Apelin-APJ Signaling Is a Critical Regulator of Endothelial MEF2 Activation in Cardiovascular Development


Rationale: The peptide ligand apelin and its receptor APJ constitute a signaling pathway with numerous effects on the cardiovascular system, including cardiovascular development in model organisms such as xenopus and zebrafish.

Objective: This study aimed to characterize the embryonic lethal phenotype of the Apj−/− mice and to define the involved downstream signaling targets.

Methods and Results: We report the first characterization of the embryonic lethality of the Apj−/− mice. More than half of the expected Apj−/− embryos died in utero because of cardiovascular developmental defects. Those succumbing to early embryonic death had markedly deformed vasculature of the yolk sac and the embryo, as well as poorly looped hearts with aberrantly formed right ventricles and defective atrioventricular cushion formation. Apj−/− embryos surviving to later stages demonstrated incomplete vascular maturation because of a deficiency of vascular smooth muscle cells and impaired myocardial trabeculation and ventricular wall development. The molecular mechanism implicates a novel, noncanonical signaling pathway downstream of apelin-APJ involving Gα13, which induces histone deacetylase (HDAC) 4 and HDAC5 phosphorylation and cytoplasmic translocation, resulting in activation of myocyte enhancer factor 2. Apj−/− mice have greater endocardial Hdac4 and Hdac5 nuclear localization and reduced expression of the myocyte enhancer factor 2. We identify a number of commonly shared transcriptional targets among apelin-APJ, Gα13, and MEF2 in endothelial cells, which are significantly decreased in the Apj−/− embryos and endothelial cells.

Conclusions: Our results demonstrate a novel role for apelin-APJ signaling as a potent regulator of endothelial MEF2 function in the developing cardiovascular system. (Circ Res. 2013;113:22-31.)

Key Words: Apelin ■ APJ ■ developmental biology ■ Gα13 ■ G proteins ■ HDAC4 ■ HDAC5 ■ MEF2A ■ MEF2C

Various experimental approaches have indicated that the apelin-APJ pathway is a potent regulator of both cardiac and vascular functions.14 The apelin (also known as APLN) ligand is translated as a 77-amino-acid pre-pro peptide and cleaved to shorter peptides that bind the G-protein–coupled receptor APJ (also known as AGTRL1/APLNR).5,6 Apelin is expressed primarily in the endothelium and acts both locally and in a paracrine manner to activate APJ.7,8 In the vasculature, studies support a vasodilator role in both the arterial and venous circulation and suggest that these effects are mediated at least in part by nitric oxide.2,9-12 Direct inotropic effects have also been demonstrated in isolated rodent hearts and cardiomyocytes.1,13

Studies in developmental model organisms have suggested that the apelin-APJ pathway has a critical role in cardiovascular development. In vivo perturbation of apelin and APJ gene expression in xenopus was found to disrupt blood vessel development, and in vitro studies showed apelin to be a potent angiogenic factor and regulator of endothelial cell (EC) migration, cell division, and apoptosis.14,15 Previous studies have provided evidence that the apelin-APJ pathway modulates blood vessel size by regulating proliferation and assembly of vascular cells.16 Experiments in zebrafish found that apelin-APJ

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Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<td>CX37</td>
<td>connexin 37</td>
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<tr>
<td>CX40</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>HDAC4</td>
<td>histone deacetylase 4</td>
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<td>HUVEC</td>
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<td>MEF2</td>
<td>myocyte enhancer factor 2</td>
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signaling regulates migration of myocardial progenitors to the midline from the anterior lateral plate mesoderm, and that disruption of this pathway leads to defective migration, loss of critical inductive differentiation signals, and failure of myocardial progenitor cell development.15–19 Despite these studies in model organisms, null mutations in the Apn and Apj locus have failed to provide definitive evidence for a role in higher vertebrate cardiovascular development.1,10,20–21 Here, we characterize the cardiovascular developmental phenotype of the Apj null mice. Homozygous targeted animals failed to be delivered in a mendelian pattern, and embryos exhibited a spectrum of cardiac and vascular developmental defects. Moreover, we describe a novel, noncanonical signaling cascade by which apelin-APJ signaling, via involvement of Gtx13, histone deacetylase (HDAC) 4, and HDAC5, activates the myocyte enhancer factor 2 (MEF2) transcription factors, which have critical roles in cardiovascular development.22–24 Our results implicate a key role for apelin-APJ signaling as an important regulatory switch that controls the activation of MEF2 transcription factors in cardiovascular development.

Methods

An expanded Methods section is available in the Online Data Supplement.

Mice

All animal experiments were conducted with approval of the Yale University and Stanford University Institutional Animal Care and Use Committees. The global Apj−/− mice were previously described.21 In brief, a single targeting construct was made containing the C57BL/6 Apj homology region, 3 LoxP sites, and the neomycin and diphtheria toxin genes. The construct was transfected into B6-3 embryonic stem cells and targeted embryonic stem cell clones transfected with pB-SCre, and resulting clones found to have deleted both the neo and Apj genes were injected into C57BL/6J-Tyr−2J blastocysts to establish the targeted allele fully in the C57BL/6 background.

Cell Culture and Transfection

Mouse heart ECs were isolated by digesting whole hearts with collagenase (2 mg/mL) with gentle agitation for 45 minutes at 37°C. The cell suspension was triturated 12 times, filtered through 70-μm cell strainers, and then centrifuged at 400g for 5 minutes at 4°C. Cells were resuspended in 2 mL cold PBS with 0.1% BSA, and the cell suspension was incubated with Dynabeads (110.35-mouse, Invitrogen) coated with purified antibody to CD31 (553370 [mouse], 5 μL per 50 μL bead suspension, BD Pharmingen). We performed a second sorting step to ensure the purity of the ECs. Human umbilical vein ECs (HUVECs; Yale VBT Core) and COS7 cells (Lonza) were cultured at 37°C in a 5% CO2 incubator. For HUVECs, growth medium was EGM-2 (Lonza) containing 2% fetal bovine serum. DMEM (Gibco) with 10% fetal bovine serum was used for COS7. For experimental treatments, HUVECs (passages 3–7) were grown to 70% to 90% confluence. Transient transfections of plasmids were performed with Fugene HD (Promega) using the manufacturer’s protocol. For gene silencing, small interfering RNAs (Stealth siRNA, Invitrogen) were transfected with RNAiMAX (Invitrogen) using the manufacturer’s protocols.

