Received: 2 December 2021 | Revised: 23 August 2022 | Accepted: 29 August 2022

DOI: 10.1096/fj.202101856RRRR

THE FASEB JOURNAL

RESEARCH ARTICLE

Wnt11 in regulation of physiological and pathological cardiac growth

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Funding information
Aarne Koskelon Säätiö (Aarne Koskelo Foundation); Academy of Finland (AKA), Grant/Award Number: 268505 and 297094; Paavo Nurmen Säätiö (Paavo Nurmi Foundation); Sydäntutkimussäätiö (Finnish Foundation for Cardiovascular Research)

Abstract

Wnt11 regulates early cardiac development and left ventricular compaction in the heart, but it is not known how Wnt11 regulates postnatal cardiac maturation and response to cardiac stress in the adult heart. We studied cell proliferation/maturation in postnatal and adolescent Wnt11 deficient (*Wnt11*−/−) heart and subjected adult mice with partial (*Wnt11*+/−) and complete Wnt11 (*Wnt11*−/−) deficiency to cardiac pressure overload. In addition, we subjected primary cardiomyocytes to recombinant Wnt proteins to study their effect on cardiomyocyte growth. Wnt11 deficiency did not affect cardiomyocyte proliferation or maturation in the postnatal or adolescent heart. However, Wnt11 deficiency led to enlarged heart phenotype that was not accompanied by significant hypertrophy of individual cardiomyocytes. Analysis of stressed adult hearts from wild-type mice showed a progressive decrease in Wnt11 expression in response to pressure overload. When studied in experimental cardiac pressure overload, Wnt11 deficiency did not exacerbate cardiac hypertrophy or remodeling and cardiac function remained identical between the genotypes. When subjecting cardiomyocytes to hypertrophic stimulus, the presence of recombinant Wnt11 together with Wnt5a reduced protein synthesis. In conclusion, Wnt11 deficiency does not affect postnatal cardiomyocyte proliferation but leads to cardiac growth. Interestingly, Wnt11 deficiency alone does not substantially modulate hypertrophic response to pressure overload in vivo. Wnt11 may require cooperation with other noncanonical Wnt proteins to regulate hypertrophic response under stress.

KEYWORDS
cardiac hypertrophy, left ventricle remodeling, pressure overload, Wnt11 protein

Abbreviations: LV, left ventricular; TAC, thoracic aortic constriction.

1 | INTRODUCTION

In embryogenesis, the heart development from mesodermal stem cells is guided and regulated by secreted morphogens including Wnt proteins, fibroblast growth factors (FGFs), and members of the transforming growth factor-β (TGFβ) superfamily.[1] The Wnt family of secreted glycoproteins, encoded by 19 different genes, has been shown to regulate a number of cell functions, including survival, proliferation, migration, cell fate, and differentiation.[2] Several Wnt proteins through canonical and noncanonical type of signaling, work in sequential stages in human embryonic heart development.[3] During heart development, canonical Wnt pathway, which activates β-catenin signaling, is required for the expansion of cardiac progenitor pools, enabling the formation of cardiac chambers and main arteries, In the subsequent phase, inhibition of canonical Wnt/β-catenin signaling is needed for cardiomyocyte differentiation.[2,4]

Non-canonical Wnt signaling, by Wnt11, has been reported to induce cardiogenic differentiation by multiple mechanisms: inhibiting canonical Wnt/β-catenin signaling in caspase-dependent manner,[5] activating protein kinase C (PKC)-mediated JNK pathway[6] and cell polarity pathways[7] or by modulating electrical gradient in the myocardium via calcium signaling.[8] Wnt11 signaling can also compete with canonical Wnt proteins by binding to the same frizzled receptors.[9] Wnt11 is expressed in the heart in early cardiac development,[7] and is involved in atrioventricular septation and outflow tract morphogenesis in the developing heart.[10,11] In addition to these, Wnt11 localizes to ventricles in the embryonic heart at E10[12] where it controls cardiomyocyte adhesion and organization forming the compact functional left ventricular (LV) myocardium. In 129SV mouse strain, Wnt11 deficiency led to downregulation of cardiac transcription factors Gata4, Nkx2.5, and Mef2c as well as expression of atrial natriuretic peptide, and led to severe malformation of the heart, causing lethality from mid-gestation onwards.[12] In addition, hypoxia-induced or morpholino-induced Wnt11 deficiency increased cardiomyocyte proliferation at late embryonic/neonatal stage,[13] but it is not known how Wnt11 regulates cardiomyocyte proliferation and cardiac maturation postnatally, in adolescence as well as in adulthood.

In this study, we investigated how Wnt11 deficiency affects the postnatal and adolescent heart as well as the response to cardiac stress in adult mouse hearts. We determined cardiomyocyte proliferation and hypertrophy in the hearts of postnatal and adolescent Wnt11 deficient mice. To study the role of Wnt11 in cardiac hypertrophy and fibrosis in the stressed heart, Wnt11 deficient mice were subjected to cardiac pressure overload. Modulation of Wnt11 might be a potential drug target for the treatment of heart disease and more detailed information on this signaling protein is therefore needed.

2 | MATERIALS AND METHODS

2.1 | Animals

The generation of *Wnt11*−/− (Wnt11 KO) deficient mouse line has been described earlier.[14] In 129SV strain, Wnt11 deficiency is embryonic lethal, where viability is substantially reduced from E13 onwards.[12] Given that genetic modifiers are known to modulate the penetrance or severity of cardiac disease in the mouse,[15] we crossed the Wnt11 KO allele to another mouse genetic background. Therefore, Wnt11 deficient 129SV mice were backcrossed to C57BL6J strain for a minimum of 10 generations.[16] However, even in C57BL background 60% of the Wnt11 KO mice die in utero or until first weeks of age while *Wnt11*+/− (Wnt11 HET) mice show normal viability. Animals were maintained on a 12 h light/dark cycle with unlimited access to water and chow at the Oulu Laboratory Animal Center. All experiments were performed with the approval from the National Animal Experiment Board of Finland (consent number ESAVI/8134/04.10.07/2017) as well as by local authorities.

2.2 | Myocardial stress models and echocardiography

Thoracic/transverse aortic constriction (TAC) model was utilized to study the effect of Wnt11 to pressure overload-induced cardiac hypertrophy and heart failure in mice. At the age of 7–9 weeks, the thoracic aorta of wild-type (*Wnt11*+/+, WT, $n = 13$), Wnt11 heterozygote (*Wnt11*+/−, Wnt11 HET, $n = 20$), or Wnt11 knockout (*Wnt11*−/−, Wnt11 KO, $n = 4$) mice were constricted as previously described.[17] Briefly, male mice were anesthetized with a mixture of 110 mg/kg ketamine (Ketaminol Vet; Intervet International, Boxmeer, Netherlands) and 15 mg/kg xylazine (Rompun Vet; Orion Pharma, Espoo, Finland) administered i.p., intubated and ventilated during the surgery. Medial sternotomy was performed, and a 7–0 silk suture was applied to band the aorta to a size matching a 27G (diameter of 0.4 mm) needle. Sham-operated animals ($n = 5$) were subjected to a similar procedure with opening the thoracic cavity, but no suture was applied to band the aorta. After the operation, 2 mg/kg atipamezole (Antisedan; Orion Pharma, Espoo, Finland) was administered i.p. Pain alleviation medication buprenorphine (Vetergesic; Ceva Santé Animale, Libourne, France) at the

dosage of 0.05–0.1 mg/kg s.c. twice per day and carprofen (Rimadyl; Zoetis, Louvain-la-Neuve, Belgium) at the dosage of 5 mg/kg s.c. once per day was administered for 3 days after the operation. Mortality after TAC operation was 1/13 for wild-type and 1/20 for Wnt11 heterozygotes; none of the Wnt11 KO mice were lost after the operation. After 8 weeks, mice were euthanized with CO_2. The hearts were perfused with saline, quickly excised, and samples collected for histology and biochemical analysis.

The cardiac structure and function were analyzed by echocardiography at baseline, 4 weeks, and right before sacrificing at 8 weeks after TAC. Echocardiography measurements were carried out under isoflurane (Baxter, Deerfield, IL, USA) anesthesia with a Vevo 2100 (VisualSonics Toronto, ON, Canada) high frequency, high-resolution linear array ultrasound system using an MS-550D transducer (40 MHz, axial resolution 40 μm, lateral resolution 90 μm). B-mode and M-mode images were recorded and carefully analyzed with Vevo Workstation software 1.7 by a blinded observer.

Ischemic myocardial stress was induced in 8-week-old C57BL6J male mice by ligation of left anterior descending coronary artery either transiently as ischemia–reperfusion (IR) injury (30 min ischemia), followed by 24 h reperfusion ($n = 10$), or permanently as myocardial infarction (MI) model to evaluate longer timepoints ($n = 19$).[18]

2.3 | Telemetry for monitoring blood pressure

Determination of blood pressure was performed with radio-telemetry as described.[19] Briefly, 4-month-old male WT ($n = 4$) and Wnt11 KO ($n = 4$) mice were anesthetized with ketamine and xylazine and a fluid-filled catheter (DSI Instruments, Harvard Bioscience, St. Paul, MN, USA) was inserted via the left carotid artery into the aortic lumen, and the transducer with battery was placed in the subcutaneous layer of the subscapular region. After recovery of 4 days, hemodynamics were recorded via telemetry in conscious, unrestrained animals every 5 min throughout the experiment for 10 days.

