Lactation Is a Risk Factor of Postpartum Heart Failure in Mice with Cardiomyocyte-specific Apelin Receptor (APJ) Overexpression

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The G protein-coupled receptor APJ and its ligand apelin are highly expressed in cardiovascular tissues and are associated with the regulation of blood pressure and cardiac function. Although accumulating evidence suggests that APJ plays a crucial role in the heart, it remains unclear whether up-regulation of APJ affects cardiac function. Here we generated cardiomyocyte-specific APJ-overexpressing (APJ-TG) mice and investigated the cardiac phenotype in APJ-TG mice. Male and non-pregnant APJ-TG mice showed cardiac hypertrophy, contractile dysfunction, and elevation of B-type natriuretic peptide gene expression in the heart but not cardiac fibrosis and symptoms of heart failure, including breathing abnormality and pleural effusion. We further examined the influence of APJ overexpression in response to physiological stress induced by pregnancy and lactation in the heart. Interestingly, repeating pregnancy and lactation (pregnancy-lactation cycle) exacerbated cardiac hypertrophy and systolic dysfunction and induced cardiac fibrosis, lung congestion, pleural effusion, and abnormal breathing in APJ-TG mice. These data indicate that female APJ-TG mice develop postpartum cardiomyopathy. We showed that lactation, but not parturition, was critical for the onset of postpartum cardiomyopathy in APJ-TG mice. Furthermore, we found that lactating APJ-TG mice showed impaired myocardial angiogenesis and imbalance of pro- and antiangiogenic gene expression in the heart. These results demonstrate that overexpression of APJ in cardiomyocytes has adverse effects on cardiac function in male and non-pregnant mice and that lactation contributes to the development of postpartum cardiomyopathy in the heart with APJ overexpression.

Pregnancy and lactation are essential processes in the reproduction of mammals and induce marked changes in systemic hormone and hemodynamic status, consequently affecting cardiac function (1, 2). Pregnant women show increases in circulating blood volume, heart rate, and cardiac output to provide sufficient blood to their fetuses (3). It has been reported that elevation of cardiac output and adaptive hypertrophy are observed in lactating rats for supplying blood to the mammary gland (4–6). Furthermore, we reported previously that lactation causes contractile dysfunction in pregnancy-associated hypertension in mice (7). Thus, pregnancy and lactation are closely related to alteration of cardiac function.

Although maternal cardiac function is normally maintained throughout the peripartum period, in some women, heart failure of unknown etiology occurs between the last month of pregnancy and the early postpartum period, known as postpartum cardiomyopathy, which is called postpartum cardiomyopathy in the case of developing heart failure during the postpartum period (8, 9). Several factors, such as viral infection, autoimmune responses, and hypertensive complications in pregnancy, have been considered triggers of peripartum cardiomyopathy (10–12). Recent works have demonstrated the existence of a familial form of peripartum cardiomyopathy (13–15), whereas, formerly, peripartum cardiomyopathy was defined as the non-familial form of cardiomyopathy. Moreover, studies using genetically engineered mouse models revealed that cardiomyocyte-specific overexpression of Gαq, one of the G proteins, induces marked cardiomyocyte apoptosis in the peripartum period, resulting in peripartum cardiomyopathy (16–18). Other studies have shown that cardiomyocyte-specific deletion of STAT3 or PGC1α (peroxisome proliferator-activated receptor γ, coactivator 1 α) in mice causes impaired cardiac angiogenesis and leads to onset of postpartum cardiomyopathy (19, 20). Thus, the pathogenesis of peripartum cardiomyopathy is highly complex and not fully understood.

APJ (also known as apelin receptor, APLNR, or AGTRL1) is one of the G-protein coupled receptors and is highly expressed in cardiovascular tissues (21, 22). It has been reported that APJ and its ligand apelin are involved in the regulation of blood pressure, angiogenesis, and maintenance of cardiac function (21–24). Apelin administration increases cardiac contractility in isolated working heart models (25). Furthermore, apelin KO mice and APJ-KO mice show reduced cardiac contractility (26, 27). In addition to a positive inotropic effect, the apelin-APJ system also has cardioprotective effects against ischemia-reperfusion injury and anticancer drug-induced cardiotoxicity (28, 29). Although several lines of evidence suggest critical roles of the apelin-APJ system in the heart, the effect of increased APJ expression on cardiac function is not elucidated.