Statistical Analysis

All in vitro experiments (chromatin immunoprecipitation assays, immunoprecipitations, Western blots, and quantitative polymerase chain reaction [qPCR] assays) are representative of 3 independent experiments. Results are reported as mean±SEM. An unpaired Student t test or 1-way ANOVA test was used as appropriate to determine statistical significance. Post hoc analysis was performed with the Bonferroni method. Values of P<0.05 were considered significant.

Results

Apj Deficiency Results in Incomplete Embryonic Lethality

Our previous findings, as well as Apj knockout (Apj−/−) mice generated by other groups, demonstrate a significantly decreased number of Apj−/− mice that are born (although not discussed, we found the offspring genotype ratio of Ishida et al22 to be statistically significant by χ² test with P<0.03).20,21 Heterozygous Apj+/- animals were fully viable and fertile. Mating of Apj−/− mice resulted in a lower-than-expected number of Apj+/- mice at the time of weaning (238 offspring studied, with 139 heterozygotes, 77 wild type, and 22 null mutants), reflecting statistically significant fewer Apj−/− mice (χ², P=1.05×10⁻⁷). Further embryonic evaluation demonstrated that lethality began at embryonic day (E) 10.5 and continued through to E12.5 (Table). Moreover, >20% of Apj−/− pups died immediately after birth. Based on a 25% expected homozygous null mice, we saw only 9.2% a live Apj−/− mice at the time of weaning. We did not observe any embryonic or postnatal death in the wild-type or the heterozygous mice.

Apj−/− Embryos Have Vascular Defects

The Apj−/− embryos showed a spectrum of vascular deficits at early embryonic stages. Roughly 22% (7 of 32) of the surviving Apj−/− embryos at E10.5 had impaired maturation of the yolk sac vasculature, with a paucity of developed vascular structures (Figure 1A). Moreover, the same percentage (7 of 32) had anterior cardinal veins and dorsal aorta that were either delayed or defective vascular smooth muscle cell layers surrounding the aortic endothelium, suggesting either delayed or defective vascular smooth muscle cell layer recruitment (Figure 1C).
Apj−/− Embryos Demonstrate Cardiac Developmental Defects

Dissection of embryos at E10.5 revealed that ≈19% (6 of 32) of the surviving Apj−/− embryos had cardiac abnormalities, with the most severe mutants exhibiting enlarged and abnormally formed hearts that had not completed looping, with large pericardial effusions (Figure 2A, left and middle). In these embryos, there were no bulboventricular groove and no apparent demarcation of a bulbous cordis, with the outflow tract arising from the common ventricle and showing decreased mesenchymal formation. Formation of the atrioventricular cushion was delayed in all of the surviving Apj−/− embryos evaluated at this stage (Figure 2A, right). Apj−/− embryos surviving to the later stages (E12.5 and E15.5), despite their grossly normal size and appearance, were all found to have myocardial defects, including thinning of the myocardium (in all Apj−/− embryos evaluated) and a high prevalence of ventricular septal defects (≈20% of Apj−/− embryos; Figure 2B; Online Figure III). We also found significantly decreased proliferating cell nuclear antigen staining in the hearts of E12.5 Apj−/− embryos compared with wild-type littermates (Figure 2C). Moreover, we found in E15.5 Apj−/− embryos significantly decreased capillary densities compared with their wild-type littermates (Figure 2D). Finally, we also examined the hearts of Apj−/− mice surviving to adulthood. We found a number of various cardiac malformations, including enlarged right ventricles (4 of 10 Apj−/− mice) and ventricular septal defects (2 of 10 Apj−/− mice), which were not seen in wild-type or heterozygous littermates (Online Figure IV).

Apelin-APJ Regulates Endothelial MEF2 Activation in a Go13-Dependent Manner

On the basis of the observed embryonic lethal phenotype of the Apj−/− mice, we sought to investigate the downstream targets of apelin-APJ signaling in cardiovascular development. Previous analyses of Apj expression in developing mouse, xenopus, and zebrafish embryos demonstrated that APJ is expressed predominantly in the endothelial layers of arteries and veins, as well as in the endocardial layer in the hearts, at E9.5 to E10.5.15,25,26 We confirmed by in situ hybridization in E10.5 embryos that the predominant expression of Apj was in the endocardium and ECs (data not shown). We previously demonstrated that apelin-APJ signaling regulates Krüppel-like factor 2 (Klf2) transcription.4,27 In concurrence with these

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Table. Embryonic Stage Genotypes From Apj+/− Mating

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*Dead embryos defined by absence of heart beating.