2.4 | Cardiac cell fractionation

Adult ventricular myocytes were isolated from 5-month-old C57BL6 mice as described (sham $n = 5$, TAC 2 weeks $n = 5$, TAC 3 weeks $n = 4$).[20] Briefly, mice were deeply anesthetized with isoflurane and the heart was excised rapidly, cannulated through the aorta, and retrogradely perfused with HEPES-buffered Tyrode's solution supplemented with 0.1% collagenase type 2 (Worthington, Lakewood, NJ, USA) and 2,3-butandione-monoxime (MP Biochemicals, Irvine, CA, USA). The ventricular tissue was homogenized, then myocytes were collected by low-speed centrifugation (18*g* for 2 min) and frozen at-70°C. The supernatant was further centrifuged at 300 g for 5 min to pellet the fibroblasts and endothelial cells. These cells were resuspended in phosphate-buffered saline (PBS) supplemented with 2 mM EDTA, 2% FBS, and filtered through 40 μm nylon mesh. The endothelial cells were isolated by immunomagnetic cell separation with CD31 mouse microbeads with MS columns and a VarioMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD31-positive endothelial fraction and the CD31-negative fibroblast fraction were frozen at −70°C.

2.5 | Cardiomyocyte cell culture

Neonatal rat ventricular cardiomyocytes (NRVM) used for in vitro experiments were isolated from 2 to 4 days-old Sprague–Dawley rats. After decapitation, ventricles were rapidly excised, cut into small pieces, and incubated with 2 mg/ml collagenase type 2 (Worthington, Lakewood, NJ, USA), 50 μM $CaCl_2$ in PBS for approximately 2 h at 37°C with gentle shaking while collecting the detached cells every 10–25 min to a tube containing DMEM/F12 with L-glutamine (Sigma, Darmstadt, Germany) supplemented with 30% FBS (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin–streptomycin (PS) (Sigma). Cells were centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended in 10% FBS and 1% PS in DMEM/F12 (Sigma). Centrifugation was repeated and the cells were plated on 100 mm cell culture dishes (2–3 hearts per plate) and incubated at 37°C/5% CO_2 for 2 h to let cardiac fibroblasts attach to the cell dishes. Thereafter, the non-attached cell fraction containing cardiomyocytes was collected, plated at 150 000 cells/cm^2, and cultured at 37°C/5% CO_2. The experiments were started 2 days after isolation in complete serum-free medium (CSFM) containing 2.5 mg/ml bovine serum albumin (BSA) (Sigma), 5 μg/ml–5 μg/ml–5 ng/ml insulin–transferrin–sodium selenite (ITS) (Roche, Basel, Switzerland), 2.8 mM Na-pyruvate, 0.1 nM T_3 (Sigma), and 1% PS in DMEM/F12.

Recombinant Wnt11 and Wnt5a proteins (R&D Systems, Minneapolis, MN, USA) were added as 200 ng/ml. Tritium [^{3}H]-labeled leucine was used to measure the amount of incorporated leucine as an indicator of protein synthesis. NRVM were cultured in 24-well plates and cells supplemented with [^{3}H]-leucine (1 μCi/ml, PerkinElmer, Waltham, MA, USA) in CSFM. When appropriate, cells were treated with ET-1 (100 nM) (Sigma, Darmstadt, Germany) or bFGF (20 ng/ml) (Peprotech, London, UK). After 24 h cells were lysed and processed

for measurement of incorporated [^{3}H]-leucine by Tri-Carb 2900TR liquid scintillation counter (PerkinElmer, Waltham, MA, USA).

2.6 | Proliferation assay

To analyze the number of cells undergoing cell cycle, female Wnt11 HET, Wnt11 KO mice, and their WT littermates were injected 4 days prior to sacrifice[21] i.p. with a dose of BrdU analog 5-ethynyl-2′-deoxyuridine (EdU, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) 25 mg/kg (stock 5 mg/ml in PBS), which efficiently binds to DNA of proliferating cells.[22] Mice were sacrificed at P16-P20, hearts excised, and prepared as formalin-fixed paraffin-embedded sections. Incorporation of EdU was analyzed with Click-iT chemistry (Alexa Fluor 555 Imaging kit, Molecular Probes/Thermo Fisher Scientific, Waltham, MA, USA) by imaging from left ventricle and septum from three fields with fluorescence microscope with 20× objective.

2.7 | Histological stainings

Hearts were fixed overnight in phosphate-buffered 10% formalin (pH 7.0) and prepared as paraffin-embedded transversal cardiac tissue sections (thickness of 5 μm), or frontal sections when examining heart morphology. For determination of cell size, the transversal sections were stained with 5 μg/ml wheat germ agglutinin (WGA) Alexa Fluor 488-conjugate (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). Cross-sectional myocyte cell size was quantified (Adobe Photoshop CS5) as an average of 50 cardiomyocytes/section obtained from five representative fields of each section from LV sites or septum, when indicated. The amount of cardiac fibrosis was assessed with Picrosirius Red (Direct Red 80, Sigma, Darmstadt, Germany) staining, which can be analyzed for its birefringence under polarized light or by its red fluorescence.[23] The sections were viewed under fluorescent light (with 540/25 nm filter) and imaged for multiple fields of each section from the LV and septum sites which represented increased interstitial fibrosis.

To visualize mitotic cells, the heart sections were stained overnight with phosphohistone-H3 (PHH3, Cell Signaling Technology, Danvers, MA, USA, #9701, 1:100), then double-stained overnight with α-actinin (Sigma, Darmstadt, Germany, ab7811, 1:100). The number of mitotic cardiomyocytes were determined from ventricles and septum from four fields (P0 mice) and from left ventricle and septum from six fields (P16-P20 mice) with fluorescence microscope with 40× objective.

2.8 | RNA isolation and quantitative real-time PCR

RNA was isolated from the left ventricle with Trizol (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).[24] cDNA was synthesized from 500 ng of RNA with First Strand cDNA synthesis kit (GE Healthcare, Chicago, IL, USA). The expression levels were evaluated on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with TaqMan or SYBR Green chemistry. Oligonucleotide primers and probes were obtained from Sigma unless otherwise stated. The expression levels of Wnt11 were determined with TaqMan gene expression kit (Applied Biosystems, Mm00437328_m1, VIC/MGB probe, primer limited), whereas Wnt5a levels were evaluated with SYBR Green chemistry (primers according to Harvard Primer Bank, PrimerBank ID 6678597a1, sequence (5′ to >3′) forward (For) CAACTGGCAGGACTTTCTCAA, reverse (Rev) CATCTCCGATGCCGGAACT). Sequences (5′ to >3′) for the main factors studied from mouse hearts were: ANP probe FAM-TCGCTGGCCCTCGGAGCCT-TAMRA, For GAAAAGCAAACTGAGGGCTCTG, Rev CCTACCCCCGAAGCAGCT; BNP probe FAM-CATCA TTGCCTGGCCCATCGC-TAMRA, For AGGCGAGACA AGGGAGAACA, Rev GGAGATCCATGCCGCAGA; β-MHC probe FAM-AGCCCTCAGACCTGGAGCCTTT GC-TAMRA, For AGCTCTAAGGGTGCCCGTG, Rev TGCTTCCACCTAAAGGGCTG; c-myc probe FAM-TG CCCTGCGCGACCAGATCC-TAMRA,ForGTCAGAGGA GAAACGAGCT, Rev GGGCCTTTTCGTTGTTTTCCA; c-kit probe FAM-CAAACCCCAAGGCTGGCATCACC-TAMRA, For CCACGGACCTGACGTTTGTC, Rev CGCGCTTCACGTTTTTGA. Mouse p53 expression levels were determined with TaqMan gene expression kit (Applied Biosystems, Mm01731290_g1, VIC/MGB probe). Sequences (5′ to >3′) to study gene expression levels in rat cardiomyocyte cultures were: ANP probe FAM-TCGCTGGCCCTCGGAGCCT-TAMRA,ForGAAAAGCA AACTGAGGGCTCTG, Rev CCTACCCCCGAAGCAGCT; BNP probe FAM-CGGCGCAGTCAGTCGCTTGG-TAM RA, For TGGGCAGAAGATAGACCGGA, Rev ACA ACCTCAGCCCGTCACAG; β-MHC probe FAM-TGTGA AGCCCTGAGACCTGGAGCC-TAMRA, For GCTACCC AACCCTAAGGATGC, Rev TCTGCCTAAGGTGCTGTT TCAA. 18S was used as a reference gene, probe FAM-CCTGGTGGTGCCCTTCCGTCA-TAMRA, For TGG TTGCAAAGCTGAAACTTAAAG, Rev AGTCAAATTAA GCCGCAGGC for TaqMan and For CGCCGCTAGAGG TGAAATTC,RevCCAGTCGGCATCGTTTATGGforSYBR primer sets. Other oligonucleotide primer sequences used for mRNA quantification are presented in our previous studies.[24,25]

2.9 | Western blotting

Total protein content was extracted from left ventricle.[26] The protein concentrations were determined with colorimetric protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (40 μg) were denatured and loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), run at 150 V, and transferred to 0.2 μm nitrocellulose membrane (Trans-Blot Turbo RTA transfer kit) using Trans-Blot Turbo Transfer System (Biorad Laboratories, Hercules, CA, USA). The membranes were incubated for 1 h at RT in blocking buffer (Odyssey, LI-COR Biosciences, Lincoln, NE, USA) diluted 1:1 into Tris-buffered saline (TBS, 50 mM Tris, 200 mM NaCl, pH 7.4). Membranes were then incubated overnight with primary antibody pGSK-3β (Ser9) (Cell Signaling Technology, Danvers, MA, USA, #9336, 1:1000), pS6 (Ser240/244) (Cell Signaling #2215, 1:1000), or GAPDH (Millipore, Sigma, Darmstadt, Germany, #MAB374, 1:100 000) in blocking buffer, washed with TBS-0.05% Tween-20 and incubated with secondary antibody (goat-anti-rabbit Alexa Fluor 680; Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA or goat-anti-mouse IRDye 800; Rockland Immunochemicals, Pottstown, PA, USA) for 1 h at RT. Antibody binding was detected by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified using the public domain NIH Image program (developed at the U.S. National Institutes of Health, Bethesda, MD).

