

WIF1 causes dysfunction of heart in transgenic mice

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Abstract Wnt activity is a key regulator of cardiac progenitor cell self-renewal, differentiation and morphogenesis. However, Wnt inhibitory factor 1 (WIF1), a antagonists of Wnt signaling activity, its potential effects on heart development has not yet been approached by either in vivo or in vitro studies. Here, the expression of WIF1 was regulated in a different way in the dilated and hypertrophic cardiomyopathy heart from transgenic mice by mutations in cardiac troponin T, cTnT^{R141W} and cTnT^{R92Q}. The heart tissue specific transgenic mice of WIF1 was studied using M-mode echocardiography and histologic analyses.

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Production levels of an array of effectors and transcription factors that impact cellular organization and tissue morphology were measured. The effects of WIF1 on β -catenin pathway could be reversed by LiCl regarding signaling pathways and effector and respondent molecules in H9c2 cells, consistent with the expression levels of c-myc, natriuretic peptide precursor type B and skeletal muscle actin $\alpha 1$. Among the most noteworthy findings were that WIF1 impaired the function and structure of heart, and the effects on β -catenin pathway maybe the course of the former. It is anticipated that our findings will contribute to expansion of our understanding of WIF1 biological function on heart development and possible modes of treatment of heart diseases.

Keywords WIF1 · Heart · Transgene · Mice

Introduction

Wnt is a key regulator of cardiac progenitor cell self-renewal, differentiation and morphogenesis (Cohen et al. 2008). Various secreted Wnt antagonists interact directly or indirectly to affect Wnt signaling and influence its regulatory processes (Kawano and Kypta 2003), and their modulating capacities are thus important in cardiac development. Wnt signaling can be mediated through either canonical β -catenin-mediated Lef/Tcf transcriptional activity or other non-canonical pathways (Macdonald et al. 2007; Semenov

et al. 2007). Extracellular antagonists of the Wnt signaling pathway include the secreted frizzled-related protein family, Wnt inhibitory factor 1 (WIF1), and the Cerberus and Dickkopf (Dkk) family.

Wnt inhibitory factor 1 was first identified as an expressed sequence tag from human retina with highly conserved among species (Hsieh et al. 1999). The WIF1 binds directly to extracellular Wnt ligands, preventing their interaction with the receptors and leading to β -catenin degradation (Hsieh et al. 1999). WIF1 is present across vertebrate families and consists of an N-terminal secretion signal sequence, the WIF domain, five EGF-like domains and a hydrophilic C terminus (Malinauskas et al. 2011). Human WIF1 binds through to eight Wnts [3a,4,5a,7a,9a,11 (Surmann-Schmitt et al. 2009), wingless and *Xenopus* Wnt8] and a protein involved in neuronal differentiation, olfactomedin 1 (Nakaya et al. 2008).

During embryogenesis in *Xenopus* and zebrafish, expression of WIF1 is first detectable at the start of somitogenesis in the paraxial mesoderm, and WIF1 expression continues in adults in the heart, lungs and cartilage-mesenchyme interfaces of various species (Surmann-Schmitt et al. 2009). Moreover, WIF1 plays an essential role in the regulation of Wnt signals in development of central nervous system (Hu and Zhao 2010) [9], and it is indicated that WIF1 enhances cardiomyogenesis (Buermans et al. 2010).

The downregulation of WIF1 by promoter hypermethylation has been reported in various human malignancies including carcinoma of urinary bladder, lung, breast, esophagus and stomach (Ding et al. 2009; Urakami et al. 2006; Mazieres et al. 2004; Ai et al. 2006; Clément et al. 2008; Chan et al. 2007; Taniguchi et al. 2005). In addition, it has been shown that WIF1 functions as a tumor suppressor in melanoma, nasopharyngeal, esophageal, stomach, breast, and lung cancers (Clément et al. 2008; Chan et al. 2007; Taniguchi et al. 2005; Lin et al. 2007, 2008; Kim et al. 2007; Wissmann et al. 2003), and overexpression of WIF1 inhibits the growth of cells from lung and bladder cancers (Tang et al. 2009).

We reported previously that cTnT^{R141W} transgenic mice manifest progressive chamber dilation and contractile dysfunction, and have a pathologic phenotype similar to that of human dilated cardiomyopathy (DCM) (Watkins et al. 1995; Juan et al. 2008). In addition, cTnT^{R92Q} transgenic mice manifest ventricular wall hypertrophy, reduced ventricular chamber

and diastolic dysfunction, and have a pathologic phenotype similar to that of human hypertrophic cardiomyopathy (HCM) (Thierfelder et al. 1994; Tardiff et al. 1999; Javadpour et al. 2003). Interestingly, the expression of mutant cTnT^{R92Q} and cTnT^{R141W} genes causes divergent pathologic phenotypes, as reported for both the human disorder and mouse models of cardiomyopathy (Watkins et al. 1995; Thierfelder et al. 1994; Tardiff et al. 1999; Javadpour et al. 2003).

Recently, we found that WIF1 was strongly expressed in heart from mice at embryonic 16.5–7 days of age, whereas their expression exhibited down-regulation at 14 days and expression continued to decrease thereafter with age. Moreover, the expression of WIF1 increased in the heart tissue of cTnT^{R141W} transgenic mice, while decreased in the cTnT^{R92Q} transgenic mice.

These results suggest that WIF1 may be involved in the development of the heart and may have important regulation on the pathogenesis of cardiomyopathy.