Figure 1. Vascular defects in Apj−/− embryos. A, Yolk sac from E10.5 Apj−/− embryos show abnormal vasculature compared with wild-type (WT) littermates. Hematoxylin and eosin stain of the yolk sac section shows immature vascular plexus formation. Mes indicates mesoderm; and end, endoderm. White bars indicate 1 mm; black bars indicate 25 µm. B, CD31 immunohistochemistry of E10.5 embryo sections show defective major vessel development in a subset of Apj−/− embryos. Dorsal aorta (white arrows) and anterior cardinal veins (black arrows) are identified in the wild-type embryo. Bars indicate 100 µm. C, CD31 and smooth muscle actin (SMA) stains of E12.5 and E15.5 sections show thinner vascular smooth muscle layers surrounding the developing aorta in Apj−/− embryos. SMA staining is shown in red; CD31 staining is shown in green; and DAPI nuclear staining is shown in blue. Wall thickness was calculated from the inner and outer media (SMA positive) circumference in 3 sections from each embryo (n=4–6 embryos per group). Bars indicate 20 µm (E12.5) and 40 µm (E15.5). **P<0.01 vs wild type.
data, Klf2 expression as detected by in situ hybridization was decreased in the endocardium of the heart and the endothelial layer of the aorta in E10.5 Apj−/− embryos (Figure 3A and 3B). Given the extent of evidence demonstrating the role of the MEF2 family of transcription factors regulating KLF2 transcription,30–33 we addressed the hypothesis that apelin-APJ signaling induces MEF2 activation. We evaluated the effect of apelin-APJ on 3 MEF2 responsive luciferase constructs: (1) MEF2 luciferase reporter containing 3 tandem MEF2 binding sites (3X-MEF2); (2) a 221-bp promoter region of the KLF2 gene31; and (3) a shorter, 41-bp promoter construct containing the minimal MEF2 binding site from the KLF2 promoter as previously described (Online Figure V).30 We found that overexpression of MEF2A and MEF2C in COS7 cells resulted in a robust induction of the 3X-MEF2 luciferase reporter (Figure 3C). Overexpression of apelin and APJ in this context resulted in a greater induction of the 3X-MEF2 luciferase reporter activity (Figure 3C). Furthermore, overexpression of apelin and APJ in HUVECs induced luciferase reporter activity from all 3 MEF2 reporter constructs, including 2 derived from the KLF2 promoter (Figure 3D and 3E; Online Figure VI). APJ transfection alone was also able to significantly induce the 221-bp KLF2 promoter (Figure 3D). In addition, either mutagenesis of the MEF2 binding site or concurrent knockdown of MEF2A/C with apelin-APJ–transfection resulted in abrogation of apelin-APJ induced MEF2 luciferase reporter activity (Figure 3D). To further assess whether apelin-APJ signaling can regulate MEF2 binding on the KLF2 promoter, we conducted chromatin immunoprecipitation assays. The level of MEF2 binding to the KLF2 promoter in HUVECs was markedly decreased by apelin-APJ knockdown (Figure 3F).

APJ has previously been found to signal through 2 G proteins, namely Gαq and Gz1.34,35 To evaluate whether apelin-APJ–mediated induction of MEF2 activity involves either of these G proteins, we overexpressed constitutively active forms of Gαq (Gαq-QL) and Gz1 (Gz1-QL) with MEF2A, MEF2C, and the MEF2 luciferase reporter. We found no induction of the MEF2 reporter activity, suggesting that these G proteins were not involved in regulation of MEF2 activity (Figure 3C). Gz13, another member of the G protein family, has recently been described to target MEF2 but has not previously been associated with apelin-APJ signaling.33 We found that constitutively active Gz13 can robustly induce MEF2 activity on the basis of all of the luciferase reporters tested in both COS7 cells (Figure 3C) and HUVECs (Figure 3E; Online Figure VI). We next evaluated whether Gz13 is a bona fide target of apelin-APJ signaling. Overexpression of APJ in COS7 cells was able to induce Gz13 activity as measured by guanosine-5′-triphosphate-γS bound–Gz13, which was further augmented by stimulation with the apelin 13 peptide (Figure 3G). Moreover, stimulation

Figure 2. Myocardial defects in Apj−/− embryos. A, E10.5 Apj−/− embryos with cardiac developmental defects, including abnormal chamber development and looping, and large pericardial effusions (PE). Hematoxylin and eosin-stained serial sections show E10.5 Apj−/− embryo with outflow tract (OT) arising from the common ventricle (CV) lacking bulbus cordis (BC) and thin ventricular wall with minimal trabeculation. Right, whole-mount CD31 staining (green) of E10.5 embryos showing defective atrioventricular cushion formation in Apj−/− embryons (n=6 per group). A indicates atrium; and V, ventricle. Scale bars represent 170 μm (low magnification) and 100 μm (high magnification). B, E12.5 and E15.5 Apj−/− embryos (n=6 per group). A indicates atrium; and V, ventricle. Scale bars show 500 μm (low magnification) and 100 μm (high magnification). C, E12.5 Apj−/− hearts have a significant reduction in the number of proliferating cell nuclear antigen positive (red) cells (n=4–5 per group). CD31 staining is shown in green. **P<0.01 vs wild type (WT). Bars indicate 200 μm (low magnification) and 100 μm (high magnification). D, E15.5 Apj−/− hearts have a significantly decreased capillary densities in their ventricular walls compared with their wild-type littermates (n=4–6 per group). CD31 staining is shown in green; smooth muscle actin staining is shown in red. **P<0.01 vs wild type. Scale bars show 500 μm (low magnification) and 100 μm (high magnification).
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of HUVECs with apelin 13 led to a robust increase in G \(\alpha_{13}\) activity, as assessed by immunoprecipitation of GTP-bound \(\alpha_{13}\) (Figure 3H). Finally, transfection of HUVECs with increasing concentrations of \(\mathrm{APJ}\) expression plasmid also led to increased \(\alpha_{13}\) activity, suggesting an apelin-independent ability of \(\mathrm{APJ}\) to activate \(\alpha_{13}\) (Figure 3I).

**Apelin-APJ Activates MEF2 Activity Via Phosphorylation and Nuclear Export of HDAC4 and HDAC5 in ECs**

MEF2 is known to be regulated by multiple mechanisms, including class II HDAC–mediated inhibition.\(^{34}\) We next evaluated whether apelin-APJ signaling–mediated regulation of MEF2 activity involves HDACs. Stimulation of HUVECs with apelin 13 led to a robust induction of HDAC4 and HDAC5 translocation to the cytoplasm from the nucleus (Figure 4A; Online Figure VII). Moreover, overexpression of \(\mathrm{APJ}\) alone in HUVECs also induced translocation of HDAC4 and HDAC5 to the cytoplasm (Figure 4B).

