Published in final edited form as:
Circ Res. 2013 June 21; 113(1): 22–31. doi:10.1161/CIRCRESAHA.113.301324.

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

Yujung Kang[1], **Jongmin Kim**[1], **Joshua P. Anderson**[2], **Jingxia Wu**[1], **Scott R. Gleim**[1], **Ramendra K. Kundu**[2], **Danielle L. McLean**[1], **Jun-dae Kim**[1], **Hyekyung Park**[1], **Suk-won Jin**[1], **John Hwa**[1], **Thomas Quertermous**[2], and **Hyung J. Chun**[1]

[1]Yale Cardiovascular Research Center, Section of Cardiovascular Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06511

[2]Divivsion of Cardiovascular Medicine, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305

Abstract

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 define the involved downstream signaling targets.

Methods and Results—We report the first characterization of the embryonic lethality of the *Apj*−/− mice. Greater than half of the expected *Apj*−/− embryos died *in utero* due to 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 due to a deficiency of vascular smooth muscle cells, and impaired myocardial trabeculation and ventricular wall development. The molecular mechanism implicates a novel, non-canonical signaling pathway downstream of apelin-APJ involving Gα13, which induces histone deacetylase (HDAC) 4 and HDAC5 phosphorylation and cytoplasmic translocation, resulting in activation of MEF2 (myocyte enhancer factor 2). *Apj*−/− mice have greater endocardial Hdac4 and Hdac5 nuclear localization, and reduced expression of the MEF2 transcriptional target *Klf2*. 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.

Address correspondence to: Dr. Hyung J. Chun, Yale University School of Medicine, Section of Cardiovascular Medicine, 300 George Street, Room 770H, New Haven, CT 06511, Tel: (203) 737-6389, Fax: (203) 737-6118, hyung.chun@yale.edu, Dr. Thomas Quertermous, Stanford University School of Medicine, Division of Cardiovascular Medicine, Stanford University, 300 Pasteur Drive, Stanford, California 94305, Tel: (650) 723-5013, Fax: (650) 725-2178, tomq1@stanford.edu.

DISCLOSURES
None.

Keywords

Apelin; APJ; Gα13; HDAC4; HDAC5; MEF2A; MEF2C; developmental biology; G proteins

INTRODUCTION

Various experimental approaches have indicated that the apelin-APJ pathway is a potent regulator of both cardiac and vascular functions.[1–4] 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 vasodepressor 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 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 blood vessels.[16] Experiments in zebrafish found that apelin-APJ 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.[17–19] Despite these studies in model organisms, null mutations in the *Apln* 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, non-canonical signaling cascade by which apelin-APJ signaling, via involvement of Gα13, HDAC4 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 in providing 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, three LoxP sites, and the neomycin and diphtheria toxin genes. The construct was transfected into B6-3 embryonic stem (ES) cells, targeted ES cell clones transfected with pBSCre, and resulting clones found to have deleted both the neo and *Apj* genes were injected into C57BL/6J-Tyrc-2J blastocysts to establish the targeted allele fully in the C57BL/6 background.

Cell culture and transfection

Mouse heart endothelial cells (ECs) were isolated by digesting whole hearts with collagenase (2 mg/mL) with gentle agitation for 45 min at 37 °C. The cell suspension was triturated 12 times, filtered through 70-μm cell strainers and then centrifuged at 400g for 5 min at 4 °C. Cells were resuspended in 2 mL of 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 of bead suspension, BD Pharmingen). We performed a second sorting step to ensure the purity of the ECs. HUVECs (Yale VBT Core) and COS7 cells (Lonza) were cultured at 37°C in a 5% CO_2 incubator. For HUVECs, growth medium was EGM-2 (Lonza) containing 2% fetal bovine serum (FBS). DMEM (Gibco) with 10% FBS was used for COS7. For experimental treatments, HUVECs (passage 3–7) were grown to 70–90% confluence. Transient transfections of plasmids were performed using Fugene HD (Promega) using manufacturer's protocol. For gene silencing, siRNAs (Stealth siRNA, Invitrogen) were transfected using RNAiMAX (Invitrogen) using manufacturer's protocols.

Statistical analysis

All in vitro experiments (ChIP assays, immunoprecipitations, western blots, and quantitative PCR assays) are representative of three independent experiments. Results are reported as mean ± SEM. An unpaired Student's *t*-test or one way ANOVA test were used as appropriate to determine statistical significance. Post hoc analysis was performed using the Bonferroni method. $P < 0.05$ was 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.al.* to be statistically significant by Chi-square test with $P<0.03$).[10, 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 heterzygotes, 77 wildtype, and 22 null mutants), reflecting a statistically significant fewer *Apj*−/− mice (Chi square $P=1.05\times10^{-7}$). Further embryonic evaluation demonstrated that lethality began at embryonic day (E) 10.5 and continued through to E12.5 (Table 1). Moreover, more than 20% of *Apj−/−* pups died immediately after birth. Based on a 25% expected homozygous null mice, we saw only 9.2% live *Apj−/−* mice at the time of weaning. We did not observe any embryonic or postnatal death in the wildtype 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 out 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, same percentage (7 out of 32) had anterior cardinal veins and dorsal aorta that were either lacking or were small and aberrantly located (Figure 1B). Staining of whole embryos (E9.5) with an anti-CD31 antibody suggested that endothelial cells were present at a comparable level, but contributed to vessels that were not formed appropriately (Online Figure I).

We further evaluated those embryos that survived to later stages (E12.5–E15.5). We found that the majority of those *Apj−/−* embryos surviving to these stages appeared grossly normal (Online Figure II), although a small number of dead *Apj−/−* embryos were also identified at

E12.5 (Table 1). Despite the normal appearance, evaluation of their developing vessels demonstrated significantly decreased 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 approximately 19% (6 out 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 center panels). In these embryos there was no bulbo-ventricular 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 atrio-ventricular cushion was delayed in all of the surviving *Apj−/−* embryos evaluated at this stage (Figure 2A, right panels). *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 high prevalence of ventricular septal defects (~20% of *Apj−/−* embryos) (Figure 2B and Online Figure III). We also found significantly decreased PCNA staining in the hearts of E12.5 *Apj−/−* embryos compared to wildtype littermates (Figure 2C). Moreover, we found in E15.5 *Apj−/−* embryos significantly decreased capillary densities compared to their wildtype littermates (Figure 2D). Lastly, we also examined the hearts of *Apj−/−* mice surviving to adulthood. We found a number of varying cardiac malformations, including enlarged right ventricles (4 out of 10 *Apj−/−* mice) and ventricular septal defects (2 out of 10 *Apj−/−* mice), which were not seen in wildtype or heterozygous littermates (Online Figure IV).

