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Transcriptome Characterization of Estrogen-Treated Human Myocardium Identifies Myosin Regulatory Light Chain Interacting Protein as a Sex-Specific Element Influencing Contractile Function

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Objectives This study investigated the effects of 17β-estradiol (E2) on gene regulation in human cardiac tissues. We hypothesized that a candidate E2 effect is cardiomyocyte (CM)- and sex-specific, conserved between humans and mice, and that E2 impairs contractile function in male CMs only.

Background Both men and women produce E2 locally from androgenic precursors. E2 regulates cardiovascular function, but specific mechanisms, protective or harmful, are not fully understood.

Methods We performed genome-wide expression profiling of E2-treated cardiac tissues from men and women, and studied gene expression and function in CMs from hearts of male and female E2-treated mice.

Results We found 36 E2-dependent genes regulated in a sex-specific manner. Of these, after E2 exposure, the myosin regulatory light chain interacting protein (*MYLIP*) gene was induced in tissues of men only. Focusing on Mylip and employing isolated mouse CMs, we confirmed our hypotheses that the E2 effect is CM- and sex-specific and conserved between humans and mice. The E2-treatment led to impaired contractile function in male CMs only, which was characterized by increased Mylip mRNA and protein levels, and decreased myosin regulatory light chain (Mrlc) protein. Our report is the first to our knowledge to show that cardiac Mrlc is an in vivo substrate for Mylip, leading to augmented Mrlc ubiquitination. Of relevance, we found that *MYLIP* expression levels rise with increasing age in hearts of men.

Conclusions E2 directly influences cardiac gene regulation, and E2 actions may be different between the sexes. Since E2 levels rise in older and/or obese men, pharmacological targeting of MYLIP in men with elevated E2 levels could possibly decrease their risk for the development or progression of cardiovascular disease. (J Am Coll Cardiol 2012;59:410–7) © 2012 by the American College of Cardiology Foundation

Sex- and menopause-related differences in cardiovascular diseases have drawn considerable attention to the sex steroid hormone 17β-estradiol (E2), which has been largely believed to be beneficial for the heart (1). However, unexpected negative findings from large randomized clinical trials (2,3) and conflicting results from animal studies have led to controversy about the role and actions of E2. Additionally, recent studies have revealed that E2 is also relevant for cardiovascular function in men (4). A better understanding of the effects of E2 on molecular and cellular physiology in the cardiovascular system is necessary.

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The primary source of E2 production is the ovary. However, E2 is also produced locally in both sexes through the conversion of testosterone by aromatase (5), with the adipose tissue contributing significantly to its circulating pool (6). E2 modulates a number of biological processes, such as reproduction, development, cell proliferation, dif-

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ferentiation, apoptosis, and metabolism, via gene regulation. E2 directly influences gene expression through the classical cytosolic estrogen receptors (ER) α and β, although rapid nongenomic actions not requiring the ER have also been reported. In the genomic effects, E2 binds to the ER, and the newly formed complex translocates to the nucleus. Subsequently, E2-ER complexes interact with a number of coactivator and corepressor proteins that are recruited to the receptor after ligand binding, to mediate or inhibit transcription (7,8).

We investigated the effect of E2 on the regulation of gene expression in human cardiac tissues. Following genome-wide expression profiling, we selected myosin regulatory light chain interacting protein (*Mylip*) as our candidate, and we tested the hypothesis that this estrogenic effect occurs in the cardiomyocyte (CM) using isolated CMs from hearts of E2-treated mice. We found that the effect is conserved between humans and mice, and that E2 modulates the expression of Mylip to control cellular contraction. We have therefore identified an effect of E2 on the regulation of gene expression occurring directly in the CM. This effect differs between the sexes, and we report that E2 impairs contractile function in male, but not in female, CMs. Notably, we also found that *MYLIP* expression is higher in the hearts of men older than 50 years than in the hearts of men younger than 40 years. Considering that E2 levels rise in older and/or obese men and, particularly, that elevated E2 levels in men with heart failure are associated with poor prognosis (4), our findings could be clinically relevant. Further confirmation of these findings at a clinical level could help to develop sex-specific treatments as part of a more individualized medical care, tailored to the specific needs of men and women.

Methods

Human cardiac tissue studies. Tissue harvest, culture, and treatment were recently described (9) and are summarized in the Online Methods. Tissues from 6 patients (50% women) undergoing coronary bypass surgery were treated with 10^{-8} mol/l E2 or 2-hydroxypropyl-β-cyclodextrin (HBC) (vehicle control for E2 treatment) for 24 h. This concentration was chosen to obtain an E2 level of physiological range. The study was approved by the Charite University Hospital Ethics Committee, it complies with the principles outlined in the Declaration of Helsinki, and written consent was obtained.