2.10 | Statistical analysis

Data are presented as mean ± SD. Results were statistically analyzed with IBM SPSS software using Student's *t* test or Mann–Whitney *U* test, or in case of multiple groups, with one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test. Differences were considered significant with p value ≤ .05.

3 | RESULTS

3.1 | Wnt11 deficiency does not significantly affect postnatal cardiomyocyte proliferation/maturation

Wnt11 plays a substantial role in the cardiac development by contributing to cell differentiation but its actions in the postnatal, adolescent, and adult heart are not known. It has been reported that silencing Wnt11 at embryonic stage increases cardiomyocyte proliferation in neonatal heart.[13] First, we aimed to determine how Wnt11 affects cell proliferation in the postnatal and adolescent heart. Newborn Wnt11 KO mice and their WT littermates were sacrificed on the day of birth (postnatal day P0) and hearts were stained for phosphohistone H3 (PHH3) to visualize the mitotic cells. The proportion of mitotic PHH3 positive cardiomyocytes was not altered in hearts of newborn Wnt11 KO mice compared to their WT littermates (Figures 1A and S1).

It has been reported that cardiomyocyte proliferation bursts during preadolescence, to establish the final cardiomyocyte number.[21] This finding has also been challenged and reported to rather indicate cardiomyocyte multinucleation as well as cardiomyocyte nuclear polyploidization and that the final cardiomyocyte number is mainly established within the first postnatal week.[27] To study the role of Wnt11 in regulation of cardiomyocyte proliferation/binucleation/polyploidization processes in preadolescence, we administered one dose of EdU to young mice 4 days prior to sacrifice at days P16, P18, or P20. The highest EdU incorporation into DNA was detected at P16 while it steadily reclined from timepoint P16 to P20 (Figure 1B). When assessing EdU incorporation at this timepoint from different genotypes, the number of EdU-positive cells in the heart did not differ between Wnt11 HET and Wnt11 KO and their WT littermates (Figure 1C). To confirm the EdU incorporation results, we analyzed the same sections with PHH3 stain. Similar to EdU analysis, there was no difference between genotypes in PHH3 analysis (Figures 1D and S1). Cell proliferation profile associates with similarly altered expression levels of growth suppressor gene p53 in the myocardium (Figure 1E).

C-myc controls transcriptional programs for cell proliferation. While the juvenile heart is permissive to c-myc-mediated proliferation, adult cardiomyocytes can also re-enter the cell cycle if c-myc signaling is manipulated.[28] C-kit is a receptor in cardiac stem cells, and its expression is highest in embryonic stage declining to low levels in the adult heart. C-kit signaling has been shown to contribute to cardiac homeostasis and regeneration and potentially contributes to myocyte terminal differentiation in neonatal heart.[29] Cardiac expression of c-myc was highest in neonatal stage, whereas its expression was substantially downregulated in preadolescent stage, and Wnt11 deficient mice represented more sustained levels of c-myc as well as cardiogenic factor c-kit (Figure 1F). Despite of the higher expression level of c-myc remaining in Wnt11 deficient mice, the proliferation was not induced at preadolescent stage (Figure 1C,D). Furthermore, Wnt11 deficiency did not significantly affect the expression levels of cardiac transcription factors Gata4, Nkx2.5, or Mef2c in neonatal or preadolescent stage (Figure 1F).

In addition, c-myc can also mediate cardiomyocyte hypertrophy.[30] In line with this, elevated c-myc expression in Wnt11 deficient heart was accompanied by mildly

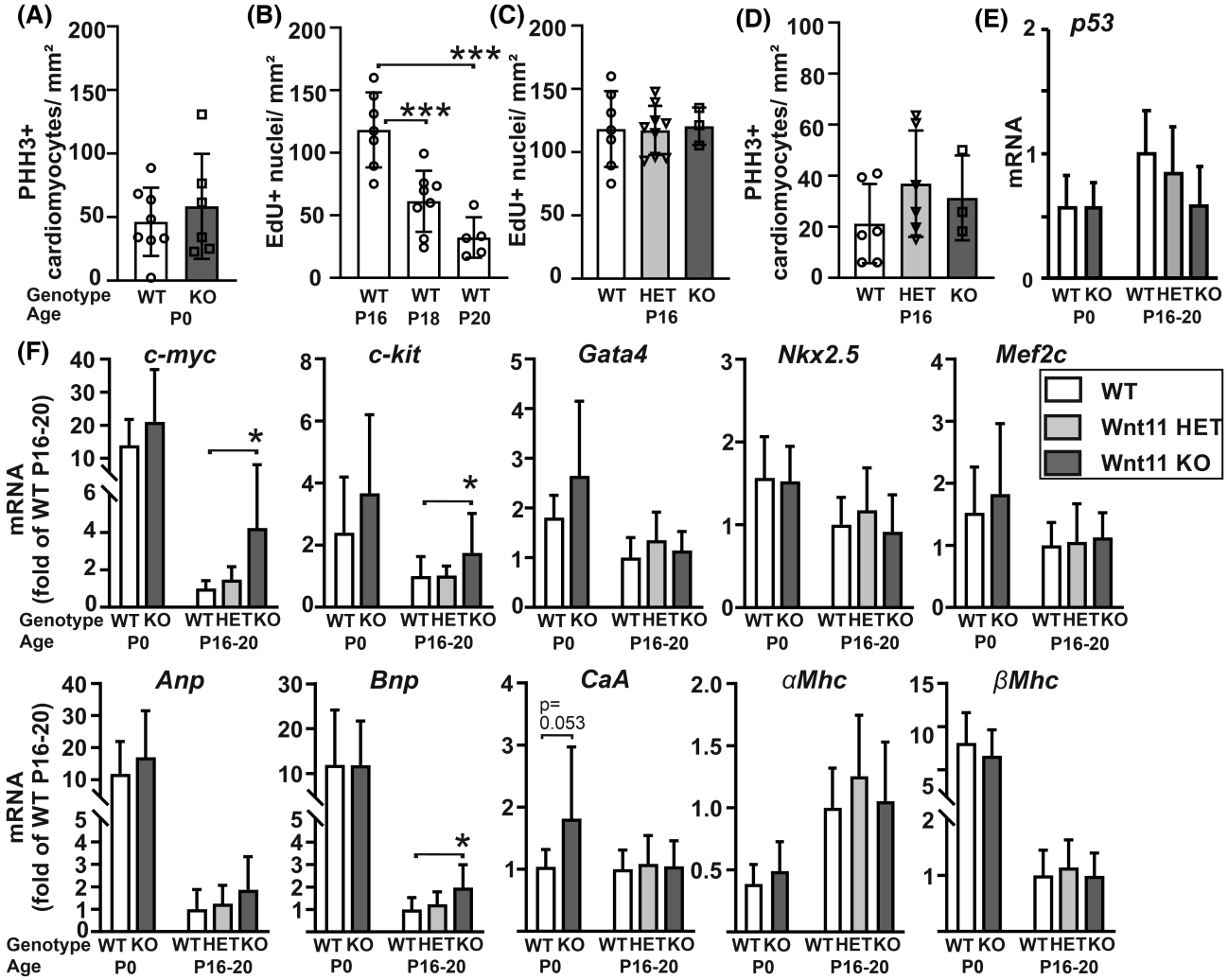

FIGURE 1 Wnt11 deficiency does not affect cardiomyocyte proliferation/maturation but induces prohypertrophic gene expression in the juvenile heart. (A) The number of mitotic cardiomyocytes was analyzed in newborn hearts with phosphohistone H3 (PHH3)/α-actinin double-stain at postnatal day 0 (P0). (B) The number of cells under DNA synthesis was determined with incorporation of EdU and analyzed at either P16, P18, or P20 from left ventricle and septum. (C) EdU incorporation was analyzed from different genotypes at P16, when the highest DNA synthesis was observed. (D) The number of mitotic cardiomyocytes was determined with PHH3/α-actinin immunostain at P16 from left ventricle and septum. (E) Expression of cell proliferation regulator p53. (F) Expression of cardiogenic factors, genes associated with proliferation and hypertrophy, and cardiac myofiber thick and thin filaments were analyzed from P0 and P16-P20 left ventricles; $n = 8, 6$ (A), $n = 7, 8, 5$ (B), $n = 7, 9, 3$ (C), $n = 6, 6, 3$ (D), $n = 7$ (E-F, P0), $n = 6–9$ (E-F, P16-20); $^{*}p < .05$, $^{***}p < .001$.

increased expression levels of natriuretic peptides ANP, BNP as well as cardiac α-actin, indicating the hypertrophic potential of Wnt11 KO. However, when studied at the molecular level, this prohypertrophic response is only shown on the gene expression level. In early postnatal period, cardiomyocytes undergo fiber type transition from β-MHC to α-MHC, which is the predominant fiber type present in the adult mouse heart. Wnt11 deficiency had no effect on this transition (Figure 1F). To conclude, Wnt11 deficiency does not significantly induce postnatal cardiomyocyte proliferation or restrict cardiomyocyte maturation in early postnatal/preadolescent stage. Instead, Wnt11 deficiency induces prohypertrophic gene expression in the postnatal heart.