Apelin-APJ regulates endothelial MEF2 activation in a Gα13 dependent manner

Based on 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 predominantly expressed in the endothelial layers of arteries and veins, as well as the endocardial layer in the hearts at E9.5–10.5.[15, 25, 26] We confirmed by *in situ* hybridization in E10.5 embryos that the predominant expression of *Apj* was in the endocardium and endothelial cells (data not shown). We previously demonstrated that apelin-APJ signaling regulates KLF2 transcription.[4, 27] In concurrence with this 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,[28–30] we addressed the hypothesis that apelin-APJ signaling induces MEF2 activation. We evaluated the effect of apelin-APJ on three MEF2 responsive luciferase constructs: 1) MEF2 luciferase reporter containing three tandem MEF2 binding sites (3X-MEF2), 2) a 221 basepair promoter region of the KLF2 gene,[31] and 3) a shorter, 41 basepair 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 human umbilical vein endothelial cells (HUVECs) induced luciferase reporter activity from all three MEF2 reporter constructs, including two derived from the KLF2 promoter (Figures 3D, 3E, and 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 (ChIP) 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 two G proteins, namely Gαq and Gαi.[13, 32] 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 Gαi (Gαi-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 apelin-APJ mediated regulation of MEF2 (Figure 3C). Gα13, another member of the G protein family, has recently been described to target MEF2, but has not been previously associated with apelin-APJ signaling.[33] We found that constitutively active Gα13 can robustly induce MEF2 activity based on all of the luciferase reporters tested in both COS7 cells (Figure 3C) and HUVECs (Figures 3E and Online Figure VI). We next evaluated whether Gα13 is a bona fide target of apelin-APJ signaling. Overexpression of APJ in COS7 cells was able to induce Gα13 activity as measured by GTP-γS bound Gα13, which was further augmented by stimulation with the apelin 13 peptide (Figure 3G). Moreover, stimulation of HUVECs with apelin 13 led to a robust increase in Gα13 activity, as assessed by immunoprecipitation of GTP bound Gα13 (Figure 3H). Lastly, transfection of HUVECs with increasing concentration of *APJ* expression plasmid also led to increased Gα13 activity, suggesting an apelin-independent ability of APJ to activate Gα13 (Figure 3I).

Apelin-APJ activates MEF2 activity via phosphorylation and nuclear export of histone deacetylases 4 and 5 in endothelial cells

MEF2 is known to be regulated by multiple mechanisms, including class II histone deacetylase (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 and Online Figure VII). Moreover, overexpression of APJ alone in HUVECs also induced translocation of HDAC4 and HDAC5 to the cytoplasm (Figure 4B).

In conjunction with regulation of HDAC4/5 cellular localization, apelin and 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 siRNA mediated APJ knockdown (Figure 4D). Moreover, APJ knockdown led to decreased HDAC4/5 phosphorylation at baseline. We further validated the role of Gα13 in this signaling cascade by demonstrating that knockdown of Gα13 in HUVECs abrogated the increased HDAC4/HDAC5 phosphorylation in response to APJ overexpression (Figure 4E). Moreover, concurrent overexpression of HDAC4 or HDAC5 with apelin and APJ in HUVECs led to inhibition of MEF2 transcriptional activity on the KLF2 promoter driven luciferase reporter (Figure 4F).

Increased nuclear localization of HDAC4 AND HDAC5 in Apj−/− endocardium and endothelial cells

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 to wildtype littermates (Figure 5A).

We also evaluated the isolated heart ECs of *Apj−/−* mice. We found in ECs isolated from wildtype mice a robust cytoplasmic translocation of both Hdac4 and Hdac5 in response to apelin 13 stimulation (Figure 5B). However, in ECs isolated from *Apj−/−* hearts, we found that the percent of ECs without nuclear Hdac4 and Hdac5 staining was significantly reduced at baseline, and was not affected by apelin 13 stimulation. Moreover, we found decreased levels of phosphorylated Hdac4 and Hdac5 in heart ECs from *Apj−/−* mice compared to their wildtype littermates (Figure 5C).

Shared transcriptional targets of apelin-APJ, Gα13, and MEF2 are decreased in Apj−/− embryos and endothelial cells

Given the preferential endothelial/endocardial expression of *Apj* in the developing embryo, we further pursued the endothelial based relationship between apelin-APJ, Gα13, and MEF2 by conducting microarray analyses using HUVECs subjected to following knockdown conditions: 1) apelin and APJ, 2) Gα13, and 3) MEF2A and MEF2C (Online Figure VIII). We found a large number of genes that were similarly regulated in the three conditions, including a number of genes with known roles in cardiovascular development (Online Table I). We validated a subset of the downregulated genes by qPCR analyses (Figure 6A and Online Table II). We found that knockdown of APJ alone (without apelin) also resulted in decreased expression of these targets, although some transcripts were only minimally reduced (Figure 6A). To evaluate whether these downregulated genes were also similarly regulated in the *Apj−/−* mice, we conducted qPCR analyses of E10.5 *Apj−/−* embryos. The genes confirmed by qPCR analyses in HUVECs, including connexin 37 (CX37), connexin 40 (CX40) and VCAM-1, were also significantly downregulated in the *Apj−/−* embryos (Figure 6B). Moreover, we conducted additional qPCR assays in isolated heart ECs from wildtype and *Apj−/−* mice and found that the majority of these genes were also significantly downregulated (Figure 6C). With respect to Klf2, we found in isolated heart ECs no difference in the baseline expression, likely due to the low basal level of expression in mature heart ECs that was detected; however, when these cells were stimulated with rosuvastatin (member of HMG-CoA reductase inhibitor class of drugs, which are known to induce endothelial KLF2 expression[31, 36, 37]), ECs from wildtype 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 two 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, Cx37 and Cx40 expression were 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 to wildtype 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 current findings shed 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 GPCR knockout mice, including the *Par1* knockout mice[40] and the *Cxcr7* knockout mice,[41, 42] and suggest the possibility of alternate GPCR signaling cascades compensating for the loss of apelin-APJ signaling. A nitrosourea induced inactivating single basepair loss of function mutation in zebrafish *agtrl1b*, 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 embryos in the homozygous *Apj−/−* mouse model described here. Moreover, the zebrafish mutant phenotype was found to be a function of the genetic background, as 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 due to 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, based on our mechanistic data as well as 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. Since 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 that ligand independent activation of APJ such as that recently described[1] may be contributing to the developmental context. Indeed, our findings suggest that induction of APJ alone, independent 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.

Based on the predominance of *Apj* expression in the endothelium and the endocardium of the developing mouse embryos,[25] we chose to focus on the downstream targets of apelin-APJ signaling in the endothelial cells 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.[18, 19, 45] 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 noninvasive imagining studies and isolated cardiomyocyte studies have shown that this is due at least in part to a primary defect in cardiac contraction.[13, 21] The presence of apelin in tissue and blood likely has a tonic effect on contractile function in adult mice.[13, 46] 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 as well as 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

SOURCES OF FUNDING

This work was supported by National Institutes of Health grants (HL095654 and HL113005 to H.J.C., HL074190 to J.H., and HL077676 to T.Q.) and Howard Hughes Medical Institute Physician Scientist Early Career Award (to H.J.C.).