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* This article contains supplemental Movies S1–S3.
† This article contains supplemental Movies S4–S5.
‡ The G protein-coupled receptor APJ and its ligand apelin are highly expressed in cardiovascular tissues and are associated with the regulation of blood pressure and cardiac function. Although accumulating evidence suggests that APJ plays a crucial role in the heart, it remains unclear whether up-regulation of APJ affects cardiac function. Here we generated cardiomyocyte-specific APJ-overexpressing (APJ-TG) mice and investigated the cardiac phenotype in APJ-TG mice. Male and non-pregnant APJ-TG mice showed cardiac hypertrophy, contractile dysfunction, and elevation of B-type natriuretic peptide gene expression in the heart but not cardiac fibrosis and symptoms of heart failure, including breathing abnormality and pleural effusion. We further examined the influence of APJ overexpression in response to physiological stress induced by pregnancy and lactation in the heart. Interestingly, repeating pregnancy and lactation (pregnancy-lactation cycle) exacerbated cardiac hypertrophy and systolic dysfunction and induced cardiac fibrosis, lung congestion, pleural effusion, and abnormal breathing in APJ-TG mice. These data indicate that female APJ-TG mice develop postpartum cardiomyopathy. We showed that lactation, but not parturition, was critical for the onset of postpartum cardiomyopathy in APJ-TG mice. Furthermore, we found that lactating APJ-TG mice showed impaired myocardial angiogenesis and imbalance of pro- and antiangiogenic gene expression in the heart. These results demonstrate that overexpression of APJ in cardiomyocytes has adverse effects on cardiac function in male and non-pregnant mice and that lactation contributes to the development of postpartum cardiomyopathy in the heart with APJ overexpression.

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Postpartum Heart Failure Induced by APJ and Lactation

In this study, to investigate the effect of increased APJ expression on cardiac function, we generated cardiomyocyte-specific APJ-overexpressing (APJ-TG) mice. Surprisingly, we found that female APJ-TG mice develop severe heart failure in the postpartum period and that lactation is a key factor in the pathogenesis of heart failure in postpartum APJ-TG mice.

Experimental Procedures

Animals—Human APJ cDNA (30) was subcloned into α-myosin heavy chain promoter-containing expression vector (a gift from Prof. Jeffrey Robbins, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH) (31). The linearized DNA (10 kb) was microinjected as a transgene into the pronuclei of eggs from C57BL/6J mice. Mice were genotyped by Southern blotting. Briefly, the genomic DNA was prepared from the tails of mice, and 1 µg of DNA was digested with EcoRI and BglII. Digested DNA was separated by 0.7% agarose gel electrophoresis, transferred to a positively charged nylon membrane using alkaline buffer, and hybridized with the [α-32P]dCTP-labeled probe for the 5’ side of the mouse APJ coding sequence (APJ probe, Fig. 1A). This probe can recognize both the mouse and human APJ gene because of the high homology of the DNA sequence. After washing and drying, the membrane was exposed to the imaging plate. The image was obtained using Typhoon 8600 and ImageQuant software (GE Healthcare). To analyze cardiac function, echocardiography was performed as described previously (7). Animal experiments were performed in a humane manner and approved by the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were conducted in accordance with the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for the Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Histological Analysis—Harvested hearts were fixed in 4% paraformaldehyde for 48 h at 4 °C, washed with PBS, dehydrated, and embedded in paraffin. H&E and Masson trichrome stains were performed as described previously (29, 7). Immunohistochemistry for APJ was carried out as described previously (29). Briefly, fresh-frozen hearts were sectioned into 10-µm sections and dried at room temperature. Heart sections were fixed in 4% paraformaldehyde for 10 min at room temperature and blocked in 5% BSA for 30 min at room temperature. After endogenous avidin-biotin blocking (415041, Nichirei Biosciences, Tokyo, Japan), sections were incubated with anti-APJ antibody (1:10, rabbit polyclonal, homemade) at 4 °C overnight. Sections were washed in 0.5 M NaCl/0.05% Tween 20/PBS (−) solution three times and incubated with biotinylated donkey anti-rabbit antibody (111-065-144, Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min at room temperature. For detecting biotinylated antibody, sections were incubated with CF488A streptavidin conjugate (29034, Biotium, Hayward, CA) for 30 min at room temperature. To visualize the plasma membrane and nuclei, we used CF594 wheat germ agglutinin (WGA, 29023, Biotium) and Hoechst 33258. Using a confocal laser-scanning microscope (Fluoview FV10i, Olympus, Tokyo, Japan), we obtained fluorescence images. For measuring cross-sectional areas, deparaffinized cardiac sections were stained with CF594-conjugated WGA. Fluorescence images were obtained using a confocal microscope and analyzed with ImageJ software (http://imagej.nih.gov/ij/). Fifty cardiomyocytes per section were evaluated.