In conjunction with the regulation of HDAC4/5 cellular localization, apelin and \(\mathrm{APJ}\) overexpression in HUVECs resulted in a robust increase in HDAC4 and HDAC5 phosphorylation, which is known to be a critical step leading to their nuclear export (Figure 4C).\(^{35}\) Apelin 13 stimulation was able to induce phosphorylation of HDAC4 and HDAC5 in HUVECs, which was abrogated in the context of small interfering RNA–mediated \(\mathrm{APJ}\) knockdown (Figure 4D). Moreover, \(\mathrm{APJ}\) knockdown led to decreased HDAC4/5 phosphorylation at baseline. We further validated the role of \(\alpha_{13}\) in this signaling cascade by demonstrating that knockdown of \(\alpha_{13}\) in HUVECs abrogated the increased HDAC4/HDAC5 phosphorylation in response to \(\mathrm{APJ}\) overexpression (Figure 4E). Moreover, concurrent overexpression of HDAC4 or HDAC5 with apelin and \(\mathrm{APJ}\) in HUVECs led to inhibition of MEF2 transcriptional activity on the KLF2 promoter–driven luciferase reporter (Figure 4F).

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**Figure 3.** Apelin and \(\mathrm{APJ}\) induce myocyte enhancer factor 2 (MEF2)A/C transcriptional activity. A, In situ hybridization for \(Klf2\) in E10.5 embryos shows decreased endocardial (black arrows) \(Klf2\) expression in \(\mathrm{Apj}^{-/-}\) embryos vs wild-type (WT) littermates (n=3 embryos per group). Scale bars show 50 μm (low magnification) and 100 μm (high magnification). B, Decreased \(Klf2\) expression in the endothelial layer of the E10.5 dorsal aorta of \(\mathrm{Apj}^{-/-}\) embryos vs wild-type littermates (n=3 embryos per group). Bars indicate 100 μm. C, MEF2 luciferase reporter containing 3 tandem MEF2 binding sites is induced by MEF2A/C overexpression in COS7 cells and induced further by apelin-APJ or constitutively active \(\alpha_{13}\) (\(\alpha_{13}\)-QL), but not by constitutively active \(\alpha_{i}\) (\(\alpha_{i}\)-QL) or \(\alpha_{q}\) (\(\alpha_{q}\)-QL). ***P<0.001 vs MEF2A/C alone; ††P<0.001 vs control. D, Transfection of apelin and \(\mathrm{APJ}\) or \(\mathrm{APJ}\) alone in human umbilical vein endothelial cells (HUVECs) can induce the 221-bp KLF2 promoter–driven luciferase reporter containing the MEF2 binding site. This effect is abrogated by either mutation of the MEF2 binding site (MEF2 mt) or concurrent knockdown of MEF2A and MEF2C (MEF2A/C siRNA). †P<0.01 and ††P<0.001 vs vector control. E, Apelin-APJ or \(\alpha_{13}\)-QL overexpression in HUVECs leads to a significant induction of the 41-bp KLF2 promoter containing the minimal MEF2 binding site. ***P<0.001 vs control. F, Chromatin immunoprecipitation assay shows that MEF2 binding to the KLF2 promoter is reduced in the context of apelin-APJ knockdown in HUVECs. G, \(\mathrm{APJ}\) overexpression leads to a significant increase of \(\alpha_{13}\) activation in COS7 cells transfected with wild-type \(\alpha_{13}\) as measured by guanosine-5’-triphosphate-γ-S bound–\(\alpha_{13}\). Stimulation with apelin 13 leads to further activation of \(\alpha_{13}\) in \(\mathrm{APJ}\)-transfected cells. †P<0.05 vs vector control; ††P<0.05 vs \(\mathrm{APJ}\) without apelin 13 stimulation. H, Stimulation with apelin 13 leads to an increase in active \(\alpha_{13}\) in HUVECs. I, Transfection of \(\mathrm{APJ}\) in HUVECs leads to increased levels of active \(\alpha_{13}\) in a dos-dependent manner.

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Increased Nuclear Localization of HDAC4 and HDAC5 in Apj−/− Endocardium and ECs

To further validate our in vitro signaling mechanism in our mouse model, we evaluated the localization of Hdac4 and Hdac5 in the Apj−/− embryos. We found that the endocardial cells of E10.5 Apj−/− embryos had a significantly higher percentage of cells with positive nuclear staining for Hdac4 and Hdac5 compared with wild-type littermates (Figure 5A).

We also evaluated the isolated heart ECs of Apj−/− mice. We found that the endothelial cells of E10.5 Apj−/− embryos had a significantly higher percentage of cells with positive nuclear staining for Hdac4 and Hdac5 compared with wild-type littermates (Figure 5A).

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Shared Transcriptional Targets of Apelin-APJ, Gα13, and MEF2 Are Decreased in Apj−/− Embryos and ECs

Given the preferential endothelial/endocardial expression of Apj in the developing embryo, we further pursued the endothelium-based relationship between apelin-APJ, Gα13,
of HMG-CoA reductase inhibitor class of drugs, which are known to induce endothelial KLF2 expression), 31,36,37 ECs from wild-type mice demonstrated a significant induction of Klf2, whereas ECs from Apj−/− mice failed to respond in a similar manner (Figure 6D).

To further validate these data, we evaluated the protein expression of 2 putative targets of this signaling axis: CX37 and CX40. HUVECs subjected to APJ knockdown expressed decreased levels of CX37 and CX40 as detected by Western blots (Figure 6E). Moreover, expression of Cx37 and Cx40 was decreased in the aortic endothelium of the E10.5 Apj−/− embryos (Figure 6F). We also found reduced expression of Cx37 and Cx40 in isolated heart ECs from Apj−/− mice compared with wild-type littermates (Figure 6G).

**Discussion**

Here, we provide the first description of the cardiovascular developmental defects in Apj−/− mouse embryos. We identified a novel endothelial signaling cascade that originates from apelin-APJ and targets Gα13, resulting in HDAC4/5 cytoplasmic translocation and MEF2 activation. We validate this pathway in vivo by demonstrating increased nuclear localization of Hdac4 and Hdac5 and decreased expression of Klf2, a validated MEF2 target, in Apj−/− embryos. Moreover, we report a number of novel, putative endothelial MEF2 targets, including Cx37 and Cx40, that will require further investigation. Given the known critical roles of Gα13 and MEF2 in embryonic cardiovascular development,22–24,38,39 our present findings provide further insights into the upstream signaling mechanism that regulates the activation of these intracellular signaling components in cardiovascular development.