Nonstandard Abbreviations

EC	Endothelial cell
HUVEC	Human umbilical vein endothelial cell
HDAC4	Histone deacetylase 4
HDAC5	Histone deacetylase 5
MEF2	Myocyte enhancer factor 2
CX37	Connexin 37
CX40	Connexin 40

REFERENCES

1. Scimia MC, Hurtado C, Ray S, Metzler S, Wei K, Wang J, Woods CE, Purcell NH, Catalucci D, Akasaka T, Bueno OF, Vlasuk GP, Kaliman P, Bodmer R, Smith LH, Ashley E, Mercola M, Brown JH, Ruiz-Lozano P. Apj acts as a dual receptor in cardiac hypertrophy. Nature. 2012; 488:394–398. [PubMed: 22810587]
2. Chun HJ, Ali ZA, Kojima Y, Kundu RK, Sheikh AY, Agrawal R, Zheng L, Leeper NJ, Pearl NE, Patterson AJ, Anderson JP, Tsao PS, Lenardo MJ, Ashley EA, Quertermous T. Apelin signaling antagonizes ang ii effects in mouse models of atherosclerosis. The Journal of clinical investigation. 2008; 118:3343–3354. [PubMed: 18769630]
3. Kim J, Kang Y, Kojima Y, Lighthouse JK, Hu X, Aldred MA, McLean DL, Park H, Comhair SA, Greif DM, Erzurum SC, Chun HJ. An endothelial apelin-fgf link mediated by mir-424 and mir-503 is disrupted in pulmonary arterial hypertension. Nature medicine. 2013; 19:74–82.
4. Chandra SM, Razavi H, Kim J, Agrawal R, Kundu RK, de Jesus Perez V, Zamanian RT, Quertermous T, Chun HJ. Disruption of the apelin-apj system worsens hypoxia-induced pulmonary hypertension. Arteriosclerosis, thrombosis, and vascular biology. 2011; 31:814–820.
5. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL, Shi X, Petronis A, George SR, Nguyen T. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. Gene. 1993; 136:355–360. [PubMed: 8294032]
6. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, Kawamata Y, Fukusumi S, Hinuma S, Kitada C, Kurokawa T, Onda H, Fujino M. Isolation and characterization of a novel endogenous peptide ligand for the human apj receptor. Biochemical and biophysical research communications. 1998; 251:471–476. [PubMed: 9792798]

7. Kleinz MJ, Davenport AP. Immunocytochemical localization of the endogenous vasoactive peptide apelin to human vascular and endocardial endothelial cells. Regulatory peptides. 2004; 118:119–125. [PubMed: 15003827]
8. Kleinz MJ, Skepper JN, Davenport AP. Immunocytochemical localisation of the apelin receptor apj, to human cardiomyocytes, vascular smooth muscle and endothelial cells. Regulatory peptides. 2005; 126:233–240. [PubMed: 15664671]
9. Cheng X, Cheng XS, Pang CC. Venous dilator effect of apelin, an endogenous peptide ligand for the orphan apj receptor, in conscious rats. European journal of pharmacology. 2003; 470:171–175. [PubMed: 12798955]
10. Ishida J, Hashimoto T, Hashimoto Y, Nishiwaki S, Iguchi T, Harada S, Sugaya T, Matsuzaki H, Yamamoto R, Shiota N, Okunishi H, Kihara M, Umemura S, Sugiyama F, Yagami K, Kasuya Y, Mochizuki N, Fukamizu A. Regulatory roles for apj, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. The Journal of biological chemistry. 2004; 279:26274–26279. [PubMed: 15087458]
11. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, Osmond DH, George SR, O'Dowd BF. Characterization of apelin, the ligand for the apj receptor. Journal of neurochemistry. 2000; 74:34–41. [PubMed: 10617103]
12. Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, Fujimiya M. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. Regulatory peptides. 2001; 99:87–92. [PubMed: 11384769]
13. Szokodi I, Tavi P, Foldes G, Voutilainen-Myllyla S, Ilves M, Tokola H, Pikkarainen S, Piuhola J, Rysa J, Toth M, Ruskoaho H. Apelin, the novel endogenous ligand of the orphan receptor apj, regulates cardiac contractility. Circulation research. 2002; 91:434–440. [PubMed: 12215493]
14. Cox CM, D'Agostino SL, Miller MK, Heimark RL, Krieg PA. Apelin, the ligand for the endothelial g-protein-coupled receptor apj, is a potent angiogenic factor required for normal vascular development of the frog embryo. Developmental biology. 2006; 296:177–189. [PubMed: 16750822]
15. Inui M, Fukui A, Ito Y, Asashima M. Xapelin and xmsr are required for cardiovascular development in xenopus laevis. Developmental biology. 2006; 298:188–200. [PubMed: 16876154]
16. Kidoya H, Ueno M, Yamada Y, Mochizuki N, Nakata M, Yano T, Fujii R, Takakura N. Spatial and temporal role of the apelin/apj system in the caliber size regulation of blood vessels during angiogenesis. The EMBO journal. 2008; 27:522–534. [PubMed: 18200044]
17. Quertermous T. Apelin and its g protein-coupled receptor regulate cardiac development as well as cardiac function. Developmental cell. 2007; 12:319–320. [PubMed: 17336895]
18. Scott IC, Masri B, D'Amico LA, Jin SW, Jungblut B, Wehman AM, Baier H, Audigier Y, Stainier DY. The g protein-coupled receptor agtrl1b regulates early development of myocardial progenitors. Developmental cell. 2007; 12:403–413. [PubMed: 17336906]
19. Zeng XX, Wilm TP, Sepich DS, Solnica-Krezel L. Apelin and its receptor control heart field formation during zebrafish gastrulation. Developmental cell. 2007; 12:391–402. [PubMed: 17336905]
20. Roberts EM, Newson MJ, Pope GR, Landgraf R, Lolait SJ, O'Carroll AM. Abnormal fluid homeostasis in apelin receptor knockout mice. The Journal of endocrinology. 2009; 202:453–462. [PubMed: 19578099]
21. Charo DN, Ho M, Fajardo G, Kawana M, Kundu RK, Sheikh AY, Finsterbach TP, Leeper NJ, Ernst KV, Chen MM, Ho YD, Chun HJ, Bernstein D, Ashley EA, Quertermous T. Endogenous regulation of cardiovascular function by apelin-apj. American journal of physiology. Heart and circulatory physiology. 2009; 297:H1904–H1913. [PubMed: 19767528]
22. Lin Q, Lu J, Yanagisawa H, Webb R, Lyons GE, Richardson JA, Olson EN. Requirement of the mads-box transcription factor mef2c for vascular development. Development. 1998; 125:4565–4574. [PubMed: 9778514]
23. Naya FJ, Black BL, Wu H, Bassel-Duby R, Richardson JA, Hill JA, Olson EN. Mitochondrial deficiency and cardiac sudden death in mice lacking the mef2a transcription factor. Nat Med. 2002; 8:1303–1309. [PubMed: 12379849]