Nondiseased male human hearts (n = 6 for age <40 years, n = 9 for age >50 years) technically unusable for transplantation were obtained from general organ donors. Experimental protocols were approved by the Scientific and Research Ethical Committee of the Medical Scientific Board at the Hungarian Ministry of Health (ETT-TUKEB) under ethical approval No. 4991-0/2010-1018EKU (339/PI/010).

Hybridization and microarray profiling. Detailed protocols are provided in the Online Methods. Microarray data are deposited in the ArrayExpress database (accession No. E-MEXP-2971).

Microarray data analysis. Data were analyzed as described recently (10,11) and as summarized in the Online Methods.

Mouse experiments. Middle-aged (11- to 14-month-old) male (n = 20) and female (n = 22) C57BL/6J mice (Charles River Laboratories, Sulzfeld, Germany) were treated in vivo with 0.2 mg/kg E2, a dose reported to lead within 2 h to a 2.5-fold increase in E2 levels up to 129 pg/ml and within the physiological range for mice, returning to baseline after 22 h as E2 gets metabolized (12). Control mice were treated with HBC. The peak serum levels in elderly men are also reported to be up to 129 pg/ml, supporting the choice of E2 dose (13). Five hours after injection, mice were sacrificed and divided into 2 sets. In the first set (n = 4 male HBC, 6 male E2, 5 female HBC, 7 female E2), CMs were isolated for contraction and mRNA measurements. The second set (n = 5/group) was used to extract protein. Unloaded cell shortening was measured with a video-edge detector (Ionoptix, Milton, Massachusetts) at 1, 2, and 4 Hz using field stimulation. Detailed protocols are provided in the Online Methods. The study was approved by the Ethics Committee on Animal Use of the University of Leuven.

Quantitative real-time reverse transcription PCR. Reactions were performed as described previously (9) and as summarized in the Online Methods.

Immunoblotting and coimmunoprecipitation. Detailed protocols are provided in the Online Methods.

Statistical analysis. All data were analyzed statistically using the R version 2.11.0 software. Data are shown as the mean ± SEM. Comparisons between 2 groups were performed with unpaired *t* test and between multiple groups using 2-way analysis of variance with Tukey's post-hoc test adjusting for multiple comparisons, considering $p \leq 0.05$ significant.

Abbreviations and Acronyms

- **CM** = cardiomyocyte
- **E2** = 17β-estradiol
- **ER** = estrogen receptor
- **HBC** = 2-hydroxypropyl-β-cyclodextrin
- **Mrlc** = myosin regulatory light chain
- **Mylip** = myosin regulatory light chain interacting protein

Results

E2-dependent regulation of gene expression in human cardiac tissues. To investigate the effects of E2 on global human cardiac gene expression, we treated heart tissues from men and women with E2 or HBC (vehicle), and we performed microarray analysis. After verifying the quality of the microarray data, we fitted a linear model with an empirical Bayesian method (14) to determine differential gene expression. To extract biologically useful information, we examined those probe sets with an unadjusted p value of up to 0.001. This resulted in 77 significant probe sets (Online Table 1). Unsupervised hierarchical clustering of these probe sets revealed that the treatment with E2 was not

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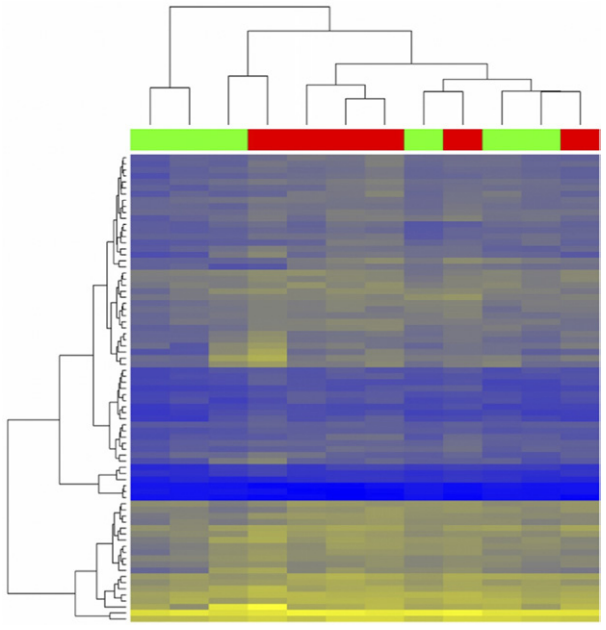