However, potential effects of WIF1 on heart development, especially on cardiomyopathy have not yet been approached by either in vivo or in vitro studies. Herein we present our investigations, using α -MHC-WIF1 transgenic mice, as well as corresponding cell line, of the effects of WIF1 on heart geometry and function and its probable mechanisms which could be important in documenting underlying regulation of Wnt signaling and Wnt antagonists in heart development and heart diseases.

Materials and methods

Animals

The α -MHC-cTnT^{R141W} and α -MHC-cTnT^{R92Q} transgenic mice produced for the present study exhibited phenotypic characteristics consistent with those presented in previous reports (Juan et al. 2008; Tardiff et al. 1999). Genotyping of the transgenic mice was facilitated by the polymerase chain reaction (PCR; cTnT forward, 5'GAACAGGAGGAAGGCTGAGGATGAG and reverse, 5'TATTTCCAGCGCCCGGTGACTTTAG).

The cDNAs encoding human WIF1 (Genbank accession no. NM_007191) were cloned into an expression plasmid under the α -MHC promoter. The

construct was microinjected into male pronuclei of fertilized mouse oocytes and implanted into pseudo-pregnant females to generate the transgenic mouse lines. Genotyping was performed by PCR analysis of genomic DNA and the expression of the target gene was analyzed by western blot analysis using antibody to WIF1 (R&D). Genotyping of transgenic mice was facilitated by the PCR (forward, 5'AGGCATCAGTTGTTCAAGTTGGTT and reverse, 5' GCAGTTTGC TTTGTCACAGTTCAC) under standard conditions. The WIF1 transgenic mice were maintained on a C57BL/6 J genetic background. All the mice were bred in an AAALAC-accredited facility and the use of animals was approved by the Animal Care and Use Committees of The Institute of Laboratory Animal Science of Peking Union Medical College (GC08-2001).

Mouse echocardiography

M-mode echocardiography was performed at 1, 3, 6 and 10 months of age on each transgenic mouse with the small animal echocardiography analysis system (Vevo770, Canada) as previously described (Dan et al. 2010; Lu et al. 2012). Briefly, mice were lightly anesthetized by intraperitoneal injection of tribromoethanol at a dose of 180 ml/kg body weight. M-mode echocardiography of the left ventricle was recorded at the tip of the mitral valve apparatus with a 30 MHz transducer. LVAW, LVPW, LVID and EF % were measured. The FS % was calculated as (LVIDD—LVISD)/LVIDD.

Histological analysis

For light microscopy, cardiac tissue from mice at 6 months of age was fixed in 4 % formaldehyde and mounted in paraffin blocks, and sections were stained with H&E or Masson trichrome as previously described (Javadpour et al. 2003). For transmission electron microscopy (TEM), cardiac tissues were routinely fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixated in buffered 1 % osmium tetroxide for 1 h. Samples were then dehydrated using a series of ethanol immersions and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined under a JEM-1230 TEM (Dan et al. 2010; Lu et al.

2012). Myocytes were analysed by an observer blinded to the genotype of the mice.

RNA extraction, quantification, and reverse-transcription (RT)-PCR

Total RNA was isolated from the tissue or the cell line using TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from 2 µg of total RNA using random hexamer primers according to the Superscript III reverse transcriptase manufacturer's protocol (Invitrogen). Detection of mRNA for (nppb), skeletal muscle (act α 1) and procollagen type III α 1 (Col3 α 1) were carried out by the RT-PCR using GAPDH as normalization under standard conditions (For Nppb forward, 5'TAGCCAGTCTCCAGAACAA and reverse, 5'AA CAACCTCAGCCCGTCA; for Acta1 forward, 5'GG CGACTGGAGTCAATAC and reverse, 5'CTCTAC AAGCCCGTCATAC; for Col3 α 1 forward, 5' CT CAAGAGCGGAGAATACTGG and reverse, 5'CA ATGTCATAGGGTGCGATA; for GAPDH forward, 5'CAAGGTCATCCATGACAACCTTTG and reverse 5'GTCCACCACCCTGTTGCTGTAG).

Protein extraction and immunoblotting

Total protein lysates from heart tissue of each transgenic mouse aged 6 months, as well as of cells, were prepared as previously described (Lu et al. 2012). The cytosolic and nuclear fractions were derived following the Nuclear/Cytosol Fractionation Kit manufacturer's protocol (BioVision). After performing SDS-PAGE and transfer to nitrocellulose (Millipore), the membranes were incubated overnight with antibody to WIF1 (Santa Cruz), β -catenin (Abcam) or c-Myc (Cell Signaling). After incubation with the appropriate secondary antibody for 1 h at room temperature, antibody binding was detected with a HRP-conjugated immunoglobulin G (Santa Cruz) using a chemiluminescent detection system (Westernblotting luminal reagent, Santa Cruz). Bands intensities were quantified using the ImageJ software, and GAPDH was used for normalisation.

Cell culture

Cells of the rat embryonic ventricular myocyte cell line H9c2 were grown in high glucose DMEM supplemented with 10 % defined fetal bovine serum

(HyClone), 100 U/ml penicillin and 100 g/ml streptomycin (HyClone) in a humidified 5 % CO₂ incubator at 37 °C. The expression construct for WIF1 were generated by cloning full-length human WIF1 cDNA fragment into the pCDNA3.1 (+) vector (Invitrogen). The presence of insert was verified using restriction digestion and DNA sequencing. H9c2 cells were transfected with the constructs using Lipofectamine 2000 (Invitrogen). Stable cell line was established by subsequent selection with 800 µg/ml G418 (Amresco). The cell line was treated for 6 h with LiCl (Sigma) at a final concentration of 50 mM, or Fz-8/Fc (R&D) at a final concentration of 500 ng/ml for 3 h, then cells were harvested for RT-PCR and western bolt analysis.