3.2 | Wnt11 deficiency modulates heart morphology in adolescent and adult mice

While Wnt11 KO leads to severe embryonic lethality in the 129SV genetic background due to heart failure,[12,14] Wnt11 KO allele in the C57BL background creates less severe phenotype allowing the studies with Wnt11 KO mice postnatally throughout adulthood.[16] Wnt11 deficient adolescent hearts showed cardiac growth as indicated by increased heart weight to body weight ratio (Figure 2A). In 4-month-old adult mice, the papillary muscle hypertrophy as well as enlargement of the myocardial LV and septum area were evident in Wnt11 KO hearts compared to WT littermates (Figure 2B). This enlarged heart phenotype

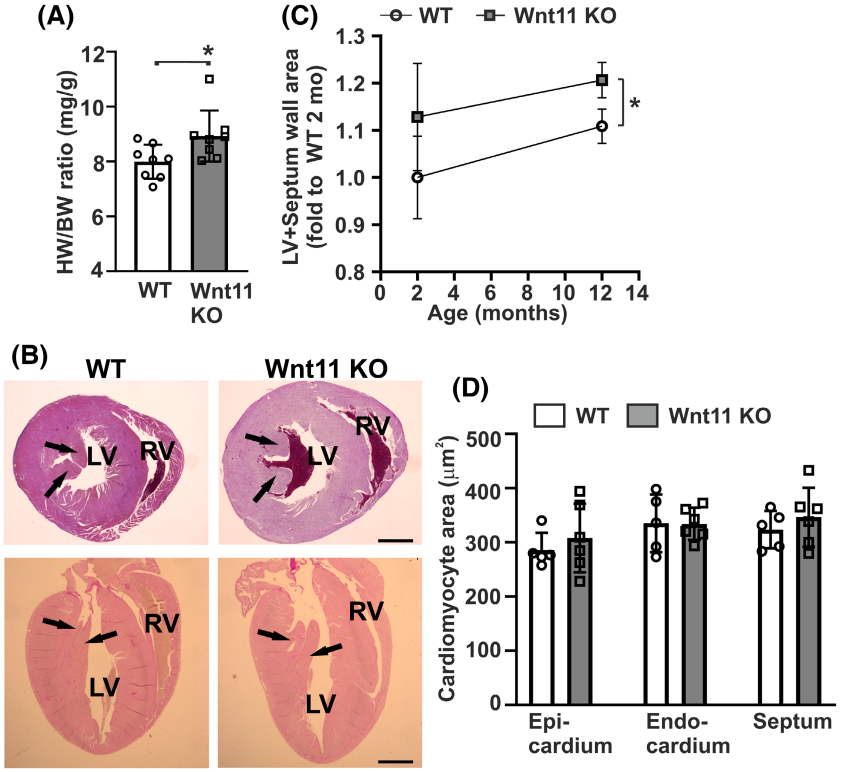

FIGURE 2 Wnt11 deficiency leads to cardiac growth. (A) Heart weight to body weight ratios determined from adolescent mice (2–3 weeks old) shows cardiac hypertrophy already at young age. (B) Transversal (upper row) and longitudinal (bottom row) cardiac sections from WT and Wnt11 KO mice (4 months old) show hypertrophied LV and septal areas and papillary muscles (arrows). (C) Hypertrophic phenotype is sustained throughout the adulthood as determined in LV and septal myocardial area (2 and 12 months old). (D) Cardiomyocyte cross-sectional areas quantified from epicardial, endocardial, and septal sites with wheat germ agglutinin (WGA) stain (2–4 months old); scale bar 1 mm (B); $n = 8$ (A), $n = 3$–4 (C, 2 months), $n = 2$–3 (C, 1 year), $n = 5$–6 (D); $^*p < .05$.

was sustained throughout adulthood (Figure 2C). When examined with echocardiography, Wnt11 KO mice represented prominent hypertrophied LV posterior wall (Table 1). Although the morphological changes caused by deficiency of Wnt11 were evident, the cardiac systolic function or LV chamber dimensions were preserved (Table 1).

Even in the C57BL background with generally milder cardiac phenotype, Wnt11 deficiency may lead to severe morphological defects and embryonic lethality. One survivor was found to have atrioventricular septation defect with transposition of the great arteries as reported for Wnt11 KO earlier.[11,12] However, other Wnt11 KO hearts had no septal defects and showed enlarged phenotype with varying severity, which included occasional gross cardiomegaly phenotype (1/10 of KO hearts) (Figure S2).

Next, we determined whether enlargement of Wnt11 KO hearts is due to hypertrophy of individual or regional cardiomyocytes. To examine the possible site-specific effects of Wnt11 in the heart, cardiomyocyte sizes were analyzed separately from epicardial, endocardial, and septal myocardium (Figure 2D), and a mild regional hypertrophy was detected in endocardial and septal myocardium (Figure 2B,D). The cellular hypertrophy was not homogenous within the ventricular wall, but more evident in some Wnt11 deficient cardiomyocytes compared to other, and this affected the statistical evaluation. Finally, to exclude that cardiac hypertrophy is due to possible kidney defects observed in Wnt11 KO mice,[16] blood pressure was measured. As determined by radiotelemetry in freely moving mice throughout 10-day period, no difference between the genotypes was detected (systolic and diastolic blood pressure in WT 140 ± 4 and 108 ± 5 mmHg, Wnt11 KO 141 ± 4 and 102 ± 6 mmHg, respectively, $n = 4$/genotype).

To conclude, Wnt11 deficiency induces moderate cardiac growth evident in adolescent mice and throughout adulthood. Occasionally, cardiac growth via hypertrophy and embryonic cardiomyocyte hyperplasia[13] led to severe cardiomegaly in Wnt11 deficient mice.

3.3 | The expression of Wnt11 in cardiac stress models

Regarding the contribution of Wnt11 to cardiac pathophysiology, our first aim was to define how Wnt11

expression is altered in cardiac stress. We determined Wnt11 expression in LV myocardium after experimental myocardial ischemia or pressure overload. Expression of Wnt11 after 24 h of cardiac ischemia–reperfusion injury was substantially downregulated in the surviving left ventricle (Figure S3). Wnt11 expression was restored to control levels during the inflammation and its resolution phases of post-infarction cardiac repair and thereafter modestly decreased during the ischemic remodeling (Figure S3). It should be noted that Wnt11 expression is not significantly downregulated during the proliferative phase at 7 days after ischemic injury. In chronic pressure overload model by thoracic aortic constriction (TAC), Wnt11 expression was downregulated at 2 weeks after induction of pressure overload and further decreased at 8 weeks after TAC (Figure 3A).

To determine the cell types expressing Wnt11, we fractioned cardiac cell types from the hearts subjected to pressure overload. In cell population level, Wnt11 was expressed in all resident cardiac cell types while more highly expressed in fibroblasts (Figure 3B). In response to pressure overload, Wnt11 expression in nonmyocytes was downregulated at 2 weeks after TAC, and a similar trend was also observed in cardiomyocytes (Figure 3C).

TABLE 1 Wnt11 deficiency induces cardiac growth but does not compromise cardiac function

	WT $n = 5$	Wnt11 KO $n = 3$
EF (%)	53.8 ± 3.7	53.4 ± 8.3
FS (%)	27.8 ± 2.3	27.7 ± 5.1
HR (BPM)	438 ± 32	442 ± 12
LVID;d (mm)	4.77 ± 0.32	4.93 ± 0.97
LVID;s (mm)	3.45 ± 0.29	3.59 ± 0.91
LVPW;d (mm)	0.69 ± 0.06	0.87 ± 0.08*
LVPW;s (mm)	1.06 ± 0.04	1.28 ± 0.15*
LVW/BW	3.10 ± 0.45	3.92 ± 1.51
LV Vol;d (μl)	106.6 ± 16.4	118.6 ± 55.7
LV Vol;s (μl)	49.5 ± 9.8	57.6 ± 35.3
BW (g)	31.8 ± 1.3	31.1 ± 0.6

Note: Mice were subjected to echocardiography analysis at the age of 4 months. Ejection fraction (EF), fractional shortening (FS), heart rate (HR), LV internal diameter in diastole (LVID;d) and systole (LVID;s), LV posterior wall thicknesses (LVPW;d, LVPW;s), LV weight versus body weight (LVW/BW), LV volumes (LV Vol;d, LV Vol;s), and body weights (BW) are represented as mean ± SD; *$p < .05$, versus WT.

