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ORIGINAL ARTICLE

Pregnancy-induced physiological hypertrophic preconditioning attenuates pathological myocardial hypertrophy by activation of FoxO3a

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Abstract

Previous studies show a woman's pregnancy is correlated with post-reproductive longevity, and nulliparity is associated with higher risk of incident heart failure, suggesting pregnancy likely exerts a cardioprotection. We previously reported a cardioprotective phenomenon termed myocardial hypertrophic preconditioning, but it is unknown whether pregnancy-induced physiological hypertrophic preconditioning (PHP) can also protect the heart against subsequent pathological hypertrophic stress. We aimed to clarify the phenomenon of PHP and its mechanisms. The pluripara mice whose pregnancy-induced physiological hypertrophy regressed and the nulliparous mice underwent angiotensin II (Ang II) infusion or transverse aortic constriction (TAC). Echocardiography, invasive left ventricular hemodynamic measurement and histological analysis were used to evaluate cardiac remodeling and function. Silencing or overexpression of *Foxo3* by adeno-associated virus was used to investigate the role of FoxO3a involved in the antihypertrophic effect. Compared with nulliparous mice, pathological cardiac hypertrophy induced by Ang II infusion, or TAC was significantly attenuated and heart failure induced by TAC was markedly improved in mice with PHP. Activation of FoxO3a was significantly enhanced in the hearts of postpartum mice. FoxO3a inhibited myocardial hypertrophy by suppressing signaling pathway of phosphorylated glycogen synthase kinase-3β (p-GSK3β)/β-catenin/Cyclin D1. Silencing or overexpression of *Foxo3* attenuated or enhanced the anti-hypertrophic effect of PHP in mice with pathological stimulation. Our findings demonstrate that PHP confers resistance to subsequent hypertrophic stress and slows progression to heart failure through activation of FoxO3a/GSK3β pathway.

Keywords Physiological myocardial hypertrophy · Heart failure · Pregnancy hypertrophic preconditioning · FoxO3a

Introduction

Pathological cardiac hypertrophy has detrimental effects on cardiac function and makes a major contribution to the eventual onset of heart failure [1]. We previously reported a phenomenon termed hypertrophic myocardial preconditioning, in which short-term imposition of pathological hypertrophic stress on the heart has a protective effect against subsequent hypertrophic stress and slows progression to heart failure [2], and these results were verified by other researchers [3–6]. Recently, we found that myocardial hypertrophy preconditioning induced by exercise exerts a cardioprotective effect on

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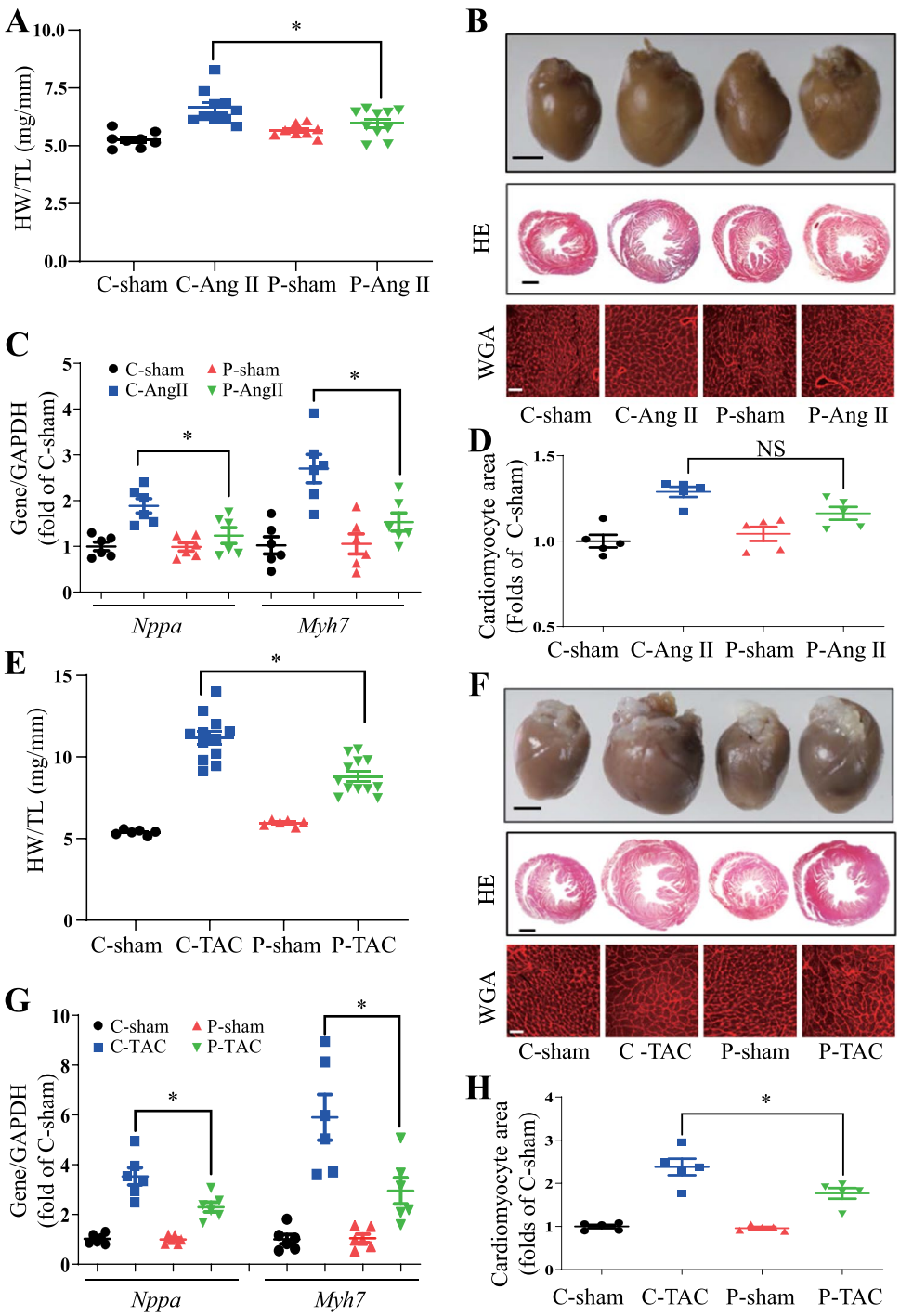


pathological stress via antihypertrophic memory [7, 8], indicating a cardioprotection can be induced by preconditioning of physiological myocardial hypertrophy. However, there has been little investigation into the effects of pregnancy related physiological myocardial hypertrophy on subsequent resistance to pathological hypertrophic stress.