There is considerable evidence for related and overlapping compensatory pathways that account for a number of features of the phenotype of Apj−/− animals in both zebrafish and mouse. The variable penetrance of embryonic lethality in the Apj−/− mice is not unlike what has been described for other G protein–coupled receptor knockout mice, including the Par1 knockout mice40 and the Cxcr7 knockout mice,41,42 and suggest the possibility of alternative G protein–coupled receptor signaling cascades compensating for the loss of apelin-APJ signaling. A nitrosourea-induced inactivating single-base-pair loss-of-function mutation in zebrafish agtr1lb, the grinch mutant, was found to exhibit the severe loss of function phenotype in only half their offspring.18 This finding is similar to the incomplete loss of HMG-CoA reductase inhibitor class of drugs, which are known to induce endothelial KLF2 expression).31,36,37

**Figure 5. Apj−/− embryos have increased nuclear Hdac4 and Hdac5.** A, A significantly greater number of endocardial cells from E10.5 Apj−/− embryos have nuclear Hdac4 and Hdac5 (red) staining compared with wild-type (WT) embryos. DAPI (blue) and CD31 (green) staining is shown (n=4–5 embryos per group). **P<0.01 and ***P<0.001. B, Isolated heart endothelial cells (ECs) demonstrate a greater percentage of cells lacking nuclear Hdac4 or Hdac5 staining from wild-type mice vs Apj−/− mice. Only isolated ECs from wild-type mice respond to apelin by translocation of Hdac4 and Hdac5 to the cytoplasm. ECs from Apj−/− demonstrate no response to apelin 13. **P<0.01 vs no apelin 13 stimulation; ††P<0.01 vs unstimulated wild type; †††P<0.001 vs apelin 13–stimulated wild type. C, The levels of phosphorylated Hdac4 and Hdac5 are decreased in heart ECs from Apj−/− mice.
of embryos in the homozygous Apj−/− mouse model described here. Moreover, the zebrafish mutant phenotype was found to be a function of the genetic background, because different pairs of mutant carriers reproducibly yielded embryos with differing severities of phenotype. There are also anecdotal reports that the embryonic lethality of Apj−/− mice is most severe when examined on specific inbred backgrounds. The observed incomplete penetrance is likely a result of stochastic activation of related pathways, which rescues heart and vascular development, and the observed alteration of this ratio by genetic background likely reflects differences in the ability of the compensatory pathways to be activated when apelin-APJ signaling is absent.

Our findings also suggest that both MEF2A and MEF2C are likely involved in apelin-APJ signaling on the basis of our mechanistic data and the variable phenotype of the Apj−/− mice, some of which mimic the early embryonic lethality of the Mef2c null embryos (Figures 1A, 1B, and 2A)22,24 with others sharing the phenotype of the Mef2a null mice (Online Figure IV).23

Three Apln−/− mouse lines have been generated and reported in the literature.16,43,44 In each case, the Apln−/− mice were found to be viable with Mendelian inheritance of the null allele. Because apelin is considered to be the only ligand for APJ and APJ to be the only receptor for apelin, the expectation is that the phenotype generated by functional deletion of the Apln and Apj genes should be very similar, if not identical. The likely explanation for differences in the Apln versus Apj null mice is that either a secondary ligand for APJ may exist, or a ligand-independent activation of APJ such as that recently described1 may be contributing to the developmental context. Indeed, our findings suggest that induction of APJ alone, independently of apelin, may be capable of transducing the signals necessary for MEF2 activation. These findings will require additional investigation to delineate the role of apelin-dependent and apelin-independent signaling cascades that are downstream of the APJ receptor.

On the basis of the predominance of Apj expression in the endothelium and endocardium of the developing mouse
embryos, we chose to focus on the downstream targets of apelin-APJ signaling in the ECs and identified MEF2 as a targeted transcription factor. Our work leaves open the possibility that apelin-APJ signaling in other cell types may also be important for proper cardiovascular development, especially in light of recent findings demonstrating the role of APJ signaling in myocardial progenitor cells in zebrafish. Conditional, tissue-specific, Apj-deficient mice are currently being generated and will help address these unresolved questions. Moreover, the role of apelin-independent activation of APJ in the context of cardiovascular development remains to be fully elucidated.

Our previous work had demonstrated that surviving adult Apj−/− mice have a functional cardiovascular defect, and non-invasive imaging studies and isolated cardiomyocyte studies have shown that this is attributable, at least in part, to a primary defect in cardiac contraction. The presence of apelin in tissue and blood likely has a tonic effect on contractile function in adult mice. However, our findings also suggest the possibility of congenital structural defects in some adult Apj−/− mice that further impair their cardiovascular function. We have seen enlarged right ventricles and ventricular septal defects in adult Apj−/− mice that could be contributing to the impaired exercise tolerance of these mice. These congenital defects are in keeping with types of developmental defects identified in the Apj−/− embryos and likely represent survival of those animals with the less severe defects. These observations prompt human studies investigating the association of genetic variation in the APLN and APJ loci with congenital heart abnormalities.

Sources of Funding

This work was supported by National Institutes of Health grants (HL095654 and HL113005 to H.J. Chun, HL077676 to T. Quertermous) and the Howard Hughes Medical Institute Physician Scientist Early Career Award (to H.J. Chun).

Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Embryonic development of the heart and blood vessels is a complex process that depends on the actions of various signaling pathways.
- G protein–coupled receptor signaling controls multiple aspects of both vascular and cardiac development.
- Mice with genetic deletion of the G protein–coupled receptor Apj display incomplete embryonic lethality.

**What New Information Does This Article Contribute?**

- Apj knockout mice show numerous cardiac and vascular developmental abnormalities in utero.
- Apelin-APJ signaling targets the G protein G alpha 13, which results in cytoplasmic translocation of histone deacetylases (HDAC) 4 and HDAC5 and myocyte enhancer factor 2 activation.

- We identify several novel endothelial targets of apelin-APJ signaling, including connexin 37 and connexin 40.