3.4 | The effects of Wnt11 deficiency on cardiac function and hypertrophy in pressure overload

Wnt11 can contribute to cardiac stress by potentially increasing cardiomyogenesis, regulating fibroblast activity, and increasing the transcription factors of fetal gene program generally involved in cardiac hypertrophy and remodeling processes in stress. Altogether, these suggest that the potential roles of Wnt11 signaling in cardiac development and tissue homeostasis may be relevant to adult myocardium remodeling, which could potentially be counteracted by inhibition of Wnt11. Our next aim was to define whether genetic Wnt11 deficiency modulates cardiac structure and function in response to stress in the adult heart. To study the partial downregulation/deficiency of Wnt11, we included the heterozygote (HET) Wnt11

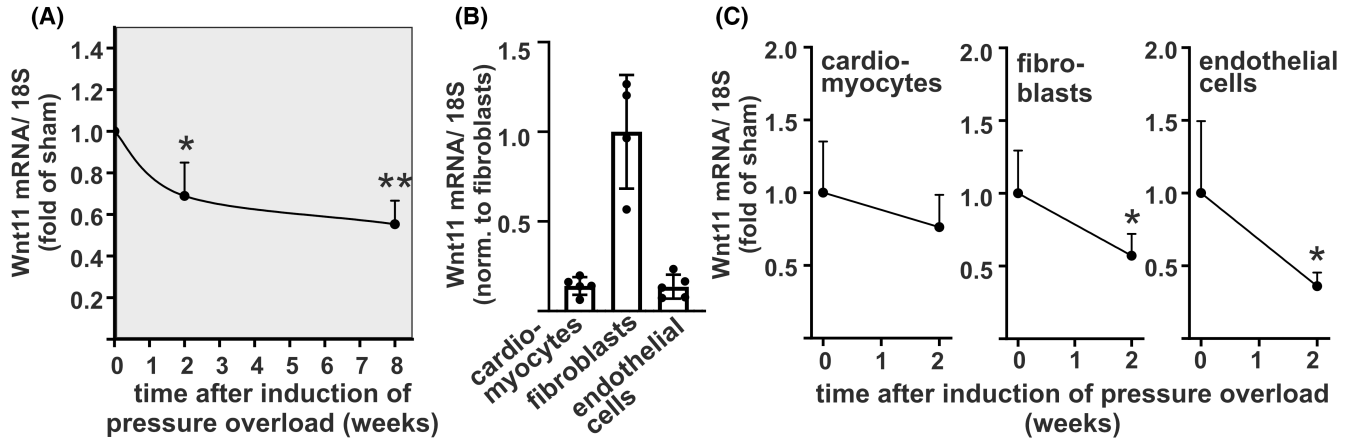

FIGURE 3 Wnt11 expression is downregulated in myocardium in response to stress. (A) Wnt11 expression was determined from LV myocardium after pressure overload induced by thoracic aortic constriction (TAC). (B) Wnt11 expression was determined in resident cardiac cells fractioned from healthy ventricular myocardium. (C) Wnt11 expression was determined in resident cardiac cells fractioned from ventricular myocardium from sham-operated hearts and 2 weeks after TAC from wild-type mice; $n = 6–7$ (A), $n = 4–5$ (B–C). qPCR result at each timepoint has been compared to its own sham-operated control group; *$p < .05$, **$p < .01$ versus sham.

deficient study group to be compared with WT and Wnt11 KO mouse groups. This improved the statistical power of our study since it was difficult to reach a sufficient number of male Wnt11 KO mice. To investigate the effect of Wnt11 deficiency in hemodynamic pressure overload, 8-week-old mice were subjected to TAC and followed for 8 weeks.

At baseline monitoring of cardiac structure and function with echocardiography before TAC surgery, there were no differences between genotypes although a mild hypertrophy was observed in Wnt11 KO hearts (Figure S4). At 8 weeks, LV posterior wall thicknesses were significantly elevated in all genotypes in response to TAC whereas LV inner diameter remained unchanged (Figure 4A, Table 2). TAC operation decreased the cardiac systolic function (Figure 4A, full echocardiography data in Table 2), but no differences between the genotypes of TAC-operated mice were observed.

Despite the finding that Wnt11 KO hearts were enlarged in healthy mice (Figure 2, Table 1), Wnt11 KO mice did not elicit greater hypertrophic response to pressure overload. This was evidenced by analysis of LV posterior wall thickness by echocardiography (Figure 4A, Table 2), determination of heart weight to body weight ratio (Figure 4B), analysis of LV area in histological sections (Figure 4C), and by measuring of cross-sectional area of individual cardiomyocytes (Figures 4D and S4). Additionally, Wnt11 KO hearts showed reduced phosphorylation of S6 ribosomal protein (Figure 4E). Given that there was a moderate prohypertrophic effect in Wnt11 KO hearts, this may represent a compensatory mechanism to curtain further hypertrophic growth. Wnt11 deficiency did not affect the phosphorylation of GSK3β (Figure 4E).

3.5 | The effects of Wnt11 deficiency on cardiac hypertrophic and fibrotic remodeling in pressure overload

The potential Wnt11 functions in the stressed heart may also involve other cell types such as fibroblasts and endothelial cells, where Wnt11 is also expressed (Figure 3B,C). We further determined the effect of Wnt11 deficiency on cardiac capillary network and extracellular matrix modeling.

Wnt11 deficiency did not have a significant effect on perivascular or interstitial fibrosis (Figure 5A). Furthermore, Wnt11 deficiency did not affect capillary area or density in LV myocardium (Figure 5B). Next, we analyzed the expression of genes involved in cardiac remodeling, including genes of myocardial hypertrophy and contractility, cardiogenesis, induction of fetal gene program and regulation of extracellular matrix homeostasis. In line with the results on cardiac function, hypertrophy, and fibrosis, Wnt11 deficiency did not affect these gene expressions in the heart (Figure 5C). However, Wnt11 KO significantly increased skeletal α-actin/β-myosin heavy chain (β-MHC) ratio (Figure 5C). Also evidenced in this dataset, Wnt11 expression was reduced in LV myocardium in response to TAC (Figure 5D) similar to results described in Figure 3A. Wnt11 expression was downregulated by TAC even further in Wnt11 HET hearts and, as confirmed, fully absent in Wnt11 KO hearts (Figure 5D). The expression of another noncanonical Wnt, Wnt5a, which may act in cooperation with Wnt11, was downregulated by TAC but not affected by Wnt11 knockdown (Figure 5D). Similarly, Wnt11 deficiency did not affect Wnt5a expression levels in neonatal, juvenile, or sham-operated mouse hearts (Figure S5). When determined from Gene expression Omnibus profile, non-canonical Wnt expressions (Wnt11, Wnt4, Wnt5a) in the heart seem to be rather downregulated after experimental cardiac pressure overload (Figure S6).

3.6 | Cooperation of noncanonical Wnt proteins in restriction of cardiomyocyte hypertrophic growth

Finally, to study the effect of recombinant noncanonical Wnt proteins on hypertrophy, we treated neonatal cardiomyocytes with Wnt11, Wnt5a, and their combination; Noncanonical Wnt proteins did not affect leucine incorporation in an unstimulated situation (Figure 6A). Interestingly, when given a hypertrophic stimulus FGF, Wnt11, or Wnt5a alone did not modulate the hypertrophic growth response, but when combined, a clear reduction of leucine incorporation was detected (Figure 6B). This suggests both Wnt11 and Wnt5a to be required for anti-hypertrophic effect in stressed cardiomyocytes. Similar results were obtained with endothelin-1 stimulus (ET-1), suggesting the phenomenon to be independent on the type of hypertrophic stimuli (Figure 6C). Furthermore, noncanonical Wnt proteins, Wnt5a alone or together with Wnt11, downregulated the expression of hypertrophic marker genes BNP and β-MHC in response to ET-1 stimuli (Figure 6D).

4 | DISCUSSION

4.1 | Wnt11 in the regulation of cardiomyocyte proliferation and maturation

Cardiomyocytes are terminally differentiated cells that were long considered unable to undergo transition