Significant physiological changes of the cardiovascular system occur during pregnancy, including cardiac hypertrophy, sustained activation of the renin–angiotensin–aldosterone system, and a marked increase in plasma volume, indicating that the heart is under stress [9]. These physiological responses to increased demand are beneficial for both the mother and fetus. There is evidence that short-term, high-intensity stress on the heart can induce cardioprotective preconditioning [2], but it is unknown whether less intense physiological stress acting over a longer period, such

◂**Fig. 1** PHP attenuates pathological myocardial hypertrophy. **A** Heart weight/tibia length (HW/TL) ratio after infusion of angiotensin II (Ang II) or the vehicle with/without PHP, n = 8–10 per group. $P_{int} = 0.007$. **B** Representative macroscopic photographs of hearts (upper panels, scale bar = 2 mm), as well as hematoxylin–eosin (HE) stained sections of hearts (middle panels, scale bar = 1 mm) and wheat germ agglutinin (WGA)-stained myocardial sections (lower panels, scale bar = 30 μm). **C** qPCR for myocardial *Nppa* and *Myh7,* n = 6 per group. *Nppa*: $P_{int} = 0.041$. *Myh7*: $P_{int} = 0.025$. **D** Quantitative analysis of cardiomyocyte cross-sectional area, n = 5 per group. $P_{int} = 0.059$. **E** HW/TL ratio of mice subjected to transverse aortic constriction (TAC) or sham surgery with/without PHP. $n = 6–12$ per group. $P_{int} < 0.001$ (**F**) Representative macroscopic photographs of hearts (upper panels, scale bar = 2 mm), as well as H&E-stained sections of hearts (middle panels, scale bar = 1 mm) and WGA-stained myocardial sections (lower panels, scale bar = 30 μm). **G** qPCR for *Nppa* and *Myh7, n* = 6 per group. *Nppa*: $P_{int} = 0.021$. *Myh7*: $P_{int} = 0.017$. **H** Quantification of cardiomyocyte cross-sectional area in each group, n = 5 per group. $P_{int} = 0.038$. *P* values were calculated by two-way ANOVA followed by Bonferroni's correction for post hoc multiple comparisons (A, C, D, E, G, H). $*P < 0.05$ vs. the Control-Ang II (C-Ang II) group or control TAC (C-TAC). NS: Not Significant. C: Control. P: Pregnancy-induced hypertrophic preconditioning. TAC: transverse aortic constriction. *Nppa*: natriuretic peptide A, *Myh7*: myosin heavy chain 7

as pregnancy, can also protect the heart against subsequent pathological hypertrophic stress.

A few studies have provided evidence for a cardioprotective effect of pregnancy. Kara et al. reported that fetal cells enter the maternal circulation and migrate to sites of injury in the maternal myocardium to assist in repair [10]. It was also reported that peripartum cardiomyopathy has the highest recovery rate among all types of heart failure [11], and pregnancy-induced hypertensive disorders are associated with a better outcome in patients with peripartum cardiomyopathy [12]. Interestingly, it was reported that the age of women at final pregnancy is correlated with their post-reproductive longevity [13], and nulliparity is associated with higher risk of incident heart failure with preserved ejection fraction [14], which suggest that senescence is slowed after pregnancy. These reports led us to postulate that pregnancy induces cardioprotective memory in the mother, possibly by activation of anti-hypertrophic factors, and increases resistance to subsequent cardiac stress, although it is unclear how long such an effect persists.

Forkhead box class O3a (FoxO3a) is an important member of the FOXO subfamily of forkhead transcription factors. Extensive researches have shown that the increased activation of FoxO3a involves with anti-hypertrophic effect in heart [15, 16]. In addition, it has been demonstrated activation of FoxO3a in the hearts of postpartum mice [17], but it is unclear whether enhanced cardiac activation of FoxO3a during the postpartum period confers resistance to pathological hypertrophic stress.

Based on the above points, this study was performed to test the hypothesis that pregnancy-induced hypertrophic preconditioning (PHP) engenders anti-hypertrophic memory and cardiac resistance to subsequent pathological hypertrophic stress, as well as slows progression to heart failure. We also explored the involvement of FoxO3a in the underlying mechanisms.

Materials and methods

All procedures were performed in accordance with our Institutional Guidelines for Animal Research, and this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication, 8th Edition, 2011). This study was approved by ethics review board of Southern Medical University. Detailed Methods section is available in the Supplemental Material (Sequences of primers for real-time PCR are shown in Table S1, and their melting analyses are shown in Fig. S1. Experimental protocols are shown in Fig. S2).

Statistical analysis

Data are reported as the mean ± SEM unless otherwise noted. Unpaired Student's *t*-test was used to compare two groups. Comparisons among multiple groups were performed using either one-way or two-way (if there are two factor levels) ANOVA followed by Bonferroni's correction for post hoc multiple comparisons (SPSS 16.0). Kaplan–Meier analysis was performed for comparison of overall survival. In all analyses, $P < 0.05$ was considered statistically significant.

Results

PHP attenuates pathological myocardial hypertrophy induced by angiotensin-II (Ang II).

We firstly investigated the characteristics of cardiac hypertrophy in pregnant mice. Consistent with previous reports, we found that tail blood pressure decreased slightly in the middle to late stages of pregnancy and returned to baseline by 3 weeks after delivery (Fig. S3A). While plasma Ang II concentrations were increased at middle-late pregnancy stage and recovered to baseline (control) at 3 weeks after delivery (Fig. S3B). Heart rate, body weight (BW), heart weight (HW), HW/tibia length (HW/TL) ratio, and cardiomyocyte surface area were all increased significantly in pregnant mice, while there were no significant changes in the expression of fetal genes *Nppa* (natriuretic peptide type A) and *Myh7* (myosin heavy chain 7) or the lung weight/TL ratio (LW/TL) (Fig. S3C-J).

We also investigated the time window for PHP. When transverse aortic construction (TAC) was performed in

postpartum 1 week and mice were observed for 8 weeks, there were no significant changes in HW/BW ratio and cardiomyocyte surface area of mice, and the cumulative survival rate of mice for 8 weeks of TAC was significantly lower in the postpartum group than in the control group (Fig. S4A-D). These findings suggest that peripartum pathological hypertrophic stress is harmful. Considering pregnancy-induced cardiac hypertrophy regressed completely to normal by 3 weeks after delivery, and pregnancy-associated cardiovascular complications such as peripartum cardiomyopathy or preeclampsia or myocardial ischemia/reperfusion injury are prone to emerge during the peripartum period [9, 18, 19], we selected 3 weeks postpartum as the time for application of pathological hypertrophic stress (Ang II infusion and pressure overload).

