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Transgenic over-expression of YY1 induces pathologic cardiac hypertrophy in a sex-specific manner

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ABSTRACT

YY1 can activate or repress transcription of various genes. In cardiac myocytes in culture YY1 has been shown to regulate expression of several genes involved in myocyte pathology. YY1 can also acutely protect the heart against detrimental changes in gene expression. In this study we show that cardiac over-expression of YY1 induces pathologic cardiac hypertrophy in male mice, measured by changes in gene expression and lower ejection fraction/fractional shortening. In contrast, female animals are protected against pathologic gene expression changes and cardiac dysfunction. Furthermore, we show that YY1 regulates, in a sex-specific manner, the expression of mammalian enable (Mena), a factor that regulates cytoskeletal actin dynamics and whose expression is increased in several models of cardiac pathology, and that Mena expression in humans with heart failure is sex-dependent. Finally, we show that sex differences in YY1 expression are also observed in human heart failure. In summary, this is the first work to show that YY1 has a sex-specific effect in the regulation of cardiac pathology.

1. Introduction

YY1 is a transcription factor that can activate or repress transcription of various genes important in differentiation, development and disease (reviewed in Ref. [1]). YY1 regulates gene expression through direct interaction with the promoter region of several genes, by interacting with proteins that mediate post-translational modification, including acetylation, methylation and ubiquitination, and by potentiating the activity of its co-factors. Several studies, including ours, have highlighted the importance of YY1 in cardiac pathology. YY1 has been shown to mediate endothelin-stimulated expression of b-type natriuretic peptide (BNP) [2], to repress fetal troponin I [3], to mediate skeletal α-actin regulation by interleukin-1β [4], to inhibit the cardiac α-actin promoter [5], and to regulate α myosin heavy chain (αMyHC) gene expression [6–8].

Cardiac hypertrophy is characterized by an increase in myocyte size and heart weight. Pathologic hypertrophy is accompanied by changes in gene expression that have been described as a recapitulation of a "fetal" gene program (FGP) because many embryonically-expressed genes that are down-regulated post-natally are reactivated, while several "adult" genes are repressed [9,10]. Of the changes that are observed in failing hearts, increases in β myosin heavy chain (β-MyHC), skeletal α-actin, atrial natriuretic peptide (ANP) and BNP, with coordinate decreases in αMyHC and sarcoplasmatic reticulum ATPase 2a (SERCA), are perhaps the most widely recognized. In contrast, physiologic hypertrophy is characterized by an increase in heart weight that is not accompanied by activation of the FGP [11].

Abbreviations: FGP, fetal gene program; Mena, mammalian enable; HF, heart failure; NF, non-failing; MyHC, myosin heavy chain; ANF, atrial natriuretic factor; BNP, b-type natriuretic peptide; SERCA, sarcoplasmatic reticulum ATPase 2a; Sk α-actin, skeletal α-actin; Cx43, connexin43; YY1, yin yang 1.

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We have previously shown that YY1 expression is higher in failing human hearts[6], and that acute over-expression of YY1 in neonatal cardiac myocytes prevents pathologic hypertrophy by interacting with the histone deacetylase (HDAC) 5 [12]. We have now developed a cardiac-specific mouse model of YY1 over-expression. Here we show that transgenic over-expression of YY1 results in an increase in left ventricle (LV) size in male and female mice. However, YY1 over-expression results in cardiac dysfunction and activation of the pathologic gene program in males but not in females. Previous work has shown that YY1 increases activity of the androgen receptor [13], and increased expression of the androgen receptor results in higher Mena levels [14], a protein highly expressed in human and animal models of heart failure [15]. We show that Mena levels are higher in the failing male human heart and YY1-transgenic mouse males, but not in the failing female human heart or female YY1-transgenic mice. Furthermore, we show that YY1 levels are higher in human male but not female HF patients compared to NF control hearts. This work is the first to demonstrate a sex-specific role for YY1.

2. Materials and Methods

2.1. Experimental animals

Transgenic mice were generated by injection of DNA into mice embryos using FVB mice that were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The resultant mice have a transgene with an attenuated cardiac-specific αMyHC promoter linked to the transgene for the human transcription factor YY1. These animals were bred to FVB mice in the animal facility at the University of Colorado. The litters contained both wild-type and heterozygote offspring. The genotype of the animals was determined by PCR at time of weaning and confirmed in the heart after euthanasia by RT-PCR. Mice were individually housed and allowed *ad libitum* access to standard mouse chow and water at all times. Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver and adhered to The Code of Ethics of the World Medical Association.