Normal cardiovascular development requires a complex integration of various signaling processes. The molecular mechanisms that control this process remain incompletely understood. We show that systemic knockout of Apj in the mouse leads to cardiovascular developmental defects. We identify novel downstream targets of apelin-APJ signaling, including the G protein G alpha 13. Apelin-APJ signaling activates G alpha 13 and leads to robust cytoplasmic translocation of HDAC4 and HDAC5 and increases transcriptional activity of the transcription factor myocyte enhancer factor 2. These findings provide greater insights into the mechanisms of cellular signaling that regulate cardiovascular development.
Apelin-APJ Signaling Is a Critical Regulator of Endothelial MEF2 Activation in Cardiovascular Development


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doi: 10.1161/CIRCRESAHA.113.301324

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Supplementary Material

Detailed Methods

**Antibodies.** Antibodies to GTP bound Ga13 and Ga13 (Kit# 80401, Neweast Biosciences), phosho-HDAC4 and phosho-HDAC5 (3443, 3424, Cell Signaling), HDAC4 (2072, Cell Signaling), HDAC5 (2082, Cell Signaling), connexin 37 (cx37A11-A, Alpha Diagnostic International), connexin 40 (20466, Santa Cruz), APJ (LS-A64, MBL), GAPDH (2118s, Cell Signaling), connexin 37 A11-A, Alpha Diagnostic International), connexin 40 (20466, Santa Cruz), APJ (LS-A64, MBL), GAPDH (2118s, Cell Signaling) were used for western blotting. Antibodies to CD31 (557355, BD Biosciences), SMA conjugated with cy3 (c-6198, Sigma), PCNA (2014-03, DAKO), connexin 37 (40-4200, Invitrogen), connexin 40 (20466, Santa Cruz), HDAC4 (2072, Cell Signaling) and HDAC5 (2082, Cell Signaling) were used for immunostaining on the cryosections. Alexa 488 (A11008 and A11001, Invitrogen), Alexa 568 (A11011 and A10037, Invitrogen) conjugated secondary antibodies and rat detection kit for anti-mouse CD31 (Biocare Medical) were used.

**Embryo harvest and Immunohistochemistry.** Embryos were collected at various stages of development from pregnant females, genotyped and photographed as per standard methodology. Embryos were fixed in 4% PFA at 4 °C overnight and cryoprotected in 30% sucrose (in 1× PBS) at 4 °C overnight. Embryos were then embedded in optimal cutting temperature medium (OCT) (Sakura Tissue-Tek), frozen solid in cryomolds, sectioned on a Leica CM1950 at 10 μm and stored at −20 °C. Cryosections were then air dried for 10 min and washed in 1× PBS for 15 min at room temperature. Sections were blocked in 5% heat-inactivated goat serum in 1× PBS for 1 h at room temperature and then probed with primary antibodies overnight at 4 °C. After incubation, slides were washed with 1× PBS, blocked for 1 h at room temperature and probed with secondary antibodies for 1 h at room temperature. After washing, slides were mounted in mounting medium with DAPI (Invitrogen). H&E staining was performed using standard methods.

**In situ hybridization.** Mouse Klf2 probe was amplified from mouse lung cDNA by PCR and cloned into the pCR2.1 TOPO vector (Invitrogen) using the following primers: Forward primer-5’-CTTGCACATGAAGCGACACA-3’; Reverse primer-5’-CCGTGATTCCTCCAAAGATC-3’ (463 base pairs). Sense and antisense digoxigenin (DIG)-labeled riboprobes were generated by PCR in the presence of DIG-dUTP M13 and T7 primers (Invitrogen). Incubation was done at 65 °C overnight. DIG was detected by a digoxigenin-specific, horseradish peroxidase–labeled antibody (Roche-11093274910). For alkaline phosphatase reaction, AP substrate (NBT/BCIP (Roche 11681451001) was used and the slides were mounted in aqueous mounting medium.

**G protein activity measurement.** COS7 cells were transfected with either pcDNA3 or APJ expression construct with wildtype Ga13 expression construct. Membranes were harvested from transfected cells by sucrose gradient differential centrifugation. Isolated membrane fractions were segregated into paired samples upon resuspension with HEM buffer (20 mM HEPES, pH 7.4, 1.4 mM EGTA, and 12.5 mM MgCl2). Samples were incubated 10 minutes at room temperature with excess GDP (2mM), followed by addition of [35S] GTPγS along with either vehicle (HEM) or apelin 13 peptide (Sigma). Samples were incubated for 1 h at 4 °C on a rotator. Mouse anti-Ga13-GTP antibody or anti-mouse IgG was added, and samples were incubated overnight at 4°C. Protein A/G PLUS-agarose beads (Santa Cruz) were pre-incubated with cold GTPγS to reduce non-specific binding. Saturated beads were washed to remove unbound cold GTP before being added to each sample, and incubated for 1 hour. Beads were then washed three times, resuspended in HEM, and transferred to scintillation plate. Samples were counted three times to produce final CPM readings.
Immunoprecipitation and immunoblotting. For the active Gα13 immunoprecipitation, HUVEC cells were either incubated with 1 µM apelin 13 for 30 min after serum starvation or transfected with an APJ expression construct. Cells were harvested in lysis buffer using the Gα13 activation assay kit (NewEast Biosciences). Active Gα13 proteins were immunoprecipitated by using anti-Gα13-GTP antibody and washed five times. Precipitated proteins were resolved by SDS/PAGE, transferred to PVDF membranes, and immunoblotted with anti-Gα13 (total) antibody. Proteins were visualized by using a chemiluminescence system (Thermo Scientific). For HDAC4/5 phosphorylation, HUVECs were transfected with either expression vectors or knockdown siRNAs along with the appropriate controls for 48 hours. A subset was stimulated with apelin 13. RIPA lysis buffer (Thermo Scientific) containing Halt Protease and Phosphatase Inhibitor cocktail (Thermo Scientific) was used for cell lysis. Protein contents were measured using a Bio-Rad DC assay kit. Western blots were carried out as previously described. Each western blot is a representative of three independent experiments of triplicate samples.