Capillary Density—For measuring capillary density, deparaffinized cardiac sections were treated with 20 µg/ml proteinase K for 30 min at 37 °C. To inactivate endogenous peroxidase, sections were treated with 3% hydrogen peroxide in methanol for 15 min at room temperature. Sections were blocked with tyramide signal amplification blocking reagent (FP1020, PerkinElmer Life Sciences) for 30 min at room temperature and incubated with anti-CD31 antibody (1:50, rat polyclonal, 550274, BD Biosciences) for 60 min at room temperature. After incubating biotinylated anti-rat IgG antibody (BA-4001, Vector Laboratories, Burlingame, CA) for 30 min at room temperature, secondary antibodies were detected using the tyramide signal amplification biotin system (NEL700A, PerkinElmer Life Sciences) according to the instructions of the manufacturer. To visualize the plasma membrane and nuclei, sections were stained with CF594-conjugated WGA and Hoechst 33258. Images were obtained using BZ-9000 (Bio-erove, Keyence, Osaka, Japan). The number of capillaries per 50 cardiomyocytes was determined in 10 different randomly chosen areas using ImageJ software.

TUNEL Assay—The TUNEL assay was performed using an in situ cell death detection kit, TMR Red (12156792910, Roche Diagnostics) according to the instructions of the manufacturer. Briefly, deparaffinized heart sections (5 µm) were incubated with 20 µg/ml proteinase K for 15 min at 37 °C. DNA fragments were labeled with tetramethylrhodamine-dUTP using terminal deoxynucleotidyltransferase for 1 h at 37 °C. For nuclear counterstaining, sections were stained with Hoechst 33258. Fluorescence images were acquired using BZ-9000. The numbers of TUNEL-positive cells were determined in 10 random fields using a BZ-II analyzer (Keyence). Data were represented as the percentage of TUNEL-positive cells per total number of nuclei.

Northern Blotting—Total RNA was extracted from frozen heart tissues using Isogen (311-02501, Nippon Gene, Tokyo, Japan). After glyoxylation of RNA, 10 µg of total RNA was separated by 1.2% agarose gel electrophoresis, transferred to a positively charged nylon membrane, and hybridized with [α-32P]dCTP-labeled APJ probe. After washing and drying, the membrane was exposed to the imaging plate. The image was obtained using Typhoon 8600 and ImageQuant software (GE Healthcare).

Quantitative Real-time PCR analysis—Quantitative RT-PCR was performed as described previously (7). The expression levels of atrial natriuretic peptide (ANP, Nppa), B-type natriuretic peptide (BNP, Nppb), collagen I (Col1a1), α-myosin heavy chain (Myh6), and β-myosin heavy chain (Myh7) were determined as the number of transcripts relative to those of lar posterior wall; NCX, sodium-calcium exchanger.

2 The abbreviations used are: WGA, wheat germ agglutinin; HW, heart weight; BW, body weight; LV, left ventricular; LVPW, left ventricular posterior wall; NP, non-pregnant; Preg, pregnant; 4W-PP, 4 weeks postpartum; NCX, sodium-calcium exchanger.

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Gapdh. The levels of Vegfa, Angpt1, Angpt2, Pdgfa, Pdgfb, Thbs1, and Thbs2 were determined as the number of transcripts relative to those of the Hprt gene. The primer sequences were as follows: mNppa, 5’-GTTAGGATTGACAGATTG-GAG-3’ (forward) and 5’-GCAGATACGTGCTTTTCTC-3’ (reverse); mNppb, 5’-GGGCTGTTAAGCCGACTGAG-3’ (forward) and 5’-ACCTCAAGTGTTCCAGAG-3’ (reverse); mCol1a1, 5’-GATGGATTGACAGGATTG-3’ (forward) and 5’-GATGGACTGCTTTTTCGTC-3’ (reverse); mMyh7, 5’-CACGAGCATCGCTAAGCGTCA-3’ (forward) and 5’-TGGGCTGGGTTGTAATGGA-3’ (reverse); mGapdh, 5’-ACTTCAAAGGTGGTCCCAGAG-3’ (forward) and 5’-TGGGCTGGGTTGTAATGGA-3’ (reverse); mThbs1, 5’-TCCGTAGATGAAGATGGTGAAG-3’ (forward) and 5’-ACCTCGCTGGTGAAGAGGAT-3’ (reverse); mMyh7, 5’-ACCTCGCTGGTGAAGAGGAT-3’ (forward) and 5’-TCATCGTACAGCAGCCCAAG-3’ (reverse); mHprt, 5’-GATGGATTGACAGGATTG-3’ (forward) and 5’-GATGGACTGCTTTTTCGTC-3’ (reverse); mHprt, 5’-TCATCGTACAGCAGCCCAAG-3’ (forward) and 5’-TCATCGTACAGCAGCCCAAG-3’ (reverse); mThbs2, 5’-TCGCGATCGTGAAGATTTC-3’ (forward) and 5’-TCATCGTACAGCAGCCCAAG-3’ (reverse); and mHprt, 5’-TCGCGATCGTGAAGATTTC-3’ (forward) and 5’-TCATCGTACAGCAGCCCAAG-3’ (reverse).