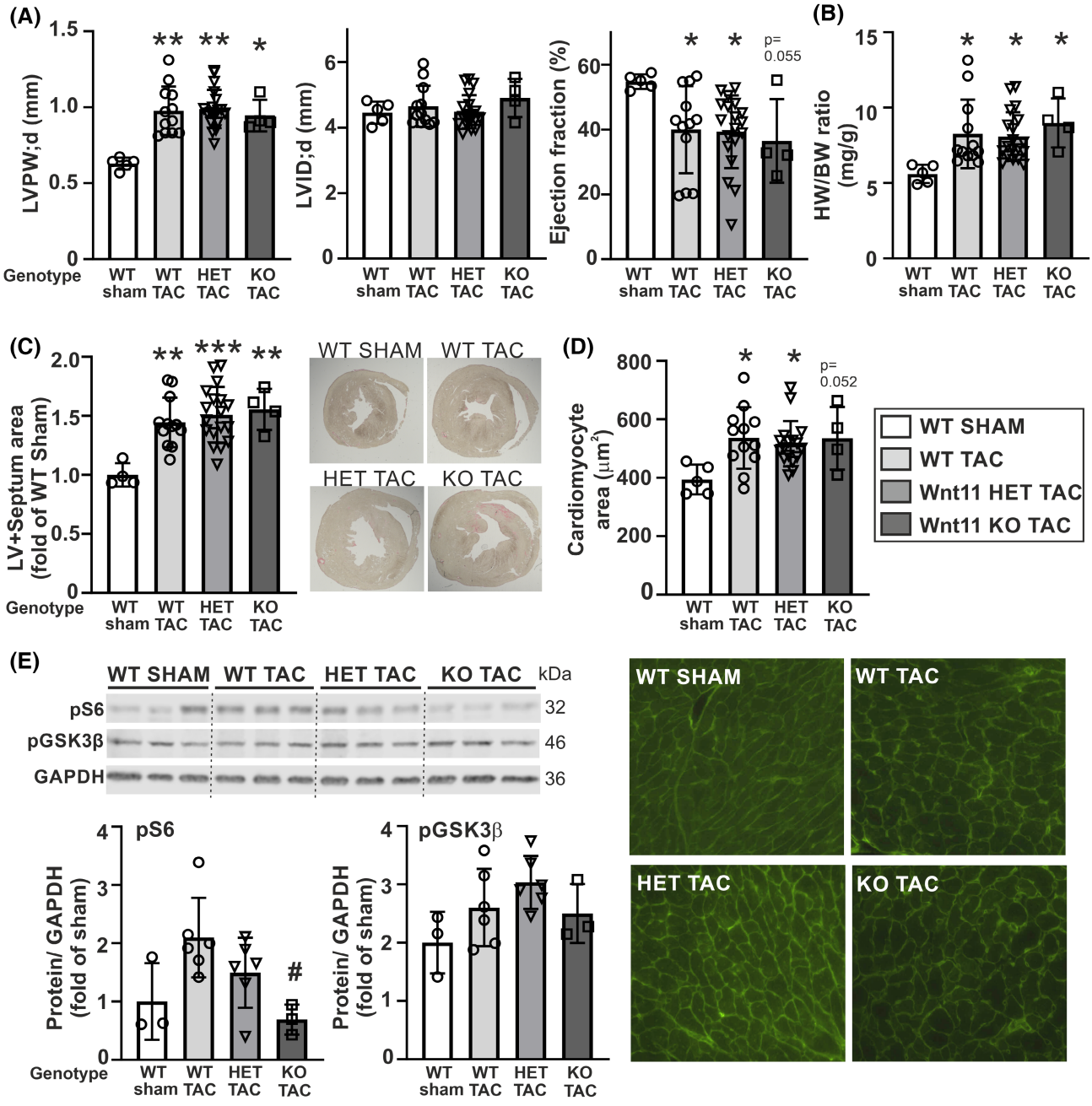

FIGURE 4 Wnt11 deficiency does not exacerbate cardiac hypertrophy in hemodynamic pressure overload. (A) Echocardiography analysis of LV dimensions and cardiac function 8 weeks after TAC (full data in Table 2). Left ventricular posterior wall thickness at diastole (LVPW;d), LV internal diameter at diastole (LVID;d). (B) Heart weight to body weight (HW/BW) ratios 8 weeks after TAC. (C) Myocardial area quantified from LV and septal wall with representative images. (D) Cross-sectional areas of individual cardiomyocytes determined from endocardial and epicardial LV myocardium with representative images below, with wheat germ agglutinin stain. (E) Analysis of hypertrophic marker phosphorylated S6, canonical Wnt, and β-catenin regulator glycogen synthase kinase 3β by western blot from LV myocardium 8 weeks after TAC; $n = 5, 12, 19, 4$ (A–D), $n = 3, 6, 6, 3$ (E); $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus sham, $\#p < .05$ versus WT TAC.

through the cell cycle. It has been demonstrated that cardiomyocyte renewal exists in the adult mammalian heart,[31] however, the signaling pathways regulating cardiomyocyte proliferation are poorly understood. Data from previous studies suggest a role for Wnt growth factors in regulating the cell cycle in various cell types. Wnt factors are a family of secreted signaling proteins that have been characterized well for their important role in tumor progression, stem cell differentiation as well as embryonic development.[32] Here, we studied the involvement of non-canonical Wnt11 protein in the regulation of postnatal cardiomyocyte proliferation and maturation.

There is a debate whether cardiomyocyte proliferation bursts during preadolescence to establish the final

TABLE 2 Wnt11 deficiency does not exacerbate cardiac hypertrophy in pressure overload

	WT sham $n = 5$	WT TAC $n = 12$	Wnt11 HET TAC $n = 19$	Wnt11 KO TAC $n = 4$
EF (%)	54.8 ± 2.2	40.0 ± 13.4*	39.3 ± 11.2*	36.5 ± 12.9
FS (%)	28.3 ± 1.3	19.7 ± 7.3*	19.2 ± 5.9*	17.8 ± 7.2*
HR (BPM)	404 ± 30	444 ± 53	436 ± 57	433 ± 65
LVID;d (mm)	4.45 ± 0.34	4.65 ± 0.63	4.48 ± 0.51	4.90 ± 0.58
LVID;s (mm)	3.20 ± 0.29	3.77 ± 0.85	3.65 ± 0.68	4.06 ± 0.80
LVPW;d (mm)	0.63 ± 0.04	0.98 ± 0.16**	0.99 ± 0.13**	0.94 ± 0.11*
LVPW;s (mm)	0.96 ± 0.07	1.27 ± 0.15***	1.27 ± 0.13***	1.17 ± 0.07*
LVW/BW	3.77 ± 0.30	6.60 ± 2.87*	5.90 ± 1.83	7.24 ± 2.03*
LV Vol;d (μl)	90.9 ± 16.0	102.2 ± 34.3	93.2 ± 25.7	114.7 ± 30.6
LV Vol;s (μl)	41.3 ± 8.9	65.1 ± 37.3	59.0 ± 28.5	75.6 ± 31.8
BW (g)	26.0 ± 0.7	27.8 ± 2.4	27.7 ± 2.3	26.1 ± 1.0

Note: Mice were subjected to a sham operation or thoracic aortic constriction (TAC) and subjected to echocardiography analysis after 8 weeks. Ejection fraction (EF), fractional shortening (FS), heart rate (HR), LV internal diameter in diastole (LVID;d) and systole (LVID;s), LV posterior wall thicknesses (LVPW;d, LVPW;s), LV weight versus body weight (LVW/BW), LV volumes (LV Vol;d, LV Vol;s), and body weights (BW) are represented as mean ± SD; *$p < .05$, **$p < .01$, ***$p < .001$, versus WT sham.

cardiomyocyte number.[21,27] It looks evident that cardiomyocyte number increases during early postnatal days by 40%; Later indication of DNA replication in preadolescence is rather due to cardiomyocyte multinucleation and polyploidism than actual cell division.[33] During fetal development, inhibition of Wnt11 by systemic maternal injection of Wnt11-morpholino at late gestation led to increased proliferation of cardiomyocytes in both left and right ventricles.[13] In neonatal cardiomyocytes, Wnt11 inhibition was shown to increase cardiomyocyte proliferation (PHH3), but to reduce their maturation (binucleation).[13] In addition, our previous study showed Wnt11 deficiency to reduce cardiogenic differentiation in embryonic stage, interfering with proper LV compaction.[12] In the current study, Wnt11 deficiency did not significantly affect the cardiomyocyte proliferation in ventricular myocardium at early postnatal stage. DNA synthesis (due to cardiomyocyte proliferation/multinucleation/polyploidization) was evident in adolescent and postnatal hearts, but Wnt11 deficiency did not increase EdU incorporation or PHH3 immunoreactivity in adolescent cardiomyocytes either. Even though Wnt11 KO hearts showed a higher degree of preservation of c-myc expression at adolescence, Wnt11 deficiency did not modulate the expression of cardiac transcription factors in postnatal or adolescent heart. These findings suggest postnatal cardiomyocyte maturation to be nearly normal despite the Wnt11 deficiency.

In humans, the number of cardiomyocytes in the left ventricle increases 3.4-fold between 1 and 20 years of life and the rate of cardiomyocyte proliferation appears to gradually decrease until the age of 20.[34] In theory, enhancing the proliferation of existing cardiomyocytes in the adult heart may provide a feasible strategy to enhance cardiac repair and improve cardiac function after injury. Although we did not investigate whether Wnt11 deficiency affects adult cardiomyocyte cell cycle or cardiomyocyte aging, our study demonstrates that Wnt11 deficiency does not remarkably induce cardiomyocyte proliferation postnatally, as analyzed at P0 and at adolescence.