Statistical analysis

All measurement data are expressed as mean ± SEM. The statistical significance of differences between groups was analyzed by Student's *t* test. Differences were considered significant at a *P* value <0.05.

Results

Detection of the expression of WIF1

Heart, liver, spleen, lung and kidney tissues from wild-type (WT) newborn mice were sampled and the expression of WIF1 mRNA was detected by the RT-PCR method. WIF1 showed high level of expression in heart tissue (Fig. 1a). Heart tissues were sampled respectively from WT mice at embryonic 16.5 days, day 0, and at 3, 7, 14, 30, 60 and 90 days of age, and the expression of WIF1 was detected. WIF1 was strongly expressed in heart from mice at embryonic 16.5–7 days of age, whereas their expression exhibited down-regulation at 14 days and expression continued to decrease thereafter with age (Fig. 1b).

The expression of mutant cTnT^{R92Q} and cTnT^{R141W} in the mouse heart evoked contrasting pathologic phenotypes, evinced as HCM and DCM. The expression of WIF1 was detected by western blot in the cTnT^{R92Q} and cTnT^{R141W} heart. WIF1 was down-regulated in the cTnT^{R92Q} heart (*P* < 0.001), but was up-regulated in the cTnT^{R141W} (*P* < 0.05; Fig. 1c, d). The results suggest that WIF1 are involved in the development of the heart and may have

contrasting different effects on the pathogenesis of cardiomyopathy.

Generation and identification of the WIF1 transgenic mice

To study the modulation of WIF1 on heart geometry and function and its probable mechanisms, C57BL/6 J mice carrying the WIF1 gene was established (Fig. 1e). Two lines of WIF1 transgenic mice, founder 17 and 21, with high levels of expression were selected from among 36 founders by the western blot procedure (Fig. 1f).

The WIF1 transgenic mice were indistinguishable from their NTG littermates at birth and in youth. No death was observed in the NTG transgenic mice, while the extent of mortality in WIF1 mice was around 4 % (1 of 25) within 10 months (*P* = 0.327 versus NTG mice).

WIF1 caused dysfunction of heart in transgenic mice

Ventricular size and function of the two transgenic lines were assessed using echocardiography. Dates from founder 21 given the similar tendency of phenotype to founder 17 for WIF1 mice at 6 months of age (Supplemental Table S1).

The deterioration was demonstrated by the 11.2 % increased left ventricular diameter at end-systole (LVESD) (*P* < 0.01, WIF1 mice versus NTG mice), 13.8 % reduced left ventricular posterior wall thickness at end-systole (LVPWS) (*P* < 0.001, WIF1 mice versus NTG mice), and 12.5 % reduced left ventricular percent fractional shortening (LVFS) (*P* < 0.01, WIF1 mice versus NTG mice) in mice at 3 months of age (Fig. 2a–c).

The representative M-mode echocardiograms from founder 17 at the age of 6 months old were shown in Fig. 2d. The other parameters of M-mode echocardiography from the NTG and WIF1 transgenic mice at 1, 3, 6 and 10 months of ages were summarized in supplemental table S2 (Founder 17 of WIF1 transgenic mice).

To sum up, the the overexpression of WIF1 in heart exhibited thin-walled and dilated left and right ventricles when compared with NTG hearts. The WIF1 transgenic mice developed a progressive LV dilation and dysfunction associated with a progressive decrease of contractile function, evidenced by