**Western Blotting—**Mouse hearts were harvested and immediately frozen in liquid nitrogen for storage at −80 °C. Cardiac tissues were powdered by a multibead shaker (Yasui Kikai, Osaka, Japan) and homogenized in radiomimmunoprecipitation assay buffer containing phosphatase and protease inhibitors (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 20 mM β-glycerophosphate, 2.1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). After incubation for 30 min, samples were centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was transferred to new tubes, and protein concentration was determined using protein assay dye reagent concentrate and protein standard I (500-0005 and 500-0006, Bio-Rad). Samples were mixed with 2× Laemmli sample buffer (100 mM Tris-HCl, 2% SDS, 100 mM DTT, 20% glycerol, and 0.01% bromphenol blue) and incubated for 5 min at 99 °C. Protein samples (50–100 μg) were loaded, subjected to SDS-PAGE, and transferred onto a PVDF membrane. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T) for 1 h at room temperature. Primary antibodies used in this study included anti-STAT3 (1:1000, 610189, BD Biosciences), anti-phospho-STAT3 (Tyr-705, 1:500, 9131, Cell Signaling Technology, Danvers, MA), anti-cathepsin D (1:1000, ab75852, Abcam, Cambridge, UK), anti-PGC1α (1:500, sc-13067, Santa Cruz Biotechnology, Dallas, TX), anti-phospho-AKT (Ser-473, 1:500, 4060, Cell Signaling Technology), anti-AKT (1:1000, 2920, Cell Signaling Technology), anti-phospho-ERK1/2 (Thr-202/Tyr-204, 1:500, 9101, Cell Signaling Technology), anti-ERK2 (1:500, 05-157, Millipore), anti-GAPDH (1:2000, 05-50118, American Research Products, Waltham, MA), and anti-α-Tubulin (1:5000, T5168, Sigma). Membranes were washed in TBS-T and incubated with horseradish peroxidase-linked secondary antibody (GE Healthcare) diluted in 0.5% nonfat dry milk in TBS-T for 1 h at room temperature. After secondary antibody incubation, the membrane was washed in TBS-T and visualized using SuperSignal West Femto chemiluminescent substrate (34094, Thermo Fisher Scientific, Waltham, MA). Chemiluminescence signals were detected using a Fuji LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

**Plasma Apelin Measurement—**Plasma samples were collected from the blood of non-pregnant and postpartum female mice by centrifugation at 3000 rpm, frozen immediately, and stored at −80 °C. Plasma apelin levels were determined by using an apelin-12 enzyme immunoassay kit (EK-057-23, Phoenix Pharmaceuticals, Burlingame, CA) according to the protocol of the manufacturer.

**Statistical Analysis—**Statistical analysis was performed using GraphPad Prism 5 (GraphPad Prism Software, La Jolla, CA). The data were analyzed with Student’s t test, one-way analysis of variance with Tukey’s post-hoc test, and two-way analysis of variance followed by Bonferroni multiple comparison test. Significant differences were defined as p < 0.05.

**Results**

**Establishment of Cardiomyocyte-specific APJ-overexpressing Mice—**We generated two lines of transgenic mice overexpressing the human APJ gene in cardiomyocytes using the α-myosin heavy chain promotor (Fig. 1A). Southern blotting analysis revealed transgene integration into the genomic DNA of line 25 and line 37 transgenic mice (Fig. 1B). Transgene-derived human APJ mRNA was detected in the hearts of both line 25 and line 37 mice (Fig. 1C). Unexpectedly, line 37 homozygous transgenic mice showed rectal prolapse with high frequency (data not shown). Therefore, we have shown data from line 25 transgenic mice in subsequent experiments unless otherwise noted. Next, we examined APJ protein expression in the heart by immunohistochemistry. Consistent with our previous report (29), in WT mice, APJ protein was expressed in the plasma membrane of cardiomyocytes in a patchy fashion (Fig. 1D, arrowheads). In the hearts of homozygous APJ transgenic mice, intense fluorescence was detected in the whole plasma membrane of cardiomyocytes but not in the coronary artery (Fig. 1D). These data demonstrate that APJ is overexpressed in cardiomyocytes and localized to the plasma membrane in the hearts of APJ-TG mice.