4.2 | Wnt11 in regulation of cardiomyocyte hypertrophy and fibrotic remodeling in response to cardiac stress

Since Wnt11 deficiency led to enlarged cardiac phenotype in the unstressed hearts, we aimed to study whether Wnt11 could regulate the cardiomyocyte growth under hypertrophic stress stimulus. Zou et al. have reported that serum Wnt5a and Wnt11 levels are increased in patients with hypertension and in the hearts of TAC mice.[35] Opposite to this, we detected Wnt11 expression to be downregulated in the myocardium in response to cardiac hemodynamic stress. To examine the role of Wnt11 in the development of cardiac hypertrophy and fibrosis, we subjected Wnt11 HET and Wnt11 KO mice to chronic pressure overload. We found the cardiac remodeling in TAC-operated Wnt11 KO hearts to be similar to that in WT mice. Wnt11 deficient hearts represented relatively normal pathophysiology in cardiomyocytes as well as in nonmyocytes as hypertrophy, fibrosis, or gene expression of known factors of remodeling were alike with those in WT TAC hearts. There is a defined actin/myosin ratio in the myocardium that is required for the maintenance of the structure and function of cardiac muscle. We observed Wnt11 KO to increase the skeletal α-actin/β-MHC ratio in response to

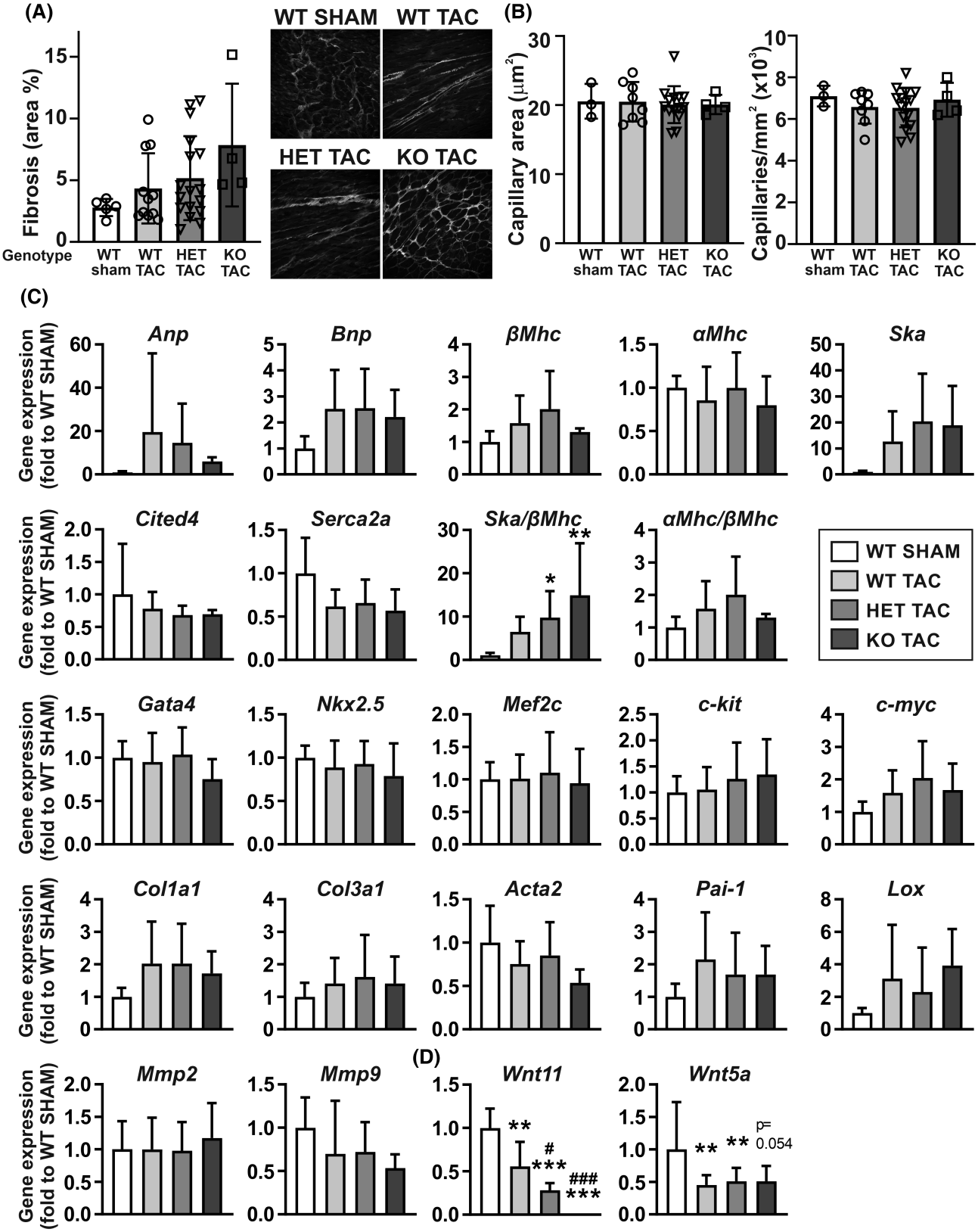

FIGURE 5 Wnt11 deficiency does not modulate hypertrophic and fibrotic remodeling in pressure overload-induced cardiac stress. (A) Interstitial fibrosis was determined with picrosirius stain from LV and septal myocardium 8 weeks after TAC. (B) The cross-sectional areas of individual capillaries and a capillary count were determined from LV myocardium with CD31 stain 8 weeks after TAC. (C). qPCR for the expression of hypertrophic, cardiogenic, and extracellular matrix remodeling genes determined from LV myocardium 8 weeks after TAC. (D) qPCR for the expression of noncanonical Wnts in LV myocardium 8 weeks after TAC; $n = 5, 12, 19, 4$ (A, C–D), $n = 3, 8, 14, 4$ (B); $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus WT sham; $\#p < .05$, $\#\#\#p < .001$ versus WT TAC.

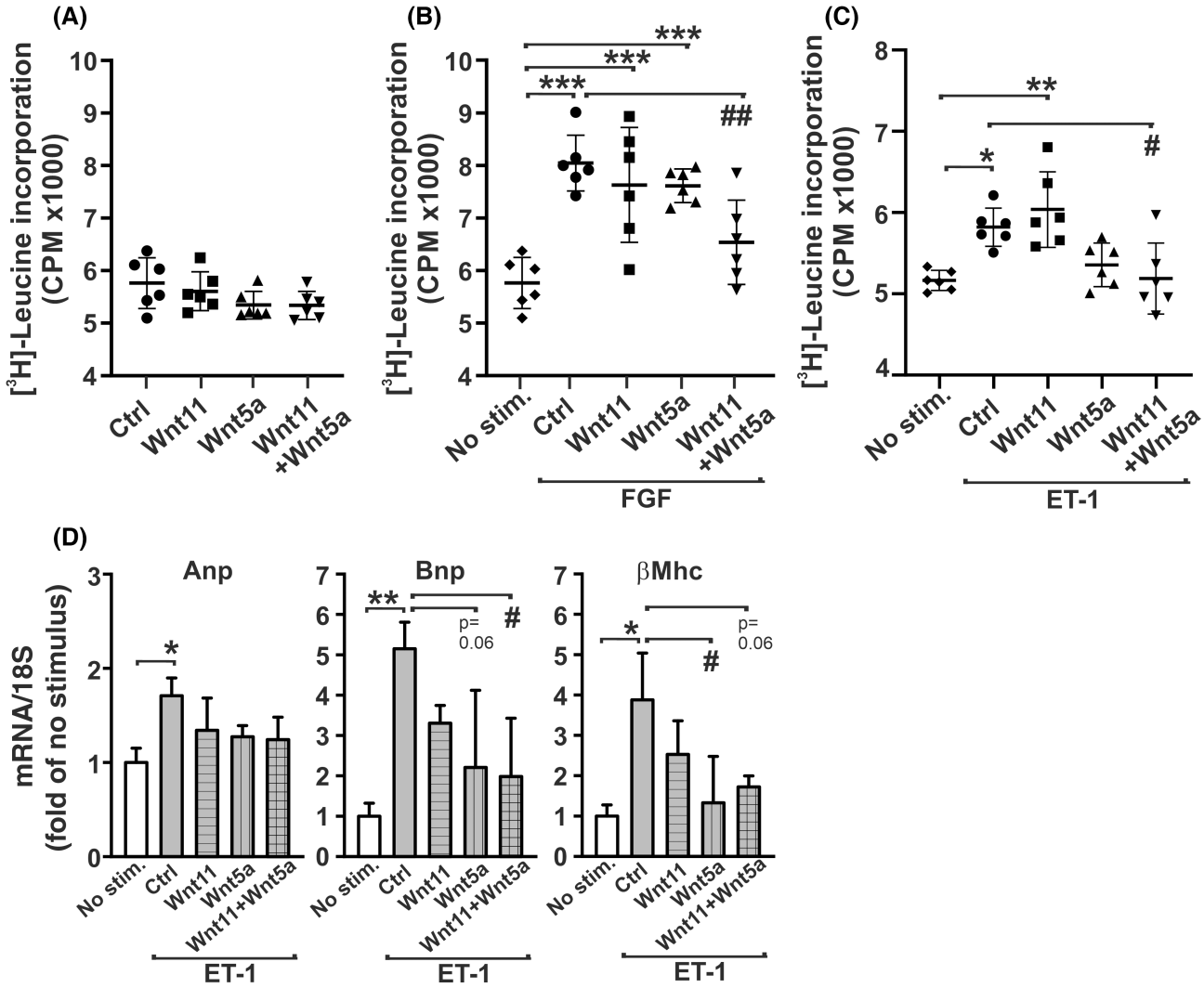

FIGURE 6 Wnt11 and Wnt5a cooperate in anti-hypertrophic effect under hypertrophic stimulus. (A) Neonatal cardiomyocytes were treated with recombinant Wnt11 and Wnt5a for 24 h in the presence of [^{3}H]-leucine. The incorporation of labeled leucine, as an indication of protein synthesis, was determined. (B) FGF increased protein synthesis, downregulated by co-addition of recombinant Wnt11 and Wnt5a. (C) Similarly, endothelin-1 (ET-1) increased protein synthesis, downregulated by combined treatment with Wnt11 and Wnt5a. (D) qPCR analysis of hypertrophic gene expression in neonatal cardiomyocytes treated with recombinant Wnt11 and Wnt5a 24 h in the presence of hypertrophic stimulus ET-1; $n = 6$ (A–C), $n = 3$ (D); *$p < .05$, **$p < .01$, ***$p < .001$ versus ctrl; #$p < .05$, ##$p < .05$ versus FGF/ET-1.

hemodynamic pressure overload. Human adults with genetic hypertrophic cardiomyopathies (HCM) often display upregulation of skeletal α-actin in the ventricles probably to compensate the decreased contractility. Increase in the skeletal α-actin/β-MHC ratio could thus reflect the compensatory mechanism in the Wnt11 KO heart to maintain LV contractile function during stress. Wnt5a and Wnt11 have also been reported as pro-fibrotic mediators. Partial silencing of Wnt11/5a proteins by the shRNA-AAV9 approach was reported to attenuate fibrotic and hypertrophic remodeling in cardiac pressure overload.[35] We did not detect antihypertrophic nor antifibrotic remodeling in either partial or full Wnt11 deficiency in pressure overload.