Realtime PCR analysis. RNA was extracted using the miRNasy® Mini kit (Qiagen) according to the manufacturer's instructions. DNase treated RNA was reverse-transcribed with iScript reverse transcriptase kit (Bio-Rad). For quantitative PCR analyses, mouse or human Taqman probes (Applied Biosystems) or designed primers (Online Table III) were used. 18S probe (Applied Biosystems) was used for an internal normalization control. Quantitative PCR was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) and Power SYBR Green (Applied Biosystems) according to the manufacturer’s protocol. The RT-PCR assays were performed on Bio-Rad CFX96 thermal cycler. Each condition was repeated three times in triplicates.

DNA constructs. The sense and antisense strands of the 41 basepair KLF2 promoter (Online Figure V) (Keck Oligo Lab, Yale University) were cloned into pGL3 vector using NheI and XhoI sites. 3X-MEF2-luciferase reporter, HDAC4-GFP, and HDAC5-GFP plasmids were kindly provided by Dr. Eric Olson. KLF2-221 bp and KLF2-221 bp-MEF2 mt constructs were kindly provided by Dr. Mukesh Jain. Expression constructs for apelin, APJ, APJ-GFP, MEF2A and MEF2C (Origene), wildtype Gα13, constitutively active Gα13, Gαi and Gαq (www.cdna.org), HDAC4-FLAG and HDAC5-FLAG (Addgene) were used.

Luciferase assays. Either COS7 cells or HUVECs were transfected and lysed in lysis buffer (Promega). Dual Luciferase Reporter System (Promega) was used according to the manufacturer’s protocol. All experiments were performed three times in triplicates.

Chromatin immunoprecipitation assay. HUVECs were transfected with control siRNA or apelin-APJ siRNAs (Invitrogen) for 48 hours and native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 minutes. Simple ChIP Plus Enzymatic Chromatin IP kit (Cell Signaling) was used per the manufacturer’s protocol. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with anti-MEF2 antibody (Santa Cruz; sc-313X), or rabbit IgG control. PCR reactions of immunoprecipitated DNA were performed to validate MEF2 binding on the KLF2 promoter. PCR primers used: FWD: TGTCAGCGCAAAGGCCCAAG and REV: GATGAGGACGAGCTCCGG. PCR products were separated by gel electrophoresis and visualized by SYBRsafe (Invitrogen).

Immunocytochemistry. For the apelin 13 effect on HDAC4/5 translocation, HUVEC cells plated on glass bottom culture dish (Mat Tek) were transfected with either FLAG or GFP-tagged HDAC4 and HDAC5 expression vectors for 24 hours. Cells were imaged using a Nikon Eclipse Ti confocal microscopy before and after treatment with apelin 13 (1 µM for 1 h at 37℃). For the effect of APJ on HDAC4/5 translocation, HUVECs were transfected with FLAG-tagged HDAC4
or HDAC5 with either GFP vector (pEGFP-N1) or APJ-GFP. 24 hours after transfection, cells were fixed with 3% paraformaldehyde, washed, and permeabilized with 0.2% Triton X-100/PBS for 10 min, and blocked with 10% fetal bovine serum in 0.1%/PBS for 10 min. Cells were incubated with Cy3 conjugated anti-FLAG antibody in blocking solution for 2 h at room temperature. Dishes were mounted using mounting medium with DAPI (Invitrogen). Analysis of fluorescent staining was performed using confocal microscopy. Isolated heart ECs from mice were treated as above and stained for Hdac4 or Hdac5. In HUVECs, the HDAC4/5 data is presented as percent cells with cytoplasmic HDAC4/5, as in basal condition the majority of cells had the transfected HDACs being localized to the nucleus. In isolated heart endothelial cells and in endocardium, this data is presented as percent cells without nuclear Hdac4/5, as the majority of cells had endogenous Hdac4/5 in both the cytoplasm and the nucleus, and responded to apelin by translocation of the nuclear fraction to the cytoplasm.

**Whole-mount immunostaining.** For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde at 4 °C overnight and dehydrated with methanol, then rehydrate through descending graded alcohols to PBS. The samples were then blocked in 1% BSA/PBS-Tween at 4 °C overnight and incubated overnight at 4°C with anti-CD31 antibody. The samples were washed 4 times for 45 min each in PBS-Tween and incubated with goat anti-rat Alexa488 antibody at 4 °C overnight.

**Microarray.** HUVECs were transfected with apelin and APJ siRNA, MEF2A and MEF2C siRNA, Ga13 siRNA or negative control siRNA (Invitrogen). After 48 hours, RNA was extracted using miRNasy® Mini Kit (Qiagen). The RNA was quantified and the RNA quality was verified. The HumanHT-12 v4 Expression BeadChip Kit (Illumina) was used according to the manufacturer's protocol by the Yale Center for Genome Analysis. Microarray results were analyzed using the bead array and limma packages in R/Bioconductor (v 2.14/2.09). Differential gene expression was defined as a two-fold difference in log intensity between samples.

**Supplemental References**


Online Table I. Gene expression analyses of HUVECs subjected to 1) apelin/APJ, 2) Gα13, or 3) MEF2A/C knockdown.