Genotype primers:

αMyHC

Forward: 5′GGT GGT GTA GGA AAG TCA GGA CTT

YY1

Reverse: 5′CCA CTG TGG TCT CGA TGG TCT

Adult male and female mice (4–4.5 months) were anesthetized with a xylazine/ketamine cocktail and euthanized by exsanguination. Body weight, whole heart, left and right ventricular weights and the length of the tibia were recorded for each animal. The tissue was flash frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Echocardiography

Transthoracic echocardiography was performed on a subpopulation of mice in order to evaluate disease by assessing LV wall thickness, chamber dimensions and fractional shortening using short-axis m-mode and long and short axis B-mode images of the LV. Images were obtained under 1.5% isoflurane anesthesia using a high resolution Visual Sonics Vevo 770 (VisualSonics Inc., Toronto, Canada) platform with a 30 MHz mechanical transducer. N = 11 transgenic negative male, 11 transgenic positive male, 6 transgenic negative female and 5 transgenic positive female animals.

2.3. Gene expression

Total RNA was extracted from LV tissue using the mirVana™ kit (Ambion). ***mRNA RT-PCR***: Reverse transcription was performed using the iScript Reverse Transcription Kit (Bio-Rad, Inc) for mRNAs according to manufacturer's recommendations and essentially as previously described [16]. mRNA expression was normalized to 18s. The primers for 18s, ANP, BNP, Serca, αMyHC and βMyHC have been previously described [12,17]. Changes in expression were determined by the ΔΔCt method [18]. N = 11 transgenic negative female; 10 transgenic negative male; 9 transgenic positive female; 11 transgenic positive male.

YY1 primers:

Forward: 5′CAG AAG CAG GTG CAG ATC AAG
Reverse: 5′GAC CAC ATG GTG ACC GAG AAC

Mena primers (mouse):

Forward: 5′CAG AAA TGA AGA TGC AGA GCC
Reverse: 5′ TGA AGG TGT GGA TTT GGG TC

Mena primers (human):

Forward: 5′CAA AGG TGA AGA TTC AGA GCC
Reverse: 5′GGG TGT GGA TTT TGG TCT GT

2.4. Protein expression

Nuclear and cytoplasmic proteins were extracted from the LV using the NEPER kit (Pierce, Pi78833) as previously described [12]. Protein expression was evaluated by western blot as previously described [18]. Briefly, 20ug of protein were separated by gel electrophoresis on a 7.5% Tris–HCl gel. YY1 (Santa Cruz Biothechnology) and calnexin (Abcam) primary antibodies were incubated overnight at 4 °C. N = 6 Female, 5 Male YY1 transgenic positive and 7 Female and 7 Male littermate controls.

2.5. Human subjects

Human subjects with end-stage heart failure (idiopathic dilated cardiomyopathy) were adult males and females of all ages, races and ethnic backgrounds who gave written consent to donate their hearts to the institutional review board-approved cardiac transplant tissue bank at the University of Colorado. The work has been carried out in accordance to The Code of Ethics of the World Medical Association. Non-failing hearts that could not be used for transplant were obtained from organ donors, with consent for research use given by family members. N = 9 NF females, 9 NF males, 13 HF males, 10 HF females (mRNA expression); N = 6 NF males, 8 HF males, 4 NF females, 6 F females (protein expression).

2.6. Statistical analysis

Statistical analyses were performed using Statview software (SAS Institute, Cary, NC). Statistical significance was set *a priori* at $p \leq 0.05$ and all data are presented as mean ± SEM. Analyses of data between two groups (HF/transgenic vs same-sex NF/transgenic negative) was done by t-test.

3. Results

3.1. YY1 transgenic mouse model generation

Transgenic animals were generated using Dr. Jeffrey Robbins' Tet-On system [19]. This system utilizes a transgenic mouse containing the mouse αMyHC promoter weakened due to mutations on the GATA4 binding sites and the thyroid responsive elements with five Tet responsive elements inserted upstream from the TATA box, and the gene of interest. Although this system was designed to be controlled by doxycycline, we noted low-level expression of ectopic YY1 in the absence of doxycycline. The rationale for the use of a conditional model was to prevent high levels of expression of YY1 that could result in toxicity. The weakened promoter resulted in moderate YY1 expression levels that were well tolerated by the animals. Two transgenic lines were generated. Due to increased progeny in Line 1, that line was used for all studies. As shown in Fig. 1A and B, YY1 expression was ~2.5-fold higher than endogenous levels in these animals.