24. Lin Q, Schwarz J, Bucana C, Olson EN. Control of mouse cardiac morphogenesis and myogenesis by transcription factor mef2c. Science. 1997; 276:1404–1407. [PubMed: 9162005]
25. Devic E, Rizzoti K, Bodin S, Knibiehler B, Audigier Y. Amino acid sequence and embryonic expression of msr/apj, the mouse homolog of xenopus x-msr and human apj. Mechanisms of development. 1999; 84:199–203. [PubMed: 10473142]
26. Kalin RE, Kretz MP, Meyer AM, Kispert A, Heppner FL, Brandli AW. Paracrine and autocrine mechanisms of apelin signaling govern embryonic and tumor angiogenesis. Dev Biol. 2007; 305:599–614. [PubMed: 17412318]
27. McLean DL, Kim J, Kang Y, Shi H, Atkins GB, Jain MK, Chun HJ. Apelin/apjsignaling is a critical regulator of statin effects in vascular endothelial cells--brief report. Arterioscler Thromb Vasc Biol. 2012
28. Wang W, Ha CH, Jhun BS, Wong C, Jain MK, Jin ZG. Fluid shear stress stimulates phosphorylation-dependent nuclear export of hdac5 and mediates expression of klf2 and enos. Blood. 2010; 115:2971–2979. [PubMed: 20042720]
29. Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone MA Jr, Garcia-Cardena G. Integration of flow-dependent endothelial phenotypes by kruppel-like factor 2. J Clin Invest. 2006; 116:49–58. [PubMed: 16341264]
30. Sohn SJ, Li D, Lee LK, Winoto A. Transcriptional regulation of tissue-specific genes by the erk5 mitogen-activated protein kinase. Molecular and cellular biology. 2005; 25:8553–8566. [PubMed: 16166637]
31. Sen-Banerjee S, Mir S, Lin Z, Hamik A, Atkins GB, Das H, Banerjee P, Kumar A, Jain MK. Kruppel-like factor 2 as a novel mediator of statin effects in endothelial cells. Circulation. 2005; 112:720–726. [PubMed: 16043642]
32. Masri B, Lahlou H, Mazarguil H, Knibiehler B, Audigier Y. Apelin (65–77) activates extracellular signal-regulated kinases via a ptx-sensitive g protein. Biochem Biophys Res Commun. 2002; 290:539–545. [PubMed: 11779205]
33. Liu G, Han J, Profirovic J, Strekalova E, Voyno-Yasenetskaya TA. Galpha13 regulates mef2-dependent gene transcription in endothelial cells: Role in angiogenesis. Angiogenesis. 2009; 12:1–15. [PubMed: 19093215]
34. Lu J, McKinsey TA, Nicol RL, Olson EN. Signal-dependent activation of the mef2 transcription factor by dissociation from histone deacetylases. Proc Natl Acad Sci U S A. 2000; 97:4070–4075. [PubMed: 10737771]
35. McKinsey TA, Zhang CL, Lu J, Olson EN. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature. 2000; 408:106–111. [PubMed: 11081517]
36. McLean DL, Kim J, Kang Y, Shi H, Atkins GB, Jain MK, Chun HJ. Apelin/apj signaling is a critical regulator of statin effects in vascular endothelial cells--brief report. Arteriosclerosis, thrombosis, and vascular biology. 2012; 32:2640–2643.
37. Parmar KM, Nambudiri V, Dai G, Larman HB, Gimbrone MA Jr, Garcia-Cardena G. Statins exert endothelial atheroprotective effects via the klf2 transcription factor. The Journal of biological chemistry. 2005; 280:26714–26719. [PubMed: 15878865]
38. Offermanns S, Mancino V, Revel JP, Simon MI. Vascular system defects and impaired cell chemokinesis as a result of galpha13 deficiency. Science. 1997; 275:533–536. [PubMed: 8999798]
39. Ruppel KM, Willison D, Kataoka H, Wang A, Zheng YW, Cornelissen I, Yin L, Xu SM, Coughlin SR. Essential role for galpha13 in endothelial cells during embryonic development. Proc Natl Acad Sci U S A. 2005; 102:8281–8286. [PubMed: 15919816]
40. Griffin CT, Srinivasan Y, Zheng YW, Huang W, Coughlin SR. A role for thrombin receptor signaling in endothelial cells during embryonic development. Science. 2001; 293:1666–1670. [PubMed: 11533492]
41. Yu S, Crawford D, Tsuchihashi T, Behrens TW, Srivastava D. The chemokine receptor cxcr7 functions to regulate cardiac valve remodeling. Dev Dyn. 2011; 240:384–393. [PubMed: 21246655]
42. Sierro F, Biben C, Martinez-Munoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M, Harvey RP, Martinez AC, Mackay CR, Mackay F. Disrupted cardiac

development but normal hematopoiesis in mice deficient in the second cxcl12/sdf-1 receptor, cxcr7. Proc Natl Acad Sci U S A. 2007; 104:14759–14764. [PubMed: 17804806]

43. Kuba K, Zhang L, Imai Y, Arab S, Chen M, Maekawa Y, Leschnik M, Leibbrandt A, Markovic M, Schwaighofer J, Beetz N, Musialek R, Neely GG, Komnenovic V, Kolm U, Metzler B, Ricci R, Hara H, Meixner A, Nghiem M, Chen X, Dawood F, Wong KM, Sarao R, Cukerman E, Kimura A, Hein L, Thalhammer J, Liu PP, Penninger JM. Impaired heart contractility in apelin gene-deficient mice associated with aging and pressure overload. Circ Res. 2007; 101:e32–e42. [PubMed: 17673668]
44. Sheikh AY, Chun HJ, Glassford AJ, Kundu RK, Kutschka I, Ardigo D, Hendry SL, Wagner RA, Chen MM, Ali ZA, Yue P, Huynh DT, Connolly AJ, Pelletier MP, Tsao PS, Robbins RC, Quertermous T. In vivo genetic profiling and cellular localization of apelin reveals a hypoxia-sensitive, endothelial-centered pathway activated in ischemic heart failure. Am J Physiol Heart Circ Physiol. 2008; 294:H88–H98. [PubMed: 17906101]
45. Paskaradevan S, Scott IC. The aplnr gpcr regulates myocardial progenitor development via a novel cell-non-autonomous, galpha(i/o) protein-independent pathway. Biology open. 2012; 1:275–285. [PubMed: 23213418]
46. Kawamata Y, Habata Y, Fukusumi S, Hosoya M, Fujii R, Hinuma S, Nishizawa N, Kitada C, Onda H, Nishimura O, Fujino M. Molecular properties of apelin: Tissue distribution and receptor binding. Biochimica et biophysica acta. 2001; 1538:162–171. [PubMed: 11336787]

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 (GPCR) signaling controls multiple aspects of both vascular and cardiac development.
- Mice with genetic deletion of the GPCR 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 Gα13, which result in cytoplasmic translocation of histone deacetylases HDAC4 and HDAC5 and MEF2 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 knock out of *Apj* in the mouse leads to cardiovascular developmental defects. We identify novel downstream targets of apelin-APJ signaling, including the G protein Gα13. Apelin-APJ signaling activates Gα13 and leads to robust cytoplasmic translocation of HDAC4 and HDAC5 and increases transcriptional activity of the transcription factor MEF2. These findings provide greater insights into the mechanisms of cellular signaling that regulate cardiovascular development.