In mice with myocardial hypertrophy induced by continuous infusion of Ang II for 4 weeks, tail blood pressure was significantly increased (Fig. S5A-B). The HW/TL ratio, cardiomyocyte cross-sectional area and the expression of fetal genes (*Nppa and Myh7*) were significantly smaller in the PHP-Ang II (P-Ang II) group than in the control-Ang II (C-Ang II) group (Fig. 1A–D). Echocardiography showed a smaller increase in diastolic left ventricular posterior wall thickness (LVPWd) in the P-Ang II group than in C-Ang II group (Fig. S6A-B), but there were no significant differences on left ventricular end-diastolic diameter (LVEDd), end-systolic diameter (LVESd), LV fractional shortening (LVFS) and the LW/BW ratio between the two groups (Fig. S6C-E).

PHP attenuates cardiac remodeling and slows progression of cardiac dysfunction induced by pressure overload

Because Ang II infusion was insufficient to induce heart failure in mice, we employed TAC to induce both cardiac hypertrophy and heart failure for further assessment of the cardioprotective effects of PHP. Four weeks after TAC, the HW/TL ratio and the cardiomyocyte cross-sectional area were significantly smaller, and the expression of *Nppa and Myh7* genes was lower in the P-TAC group than in the C-TAC group (Fig. 1E–H), and the P-TAC group also showed significantly smaller LVPWd, LVEDd and LVESd, and larger LVFS (Fig. 2A–D, Table S2). In addition, LV end-diastolic pressure (LVEDP) was lower in the P-TAC group, while LV d*p*/d*t* max (maximum rate of rising in left ventricular pressure) and LV d*p*/d*t* min (maximum rate of descending in left ventricular pressure) were higher (Fig. 2E–H). TAC induced congestive heart failure with an increase in the LW/TL. Four weeks after TAC, the LW/TL ratio was markedly smaller in the P-TAC group than in the C-TAC group (Fig. 2I). These results demonstrated that PHP could relieve cardiac remodeling and dysfunction caused by pressure-overload.

FoxO3a and its anti-growth signaling were activated in the heart of mice with PHP

According to previous reports, FoxO3a and oxytocin receptor (OTR) are involved in myocardial hypertrophy; therefore, we further confirm their expressions in PHP. We noted that myocardial protein expressions of the FoxO3a and OTR were significantly higher at a week postpartum than in control group, and their gene expressions were increased in heart of 3 weeks postpartum mice (Fig. 3A, Fig. S7A-B). Western blotting showed that phosphorylation of FoxO3a (inactivation of FoxO3) was decreased in a time-dependent manner from baseline to pregnancy at 18d and 3 weeks after delivery (Fig. 3B). Furthermore, the myocardial expression level of *Trim63*, a FOXO3a target gene, was still higher in postpartum mice at 3 weeks after delivery than in control group, while no significant changes were noted on gene expression of *Fbxo32 and Bnip3* (Fig. S8A-C). It is unknown whether a direct relationship exists between FoxO3a and GSK3β. Our CO-IP assays showed a direct binding between FoxO3a and GSK3β or between FoxO3a and Akt in the hearts of postpartum mice at 3 weeks after delivery (Fig. S9). Furthermore, Western blotting demonstrated that pressure overload in the nulliparous mice for 4 weeks significantly resulted in increased myocardial expressions of p-FoxO3a, p-GSK3β, β-catenin, and Cyclin D1, while these increases were prevented by PHP (Fig. 3C–E).

Silencing or overexpression of *Foxo3* affects growth signaling

At 2 weeks after direct intramyocardial injection of (adenovirus-associated virus) AAV-sh-*Foxo3* (short hairpin of *Foxo3*) or AAV-*Foxo3* (overexpression of *Foxo3*) in female mice, we found that silencing of *Foxo3* led to an increased myocardial expression of p-GSK3, β-catenin, and Cyclin D1 compared with the scramble group (Fig. 4A–C). In contrast, overexpression of *Foxo3* was associated with down-regulation of p-GSK3, β-catenin, and Cyclin D1 (Fig. 4D–F).

Silencing or overexpression of *Foxo3* influences cardioprotection by PHP

In postpartum mice subjected to TAC for 4 weeks, *Foxo3* knockdown in heart increased the HW/TL ratio, cardiomyocyte cross-sectional area and fetal genes (Fig. 5A–D). Correspondingly, echocardiographic measurements revealed increases in LVPWd, LVESd, and LVEDd while a reduction in LVFS in *Foxo3* knockdown mice (Fig. 5E–H, Table S3).

LVEDP were increased in *Foxo3* knockdown mice, while LV d*p*/d*t* max and LV d*p*/d*t* min were reduced (Fig. 5J–K). In addition, there is an increase in LW/TL ratio in *Foxo3* knockdown mice (Fig. 5L). In contrast, overexpression of *Foxo3* attenuated TAC-induced increases of the HW/TL ratio, cardiomyocyte cross-sectional area, fetal genes of *Nppa* and *Myh7*, LVPWd, LVESd, LVEDd, LVEDP and LW/TL ratio, and TAC-induced reduction in LVFS, LV d*p*/d*t* max and LV d*p*/d*t* min (Fig. 5A–L).