Canonical Wnt/β-catenin signaling may exacerbate cardiac stress in pressure overload. Loss of emerin, which causes hyperactivation of canonical Wnt/β-catenin signaling, increases cardiomyocyte proliferation at embryonic stage and leads to exacerbation of cardiac dysfunction in response to pressure overload.[36] Furthermore, inhibition of downstream effector of Wnt/β-catenin signaling in disheveled KO (*Dvl*−/−) mice attenuated cardiac hypertrophy in response to pressure overload.[37] In our model, Wnt11 deficiency did not exacerbate cardiac dysfunction. On the other hand, Wnt11 deficiency did not attenuate pressure overload-induced cardiac hypertrophy.

Wnt proteins may also regulate nonmyocyte cell function in the heart as shown in many other tissues.[38] Regarding canonical Wnt signaling in fibrosis, it is known that canonical Wnts are upregulated in fibrotic

diseases and that activation of canonical Wnt/β-catenin signaling induces TGFβ-mediated tissue fibrosis.[39] In aging, elevated Wnt signaling also increases skeletal muscle fibrosis.[40] While affecting cardiomyocytes at embryonic stage, Wnt11 also regulates other cell lineages in early embryonic tissue in spatio-temporal manner.[41] Furthermore, canonical Wnt/β-catenin signaling leads to enhanced proliferation of cardiac fibroblasts in vitro and modulates their extracellular matrix remodeling by upregulating matrix metalloproteinases.[42] Conditional deletion of β-catenin from resident cardiac fibroblasts or from activated myofibroblasts at the time of aortic banding preserved cardiac function, reduced cardiac hypertrophy, and attenuated fibrosis by reducing excessive extracellular matrix production.[43] However, the role of noncanonical Wnt signaling in tissue fibrosis, especially in the heart, remains largely unknown. Additionally, Wnt11 has been shown to play a role in aortic valve calcification; Wnt11 was localized to inflammatory cells and fibroblasts in areas of calcification and found to increase apoptosis as well as calcification of human aortic valve interstitial cells in vitro.[44] We found Wnt11 expression in both cardiomyocytes and nonmyocyte fraction. Despite the broad expression pattern in cardiac cells, partial, or full global Wnt11 deficiency did not affect the development of cardiac fibrosis or expression of pro-fibrotic genes in the myocardium.

Previous studies indicate that Wnt11 is induced by hypoxia in various cell types such as mesenchymal stem cells, myoblast, macrophage, and cancer cell lines in vitro.[45] On the contrary, Wnt11 is downregulated in the heart at perinatal stage in response to prolonged moderate hypoxia and is associated with cyanotic congenital heart defects.[13] In line with this, we detected a sharp downregulation of Wnt11 expression in the left ventricles after ischemia–reperfusion injury. Thus far, Wnt11 has been considered mostly to be cardioprotective in ischemia. Wnt11 gene therapy improved recovery from myocardial ischemia by modulating the inflammation.[46] In addition, transplantation of mesenchymal stem cells transduced with Wnt11 improved adjacent cardiomyocyte survival by paracrine-mediated manner.[47] It remains to be studied if Wnt11 deficiency has detrimental effects on recovery from ischemic cardiac injury.

4.3 | Reciprocal actions of noncanonical Wnt proteins Wnt11 and Wnt5a in the heart

The members of the Wnt protein family are classified into two distinct groups. The Wnt1 group including the Wnt1, Wnt3A, and Wnt8 signaling proteins mediate the canonical β-catenin pathway, whereas members of the Wnt5A group including Wnt4, Wnt5A, and Wnt11, transduce signal through the non-canonical Wnt signaling pathways. Both Wnt11 and Wnt5a are co-required for the proper second heart field development in embryogenesis where they act by restraining canonical Wnt/β-catenin signaling.[48] Furthermore, cooperative signaling of Wnt11 and Wnt5a was shown to inhibit the Wnt/β-catenin signaling through caspase-dependent degradation of Akt.[49] In the current study, we observed that augmenting both Wnt11 and Wnt5a was required to reduce neonatal cardiomyocyte protein synthesis under hypertrophic stimulus.

Opposed to what expected, cardiac hypertrophy or cardiac remodeling process in pressure overload-induced cardiac stress was not substantially affected by full or partial Wnt11 deficiency. Notably, no difference was observed in cardiomyocyte size between the genotypes after pressure overload. Therefore, the observed increase in heart weight and posterior wall area in unstressed Wnt11 KO hearts likely stems from cardiomyocyte hyperplasia in Wnt11 deficient heart during embryonic development accompanied by postnatal regional cardiac hypertrophy throughout adulthood. While Wnt5a was not reciprocally upregulated in the Wnt11 KO hearts, Wnt5a may compensate the missing actions of Wnt11 in the homeostasis of the postnatal heart as well as in cardiac stress.

4.4 | Limitations of the study and conclusions

While Wnt11 participates in embryonic development, full Wnt11 deficiency can lead to embryonic lethality in some genetic background, such as in the 129SV mouse strain.[12] When bred to C57BL6J background, although Wnt11 deficiency increased embryonic lethality, the individuals born with minor cardiac defects could normally reach maturity and be subjected to experimental cardiac pressure overload. In this context, the use of drug-inducible KO strategies with Cre-*loxP* or newer nuclease-mediated gene editing systems would have been potential approaches to study the effect of Wnt11 deficiency in the adult heart[50,51] and it is a clear limitation that we did not start with an inducible KO model. These strategies, however, are more demanding and also have their problems and challenges in terms of specificity and side effects. In addition, with the current data indicating only limited effect of global Wnt11 KO on pressure overload-induced cardiac phenotype, creating a conditional Wnt11 KO model does not appear an attractive approach, but could merely lead to recapitulation of our data. Concerning cell type-specific KO model, Wnt11 is expressed by all major cardiac cell types, making it necessary to delete Wnt11 from several

cell types simultaneously to deplete Wnt11 production in the heart. Deletion of Wnt11 only from one cell type is not likely to have drastic effects, as the model would mimic the partial Wnt11 deletion that was achieved in the heterozygous Wnt11+/− mice in the current study. It is thus plausible that the use of conditional KO models would not offer additional effects.

As an alternative approach, Wnt proteins have been targeted with small-molecule inhibitors in previous studies. Targeting porcupine, which leads to inhibition of Wnt palmitoylation and secretion from the endoplasmic reticulum, has been shown to reduce cardiac hypertrophy and remodeling in pressure overload[52] and in infarcted heart.[53] Porcupine inhibitors target canonical Wnt proteins but may also target non-canonical Wnt such as Wnt5a. Currently, the cooperation of Wnt proteins with each other and their interactions in modulation of activity of signaling pathways is poorly understood.

In conclusion, our studies demonstrate that Wnt11 deficiency does not induce cardiomyocyte proliferation in postnatal or adolescent mouse. However, Wnt11 deficiency causes mild hypertrophy, which is sustained through later cardiac development to adulthood but does not exacerbate cardiac response to hemodynamic stress. It would be of future interest to determine how cooperative signaling of Wnt11 and Wnt5a modulates cardiac stress response in vivo. Therefore, future experiments should be performed using approaches that target multiple, prespecified Wnt proteins simultaneously.

AUTHOR CONTRIBUTIONS

E.H. performed in vitro and in vivo studies, analyzed the results, wrote, and revised the manuscript; I.N. performed in vivo studies and data analysis, wrote, and critically revised the manuscript; Z.S. conducted in vivo studies and analyzed cardiac function; T.A. participated in in vitro studies and analysis, L.V. participated in in vivo studies and analysis; R.Y. and E.V. participated in histological analysis, R.L. participated in biochemical analysis and cell fractionation, L.RK. participated in cell fractionation and analysis, S.V. designed the study, participated in in vivo analysis and writing the manuscript, R.K. designed the study, participated in data analysis and critically revised the manuscript, J.M. designed the study, participated in data analysis and wrote the manuscript. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank Marja Arbelius, Sirpa Rutanen, and Kirsi Salo (University of Oulu) for excellent technical assistance. We thank Dr. Qi Xu, Center for Cell Matrix Research, University of Oulu, for mouse genotyping and dissection of neonatal hearts. This work was supported by research funding from Academy of Finland grants 268505 (J.M.) and 297094 (R.K.); Aarne Koskelo Foundation (E.H.); Paavo Nurmi Foundation (E.H.) and the Finnish Foundation for Cardiovascular Research (Z.S., R.K.).

DISCLOSURES

The authors have declared that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data that support the findings of this study are also available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Halmetoja E, Nagy I, Szabo Z, et al. Wnt11 in regulation of physiological and pathological cardiac growth. *The FASEB Journal*. 2022;36:e22544. doi: 10.1096/fj.202101856RRRR