3.2. YY1 transgenic over-expression results in increased LV/BW in males and females but decreased EF and FS only in males

As shown in Fig. 1C, heart specific YY1 over-expression resulted in higher LV/BW in 4–5 month-old male and female mice. To determine if this was associated with heart dysfunction, echocardiograms were performed. As shown in Fig. 1D and E, over-expression of YY1 resulted in lower ejection fraction (EF) and fractional shortening (FS) in males. In contrast, cardiac function was maintained in females. These results suggest that YY1 over-expression is detrimental only in males.

3.3. Changes in the fetal gene program are associated with cardiac dysfunction in males

Expression of the fetal gene program was determined in YY1 transgenic animals. As shown in Fig. 2, transgenic over-expression of YY1 resulted in no changes in the expression of αMyHC and βMyHC in females. In contrast, males showed higher βMyHC expression with no changes in αMyHC gene expression. In addition, expression of ANF was higher females ($p < 0.05$) but not males whereas expression of BNP was increased in males ($p < 0.05$) and unchanged in females compared to transgenic negative controls. Previous studies have shown that YY1 can bind to and regulate expression of skeletal α-actin [4]. Consistent with this, expression of skeletal α-actin was higher in the transgenic males and females. Finally, we did not observe changes in SERCA expression due to YY1 expression.

3.4. YY1 transgenic over-expression results in up-regulation of collagen 3 in males

Fibrosis is associated with cardiac pathology, and collagen 3 expression is increased in the setting of fibrosis [20]. Expression of collagen 3 was analyzed in YY1 transgenic over-expression animals. As shown in Fig. 2, consistent with cardiac function and gene expression results, YY1 over-expression resulted in up-regulation of collagen 3 in males but not in females, suggesting that only males develop cardiac fibrosis in response to YY1 over-expression.

3.5. Mena expression is increased in YY1 transgenic mice and in human idiopathic dilated cardiomyopathy (IDC) male patients

YY1 has been shown to potentiate the transcriptional activity of the androgen receptor [13], and transgenic mice over-expressing the