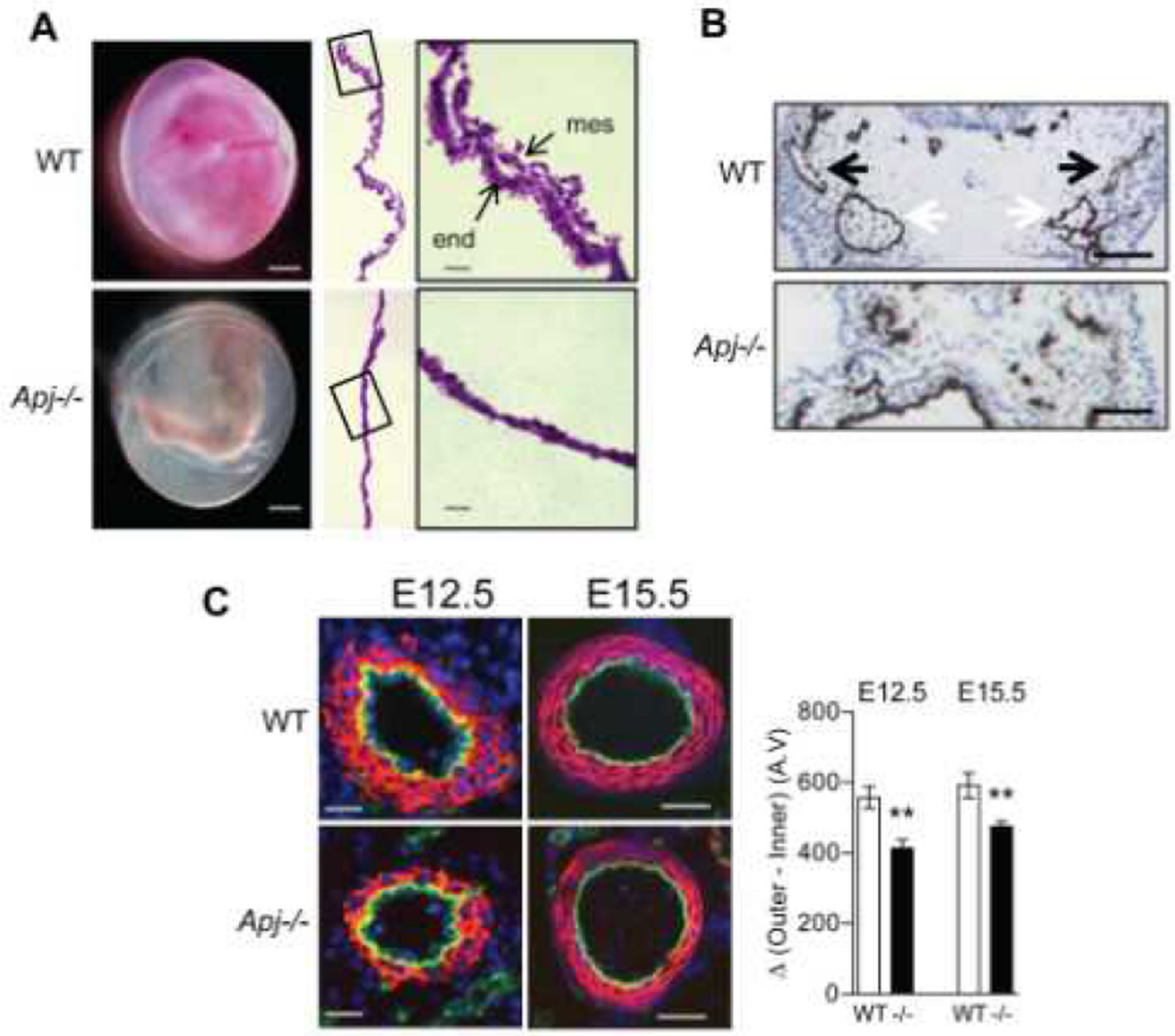

Figure 1. Vascular defects in *Apj*−/− embryos

(A) Yolk sac from E10.5 *Apj*−/− embryos show abnormal yolk sac vasculature compared with wildtype littermates. H&E stain of the yolk sac section shows immature vascular plexus formation. Mes, mesoderm and end, endoderm. White bars indicate 1 mm, black bars indicate 25 μm. (B) CD31 immunohistochemistry of E10.5 *Apj*−/− embryo section shows defective major vessel development in a subset of *Apj*−/− embryos. Dorsal aorta (white arrows) and anterior cardinal veins (black arrows) are identified in the wildtype embryo. Bars indicate 100 μm. (C) CD31 and smooth muscle actin (SMA) stain 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 is shown in blue. The 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. wildtype.

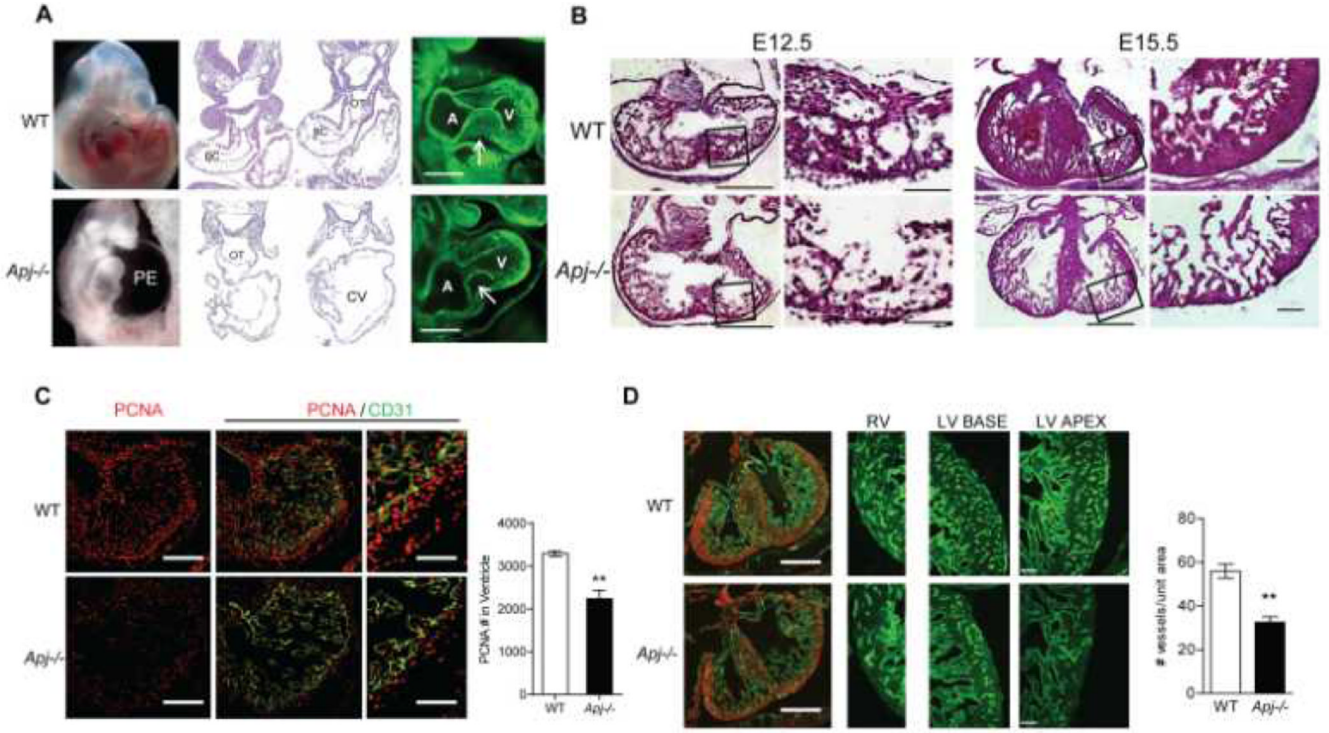

Figure 2. Myocardial defects in *Apj*−/− embryos

(A) E10.5 *Apj*−/− embryos with cardiac developmental defects, including abnormal chamber development and looping, and large pericardial effusions (PE). H&E 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 panels show whole mount CD31 staining (green) of E10.5 embryos showing defective atrioventricular cushion formation in *Apj*−/− embryos (n=6 per group). A, atrium and V, ventricle. Scale bars represent 170 μm. (B) E12.5 and E15.5 *Apj*−/− embryos have significant thinning of ventricular walls. High magnifications of left ventricular walls are shown in the right panels (n=4–7 per group at each timepoint). Scale bars show 500 μm (low magnification) and 100 μm (high magnification). (C) E12.5 *Apj*−/− hearts have significant reduction in number of PCNA+ (red) cells (n=4–5 per group). CD31 staining is shown in green. Higher magnifications of left ventricle wall are shown in the right panels. ** $P<0.01$ vs. wildtype. Bars indicate 200 μm (low magnification) and 100 μm (high magnification). D) E15.5 *Apj*−/− hearts have significantly decreased capillary densities in their ventricular walls compared to their wildtype littermates (n=4–6 per group). CD31 staining is shown in green, SMA staining is shown in red. ** $P<0.01$ vs. wildtype. Scale bars show 500 μm (low magnification) and 100 μm (high magnification).