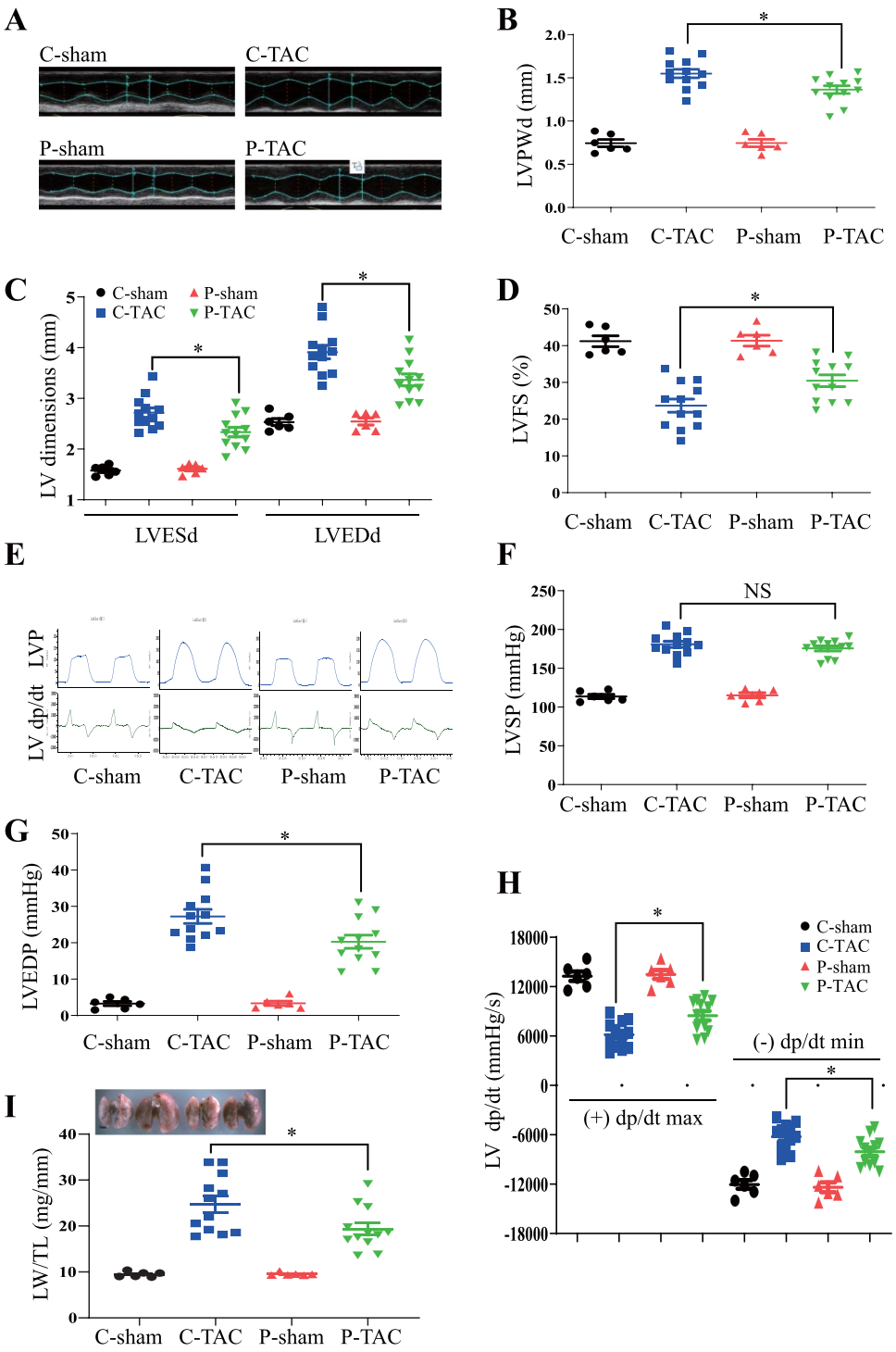


Fig. 2 PHP attenuates cardiac remodeling and slows progression of cardiac dysfunction induced by transverse aortic constriction (TAC) in mice. **A** Representative photographs of M-mode echocardiography of left ventricular (LV) chamber. **B** LV diastolic posterior wall thickness (LVPWd). $P_{int} = 0.128$. (**C**) LV end-diastolic diameter (LVEDd), $P_{int} = 0.067$, and end-systolic diameter (LVESd), $P_{int} = 0.064$. **D** LV fractional shortening (LVFS). $P_{int} = 0.070$. **E** Representative pressure curves obtained with a Millar pressure catheter. **F** LV systolic pressure (LVSP). $P_{int} = 0.685$. **G** LV end-diastolic pressure (LVEDP). $P_{int} = 0.097$. **H** Maximum rate of increase and decrease in LV pressure. dp/dt max: $P_{int} = 0.037$. dp/dt min: $P_{int} = 0.286$. **I** Lung weight/tibia length (LW/TL) ratio. $P_{int} = 0.101$. (B-D, F-I), $n = 6–2$ per group. *P* values were calculated by two-way ANOVA followed by Bonferroni's correction for post hoc multiple comparisons (B-D, F-I). $^*P < 0.05$ vs. the control TAC (C-TAC). *NS* not significant. c control. *P* pregnancy-induced hypertrophic preconditioning. *TAC* transverse aortic constriction