<table>
<thead>
<tr>
<th>Name of genes</th>
<th>List of Upregulated Genes (&gt;2 fold in all three conditions)</th>
<th>List of Downregulated Genes (&lt; 0.5 fold in all three conditions)</th>
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<tbody>
<tr>
<td>ANKRD9</td>
<td>ADAMTS9</td>
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<tr>
<td>AP1M1</td>
<td>ALOX5AP</td>
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<tr>
<td>ARMCX6</td>
<td>ANGPTL4</td>
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<tr>
<td>BCAT2</td>
<td>APLN</td>
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<tr>
<td>CENPB</td>
<td>ARSD</td>
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<tr>
<td>EIF4EBP2</td>
<td>C10orf54</td>
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<tr>
<td>FBXL18</td>
<td>C20orf160</td>
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<td>FEM1A</td>
<td>CAV2</td>
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<td>KHSRP</td>
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<td>PAK4</td>
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<td>PIP5K1C</td>
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<td>MYL12A</td>
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<td>MYL5</td>
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<td>PALM</td>
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<td>PPAP2B</td>
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<tr>
<td>Protein</td>
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<td>SH2D3C</td>
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<td>SLCO2A1</td>
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<td>TNFRSF6B</td>
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<tr>
<td>VCAM1</td>
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<tr>
<td>VIP</td>
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Online Table II. Putative endothelial apelin/APJ, Gα13, MEF2 targets identified by microarray and qPCR analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in development/gene function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF2</td>
<td>Global -/- is embryonic lethal due to impaired blood vessel maturation.</td>
<td>³, ⁴</td>
</tr>
<tr>
<td>VCAM1</td>
<td>Global -/- is embryonic lethal due to defective chorio-allantoic fusion and myocardial defects.</td>
<td>⁵, ⁶</td>
</tr>
<tr>
<td>Connexins 37 and 40</td>
<td>Double -/- mice have vascular developmental defects and hemorrhages and die between E12.5 and birth.</td>
<td>⁷</td>
</tr>
<tr>
<td>Krcc1</td>
<td>Secreted FGF binding proteins. Knockdown results in embryonic lethality within 5 days. Predicted to be a MEF2 transcriptional target.</td>
<td>⁸</td>
</tr>
<tr>
<td>Myl12a</td>
<td>Non-muscle regulatory light chain. Predicted to be a MEF2 transcriptional target.</td>
<td>⁹</td>
</tr>
<tr>
<td>Plvap</td>
<td>Global -/- is embryonic lethal due to vascular permeability. Predicted to be a MEF2 transcriptional target.</td>
<td>¹⁰</td>
</tr>
<tr>
<td>SerpinB1</td>
<td>Inhibitor of neutrophil serine proteases. Predicted to be a MEF2 transcriptional target.</td>
<td>¹¹</td>
</tr>
<tr>
<td>St3gal5</td>
<td>Catalyzes the formation of ganglioside GM3 which is important in cell differentiation, cell proliferation and integrin-mediated cell adhesion. Predicted to be a MEF2 transcriptional target.</td>
<td>¹²</td>
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</table>
**Online Table III. Primer sequences for quantitative PCR analyses for gene expression.**

<table>
<thead>
<tr>
<th>NAME of GENE</th>
<th>PRIMER SEQUENCE</th>
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</thead>
<tbody>
<tr>
<td><strong>FOR MOUSE RNA</strong></td>
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| KRCC1 (NM_145568) | Forward Sequence GAGACTCCCATCTGGAACCAAC  
Reverse Sequence TCTGAGAAGCCATCCCTGGTGTA | |
| MYL12A (NM_026064) | Forward Sequence GTTCGCCATGTTTGGACCAGTC  
Reverse Sequence CGTCCAGGTATTCGTAGTGTG | |
| PLVAP (NM_032398) | Forward Sequence GTTGACTAGCGACGTGAGT  
Reverse Sequence AGCTGTTCCTGGCACTGTTCT | |
| SERPINB1 (NM_025429) | Forward Sequence GGATGCTCCATTCCGACTGAT  
Reverse Sequence AGTTCTCCCCCTGGTAAAGGCA | |
| ST3GAL5 (NM_001035228) | Forward Sequence ACTCCAGCAAAGACCTTCAGG  
Reverse Sequence GATGTGTAGCGAAGACAAACGG | |
| VCAM1 (NM_011693) | Forward Sequence GCTATGAGGATGGAAGACTG  
Reverse Sequence ACTTGTGCAGCCACCTGAGATC | |
| **FOR HUMAN RNA** | | |
| KRCC1 (NM_016618) | Forward Sequence GAAACCATCCAGACCTACCCAAG  
Reverse Sequence CCTCGTAAGCTGACATGATC | |
| MYL12A (NM_006471) | Forward Sequence GTTTGACCAGTGCAGATTCAGG  
Reverse Sequence TAGATACTCATCAGTGGATTCTTC | |
| PLVAP (NM_031310) | Forward Sequence CAATCAGGATCATGCTCCAGCC  
Reverse Sequence CTATCTCCACCTCCAGCTT | |
| SERPINB1 (NM_030666) | Forward Sequence AGCTCAGCATGTCATGGCTCCTGCT  
Reverse Sequence CGAGATTTCAGGTTTAGCTCCAC | |
| ST3GAL5 (NM_003896) | Forward Sequence AGAGCCTCAGTGCGTGGTTTCTGG  
Reverse Sequence GAGGTCATATCBAACCCCGCCC | |
| VCAM1 (NM_080682) | Forward Sequence GATTCTGTGCCACAGTAAGGC  
Reverse Sequence TGGTCACAGGCACTTCTTG | |
Online Figure I. CD31 staining of wildtype and Apj-/- E10.5 embryos show comparable CD31 staining (n=3 per group). CD31 staining is shown in green, SMA staining is shown in red.

Online Figure II. Representative embryos from E12.5 (n=5 per group) and E15.5 (n=4 per group) showing grossly normal appearance of Apj-/- embryos.

Online Figure III. An E12.5 heart with CD31 staining showing the presence of VSD in ~20% of Apj-/- embryos (1 out of 5). Bars indicate 300 μm.
Online Figure IV. Hearts of adult Apj-/- mice demonstrated presence of ventricular septal defect (2 out of 10) as well as enlarged or deformed right ventricle (4 out of 10). Bars indicate 5 mm.

Online Figure V. Sequence of the 41 bp KLF2 promoter containing MEF2 binding sites.

Online Figure VI. Overexpression of apelin and APJ or Ga13-QL lead to significant induction of luciferase activity driven by 3X MEF2 binding site in HUVECs. *P<0.01 vs. control.
**Online Figure VII.** Stimulation of HUVECs with apelin 13 leads to cytoplasmic translocation of GFP tagged HDAC4 and HDAC5. *P<0.01.

**Online Figure VII.** Relative knockdown levels achieved for apelin, APJ, Gα13, MEF2A and MEF2C in HUVECs with the three knockdown conditions. *P<0.01 vs. control.