**Overexpression of APJ Causes Cardiac Hypertrophy and Contractile Dysfunction in Male Mice—**APJ-TG mice were born at a normal Mendelian ratio (data not shown), and the body weight was comparable between male WT and APJ-TG mice at ages of 3 and 6 months (Fig. 2A). On the other hand, the heart weight (HW) and heart weight per body weight (BW) ratio were increased in male APJ-TG mice compared with WT mice at ages of 3 and 6 months (Fig. 2 B and C). Echocardiographic
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analysis revealed that male APJ-TG mice exhibited a reduction of fractional shortening and elevation of left ventricular internal dimension relative to WT mice (Fig. 2, D–F, and supplemental Movie S1). Although there were no significant differences in diastolic left ventricular anterior wall (LVAW) and left ventricular posterior wall (LVPW) thickness between WT and APJ-TG mice at the age of 3 months, slight reductions in systolic LVAW thickness and LVPW thickness were observed in APJ-TG mice compared with WT mice at the age of 6 months (Fig. 2, G–J). Macroscopic and histological analyses showed that cardiac hypertrophy and enlargement of cardiac chamber occurred in APJ-TG mice, whereas no obvious fibrosis was seen in 6-month-old WT and APJ-TG mice (Fig. 2K). There were no significant changes in mean cross-sectional areas of myofibers between WT and APJ-TG mice (Fig. 2L). These data indicate that APJ-TG mice develop eccentric cardiac hypertrophy. Furthermore, compared with WT mice, cardiac BNP (Nppb) and β-myosin heavy chain (Myh7) gene expression levels were significantly increased in APJ-TG mice at ages of 3 and 6 months (Fig. 2M). Collagen I (Col1a1) expression was significantly increased in 3-month-old APJ-TG mice and tended to be increased in 6-month-old APJ-TG mice compared with WT mice (Fig. 2M). There were no significant differences in ANP (Nppa) and α-myosin heavy chain (Myh6) gene expression levels between WT and APJ-TG mice (Fig. 2M). These results suggest that APJ overexpression in cardiomyocytes causes pathological cardiac hypertrophy and contractile dysfunction in male mice. However, it seems likely that these pathological cardiac phenotypes are not serious because APJ-TG mice did not show cardiac fibrosis, elevation of ANP expression, and other features of heart failure, such as abnormal breathing and pleural effusion. More importantly, cardiac hypertrophy and contractile dysfunction did not get worse by aging in male APJ-TG mice (Fig. 2, C and D).

APJ Overexpression Induces Postpartum Cardiomyopathy in Female Mice—Pregnancy and lactation are considered physiological stress on the maternal heart (1, 2, 19). Next, to investigate whether APJ overexpression influences the response to physiological stress during pregnancy and lactation, we analyzed female APJ-TG mice. Non-pregnant female APJ-TG mice developed cardiac hypertrophy and contractile dysfunction, as did male APJ-TG mice (Fig. 3, A–F, and supplemental Video S2, NP). Although HW/BW was decreased in pregnant mice because of body weight gain, pregnancy did not affect HW and fractional shortening in WT and APJ-TG mice (Fig. 3, A–F, and supplemental Movie S2, Preg). 4 weeks after parturition (4 weeks postpartum (4W-PP)), WT mice that had finished breastfeeding their pups showed significant increases in HW and HW/BW compared with non-pregnant WT mice (Fig. 3, A–D, Parity 1), whereas their fractional shortening was maintained (Fig. 3, E and F, and supplemental Movie S2, Parity 1). By contrast, APJ-TG mice exhibited a reduction of cardiac contractility with increases in HW and HW/BW at 4 weeks postpartum (Fig. 3, A–F, and supplemental Movie S2, Parity 1). The cross-sectional areas of myofibers were increased in both WT and APJ-TG mice at 4 weeks postpartum compared with non-pregnant mice, whereas there were no significant changes between WT and APJ-TG mice (Fig. 3G).
Next we investigated the effect of subsequent pregnancy and lactation on cardiac function. In WT mice, subsequent pregnancy-lactation cycles increased HW, whereas HW/BW and fractional shortening were not affected, indicating that WT mice showed physiological hypertrophy (Fig. 3, A–F, and supplemental Movie S2, Parity 2 and Parity 3). However, in APJ-TG mice, a further increase in HW/BW and reduction of fractional shortening were induced by repeated pregnancy-lactation cycles (Fig. 3, A–F, and supplemental Movie S2, Parity 2 and Parity 3). Because fractional shortening of 6-month-old non-pregnant APJ-TG mice was similar to that of 2-month-old mice (Fig. 3F), the decrease of cardiac contractility in parous APJ-TG mice was not due to aging stress. Moreover, APJ-TG mice that had experienced pregnancy and lactation more than twice exhibited lung congestion and cardiac fibrosis (Fig. 3, H and I). In addition, abnormal breathing and pleural effusion were observed in APJ-TG mice that experienced three pregnancy-lactation cycles (supplemental Movie S3 and Fig. 3J).
These results indicate that physiological stress induced by pregnancy and lactation causes postpartum cardiomyopathy in APJ-overexpressing mice.