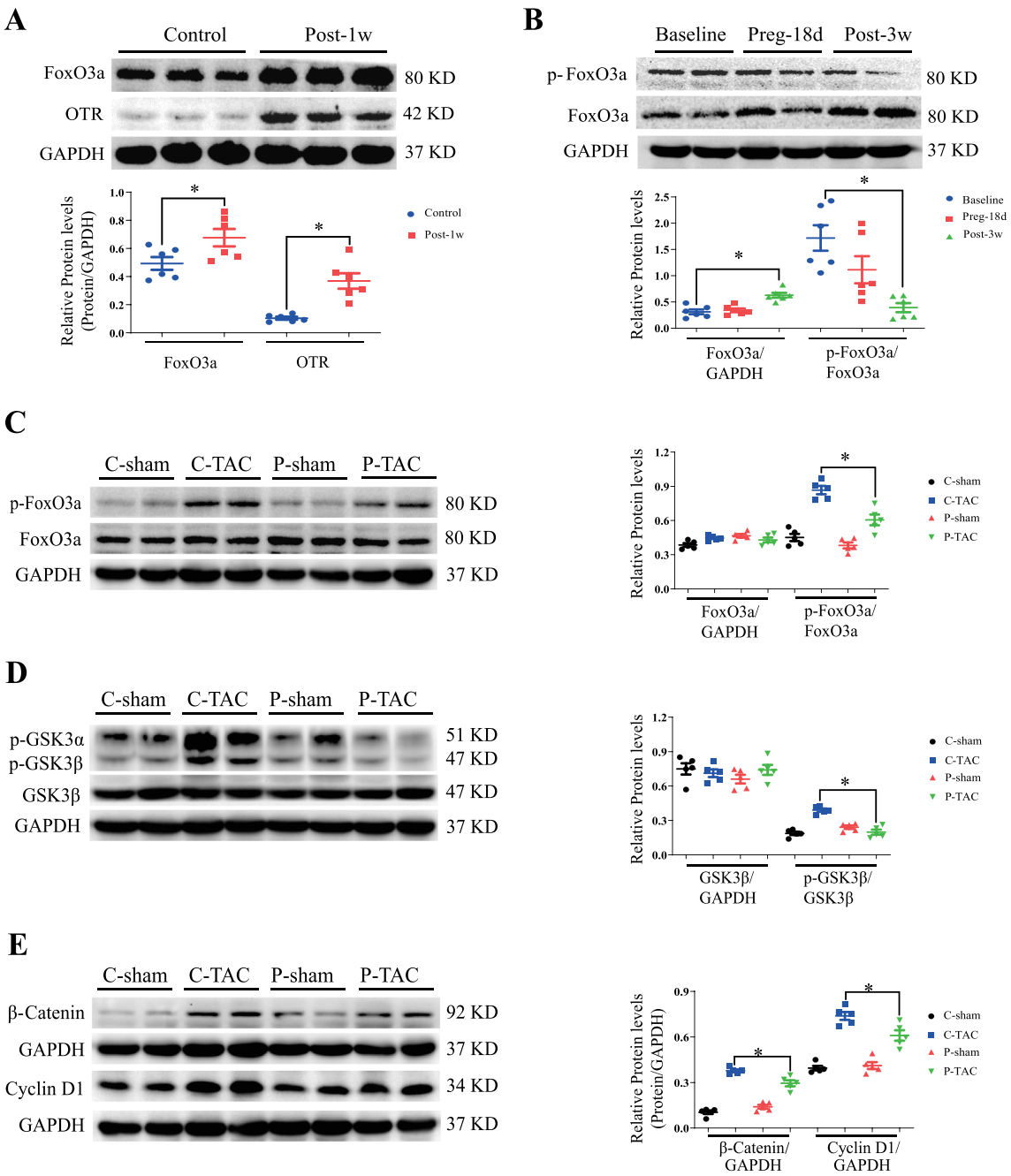


Fig. 3 FoxO3a and its anti-growth signaling were activated in the heart of mice with PHP. **A** Protein expression of FoxO3a and OTR. **B** Western blot analysis of myocardial FoxO3a and phosphorylation of FoxO3a (p-FoxO3a) (**C**) Western blot analysis of p-FoxO3a/FoxO3a. FoxO3a/GAPDH: $P_{int} = 0.018$. p-FoxO3a/FoxO3a: $P_{int} = 0.028$. **D** Western blot analysis of p-GSK3β/GSK3β. GSK3β/GAPDH: $P_{int} = 0.121$. p-GSK3β/GSK3β: $P_{int} < 0.001$. **E** Western blot analysis of β-catenin and Cyclin D1. β-catenin/GAPDH: $P_{int} = 0.001$. Cyclin D1/GAPDH: $P_{int} = 0.016$. **C**–**E** in the hearts of mice subjected to TAC for 4 weeks with/without PHP. **A**–**B** $n = 6$ per group, (**C**–**E**) $n = 5$ per group. *P* values were calculated by unpaired Student's *t*-test (**A**), one-way ANOVA (**B**) or two-way ANOVA followed by Bonferroni's correction for post hoc multiple comparisons (**C**–**E**). $^*P < 0.05$ vs C-TAC. Post-1w: 1 week postpartum. Pre-18d: pregnancy at 18d. Post-3w: 3 weeks postpartum. *C* control, *P* pregnancy-induced hypertrophic preconditioning. *TAC* transverse aortic constriction

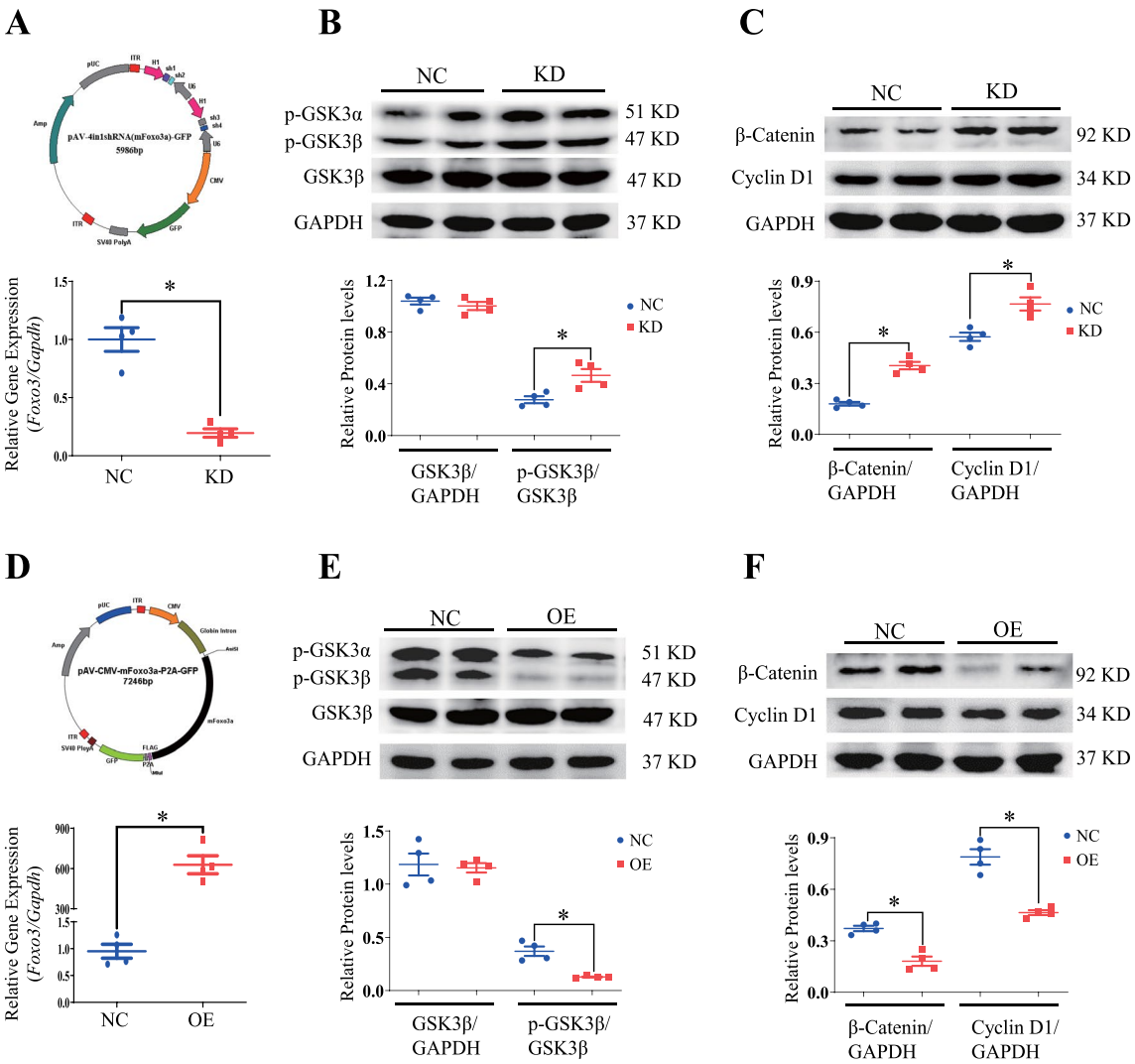
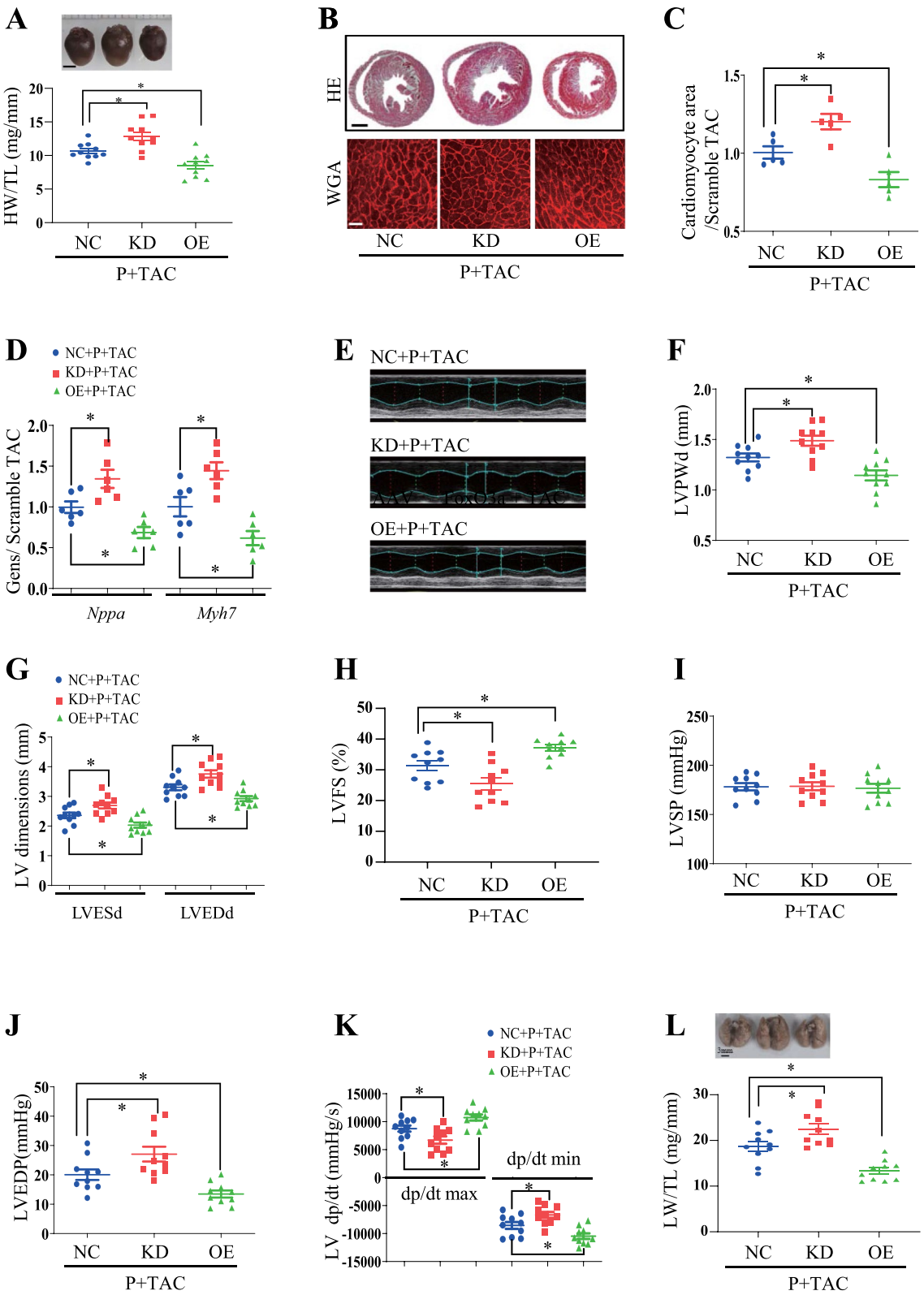