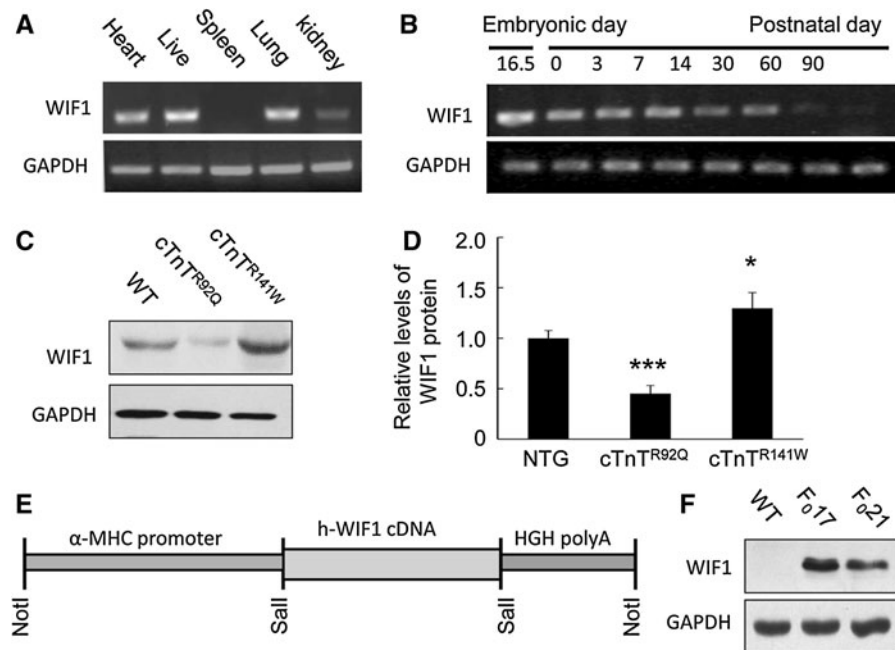


Fig. 1 Detection the expression of WIF1 and generation of the transgenic mice **(a)** The expression of WIF1 mRNA in the tissues of neonatal heart, liver, spleen, lung and kidney of WT mice was detected by RT-PCR. **(b)** Heart tissues were sampled respectively from mice of embryonic 16.5 days, at birth, and at 3, 7, 14, 30, 60 and 90 days of age, and the expression of WIF1 mRNA was detected by RT-PCR. **(c)** The protein translational level of WIF1 in the cardiomyopathic hearts from cTnT^{R92Q} and cTnT^{R141W} mice was detected by Western blot. **(d)** The

quantitative analysis of the expression levels of WIF1 using GAPDH as normalization. **(e)** The WIF1 transgenic constructs was generated by inserting the target genes under the control of the α -MHC cardiac-specific promoter and the transgenic mice were created following microinjection. **(f)** The mouse lines with WIF1 over-expression was selected by the western blot procedure using GAPDH as normalization. * $P < 0.05$; *** $P < 0.001$ versus NTG mice

decreased LV percent fractional shortening (FS %) which exhibited a significance from 1 months of age compared with NTG mice.

Morphological changes of myocytes in the WIF1 transgenic mice

By light microscopy (Fig. 3a–h), no significant changes were observed in the heart tissues of WIF1 transgenic mice, except for occurrence of myocyte disarray in the heart tissues of WIF1 transgenic mice (Fig. 3e, f). Masson trichrome staining indicated that a small proportion of collagen accumulated in the interstitial space in the WIF1 transgenic mice (Fig. 3g, h).

Ultrastructural characteristics of heart tissues are summarized in Fig. 3i, j. Slightly elongated myofibrils with swollen sarcoplasmic reticulum were observed in WIF1 mice.

Expression level of Col3 α 1 mRNA were slightly increased in WIF1 mice compared with NTG mice

($P = 0.0754$, WIF1 mice versus NTG mice; Fig. 3k, l), consistent with the result of Masson trichrome staining (Fig. 3g, h).

WIF1 inhibited β -catenin pathway hypertrophic markers in heart tissues of the transgenic mice

To assess whether functional Wnt signaling is inhibited in the heart tissues of the transgenic mice, we examined by use of the western blot procedure the occurrence of cytosolic and nuclear β -catenin, the crux of the canonical Wnt signal pathway.

Accumulation of β -catenin in nucleus and cytoplasm were decreased in WIF1 mice (Fig. 4a, b, $P < 0.01$, WIF1 mice versus NTG mice), concomitant with decreased expression of transcription factors c-myc (Fig. 4a, b, $P < 0.05$, WIF1 mice versus NTG mice), coding sequences for which are target gene for β -catenin, consistent with what is known about the β -catenin/TCF pathway (Moon et al. 2002; Nakamura et al. 2003).