Figure 1 Heat Map of E2-Regulated Human Cardiac Gene Expression

Tissues were treated with 10^{-8} mol/l E2 or HBC (vehicle) for 24 h followed by microarray analysis. Unsupervised hierarchical clustering of the identified 77 probe sets revealed that E2 did not have an exclusive effect on gene expression. **Blue** indicates low expression and **yellow** indicates high expression. In the colored horizontal side bar, **green** indicates HBC (n = 6) and **red** indicates E2 (n = 6).

sufficient to be the sole factor separating the tissues into 2 distinct groups, i.e., E2 and HBC, suggesting that the sex might play an important role in the regulation of gene expression by E2 (Fig. 1). Additionally, we found that there were small fold changes (20% to 40%) in gene expression between the 2 conditions.

Sex-specific regulation of gene expression by E2 in human cardiac tissues. We then asked whether E2 regulates cardiac gene expression in a different manner between male and female tissues. We first calculated the differences in expression between the 2 conditions of E2 and HBC treatment for each tissue. We then fitted a statistical model stratifying the data by the effect of the sex. This approach identified 41 probe sets ($p \leq 0.001$) (Online Table 2). Unsupervised hierarchical clustering of these probe sets confirmed the effect of the sex through the separate grouping of E2 effects in male and female divisions (Fig. 2A). Among these candidates, we identified the *MYLIP* gene, whose expression was induced by E2 in heart tissues of men only (Fig. 2B).

E2 regulates Mylip in a sex-dependent manner in mouse CMs. We selected *Mylip* as the most interesting candidate because of its potential influence on contraction through regulation of the myosin regulatory light chain (Mrlc) (15–18), and we used the mouse for further analysis. Due to the multicellular nature of the heart tissue, to test the hypothesis that this regulation occurred in the CM, we treated mice in vivo with E2, and we subsequently determined the level of *Mylip* in isolated CMs. In addition, this approach enabled us to test whether the regulation of *Mylip* by E2 is conserved between humans and mice. Quantitative real-time reverse transcription PCR confirmed that E2 modulated the expression of *Mylip* in a sex-specific manner (interaction $p < 0.05$). In particular, the treatment with E2 induced the expression of *Mylip* in CMs of male mice (adjusted $p \leq 0.05$), whereas the same treatment had no significant effect in female CMs (adjusted $p = 0.99$) (Fig. 3). Therefore, we have identified a novel regulatory effect of E2

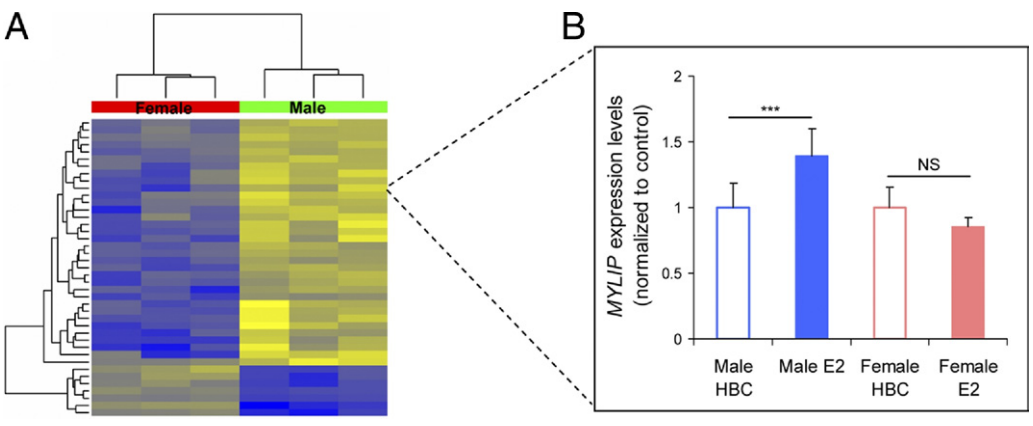

Figure 2 Heat Map of Sexually Dimorphic E2-Regulated Human Cardiac Gene Expression

(A) Unsupervised hierarchical clustering of the identified 41 probe sets divided them into 2 distinct groups. The difference of expression between HBC (n = 3/sex) and E2 (n = 3/sex) was calculated for each tissue and then the effect of the sex on this was analyzed. **Blue** indicates low expression, and **yellow** indicates high expression differences. **(B)** Microarray data analysis revealed that E2 induced the expression of *MYLIP* in cardiac tissues from men, whereas there was no significant change in cardiac tissues from women. All analyses were performed with independent tissue samples. Each tissue originated from a different individual. ***$p < 0.001$.