Fig. 4 Effects of silencing or overexpression of *FoxO3a* on growth signaling in the hearts of female mice. **A** The vector map of sh-FoxO3a and qPCR for cardiac expression of the FoxO3a in mice injected with sh-FoxO3a. **B** Western blot analysis of myocardial p-GSK3β/GSK3β. **C** Western blot analysis of myocardial β-catenin and Cyclin D1. **D** The vector map of OE-FoxO3a and qPCR for cardiac expression of the FoxO3a in mice injected with OE-FoxO3a. **E** Western blot analysis of myocardial p-GSK3β/GSK3β. **F** Western blot analysis of myocardial β-catenin and Cyclin D1. Direct intramyocardial injection of OE-FoxO3a or sh-FoxO3a by adeno-associated virus vectors carrying FoxO3a or short hairpin FoxO3a, respectively) or scramble was performed in female mice, followed by western blotting for target proteins two weeks later. (**A**–**F**) $n=4$ in each group. *P* values were calculated by unpaired Student's *t*-test (**A**–**F**). $^{*}P<0.05$ vs. NC. *NC* negative control, *KD* knockdown by sh-*Foxo3*. *OE* overexpression of *Foxo3*. *C* control. *P* pregnancy-induced hypertrophic preconditioning. *TAC* transverse aortic constriction

Effects of silencing or overexpression of *Foxo3* on growth signaling in the hearts of female mice subjected to TAC

Western blotting demonstrated that silencing of *Foxo3* in heart of mice subjected to TAC led to increased expression of p-FoxO3a, p-GSK3, β-catenin and Cyclin D1, and reduced expression of FoxO3a compared with the NC + P + TAC group (Fig. 6A–C). In contrast, overexpression of *Foxo3* was associated with a reduction in p-FoxO3a, p-GSK3, β-catenin and Cyclin D1, and an increase in FoxO3a in the hearts of female mice subjected to TAC (Fig. 6A–C).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HW/TL (mg/mm)
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LW/TL (mg/mm)
NC
KD
OE
P+TAC

◂**Fig. 5** Silencing or overexpression of *Foxo3* influences cardioprotection induced by PHP. **A** Heart weight/tibia length ratio (HW/TL) ratio, $n=10$ per group. **B** Representative macroscopic photographs of H&E-stained sections of hearts (upper panels, scale bar = 1 mm) and WGA-stained myocardial sections (lower panels, scale bar = 30 μm). **C** Quantification of cardiomyocyte cross-sectional area, $n=5$ per group. **D** QPCR analysis for *Nppa* and *Myh7,* $n=6$ per group. **E** Representative photographs of M-mode echocardiography of left ventricular chamber. **F** Left ventricular diastolic posterior wall thickness (LVPWd). **G** Left ventricular end-diastolic diameter (LVEDd) and end-systolic diameter (LVESd). **H** Left ventricular fractional shortening (LVFS). **I** LV systolic pressure (LVSP). **J** LV end-diastolic pressure (LVEDP). **K** Maximum rate of increase and decrease in left ventricular pressure, dp/dt max and dp/dt min. **L** Lung weight/tibia length (LW/TL) ratio. **F**–**L**: $n=10$ per group. Direct intramyocardial injection of AAV-*Foxo3* or AAV-sh-*Foxo3* or scramble was performed in female mice, and the mice were mated two weeks later. From 3 weeks after delivery, TAC was performed for four weeks. *P* values were calculated by one-way ANOVA followed by Bonferroni's correction for post hoc multiple comparisons (**A**, **C**, **D**, **F**–**L**). $^{*}P<0.05$ vs. the NC + P + TAC group. *Nppa*: natriuretic peptide A, *Myh7*: myosin heavy chain 7. *NC* negative control. *KD* knockdown by sh-*Foxo3*. *OE* overexpression of *Foxo3*. *P* pregnancy-induced hypertrophic preconditioning. *TAC* transverse aortic constriction