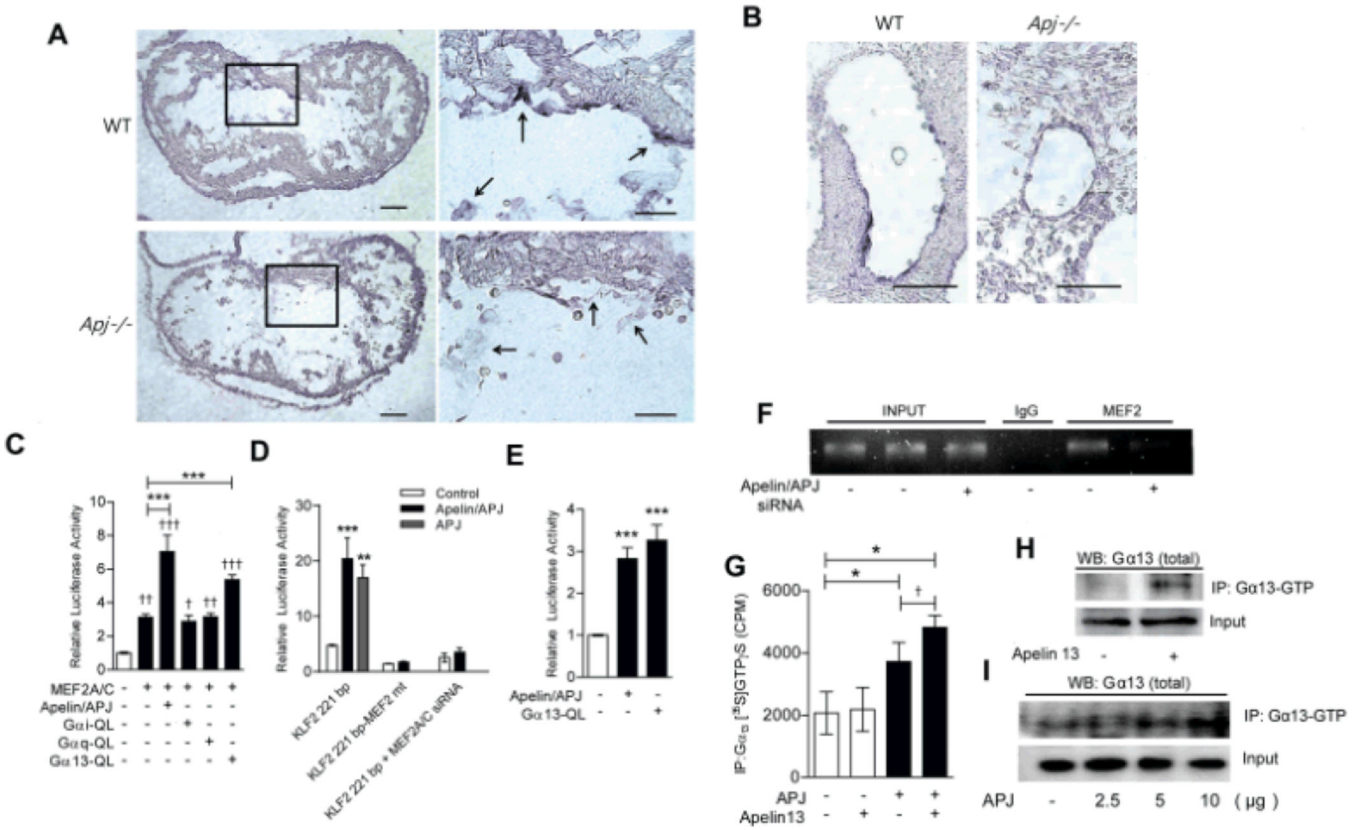

Figure 3. Apelin and APJ induce MEF2A/C transcriptional activity

(A) *In situ* hybridization for *Klf2* in E10.5 embryos show decreased endocardial (black arrows) *Klf2* expression in *Apj−/−* embryos vs. wildtype 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 *Apj−/−* embryos vs. wildtype littermates. (n=3 embryos per group). Bars indicate 100 μm. (C) MEF2 luciferase reporter containing three tandem MEF2 binding sites is induced by MEF2A/C overexpression in COS7 cells, and induced further by apelin-APJ or constitutively active Gα13 (Gα13-QL), but not by constitutively active Gαi (Gαi-QL) or Gαq (Gαq-QL). ***P<0.001 vs. MEF2A/C alone, $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 and $^{\dagger\dagger\dagger}P$<0.001 vs. control. (D) Transfection of apelin and APJ or APJ alone in 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 by concurrent knockdown of MEF2A and MEF2C (MEF2A/C siRNA). **P<0.01 and ***P<0.001 vs. vector control. (E) Apelin-APJ or Gα13-QL overexpression in HUVECs lead to a significant induction of the 41 basepair KLF2 promoter containing the minimal MEF2 binding site. ***P<0.001 vs. control. (F) ChIP assay shows that MEF2 binding to the KLF2 promoter is reduced in the context of apelin-APJ knockdown in HUVECs. (G) APJ overexpression leads to a significant increase of Gα13 activation in COS7 cells transfected with wildtype Gα13. Stimulation with apelin 13 leads to further activation of Gα13 in APJ transfected cells. *P<0.05 vs. vector control, and $^{\dagger}P$<0.05 vs. APJ without apelin 13 stimulation. (H) Stimulation with apelin 13 leads to an increase in active Gα13 in HUVECs. (I) Transfection of APJ in HUVECs lead to increased levels of active Gα13 in a dosage dependent manner.