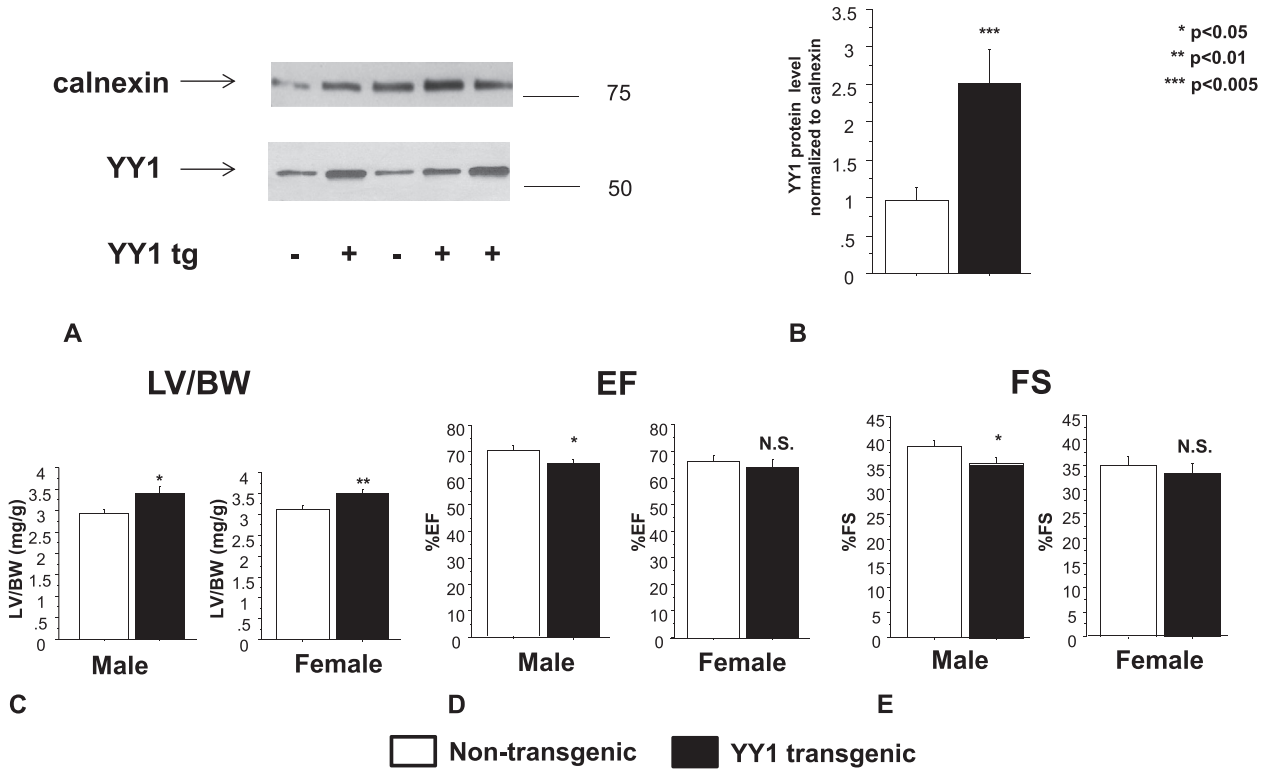

Fig. 1. YY1 protein levels are higher in transgenic animals over-expressing YY1 than transgenic negative controls and results in lower cardiac function in males but not female mice than transgenic negative controls. (A) Representative Western blot of nuclear fraction of YY1 transgenic positive and littermate negative control animals. Calnexin was used as loading control. (B) Western blot quantification. (C) Left ventricle (LV)/body weight (BW) is higher in the transgenic males and females. (D) Ejection fraction (EF) and (E) Fractional shortening (FS) are lower in male mice only. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs same-sex transgenic negative controls.