Lactation Induces Postpartum Cardiomyopathy in APJ-TG Mice—Although we found that APJ-TG mice show cardiomyopathy in the postpartum period, it remained unclear whether parturition or lactation or both induce heart failure in APJ-TG mice. To determine the critical inducer of postpartum cardiomyopathy in APJ-TG mice, we analyzed postpartum dams with or without lactation. As mentioned above, lactation induced cardiac hypertrophy in both WT and APJ-TG mice, whereas dams without lactation did not show elevation of HW/BW compared with non-pregnant mice (Fig. 4, A and B). Fractional shortening of APJ-TG mice without lactation was significantly higher than that of APJ-TG mice with lactation and was comparable with that of non-pregnant APJ-TG mice (Fig. 4C). It should be noted that, in lactating APJ-TG mice, cardiac contractility at 4 weeks postpartum did not show a fur-
ther decrease compared with the level at 3 weeks postpartum (Fig. 4C, 3W-PP, 4W-PP Lac (+)). Considering that pups began to eat food pellets rather than drinking milk around 3 weeks postpartum, the cardiac function of APJ-TG dams was not affected by lactation from 3–4 weeks postpartum. Lactation-dependent cardiac hypertrophy and reduction of cardiac contractility were also observed in line 37 APJ-TG mice (Fig. 4, B and C). Furthermore, significant increases in cardiac ANP, BNP, and collagen I (Col1a1) genes were examined. E, the number of TUNEL-positive cells in cardiac sections (n = 3–5). F, plasma apelin levels of non-pregnant and postpartum mice (n = 3–4). G, cardiac apelin (Apln) mRNA expression levels in non-pregnant and postpartum mice (n = 3–4). B–G, data are presented as mean ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

It has been reported that elevation of apoptosis in cardiac tissue is observed in mouse models of peripartum cardiomyopathy, such as Gαq transgenic mice and cardiomyocyte-specific STAT3-deficient mice (16, 19). Therefore, we further investigated the levels of apoptosis in postpartum APJ-TG mice. As shown in Fig. 4E, the number of TUNEL-positive cells was significantly increased in APJ-TG mice with lactation at 4 weeks postpartum compared with non-pregnant APJ-TG mice but not in APJ-TG mice without lactation, whereas lactation did not affect the number of TUNEL-positive cells in WT mice. These data demonstrate that lactation is critical for the onset of postpartum cardiomyopathy in APJ-overexpressing mice. Next, we investigated whether lactation...
induces apelin expression, resulting in activation of overexpressed APJ. However, compared with non-pregnant mice and mice without lactation, plasma apelin levels did not increase in both WT and APJ-TG mice with lactation at 2 and 4 weeks postpartum (Fig. 4F). Moreover, lactation did not increase cardiac apelin gene (Apiln) expression in both WT and APJ-TG mice (Fig. 4G). At 4 weeks postpartum, apelin expression was significantly decreased in APJ-TG mice with lactation compared with WT mice and non-pregnant APJ-TG mice (Fig. 4G).

**APJ Overexpression Impairs Cardiac Angiogenesis in the Postpartum Period**—Previous studies have demonstrated that the deficiency of STAT3 or PGC1α genes in cardiomyocytes causes impaired myocardial angiogenesis in the postpartum period, resulting in peripartum cardiomyopathy (19, 20). Therefore, we next investigated the capillary density and protein expression levels of STAT3 and PGC1α in the hearts of APJ-TG mice with lactation. Although there was no difference in cardiac capillary number between non-pregnant WT and APJ-TG mice, lactating APJ-TG mice had a reduced capillary number compared with WT mice, indicating that cardiac angiogenesis is impaired in postpartum APJ-TG mice (Fig. 5A). In the postpartum period, STAT3 protein levels were similar between WT and APJ-TG mice at 2 and 4 weeks postpartum (Fig. 5B, center). It has been reported that STAT3 is phosphorylated in the hearts of postpartum mice and that its level is decreased after weaning of their pups (19). Phosphorylation of STAT3 is important for its nuclear localization and activation (33). At 2 weeks postpartum, phosphorylated STAT3 was detected in both WT and APJ-TG mice at comparable levels (Fig. 5B, top). Furthermore, cardiac STAT3 deficiency causes excessive oxidative stress in the postpartum period and leads to up-regulation of cathepsin D protein levels (19). However, cathepsin D protein levels were not increased in APJ-TG mice compared with WT mice at 2 weeks postpartum (Fig. 5C). There were no differences in protein expression levels of PGC1α between WT and APJ-TG mice at 2 weeks postpartum (Fig. 5D).

We next examined the alterations in other signaling pathways. We reported previously that apelin treatment induces transient AKT and ERK1/2 phosphorylation in HEK293T cells stably expressing the human APJ gene (30). A recent work has revealed that constitutive AKT activation in the heart also contributes to the development of postpartum cardiomyopathy with decreased capillary density (34). Thus, we investigated AKT and ERK1/2 phosphorylation levels in the hearts of APJ-TG mice. However, the phosphorylation levels of AKT and ERK1/2 were comparable in the hearts of WT and APJ-TG mice (Fig. 5D). These results suggest that APJ-TG mice exhibit postpartum cardiomyopathy through the compromised angiogenesis independent of the STAT3, PGC1α, AKT, and ERK1/2 pathways.