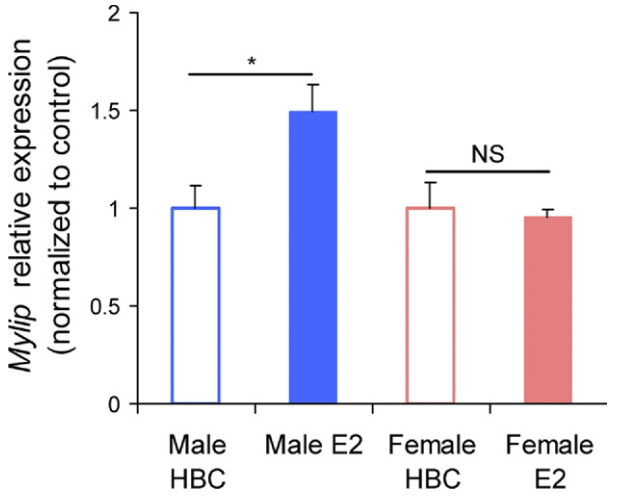

Figure 3 ***Mylip* Expression Levels in E2-Treated Mouse CMs**

Mice were treated in vivo with 0.2 mg/kg E2 or HBC for 5 h followed by cardiomyocyte (CM) isolation. Quantitative real-time PCR confirmed that E2 induced *Mylip* in CMs of male mice, whereas in female CMs, there was no effect. Data are mean ± SEM of independent biological replicates, and each was measured in duplicate. n ≥ 4/group; *p < 0.05. NS = nonsignificant.

that is CM- and sex-specific and conserved between humans and mice.

Increased Mylip and reduced Mrlc protein levels in CMs of male E2-treated mice only. Next, we investigated whether Mylip protein levels were altered in the same direction as at the transcriptional level. In particular, after E2 exposure, Mylip protein levels were significantly increased in males (adjusted $p < 0.01$), whereas they did not change in females (adjusted $p = 0.76$) (Fig. 4A). Mylip mediates ubiquitination and subsequent proteasomal degradation of Mrlc (15,18). On the basis of this, we hypothesized that E2 modulates Mrlc through increased Mylip protein levels. Immunoblotting revealed the sex-dependent abundance of Mrlc protein after treatment with E2. In line with our hypothesis, we found that there was a significant decrease (adjusted $p \leq 0.05$) in Mrlc levels in males treated with E2 compared with the male-derived control group, whereas in females, it remained stable (adjusted $p = 0.99$) (Fig. 4B).

Cardiac Mrlc is an endogenous substrate of Mylip, and increased Mylip abundance enhances in vivo Mrlc ubiquitination. Evidence supporting the notion that Mylip regulates cardiac Mrlc levels has been lacking. Our report offers novel insight into this mechanism. We hypothesized that Mrlc represents an in vivo substrate for Mylip activity in the heart. Coimmunoprecipitation analyses of male left ventricular lysates revealed the binding of Mylip to Mrlc, which was further augmented after E2 exposure (Fig. 5A). Accordingly, the expected basal ubiquitination levels of Mrlc were further increased following E2 treatment (Fig. 5B), supporting our hypothesis.

E2 impairs contractile function in male CMs only. We then postulated that the E2-triggered increase in Mylip levels characterized by the decrease in Mrlc abundance in male CMs leads to impaired contractile function in these cells. We recorded unloaded cell shortening at 1, 2, and 4 Hz. CMs from male mice treated with E2 showed a significantly smaller cell contraction compared with the HBC-treated group (Figs. 6A and 6C). On the other hand, there was an increasing trend in cell contraction in female CMs after E2 compared with controls (Figs. 6B and 6C). Similarly, there was a significant decrease in the rate of contraction of E2-treated male CMs compared with controls, whereas the opposite trend was observed in female CMs (Fig. 6D).