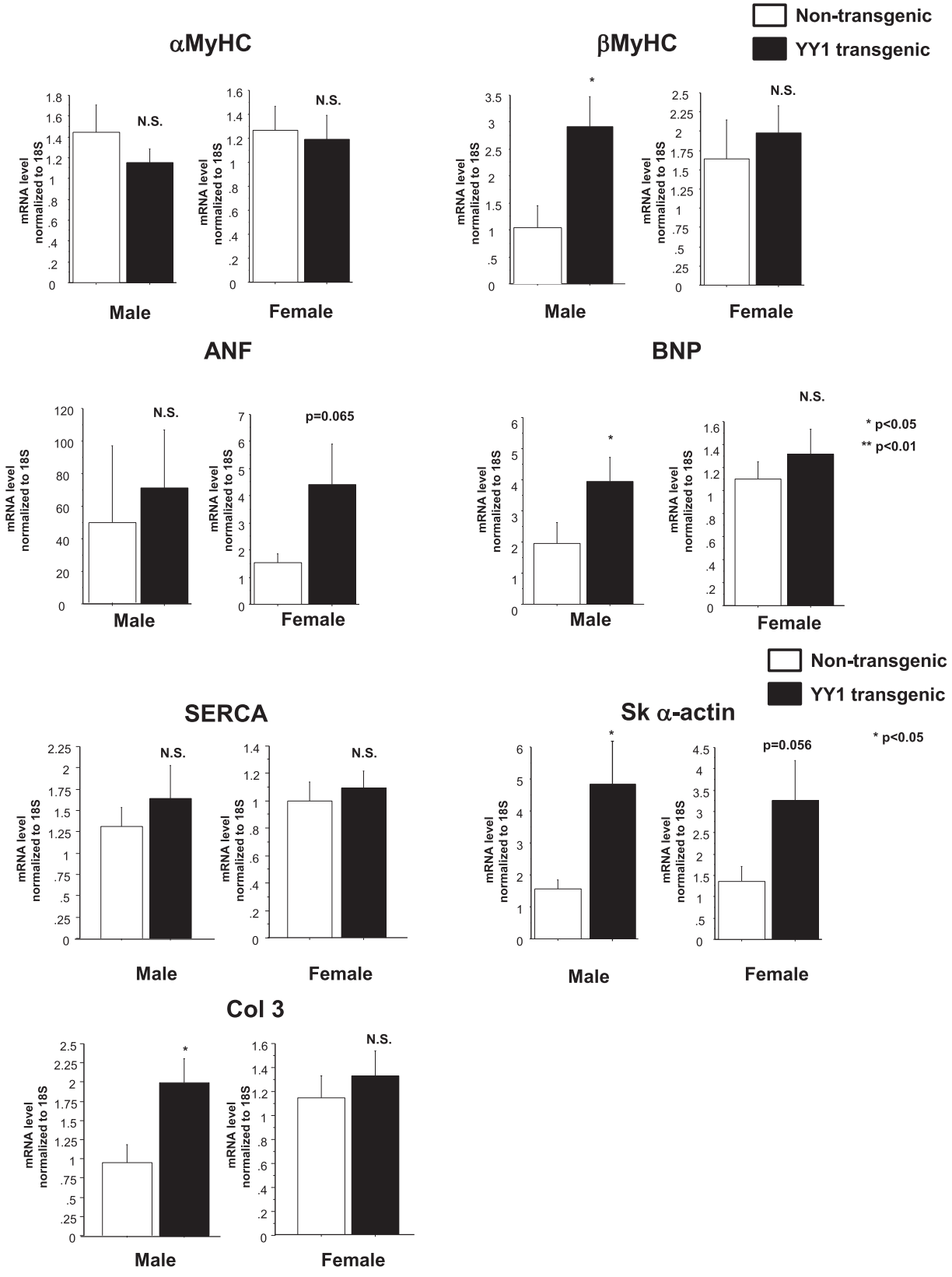

Fig. 2. Transgenic over-expression of YY1 results in up-regulation of the fetal isoforms BNP, ANF, βMyHC and skeletal α-actin, and of collagen 3 (COL3) in male mice. With the exception of skeletal α-actin and ANF expression was unchanged in female mice. Transgenic positive was compared to littermate control of the same sex. *$p < 0.05$, **$p < 0.01$ vs same-sex transgenic negative controls.

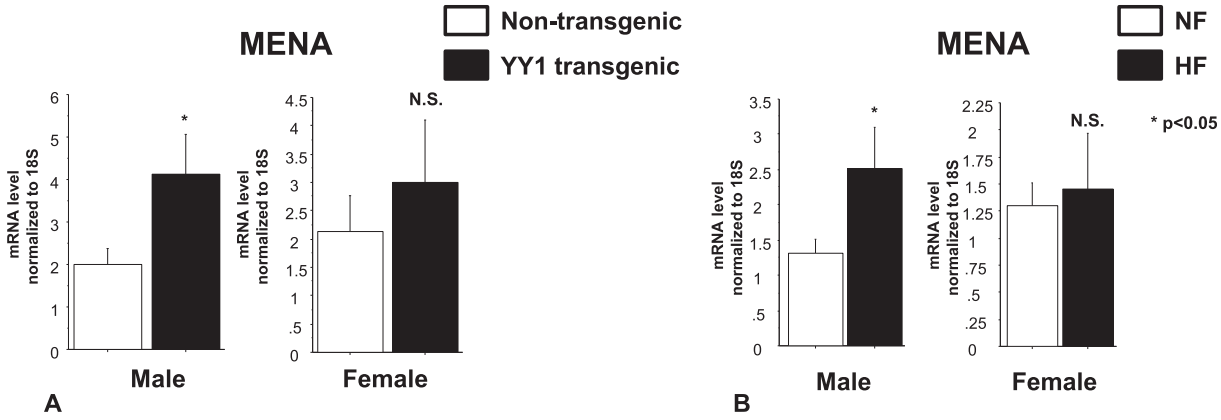

Fig. 3. Mena expression is higher in YY1 transgenic over-expression males and human male heart failure patients. (A) Mena expression is higher in YY1 transgenic positive male but not female mice. Transgenic positive was compared to littermate control of the same sex. (B) Mena expression is higher in human male but not female heart failure patients. Male and female non-failing (NF) controls were combined and compared to male and female heart failure (HF) patients. *p < 0.05 vs same-sex transgenic negative controls.

androgen receptor have increased expression of mammalian enabled (Mena) [14], a factor that regulates cytoskeletal actin dynamics and whose expression is increased in several models of cardiac pathology [15]. As shown in Fig. 3A, Mena expression is higher in male but not female YY1 transgenic mice than transgenic negative controls. We tested if expression of Mena was sex-specific in human IDC patients. As shown in Fig. 3B Mena expression is higher in male but not female IDC patients compared to NF controls.