Finally, we investigated mRNA expression levels of angiogenesis-related genes in the heart. The balance of proangiogenic and antiangiogenic factors is important for proper angiogenesis (35, 36). As shown in Fig. 5E, angiopoietin-1 (Angpt1), a proangiogenic factor (37), was induced by lactation in both WT and APJ-TG mice, whereas the levels of Angpt1 mRNA were significantly decreased in APJ-TG mice compared with WT mice in a non-pregnant state and 2 weeks postpartum. Moreover, we found that thrombospondin-1 (Thbs1), an endogenous inhibitor of angiogenesis (38, 39), was significantly elevated in lactating APJ-TG mice compared with non-lactating APJ-TG mice and lactating WT mice (Fig. 5E). These data suggest that the dysregulation of angiogenic factor expression causes impaired angiogenesis in lactating APJ-TG mice.

**Discussion**

Although it has been demonstrated that the apelin-APJ system is intimately related to cardiac development, homeostasis, and diseases, the effect of increased APJ expression in the heart remains unclear. In this study, we show that cardiomyocyte-specific APJ overexpression induces cardiac hypertrophy and contractile dysfunction in mice. In addition, we found that lactation causes postpartum cardiomyopathy in APJ-overexpressing mice.

It has been reported that apelin peptide has a positive inotropic effect and that both apelin and APJ are essential for the maintenance of cardiac contractility in mice (25–27, 29). On the other hand, we showed that APJ overexpression contributed to contractile dysfunction with eccentric hypertrophy in male and non-pregnant female mice. This discrepancy may be explained by activation of sodium-calcium exchanger (NCX). NCX has been shown to be involved in the positive inotropic effect of apelin in a working rat heart model (25). Interestingly, cardiac overexpression of NCX1, which is mainly expressed in cardiac muscle, induces eccentric hypertrophy and a decrease in cardiac contractility (40). Crucially, postpartum homozygous NCX1 transgenic mice show a significant reduction of fractional shortening compared with baseline transgenic mice (40). This raises the possibility that activation of NCX1 might be related to cardiac hypertrophy, contractile dysfunction, and postpartum heart failure in APJ-TG mice.

A previous work revealed that APJ-deficient mice exhibit resistance to pressure overload by aortic banding (41). APJ-KO mice show blunted myofiber hypertrophy after transverse aortic constriction. In addition, mechanical stretch induces cellular hypertrophy and ANP expression in APJ-expressing neonatal rat cardiomyocytes (41). In our transgenic model, overexpressed APJ may be activated by mechanical stretch under basal conditions and may contribute to cardiac hypertrophy and dysfunction in APJ-TG mice. However, male and non-pregnant APJ-TG mice did not show an increase of ANP gene expression and myofiber hypertrophy. In addition, although ANP expression was increased in the hearts of lactating APJ-TG mice, myofiber hypertrophy was not observed compared with lactating WT mice. These facts suggest that APJ activation by mechanical stretch might not contribute to pathogenesis in APJ-TG mice.

It has been suggested that the APJ receptor is involved in both Gαq- and Gαq,-mediated signaling in the heart and 3T3-L1 cells (25, 42). Cardiomyocyte-specific Gαq-overexpressing mice die from heart failure in the antepartum and postpartum periods and show massive apoptosis in the heart on day 1 postpartum (16, 18). Because inhibition of cardiac apoptosis by caspase inhibitor treatment or deletion of the NIX gene, which is a member of the Bcl2 family of proteins, improves postpar-
tum cardiac function in Gαq transgenic mice, apoptosis is thought to play a critical role in the development of peripartum cardiomyopathy in this model (17, 18). On the contrary, in our study, APJ-TG mice exhibited increased apoptosis in the heart at 4 weeks postpartum but not at 2 weeks postpartum (Fig. 4E).

Furthermore, compared with non-pregnant mice, antepartum APJ-TG mice did not show a reduction of cardiac contractility (Fig. 3E). These differences in time point of disease development between Gαq transgenic and APJ-TG mice indicates that enhanced Gαq signaling and apoptosis may not be associated with onset of postpartum cardiomyopathy in APJ-TG mice.

Lactation is thought to have beneficial effects on cardiovascular systems (43, 44). Furthermore, Safirstein et al. (45) reported that breastfeeding is related to an improvement of systolic function in peripartum cardiomyopathy patients. On the other hand, a previous work demonstrated that prolactin, which is an important hormone for milk production, is cleaved by cathepsin D and is converted into 16-kDa prolactin in the hearts of cardiomyocyte-specific STAT3-deficient mice (19). This cleaved prolactin inhibits cardiac angiogenesis, resulting in postpartum cardiomyopathy (19). Thus, the effect of lactation on peripartum cardiomyopathy remains controversial.