***MYLIP* expression levels rise with increasing age in male human hearts.** Knowing that E2 levels increase in aging men (6,13,19), we sought to support the clinical relevance of our findings. We therefore studied the expression levels of *MYLIP* in the hearts of younger and older men. Quantitative real-time reverse transcription PCR revealed that there is a higher *MYLIP* expression in the hearts of men older than 50 years than in the hearts of men younger than 40 years (Fig. 7A). Moreover, we employed 2 public microarray datasets that enabled us to stratify our analysis by age. Using this additional cohort, we further confirmed that in the hearts of men older than 50 years ($n = 4$), *MYLIP* expression was higher than in the hearts of men younger than 40 years ($n = 4$; adjusted $p = 0.002$) (Fig. 7B).

Discussion

Our novel findings are that E2 has direct effects in the heart through the regulation of gene expression; these effects can be sex- and CM-specific; E2 regulates *Mylip* in a conserved fashion in humans and mice; cardiac Mrlc is a substrate for Mylip activity; and E2 impairs cellular contractility in male CMs.

We performed genome-wide profiling of E2-treated human cardiac tissues. The variation in human material was considerable, and the changes in gene expression were not large. Small fold changes due to E2 effects have already been encountered by our group and others (20–22). Small quantitative differences in the amount of RNA might have relevant qualitative effects at a functional level. To this extent, we recently suggested that the heart is a highly regulated environment, where minor changes may have major effects (9). Here, we provide evidence supporting this notion.

Following the generation of the candidate list, the exposure of the tissues to E2 was not sufficient to be the sole factor responsible for the variation in the expression of the identified 77 probe sets between HBC- and E2-treated tissues, as demonstrated by the corresponding clustering. This finding further suggested that the sex might play an important role in the regulation of gene expression by E2. Divergent hormonal effects on gene expression in male and

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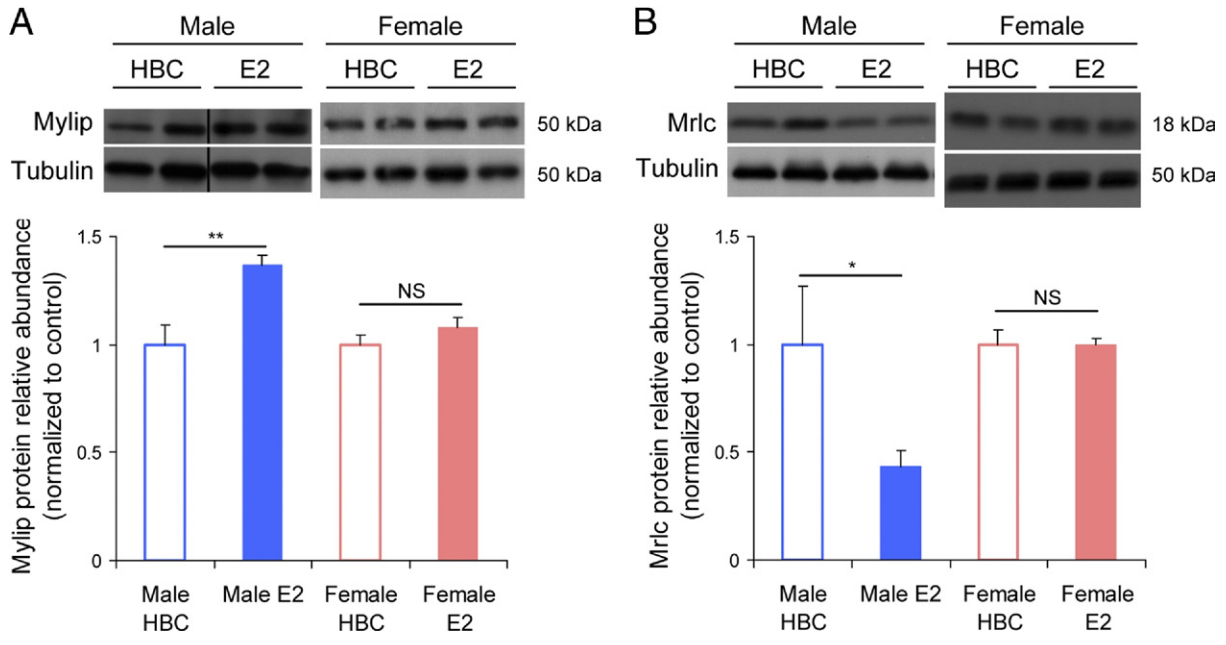