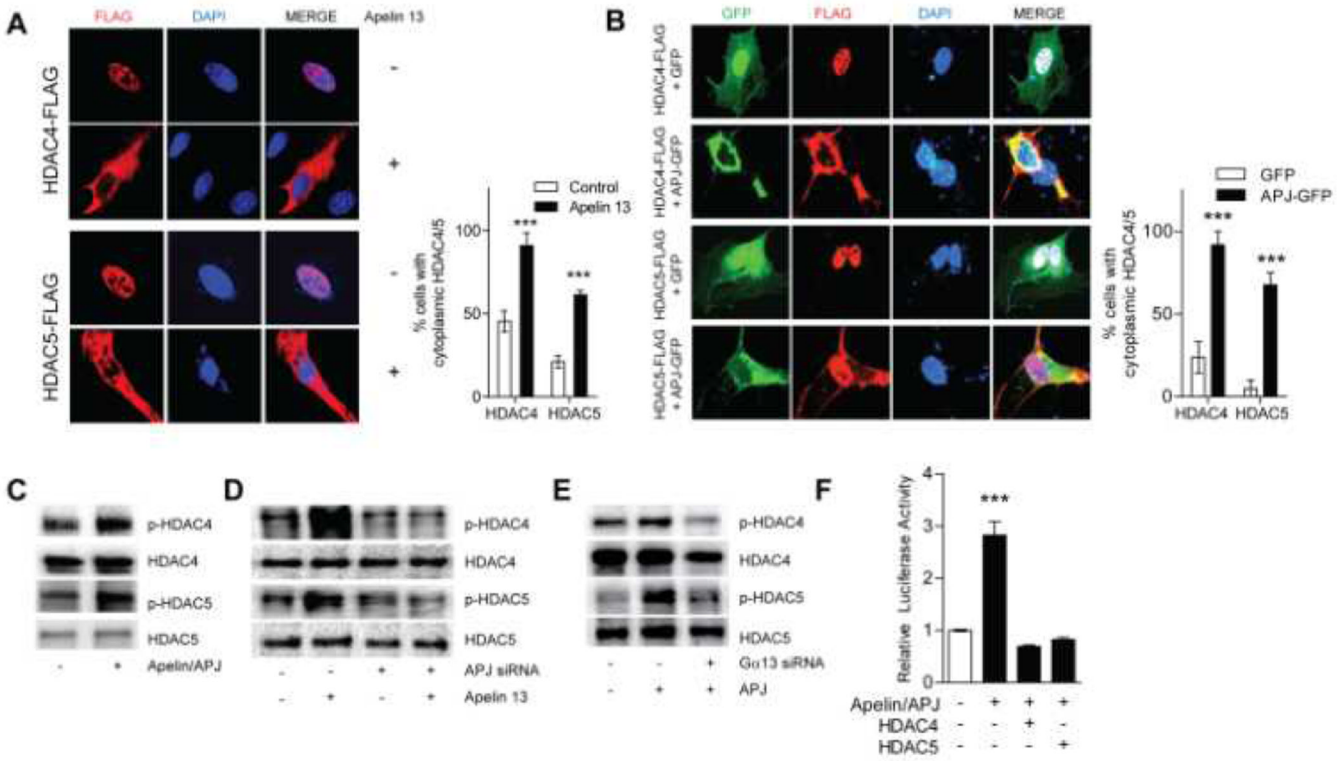

Figure 4. Apelin-APJ induces HDAC4/5 phosphorylation and cytoplasmic translocation

(A) Apelin 13 stimulation (1 μM for 1 h) leads to HDAC4 and HDAC5 (red) cytoplasmic translocation in HUVECs transfected with FLAG-tagged HDAC4/5. The percent of cells with cytoplasmic HDAC4/5 in response to apelin 13 stimulation is shown. DAPI (blue) nuclear stain is also shown. ***P<0.001. (B) Overexpression of APJ in HUVECs leads to HDAC4 and HDAC5 cytoplasmic translocation. Cells were transfected with either GFP or APJ-GFP, and co-transfected with FLAG tagged HDAC4 or HDAC5. GFP is shown in green, FLAG staining is shown in red, DAPI nuclear staining is shown in blue. The percent of cells with cytoplasmic HDAC4/5 is shown. ***P<0.001. (C) Overexpression of apelin and APJ in HUVECs lead to increase in HDAC4 and HDAC5 phosphorylation. (D) Stimulation of HUVECs with apelin 13 leads to increased HDAC4 and HDAC5 phosphorylation, which is abrogated in the context of APJ knockdown. (E) Overexpression of APJ in HUVECs leads to increased phosphorylation of HDAC4 and HDAC5, which is decreased with concurrent Gα13 knockdown. (F) Concurrent overexpression of HDAC4 or HDAC5 with apelin-APJ lead to abrogation of apelin-APJ induced activation of the KLF2–41bp luciferase reporter in HUVECs. ***P<0.001 vs. all other conditions.

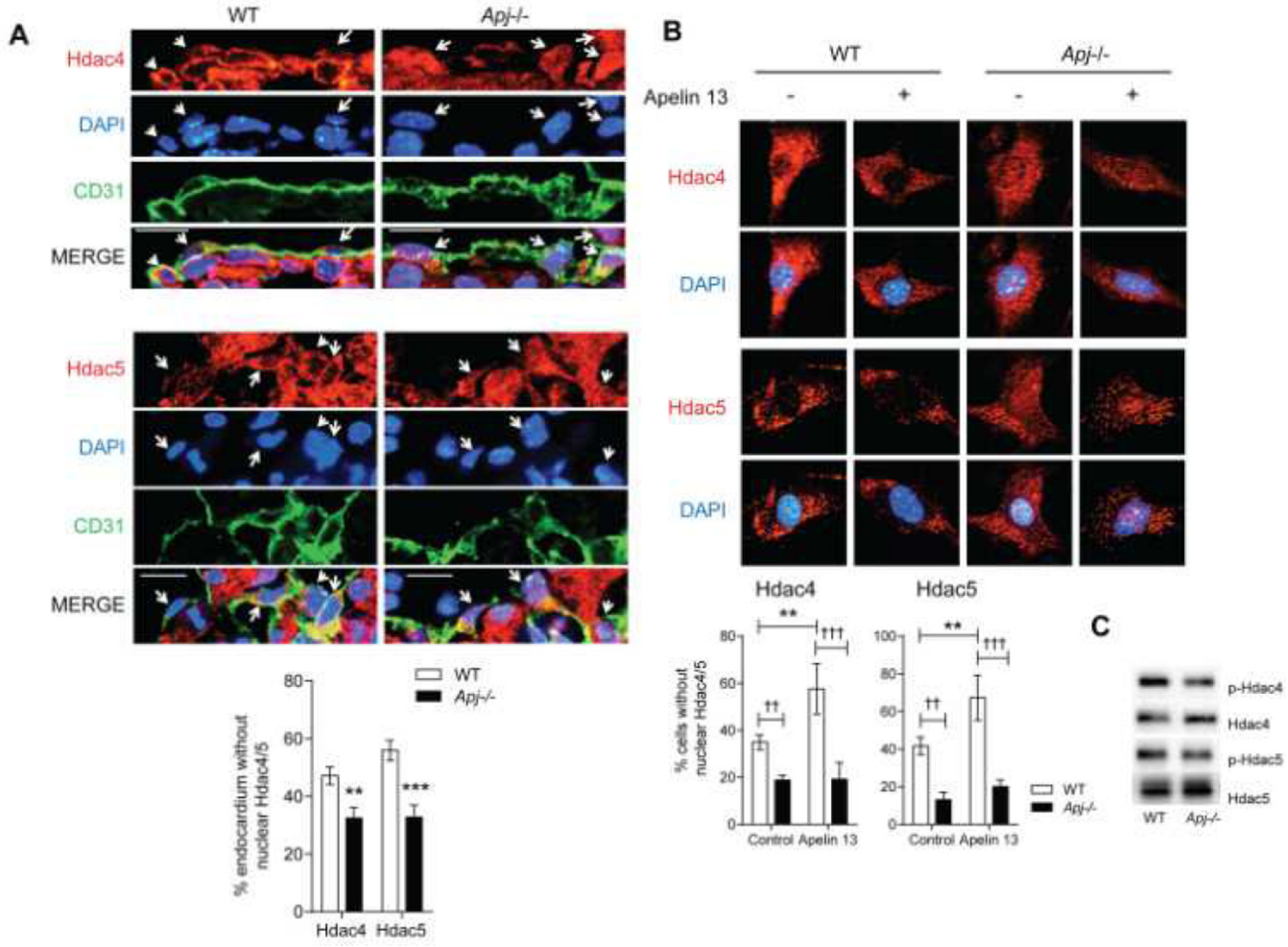

Figure 5. Apj−/− embryos have significantly higher level of nuclear Hdac4 and Hdac5