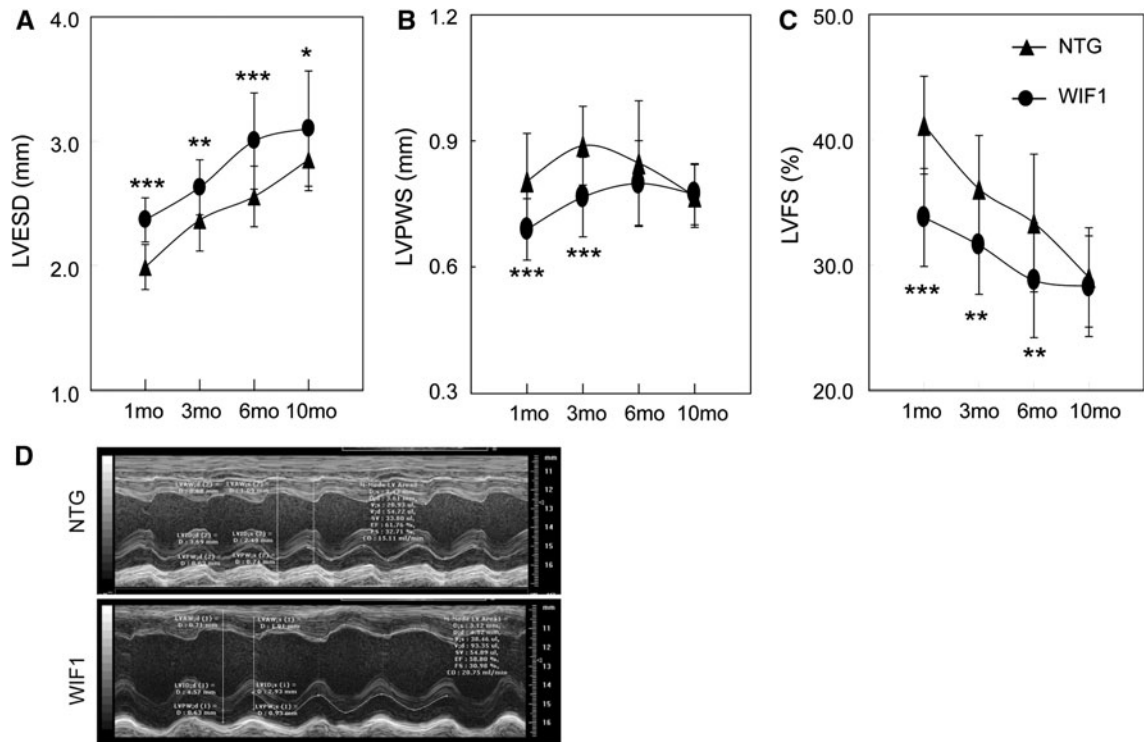


Fig. 2 The effects of WIF1 on heart dimensions and function in transgenic mice. Echocardiographic parameters of LV end-systole diameter (LVESD) (a), LV posterior wall at end-systole (LVPWS) (b) and LV fractional shortening (LVFS) (c) were compared with NTG mice at 1, 3, 6 and 10 months of age.

d Representative M-mode echocardiographic images of the left ventricle long-axis from 6 months old NTG and transgenic mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus NTG mice at the same month of age

The expression of (nppb; Fig. 4a, b, $P < 0.05$) and skeletal muscle actin $\alpha 1$ (act $\alpha 1$; Fig. 4a, b, $P < 0.05$) mRNA (both of which were commonly used as hypertrophic markers) was decreased in the WIF1 mice compared with the corresponding expression levels in NTG mice.

WIF1 blocks hypertrophic markers through inhibition of the β -catenin pathway in the H9c2 Cell Line

To assess the effect of WIF1 in vitro, H9c2 cells were transfected with construct incorporating WIF1 (Fig. 5a). Stable cell line overexpressing target gene were identified by the western blot procedure (Fig. 5b).

Accumulation of β -catenin in nucleus (Fig. 6a, b, $P < 0.01$, WIF1 cell versus vector cell) and cytoplasm (Fig. 6a, b, $P < 0.05$, WIF1 cell versus vector cell)

were decreased in WIF1 cell line, concomitant with decreased expression of c-myc (Fig. 6a, b, $P < 0.01$, WIF1 cell versus vector cell). The expression of nppb (Fig. 6c, d, $P < 0.05$) and act $\alpha 1$ (Fig. 6c and D, $P < 0.05$) mRNA were decreased in the WIF1 cell compared with the corresponding expression levels in vector cell.

LiCl can bind with and inhibit GSK-3 β and, hence, activate Wnt signaling selectively via the β -catenin/TCF pathway. Frizzled-8/Fc chimeric protein (Fz-8/Fc), an antagonist for Wnt8A, and potentially for other Wnts, decreases the expression of β -catenin to its basal level (Moon et al. 2002; Nakamura et al. 2003).

To test whether the presence of LiCl or Fz-8/Fc could worsen or reverse the effect of WIF1 on the expression of the hypertrophic markers, as has been observed in vivo (Fig. 4), the cells (stable H9c2 cell line overexpressing WIF1) were treated with LiCl or Fz-8/Fc, and the expression of β -catenin, c-myc, nppb