**FIGURE 5.** Impaired myocardial angiogenesis in APJ-TG mice. A, representative images of cardiac sections stained with CD31 antibody (microvessels, green), WGA (cell membrane, red), and Hoechst 33342 (nucleus, blue) and quantification of capillary density in the heart of non-pregnant and postpartum mice (n = 4). B–D, Western blotting analysis of STAT3 and phosphorylated STAT3 (pSTAT3, B); cathepsin D (C); and PGC1α, phospho-AKT (pAKT), AKT, phospho-ERK1/2 (pERK1/2), and ERK2 (D) in the hearts of postpartum mice with lactation. E, mRNA expression levels of angiogenesis-related genes in the heart (n = 4). Data are presented as mean ± S.E. ***, p < 0.01; ****, p < 0.001 versus non-pregnant WT mice. †, p < 0.05; ††, p < 0.01; †††, p < 0.001.
Postpartum Heart Failure Induced by APJ and Lactation

Our study, non-lactating APJ-TG mice did not develop postpartum cardiomyopathy. This result has clearly shown that lactation is critical for the onset of postpartum cardiomyopathy in APJ-TG mice. In addition, we demonstrated previously that lactation causes cardiac contractile dysfunction in pregnancy-associated hypertensive mice in the postpartum period (7). Pregnancy-associated hypertensive mice exhibit concentric cardiac hypertrophy and marked fibrosis by hypertension during late pregnancy, whereas cardiac contractility is preserved (7). Although the genetic backgrounds and antepartum maternal cardiac phenotypes of APJ-TG mice and pregnancy-associated hypertensive mice were different, both mice showed a reduction of cardiac contractility by lactation. This indicates that multiple qualitative changes in the antepartum heart are associated with disorder of adaptive response to lactation in the heart. Because our results suggest that postpartum cardiomyopathy in APJ-TG mice is not involved in decreased STAT3 activity and elevation of cathepsin D expression, APJ mice may be an effective tool for further understanding the role of lactation in postpartum cardiomyopathy. The details of the benefit and risk of lactation are poorly understood. However, our study may provide new insights into the development of postpartum cardiomyopathy associated with lactation.

In this study, capillary density was reduced in the hearts of lactating APJ-TG mice, suggesting that the impaired myocardial angiogenesis is related to the onset of lactation-induced postpartum cardiomyopathy in APJ-TG mice. Although previous studies have revealed that the apelin-APJ system in vascular cells is involved in angiogenesis (24, 46), our result suggests that APJ overexpression in cardiomyocytes disrupts the expression pattern of pro- and antiangiogenic factors such as Angpt1 and Thbs1, resulting in deteriorated angiogenesis in the hearts of lactating mice. It has been reported that PGC1α is required for expression of proangiogenic genes, including Vegfa, Angpt1, Angpt2, Pdgfa, and Pdgfb in the heart (20), whereas PGC1α is normally expressed in the hearts of APJ-TG mice. Because the Angpt1 gene is specifically decreased by APJ overexpression (Fig. 5E), it may impair myocardial angiogenesis independent of PGC1α. Further study is needed to determine how APJ overexpressed in cardiomyocytes affects expression of the Angpt1 and Thbs1 genes.

We demonstrated that APJ overexpression in cardiomyocytes contributes to postpartum heart failure in mice, whereas the cardiac expression level of APJ in patients with postpartum cardiomyopathy is unknown. Interestingly, a functional SNP has been identified in the 5′-flanking region of the APJ gene in Japanese patients of brain infarction (47). This SNP affects DNA binding of the Sp1 transcription factor and modulates APJ gene expression levels (47). It would be important to reveal the association between postpartum cardiomyopathy and gene mutations that increases APJ expression.

In our study, non-pregnant APJ-TG mice showed cardiac contractile dysfunction, eccentric hypertrophy, and increased BNP gene expression, whereas features of heart failure, such as abnormal breathing and pleural effusion, were not observed. This suggests the possibility that these pre-existing, non-severe cardiac phenotypes influence the development of lactation-induced postpartum cardiomyopathy in APJ-TG mice. Although one of the definitions of peripartum cardiomyopathy is no history of heart disease (48), non-pregnant women who are going to develop peripartum cardiomyopathy might have non-severe cardiac defects. However, in patients with peripartum cardiomyopathy, little is known about cardiac function and BNP levels before the onset of peripartum cardiomyopathy because women who have no symptoms of heart failure lack the opportunity to examine their cardiac parameters. To investigate the relationship between pre-existing non-severe cardiac defects and onset of peripartum cardiomyopathy might be important for prediction of peripartum cardiomyopathy.

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