Discussion

This study provided first experimental evidence that myocardial hypertrophic preconditioning occurs during pregnancy. We demonstrated that hypertrophic preconditioning induced by pregnancy led to an increase in cardiac resistance to subsequent pathological hypertrophic stress and delayed progression from hypertrophy to heart failure, indicating the existence of PHP as a physiological phenomenon. We also found that activation of FoxO3a was significantly enhanced in the hearts of postpartum mice and silencing or overexpression of *Foxo3* attenuated or enhanced the anti-hypertrophic effect of PHP in mice treated with Ang II infusion or TAC, which may partly explain the cardioprotective effect of PHP. Furthermore, we demonstrated that activation of FoxO3a contributed to the anti-remodeling effect of PHP by suppressing signaling pathways of GSK3β/β-catenin/Cyclin D1 involved in cell growth.

A cardioprotective effect of pregnancy has already been reported in the setting of cardiovascular disease, but not in relation to myocardial hypertrophy. During pregnancy, fetal cells readily entered the maternal circulation and colonize various maternal organs for decades as microchimeras, with beneficial effects for the mother [20]. Kara and colleagues reported that fetal cells migrated to the maternal myocardium and are recruited to sites of injury to assist in repair, which may help to explain why patients with peripartum cardiomyopathy have a high rate of recovery from heart failure [10]. Persistence of a lower mean arterial pressure and reduced arterial stiffness following pregnancy may also have a favorable effect on maternal cardiovascular remodeling [21]. In addition, it is well known that the incidence of coronary heart disease is lower in premenopausal women than in men of the same age, a difference which cannot be completely explained by the influence of female hormones. Interestingly, longevity is associated with fertility in women [13, 22], and the maternal age at final pregnancy shows a positive correlation with post-reproductive survival [23], with late first and last pregnancies being protective against all-cause mortality [24]. Besides, shorter total reproductive duration and nulliparity was associated with higher risk for incident heart failure [14]. These reports have suggested the possibility of PHP, but it was unknown whether this phenomenon had an anti-hypertrophic effect until we provided experimental evidence in the present study.

In this study, we did not investigate the duration of PHP. We previously reported that exercise-induced physiological cardiac hypertrophy regressed completely after resting for a week [7], and the molecular memory of cardioprotective preconditioning usually occurs during the regression of hypertrophy [2, 7]. Differently, in this study, we found that PHP regressed completely by 3 weeks after delivery. Therefore, we selected postpartum 3 weeks as the time point to receive pathological hypertrophic stress. Our previous study demonstrated that the cardioprotection of exercise-induced hypertrophic preconditioning weakened at 4 weeks after physiological cardiac hypertrophy regressed in mice [7]. This study showed that activation of FoxO3a mediated cardioprotection of PHP, and FoxO3a activity was weakened at 7 weeks postpartum (Fig. 3C). Therefore, it is reasonable to postulate that effect of PHP in mice may disappear at 7 weeks after delivery, that is to say the window of PHP was less than 4 weeks.

We noted that postpartum FoxO3a protein expression in the heart was upregulated at 1 week and persisted for several weeks, but the antihypertrophic effect occurred at 3 weeks rather than 1 week after delivery. Why? Although the pregnancy-induced molecular memory is largely unknown, it is reasonable to image that the peripartum weak state is associated with the harmful memory and the postpartum health state may be attributable to the persistence of beneficial memory and regression of harmful memory. This issue is needed to clarify by a lot of job in the future.