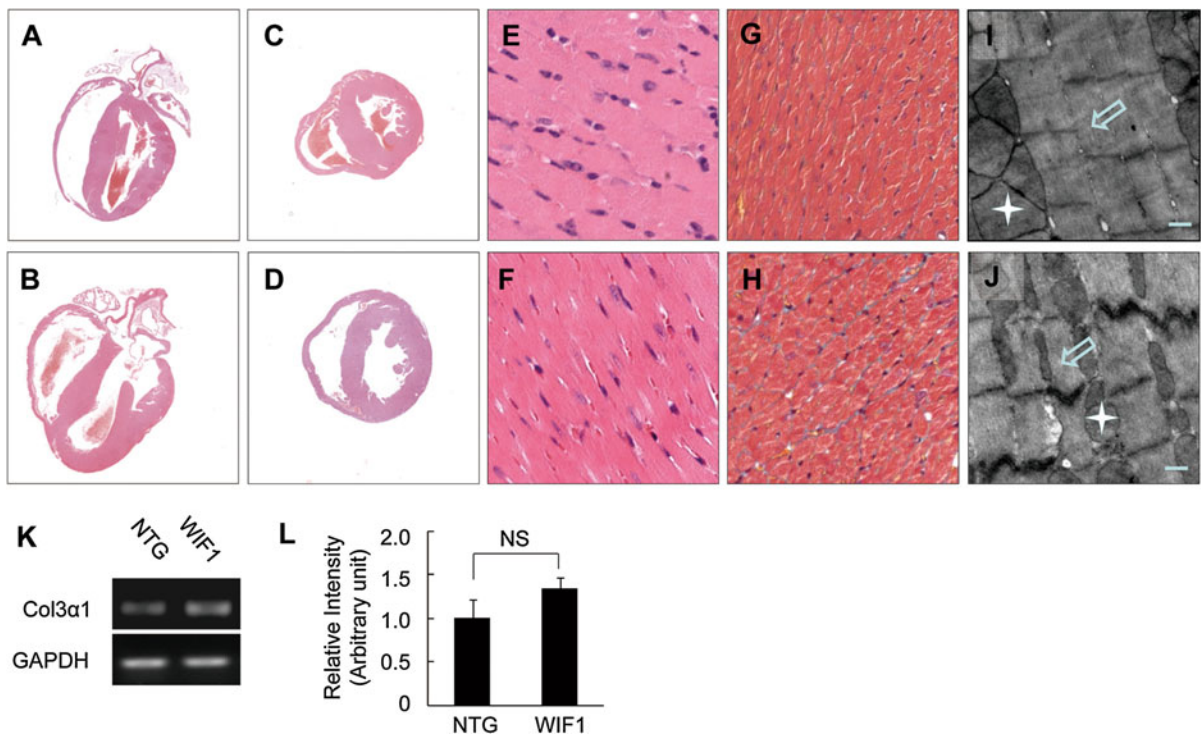


Fig. 3 Profile of histopathological and ultrastructural in hearts of transgenic mice at 6 months of age. **a–d** Shown are H&E staining patterns of whole-heart longitudinal sections and cross-sections from 6 months old NTG (**a** and **c**) and transgenic mice (**b** and **d**) (magnification, $\times 20$). **e–f** Shown are H&E stained sections of *left ventricle*, showing disparate in pathological changes from 6 months old NTG (**e**) and transgenic mice (**f**) (magnification $\times 400$). **g–h** Masson trichrome staining of sections of *left ventricle* from 6 months old NTG (**g**) and

transgenic mice (**h**); myocytes, stained red, collagenous tissue, stained green (magnification $\times 400$). **i–j** TEM showing *left ventricular* free walls from 6 months old NTG (**i**) and transgenic mice (**j**) [white scale bars = $0.5 \mu\text{m}$, sarcomeres (white hollow arrow) and mitochondria (white star)]. **k** The expression of Col3 α 1 was detected by RT-PCR. **l** The quantitative analysis of the expression of Col3 α 1 using GAPDH for normalisation (NS, no significance)

and act α 1 were determined (Fig. 6). Increase in occurrence of β -catenin in nuclear (Fig. 6a, b, no significance (NS), WIF1 cell versus vector cell) and cytoplasmic (Fig. 6a, b, NS, WIF1 cell versus vector cell) protein subfractions, as well as increase of c-myc (Fig. 6a, b, NS, WIF1 cell versus vector cell) in the WIF1 cells treated with LiCl, is consistent with observable increase in expression of nppb (Fig. 6c, d, NS, WIF1 cell versus vector cell) and act α 1 (Fig. 6c, d, NS, WIF1 cell versus vector cell) mRNAs, while the occurrence of β -catenin in nuclear (Fig. 6a, b, $P < 0.05$, WIF1 cell versus vector cell) and cytoplasmic (Fig. 6a, b, $P < 0.05$, WIF1 cell versus vector cell) protein subfractions were further inhibited as well as decreased of c-myc (Fig. 6a, b, NS, WIF1 cell versus vector cell) in the WIF1 cells treated with Fz-8/Fc, in consistent with observable decrease in the expression of nppb (Fig. 6c, d, NS, WIF1 cell versus

vector cell) and act α 1 (Fig. 6c, d, $P < 0.05$, WIF1 cell versus vector cell) mRNAs.

Discussion

The Wnt signaling pathway is a major regulator of cell proliferation, migration and differentiation, controlling embryogenesis, adult tissue homeostasis and tumor progression (MacDonald et al. 2009). WIF1 is a secreted protein that binds to Wnt proteins and inhibits their activities (Hsieh et al. 1999), and it can directly bind to Wnt proteins and inhibit Wnts from binding to their receptors in vertebrates (Hsieh et al. 1999). WIF1 inhibits rod production in retinal histogenesis in the mouse by binding to Wnt4 (Hunter et al. 2004). Most of the published reports have indicated that WIF1 is a frequent target of epigenetic

Fig. 4 The effects of WIF1 on β -catenin pathway and hypertrophic markers in the hearts at 6 months of age. **a** Immunoblots of subcellular protein extracts were probed using respective antibodies in the western blot procedure. **b** The quantitative analysis of the expression levels of proteins using GAPDH as normalization