Figure 4 **Mylip and Mrlc Protein Levels in E2-Treated Mouse Hearts**

(A) Immunoblotting analysis with antibodies specific for the indicated proteins revealed that the abundance of Mylip was increased in male hearts after E2 exposure, whereas there was no significant difference in female hearts. Black lines indicate that intervening lanes have been spliced out. **(B)** On the other hand, Mrlc protein levels were decreased in E2-treated male hearts, whereas they remained stable in female hearts. **(A and B)** Semiquantification of protein profiling by means of densitometry analysis is shown. Tubulin was used as loading control. Results are representative of 2 or more individual experiments and are presented as the mean value of 5 mice per group ± SEM. *$p < 0.05$; **$p < 0.01$. NS = nonsignificant.

female mice were suggested earlier (23), a finding that we recently confirmed in the human heart for progesterone receptor (9). We now demonstrate that E2 regulates gene expression in a sex-specific manner, particularly in CMs. We have not yet deduced the mechanisms responsible. One possibility could be differences in the expression levels of the ER between the sexes. However, the expression profiling did not reveal any significant changes. On the other hand, the E2-ER complex interacts with other transcriptional cofactors of different function, thereby modulating transcriptional regulation (7,8). We postulate that the identified sex-specific regulation of gene expression by E2 is the result of differences in the recruitment of transcriptional cofactors between the sexes.

A novel observation is that ex vivo treatment of human cardiac tissues with E2 increased the levels of *MYLIP* in

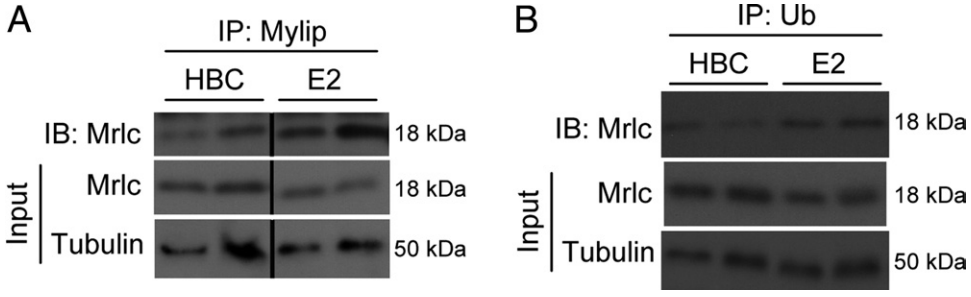

Figure 5 **Endogenous Mylip–Mrlc Interaction and In Vivo Mrlc Ubiquitination**

(A) Coimmunoprecipitation (IP) analysis revealed that Mylip interacts with Mrlc in the heart. Notably, this interaction was further enhanced after E2 exposure. Equal amounts of left ventricular lysates were incubated with anti-Mylip followed by immunoblotting of immunoprecipitates with anti-Mrlc. Input lanes, 1% of initial extracts. **Black lines** indicate that intervening lanes have been spliced out. **(B)** Following differences in overall Mrlc protein levels between male E2-treated mice and controls as shown by immunoblot (IB) analysis, it was found that ubiquitination of Mrlc was enhanced after E2 exposure. Equal amounts of proteins were immunoprecipitated using an anti-Ub antibody and analyzed by immunoblotting using an anti-Mrlc antibody. Input lanes, 1% of initial extracts. **(A and B)** Tubulin was used as loading control. Results are representative of 2 or more individual experiments.

