, which may have contributed to the differences in reported outcomes. Currently, it is not known whether ANP has any paracrine effects on CPCs and cardiomyocytes during early stages of heart development. Thus, in the present study, we have employed a homologous system (i.e., mouse ANP on mouse primary cultures) to determine the effects of ANP on proliferation of E11.5 CPCs and cardiomyocytes.

Based on second messenger analyses, we can conclude that guanylyl cyclase but not adenyl cyclase coupled ANP receptors are biologically active in E11.5 ventricular cells at ANP concentrations ranging from 100 to 1,000 ng/ml. Although ANP-mediated increases in cGMP are modest in E11.5 cells, a similar degree of induction in cGMP was reported following ANP treatment in adult Purkinje cardiomyocytes (1). It is likely that ANP increases cGMP levels in E11.5 ventricular cells via NPRA receptor since a 100–1,000 ng/ml concentration of ANP (~30–300 nM) is far below the concentration of ANP required to elicit half-maximal cGMP production via NPRB (~25 μM) (34). Results from cell cycle analyses revealed a progressive decrease in DNA synthesis of CPCs with increasing concentrations of ANP, while rates of DNA synthesis in the cardiomyocyte population remained constant. The relative increase in the number of cardiomyocytes associated with ANP treatment could be due to enhanced differentiation of CPCs rather than enhanced proliferation of cardiomyocytes. This notion is further supported by the absence of cell cycle changes in cardiomyocytes after ANP treatment. A robust increase in the nuclear localization of p27 in CPCs after ANP treatment is also consistent with the reduction in cell cycle activity of this cell type. p27 is a well-characterized cell cycle inhibitor protein, which is known to block the functions of cyclin D/CDK4 and cyclin E/CDK2 complexes and arrest cells in G1 phase (11). Notably, reductions in DNA synthesis and number of CPCs are not due to a reduction in cell viability as there is no significant difference in the levels of apoptosis between control and ANP-treated cultures. The effect of ANP on CPC cell cycle activity is further supported by our observation of lower mitotic activity (PH3-labeling index) in the ANP-rich trabecular zone vs. the compact zone (low ANP) in the left ventricular myocardium at E11.5. Interestingly, the PH3 labeling index was also significantly lower in the trabecular zone of the E11.5 right ventricle despite relatively low expression of ANP. One possible explanation for this result is that cells within the right ventricle may have a much higher sensitivity to ANP compared with those in the left ventricle as previously reported in fetal sheep heart (32).

The requirement of a 100 ng/ml (~30 nM) concentration of ANP to elicit a statistically significant effect on cell number and DNA synthesis in E11.5 CPCs is consistent with previous studies wherein a high concentration range of ANP was used for exogenous treatments in both cardiovascular and noncardiovascular cell types (13, 18, 23, 32, 39). Importantly, circulating levels of ANP in adult rat have been shown to range between 100 and 400 pg/ml (22, 33, 43). In contrast, a high plasma ANP concentration of 2.7 ng/ml has been reported in E20 fetal rat circulation (43). These circulating concentrations of ANP are around 37–1,000 times lower than the effective ANP concentrations observed in our study. Based on these circulating concentrations, the effects of ANP on CPC proliferation/differentiation observed in the present study could be considered pharmacological rather than physiological. However, the tissue concentration of ANP in fetal rat ventricles (25 ng/mg) has been shown to be at least 20 times higher than the ANP levels found in postnatal ventricles (43). Given the higher ANP concentrations present in fetal ventricles (43), we speculate that the ANP-rich zones in the developing E11.5 trabecular myocardium can create a microenvironment where ANP concentration in the interstitial fluid may be several orders of magnitude higher than that found in the general circulation. Accordingly, the ANP concentration measured in E11.5 ventricular lysate (4 ng/μg) in this study may represent an underestimated value compared with the actual concentration (ng/ml) in the tight interstitial spaces of trabecular myocardium. However, it would be technically challenging to accurately quantify ANP levels in the interstitial space of embryonic trabecular myocardium. Thus we cannot definitively state that 100 ng/ml of ANP could be considered physiological rather than pharmacological concentration.

It is likely that cell cycle activity of E11.5 CPCs is modulated via the NPRA-mediated signaling pathway because 100 ng/ml (~30 nM) of ANP are ineffective to induce cGMP production via NPRB receptors (34). Furthermore, the effects of ANP on CPC proliferation could be reversed by preincubation with an NPRA receptor-specific antagonist. The inhibitory cell cycle effects of the NPRA activation in E11.5 CPCs could be mediated by several downstream targets of cGMP such as protein kinase G, protein kinase A (via cross talk), cyclic nucleotide-gated cation channels, and phosphodiesterases (28). Further studies are required to identify specific cellular events leading to cell cycle arrest in embryonic CPCs. Results obtained in this study also suggest that disruptions in the ANP/NPRA signaling axis may cause detrimental effects on cardiac development by altering the proliferation and differentiation kinetics of embryonic CPCs. In support of this notion, studies performed on NPRA knockout mice indicate a decrease in neonatal survival at weaning due to heart abnormalities such as mesocardia and dextrocardia (35) or fetal hydrops (26). In addition, the complete absence of ANP expression in Tbx5 homozygous knockout embryos is associated with impaired cardiac differentiation, development of hypoplastic heart tube and early embryonic mortality (5).

Additional consideration must also be given to the factors, which could regulate CPC proliferation/differentiation kinetics via cross talk with the ANP/NPRA signaling axis. For example, cross talk between ANP and ET-1, nitric oxide, or VEGF receptors has been documented in noncardiomyocyte cell types (18, 24, 41). Based on these reports, it is possible that cross talk between ANP and other signaling systems may function in unison to achieve a tightly controlled balance between CPC proliferation and differentiation during cardiac ontogeny. In summary, our findings suggest that ANP produced by embryonic cardiomyocytes serves as a paracrine factor to inhibit cell cycle activity in CPCs via the NPRA/cGMP axis in midgestation stage ventricles. It is likely that perturbations in the
ANP/NPRA signaling axis could have a significant impact on heart development and incidence of congenital heart defects.

ACKNOWLEDGMENTS

We thank Dr. Richard Harvey (Victor Chang Cardiac Research Institute) for generously providing the Nkx2.5-Cre knockin mouse model.

GRANTS

This work was supported by grants from the Canadian Institutes of Health Research (MOP-62811), Faculty of Medicine, Dalhousie University, and the Canada Foundation for Innovation. T. Feridooni and A. Hotchkiss received graduate studentships from the Nova Scotia Health Research Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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