3.6. YY1 expression is increased in human IDC male patients

Since transgenic female mice over-expressing YY1 did not develop cardiac dysfunction or pathologic gene expression, we hypothesized that higher YY1 expression in human HF is only observed in male patients, and is not part of the pathologic response in females. YY1 protein levels were investigated in the nuclear fractions of NF and IDC male and female human patients. As shown in Fig. 4A and B YY1 levels are higher in male but not female HF patients compared with NF controls.

4. Discussion

YY1 has previously been demonstrated to be important for cardiomyocyte growth and differentiation in vitro [12,21]. Prior to the current study, the influence of YY1 on cardiac growth and function in vivo was unknown. The current study has therefore demonstrated for the first time that YY1 influences cardiac growth and function in a sex-dependent manner in mice. Cardiac-specific over-expression of YY1 resulted in physiologic hypertrophy in females with higher LV/BW without concomitantly lower cardiac function or higher expression of the fetal gene program. In contrast, a pathologic response was observed in males with higher LV/BW that was accompanied by lower EF/FS, and higher βMyHC, BNP, skeletal α-actin and collagen 3 gene expression. Expression of ANF was highly variable in male transgenic animals and controls, and was dramatically increased when comparing to females. The reason for this variability in expression of ANF is not presently known. In addition, we have demonstrated that a sex-difference in the expression of Mena in the YY1 overexpression mouse recapitulates

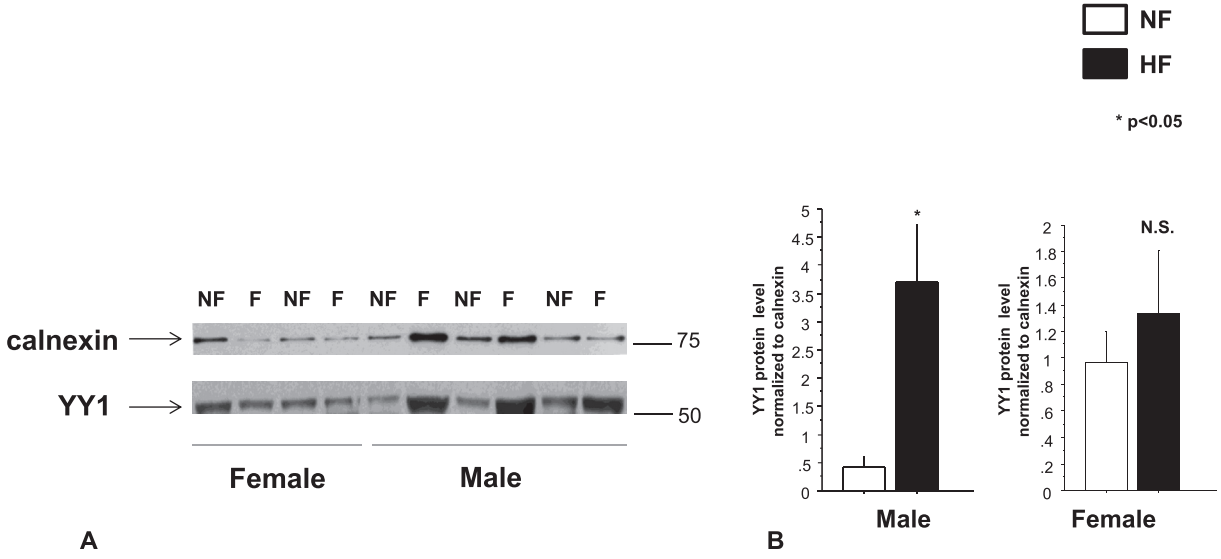

Fig. 4. YY1 protein levels are higher in male but not female human heart failure (HF) patients compared to NF control hearts. (A) Representative Western blot. Calnexin was used as a loading control. (B) Western blot quantification. *p < 0.05 vs same-sex NF.

the expression pattern in the failing male and female human heart. Finally, we showed that YY1 expression is higher in male but not female HF patients compared to NF controls.