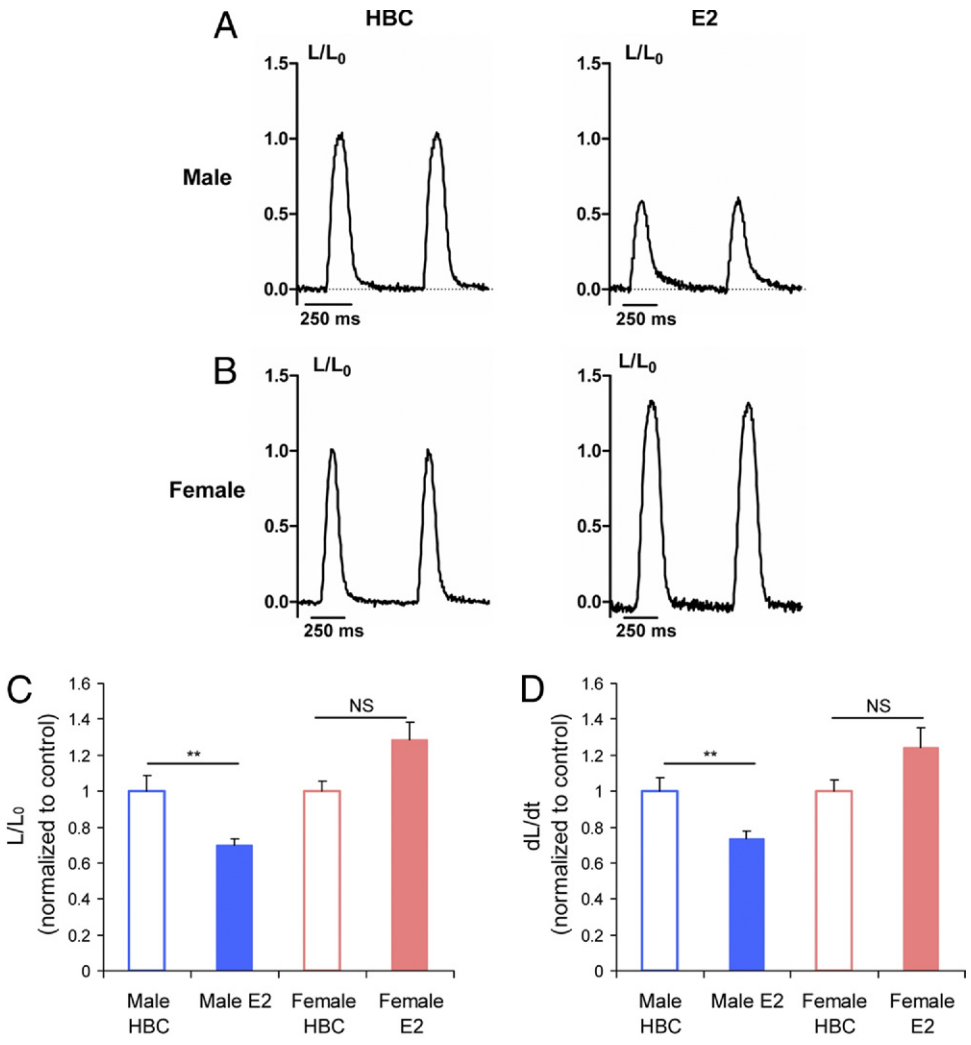

Figure 6 **Cellular Contraction of CMs From Mice Treated With E2**

The treatment with E2 led to a significant impaired contractile function in male cardiomyocytes (CMs), whereas there was an enhancing trend in female CMs. **(A and B)** Examples of a time course of unloaded cell shortening during steady-state stimulation at 2 Hz (normalized to control). **(C)** Mean data of unloaded cell shortening (L) normalized to diastolic cell length (L_0) and **(D)** rate of contraction at 2 Hz. The bars indicate SEM. n = 33 cells male HBC; 36 cells male E2; 44 cells female HBC; 34 cells female E2. **p < 0.01.

tissues from men, but not from women, compared with control tissues. To verify the idea that this regulation occurs in the CM, we treated mice in vivo with E2, and we subsequently determined the level of *Mylip* in isolated CMs. As in humans, E2 induced the expression of *Mylip* in male, but not in female, mouse CMs. We also confirmed that Mylip protein levels were altered in the same direction. Mylip is an E3 ubiquitin ligase that belongs to the ezrin-radixin-moesin (ERM) protein family, and it contains a C-terminal RING finger domain (18). In neuronal cells, Mylip has been shown to bind Mrlc, mediating its ubiquitination and subsequent proteasomal degradation (15,18). Based on this knowledge, we hypothesized that the induction of Mylip by E2 would result in decreased Mrlc levels. Indeed, there was a significant decrease in Mrlc protein levels due to E2 in male CMs, whereas there was no change in the Mrlc content of female CMs. However, the possibility that cardiac Mrlc is a substrate of Mylip had not been explored previously. We now demonstrate the endogenous interaction of Mylip with Mrlc. As expected, this binding was further augmented after E2 exposure. Subsequently, we determined that the in vivo ubiquitination of Mrlc was enhanced in E2-treated CMs.

Modulation of contraction by Mrlc in the heart has been well documented (16,17). Phosphorylation of Mrlc by myosin light chain kinase decreases the distance between myosin and actin, with an increase in Ca^{2+} sensitivity of the myofilaments and a higher rate of contraction. At baseline, there is a significant level of phosphorylation. Mutant mice for Mrlc phosphorylation show defects in heart contraction