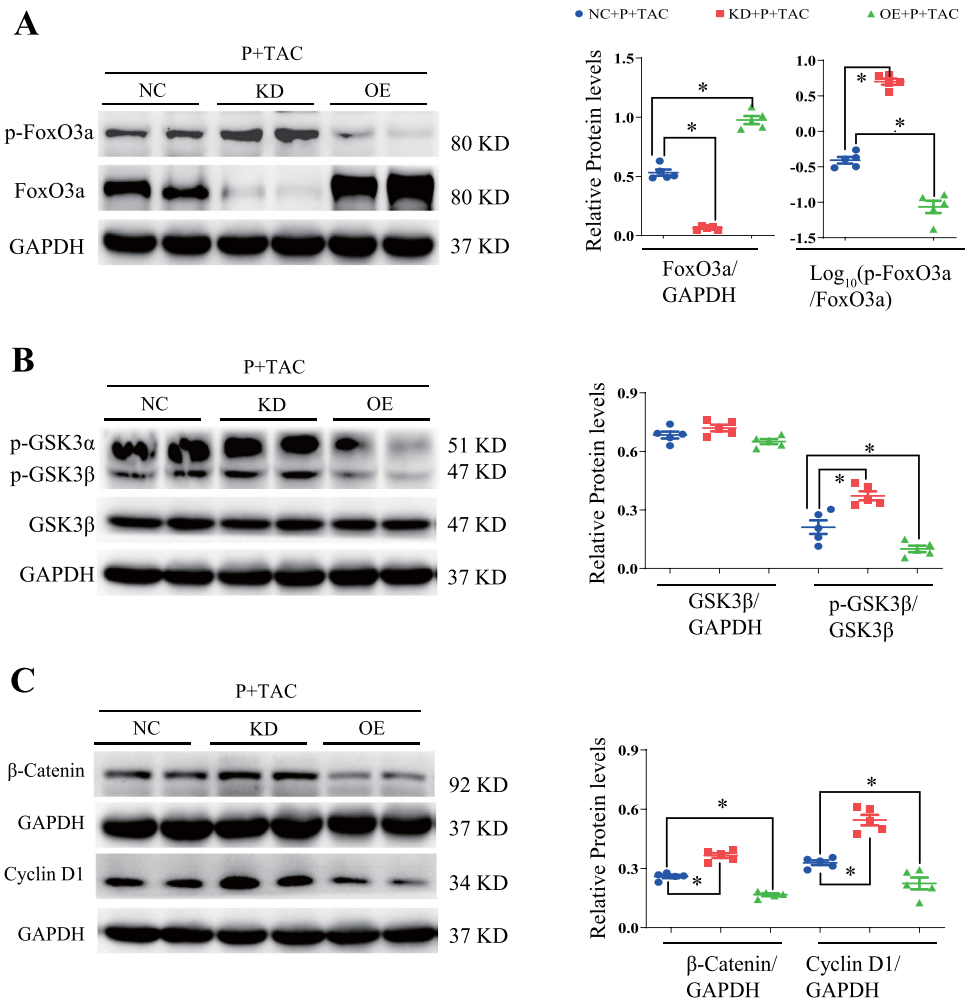

Fig. 6 Effects of silencing or overexpression of *Foxo3* on growth signaling in the hearts of mice subjected to transverse aortic constriction (TAC). **A** Western blot analysis of myocardial FoxO3a and p-FoxO3a/FoxO3a. **B** Western blot analysis of myocardial p-GSK3β/GSK3β. **C** Western blot analysis of myocardial β-catenin and Cyclin D1. **A**–**C** In mice after TAC stimulation with myocardial silencing or overexpression or scramble of *Foxo3*. *P* values were calculated by one-way ANOVA followed by Bonferroni's correction for post hoc multiple comparisons (**A**–**C**). **P* < 0.05 vs. the NC + P + TAC group. **A**–**C** *n* = 5 per group. *NC* negative control, *KD* knockdown by sh-*Foxo3*, *OE* overexpression of *Foxo3*, *P* Pregnancy-induced hypertrophic preconditioning, *TAC* transverse aortic constriction

Unloading of mechanical stress leads to regression of hypertrophy and functional recovery. Thus, regression of myocardial hypertrophy occurs in patients with aortic stenosis following aortic valve replacement, but regression is faster in women than men, partly because they are more likely to show adaptive cardiac remodeling with less fibrosis before surgery [25]. Regression of cardiac remodeling is associated with a distinct, phase-dependent gene expression profile that inhibits hypertrophic and triggers atrophic signaling pathways [26]. Maternal blood volume decreases rapidly after delivery, which represents physiological postpartum unloading.

Although we proved the cardioprotective effect of FoxO3a in female mice, it was reported antihypertrophic role of FoxO3a also existed in male mice. The increased expression of FoxO3a is associated with an anti-hypertrophy effect in the heart [15, 16], and a dominant-negative FoxO3a mutant was reported to inhibit endogenous FoxO3a activity and suppress the anti-hypertrophic effect of Sirtuin 3 [16]. Recent evidence suggests that activation of FoxO3a triggered by methyltransferases and unloading of mechanical stress is involved in muscle atrophy [26, 27]. It is worth finding more mediators in PHP as therapeutic targets of cardiac hypertrophy.

GSK3β was implicated in the growth of the heart and can block hypertrophic response by antagonizing the actions of calcineurin. In histone deacetylase-2 transgenic mice subjected to hypertrophic stress, severe cardiac hypertrophy was associated with inactivation of GSK3β, while activation of GSK3β attenuates hypertrophy [28]. It was also reported that deletion of GSK3β in cardiac fibroblasts leads to cardiac fibrosis and contractile dysfunction after myocardial infarction [29]. Moreover, intramyocardial injection of GSK3β-overexpressing stem cells improved mortality and attenuated myocardial dysfunction after myocardial infarction in mice [30]. In contrast to the anti-hypertrophic role of GSK3β, β-catenin is believed to be a pro-hypertrophic factor. Stimulation of the Wnt/Frizzled pathway activates

disheveled protein, which subsequently inhibits GSK3β, while ablation of the disheveled-1 gene attenuates pressure overload-induced cardiac hypertrophy and reduces β-catenin expression [31]. It was reported that β-catenin gene transfer increased hypertrophic stress-induced growth [32], and we have also previously demonstrated that β-catenin promotes cardiac fibrosis [33]. Additionally, Wnt signaling modulates activation of GSK3β, while our previous findings and evidence from other investigators indicate that the GSK3β/Cyclin D1 signaling pathway is closely associated with cardiovascular disease [32, 34]. Moreover, it was previously reported that FoxO3a could directly bind to Akt, while Akt could regulate GSK3β phosphorylation [35], and Wnt/β-catenin signal pathways are also classical [36]. Therefore, it is likely that Akt and Wnt also involve in FoxO3a-mediated cardioprotection of PHP. It has generally been recognized that both FoxO3a and the GSK3β/β-catenin/Cyclin D1 pathway are involved in myocardial hypertrophy, but there have been no reports of any association between them. Our CO-IP assays suggested a direct interaction between FoxO3a and Akt, as well as between FoxO3a and GSK3β. We also used overexpression and silencing of myocardial FoxO3a to reveal that FoxO3a promoted the activation of GSK3β while reduced the expression of β-catenin and Cyclin D1, and consequently was involved in the cardioprotective effect of PHP.

Although we previously confirmed that Wnt is an upstream of β-catenin [37], we also reported elsewhere that the activation of β-catenin might be independent of Wnt [32], while fibroblast growth factor receptor 4 and GSK3-β could also be acted as upstream of β-catenin [32, 33, 38]. In this study, we did not provide evidence whether Wnt is involved in PHP, but the finding that FoxO3a binding GSK-3β suggests the involvement of FoxO3a in β-catenin-related signal pathway.

In addition to FoxO3a, other mechanisms may contribute to the cardioprotective effect of pregnant myocardial hypertrophy. For example, several experimental studies have demonstrated that pregnancy has a rejuvenating effect on various organs in female mice [10, 39]. Substantial researches are needed in the future to clarify the mechanisms of pregnant myocardial hypertrophy.

In summary, myocardial hypertrophic preconditioning induced by pregnancy confers resistance to subsequent hypertrophic stress and slows progression to heart failure through activation of FoxO3a and inhibition of p-GSK3β/β-catenin/Cyclin D1 pathway.

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Author contributions JX and CZ performed experimental design and executed most of the experiments, data analyses, and wrote the manuscript. MS, WL, and HL contributed to the technical, and material support. ML, MH, LC, SM, YZ, HL, JX, WL, and JB contributed to the data interpretation and discussion. LY contributed to the concept design, data interpretation, writing and revising the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures were performed in accordance with our Institutional Guidelines for Animal Research, and this investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication, 8th Edition, 2011). This study was approved by ethics review board of Southern Medical University.

Consent for publication This study does not contain any individual person's data. All authors agree for publication.

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