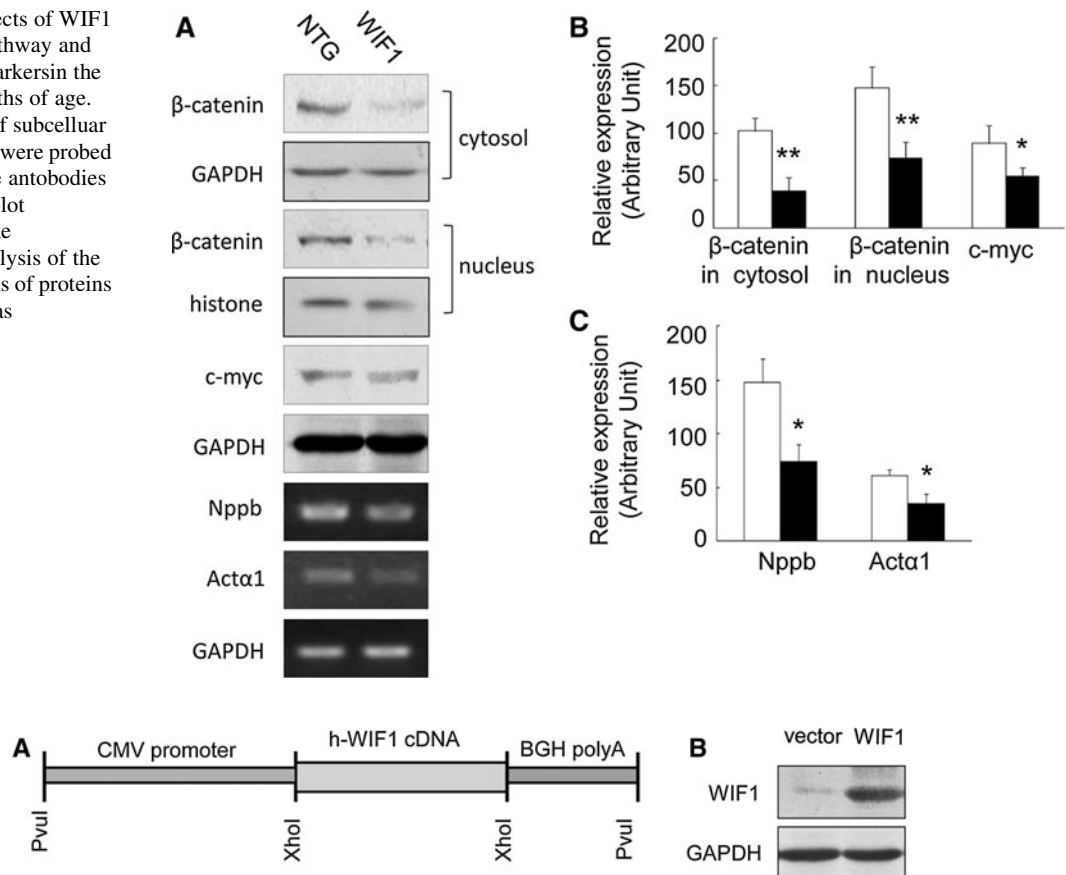


Fig. 5 The establishment of WIF1 overexpression cell line. **a** The plasmids for stable expression of WIF1 was constructed. **b** Stable cell lines were selected by Western blot with respective antibodies for WIF1. GAPDH was used as normalization

inactivation in cancers (Ding et al. 2009; Urakami et al. 2006; Mazieres et al. 2004; Ai et al. 2006; Clément et al. 2008; Chan et al. 2007; Taniguchi et al. 2005; Lin et al. 2007, 2008; Kim et al. 2007). The expression of WIF1 is first detectable at the start of somitogenesis in the paraxial mesoderm during embryogenesis in *Xenopus* and zebrafish. The WIF1 expression continues in adults in the heart, lungs and cartilage-mesenchyme interfaces of various species (Surmann-Schmitt et al. 2009), and it is indicated that WIF1 enhances cardiomyogenesis (Buermans et al. 2010).

It is indicated that WIF1 mRNA can be detected as early as the developmental stage E11, and expression persists to adulthood, and after birth, the expression level of WIF1 decreased in the cortex and diencephalon (Hu et al. 2008). In our present study, we find prominent level of WIF1 in the heart of mice at embryonic 16.5–7 days of age (Fig. 1b). Furthermore, the expression of

WIF1 was found to be regulated in the heart of transgenic mice model of cardiomyopathy (Fig. 1c, d).

To study the effect of WIF1 on heart geometry and function, we generated WIF1 transgenic mice with genetic material for this component specifically expressed in heart tissue (Figs. 2 and 3). In the present work, the most noteworthy findings were that the overexpression of WIF1 in heart exhibited thin-walled and dilated left and right ventricles when compared with NTG hearts, and the WIF1 transgenic mice developed a progressive LV dilation and dysfunction associated with a progressive decrease of contractile function, evidenced by decreased LVFS which exhibited a significance from 1 months of age compared with NTG mice. Moreover, the presence of WIF1 in the heart caused swollen sarcoplasmic reticulum and elongated myofibrils in heart tissue. Overall, WIF1 showed a similar phenotype to the cTnT^{R141W} transgenic mice of cardiomyopathy (Juan et al. 2008; Lu et al. 2012).