(A) Significantly greater number of endocardial cells from E10.5 *Apj−/−* embryos have nuclear Hdac4 and Hdac5 (red) staining compared to wildtype embryos. DAPI (blue) and CD31 (green) staining are shown (n=4–5 embryos per group). ***P*<0.01 and ****P*<0.001. (B) Isolated heart ECs demonstrate greater percentage of cells lacking nuclear Hdac4 or Hdac5 staining from wildtype mice vs. *Apj−/−* mice. Only isolated ECs from wildtype 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 wildtype, †††*P*<0.001 vs. apelin 13 stimulated wildtype. (C) The level of phosphorylated Hdac4 and Hdac5 are decreased in heart ECs from *Apj−/−* mice.

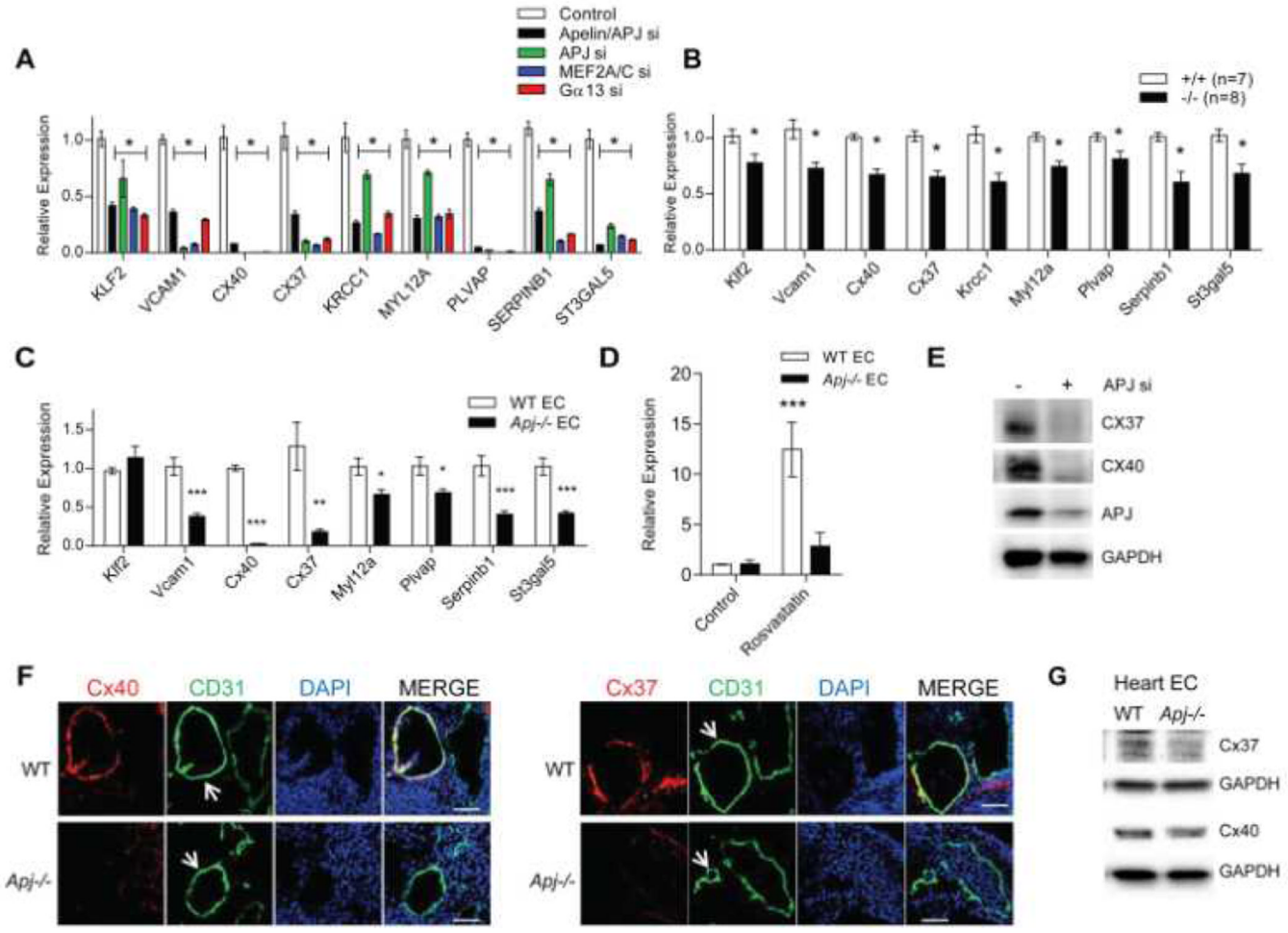

Figure 6. Shared targets of apelin-APJ, Gα13, and MEF2 in ECs are downregulated in *Apj*−/− embryos and isolated ECs

(A) Knockdown of apelin/APJ, APJ, MEF2A/C or Gα13 in HUVECs leads to decreased mRNA expression of validated and putative MEF2 target genes. **P*<0.05 for each knockdown condition compared to control. (B) Transcripts decreased in HUVECs subjected to apelin-APJ, APJ, MEF2A/C or Gα13 knockdown are also significantly decreased in E10.5 *Apj*−/− embryos. **P*<0.05 vs. wildtype. (C) Isolated heart ECs from *Apj*−/− mice demonstrate decreased expression of gene transcripts identified from the microarray analyses. **P*<0.05, ***P*<0.01 and ****P*<0.001. (D) KLF2 expression is significantly induced by rosuvastatin in isolated heart ECs from wildtype mice but not from *Apj*−/− mice. ****P*<0.001 vs. all other conditions. (E) Expression of CX37 and CX40 are decreased in HUVECs subjected to APJ knockdown. (F) Cx37 and Cx40 in CD31 staining ECs of the dorsal aorta (white arrows) are decreased in E10.5 *Apj*−/− embryos (n=4–5 embryos per group). Cx37/40 staining is shown in red, CD31 is shown in green, and DAPI is shown in blue. Bar indicates 80 μm. (G) Decreased expression of Cx37 and Cx40 in heart ECs of *Apj* −/− mice.

Table 1

Embryonic stage genotypes from *Apj+/−* mating.

Developmental stage	Total	+/+	+/−	−/−	dead −/−
E9.5	11	3	3	5	0
E10.5	143	39	61	43	11
E11.5	108	29	46	33	13
E12.5	35	7	20	8	3
E15.5	17	3	10	4	0
E17.5	18	4	13	1	0
P1	84	23	50	11	3
3-wk postnatal	238	77	139	22	0