YY1 is an ubiquitously expressed transcriptional factor involved in many cellular processes, from transcription regulation to chromatin remodeling and control of metabolic function (reviewed in Ref. [1]). A recent study showed that YY1 is important for proper cardiomyocyte differentiation, by acting as a transcriptional activator that promotes Nkx2.5 expression and cardiac progenitor cell commitment [21]. We have previously shown that YY1 protects against cardiac hypertrophy in neonatal cardiomycytes by interacting with HDAC5 and preventing its nuclear export [12]. Here we show that YY1 overexpression promotes cardiac growth in vivo. HDAC5 translocation to the cytoplasm in cell culture is an acute response, and in our previous studies, HDAC5 localization was analyzed after 2 h of hypertrophic agonist treatment. In contrast, YY1 over-expression in the mouse is present throughout the postnatal life. It is possible that acute over-expression of YY1 results in protection whereas chronic over-expression results in pathology.

It is also possible that the observed effects are due to sex differences in response to YY1 over-expression. The neonatal cardiomyocyte cell culture studies were composed of male and female cardiomyocytes and devoid of endogenous sex steroids milieu of the mouse in vivo. Both estrogen and testosterone have been demonstrated to influence cardiac growth. It may be that these important endogenous hormones interact with YY1 producing a sex-dependent difference in the cardiac response. Gonadectomy studies would be necessary to examine this possibility but are beyond the scope of this initial description.

In our previous study we showed that YY1 can promote sarcomeric organization in myocytes in culture [12]. A recent study has shown that YY1 is critical for cardiac progenitor cell commitment, and cardiac mesoderm-specific YY1 loss-of-function results in embryonic lethality [21]. These studies were performed in embryonic or post-natal systems, and results indicate that YY1 is an important factor for proper cardiomyocyte differentiation. In fact, YY1 regulation of skin gene expression has recently been associated with age- and sex-related changes [22].

We have, in addition, demonstrated that YY1 transgenic mice have higher expression of Mena that may be related to a YY1-mediated increase in androgen receptor activity. However, proper experiments to demonstrate that this is a Mena-dependent effect have not been performed and would require crossing of YY1 to a conditional Mena knockout mouse model. Interestingly, knockout of Mena results in cardiac dysfunction [15] and transgenic overexpression exacerbates heart failure [23], suggesting that Mena levels are tightly regulated in the heart. In addition, Mena is highly expressed in the young heart, and its expression is diminished in an age-related manner. The current study was performed in 4–5 month old animals, and it is possible that the observed discrepancies between the in vitro and in vivo findings are related to age-specific differences between neonatal and adult cardiomyocytes. Although speculative, it is possible that YY1 regulation of Mena is necessary or protective in development or post-natally, and detrimental in older animals.

Mena is a protein that co-localizes with cytoskeleton proteins. In a recent study Ram et al. showed that Mena regulates expression and localization of the cell-to-cell communication protein connexin43 [24]. The authors show that down-regulation of Mena results in increased connexin43 expression. Interestingly, we have recently shown that connexin43 expression is regulated in a sex-specific manner in rat cardiomyocytes, and expression in female mice is higher than in males in normal or pathological states [25]. It is unclear if these differences are due to differential expression of Mena and YY1 in these animals. These results indicate a sex-specific role of YY1 and Mena in the regulation of cytoskeleton proteins and cellular communication.

More importantly, we showed that higher YY1 expression is only observed in human IDC male patients. Since YY1 does not induce a pathologic response in transgenic female mice, it is possible that unchanged YY1 levels in human females is a consequence of lack of YY1 involvement in the pathologic response. However, this may also have a functional effect in human HF. In fact, studies have shown that male mice subjected to transverse aortic constriction developed more severe tissue fibrosis than female mice [26] and that male patients with aortic stenosis had higher levels of collagen I, III and matrix metalloproteinase 2 gene expression [27] and more fibrosis [28] than females with aortic stenosis. Although speculative, it is possible that the differences in the YY1 pathway in males and females contribute to the lesser degree of fibrosis observed in females.

In summary, our results show that transgenic over-expression of YY1 is detrimental in male animals by inducing expression of the FGP, increasing collagen 3 and Mena gene expression, and decreasing cardiac function. In contrast, although female animals have higher LV/BW, this is not accompanied by the pathological changes in gene expression or cardiac dysfunction. These results indicate that YY1 has a sex-specific function in the heart, which will be important for future studies on gene expression regulation.

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Conflict of interest

The authors have no conflict of interest.

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