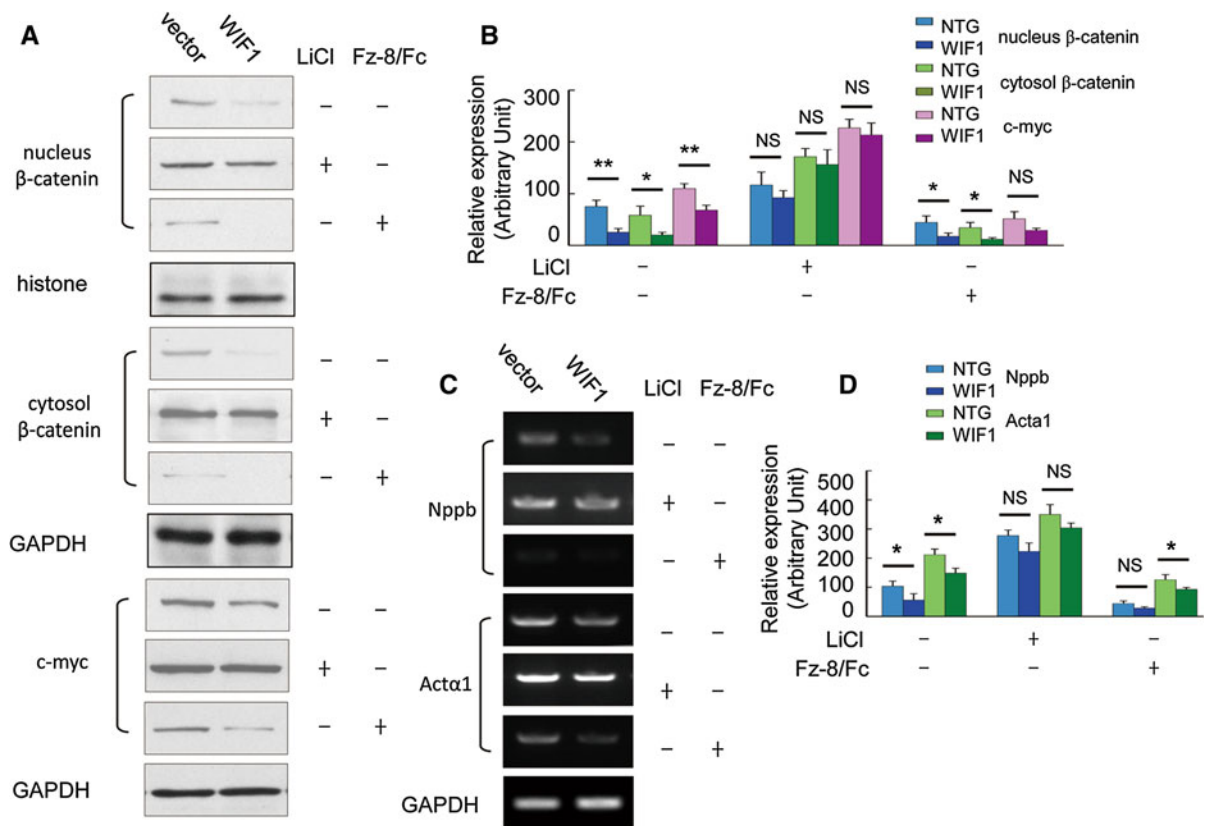


Fig. 6 WIF1 blocks hypertrophic markers through inhibition of the β-catenin pathway in the H9c2 cell Line. **a** Stable cell lines expressing the vector with no insert, or WIF1 cDNA were treated with LiCl or Fz-8/Fc, and subcellular protein extracts were analyzed using antibody to β-catenin, and c-myc was also detected in total lysates by Western blot. **b** The quantitative

analysis of the expression levels of proteins using GAPDH as normalization. **c** Transcripts for *nppb* and *acta1* were detected by RT-PCR method from stable cell lines expressing the vector with no insert, or WIF1 cDNA treated with LiCl or Fz-8/Fc. **d** The quantitative analysis of the expression levels of proteins using GAPDH as normalization

The importance of *nppb* as a diagnostic and therapeutic modality in cardiovascular disease is well known, it also acts as a local regulator of ventricular remodeling and a modifier of cardiac gene expression (Lanfear et al. 2007; Tamura et al. 2000; Tsybouleva et al. 2004). *Nppb* is a cardiac hormone produced primarily by ventricular myocytes, and its plasma concentrations are markedly elevated in patients with congestive heart failure and acute myocardial infarction. Our results indicate that WIF1 significantly decreases the expression levels of *nppb*, suggesting that WIF1 regulate cardiac hypertrophy and that remodeling is in part due to interaction with *nppb*.

Acta1 is present in the developing heart and it constitutes up to 20 % of the striated actin of the adult heart. Mice lacking skeletal actin died in the early neonatal period (Crawford et al. 2002). Our results show that levels of *acta1* mRNA are significantly

decreased in the WIF1 mice. Since *acta1* is a multifunctional protein that interacts with many proteins involved in folding, polymerisation, contractility and regulation of contractility (Feng and Marston 2009), decreased levels may affect any of those functions, suggesting that *acta1* could be involved in mechanisms by which WIF1 leads to development of heart dysfunction.

The Wnt/β-catenin pathway, which appears to antagonize cardiomyocyte differentiation and/or to restrict the size of the cardiogenic field, is critical for endocardial cushion morphogenesis, outflow tract development, and valve formation in later development (Hurlstone et al. 2003). Specific Wnt ligands bind to their target membrane receptors and interfere with the multi-protein destruction complex, resulting in downstream activation of gene transcription by β-catenin. Unphosphorylated β-catenin accumulates

and associates with nuclear transcription factors, leading to the eventual transcription and expression of target genes such as c-myc (Park et al. 2005).

Our results indicate that WIF1 decreases, the accumulation of β -catenin in the nucleus and cytoplasm, consistent with the expression of c-myc in the WIF1 transgenic mice (Fig. 4). In allied experiments, the similar results were found in the cell lines over-expressing WIF1 (Fig. 6).

LiCl can activate Wnt signaling selectively via the β -catenin/TCF pathway (Moon et al. 2002). Fz-8/Fc, an antagonist for Wnt8A and potentially for other Wnts, was shown to decrease β -catenin down to the basal level (Nakamura et al. 2003). We found that WIF1 expression alone in WIF1 mice or in H9c2 cells decreases the accumulation of β -catenin in cytoplasm and the nucleus, consistent with a decrease in c-myc expression as well as nppb and α 1 expression. Furthermore, the inhibition of the Wnt signal induced by WIF1 could be reversed by LiCl, further suggesting that WIF1 acts as a negative regulator of Wnt pathway in the myocyte and is involved in the pathological development of heart diseases.

In summary, we have found that expression of WIF1 is altered in the cardiomyopathy mouse model and that WIF1 was shown to have harmful effects on function and structure of hearts. The effects on β -catenin pathway maybe the course of the former. It is anticipated that our findings will contribute to expansion our understanding of WIF1 biological function on heart development and possible therapeutic strategy to control heart diseases.

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Conflict of interest The authors have declared that no conflict of interest exists.

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