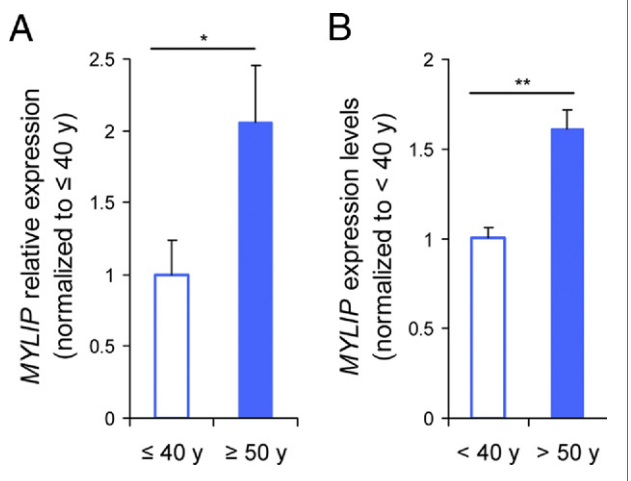

Figure 7 ***MYLIP* Expression Levels in Hearts of Younger and Older Men**

(A) Quantitative real-time PCR revealed that the expression levels of *MYLIP* are higher in the left ventricles of men older than 50 years (n = 9) compared with those of men younger than 40 years (n = 6). Data are mean ± SEM of independent biological replicates, and each was measured in duplicate. **(B)** Analysis of publicly available independent microarray datasets of cardiac tissues also revealed that in men older than 50 years, *MYLIP* expression is higher compared with men younger than 40 years (n = 4/group). Analyses were performed with independent tissue samples. Each tissue originated from a different individual. *p < 0.05; **p < 0.01. y = years.

(24). Although no data exist regarding changes in the levels of Mrlc expression, we postulated that decreased Mrlc levels would lead to impaired contractile function. Indeed, reduced Mrlc levels would preclude the positive modulatory effect of phosphorylation of Mrlc. Cell shortening measurements demonstrated that with E2 there was a significant impairment of cellular contractility in male CMs only. Our data support the regulatory role of Mrlc and reveal a novel sex-dependent mechanism. The reduced extent and rate of contraction suggest that reduction of Mrlc reproduces the effects of removal of phosphorylation.

In men, E2 is produced in significant quantities by local tissue aromatization of androgenic precursors from the testes and adrenal glands (25). In obese men, there is a marked increase of E2 production (26). In addition, E2 levels in men increase further with advancing age (6,13), and elderly men may have higher concentrations of E2 compared with age-matched women (19). Elevated E2 levels in men are associated with myocardial infarction and coronary artery disease (27) and with an increased risk of stroke (13). Notably, high E2 concentrations are a significant predictor of poor prognosis and higher mortality in men with chronic heart failure and reduced left ventricular ejection fraction (4).

Although elevated E2 levels in older and/or obese men have been associated with an increased risk and incidence of cardiovascular disease, explanations for causal pathways and putative mechanisms for this association have not been identified (4). We suggest that MYLIP could contribute to this association, and we propose that MYLIP could become a pharmacological target in this high-risk group. Relevant to this notion and to support the clinical relevance of our findings, we found that *MYLIP* expression is higher in the hearts of men older than 50 years than in the hearts of men younger than 40 years. However, no measurements of E2 levels in these same individuals exist so that a direct association could be tested. Furthermore, questions remain about the precise actions of MYLIP that could increase cardiovascular risk. However, it was recently reported that MYLIP is involved in cholesterol regulation through the ubiquitination of the low-density lipoprotein receptor (28). Clearly, avenues for further research in this area are open.

Study limitations. We acknowledge that the sample size for human material was rather small, related to limited fresh material available from closely matched individuals, as well as a low number of publicly available datasets. Yet the internal consistency across the different experiments supports the conclusions. We have also limited ourselves for mouse experiments to 1 dose of E2. The dose was chosen based on literature data that indicate that this dose leads to in vivo E2 levels that at peak are 2.5-fold higher than baseline and within the range of values found in elderly men. Studying the effect of various doses of E2 in vivo would be of interest, but it would require a large number of animals not justified by the current question, namely to confirm a CM-specific effect.

Conclusions

We have shown that E2 exerts direct effects on gene expression in the heart and these actions may be different between the sexes. The present findings may offer insight into possible molecular mechanisms responsible for some of the sex-related differences observed in the development, prognosis, and outcome of cardiovascular disease, and they underscore the potential benefit from sex-specific therapies. Lastly, our work provides evidence and grounds for the notion that E2 is a master regulator through the modulation of gene expression, strongly suggesting an explanatory path for the prominent role of E2 in the development of sexually dimorphic traits in health and disease.

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Key Words: contractility ■ estrogen ■ gene expression ■ gene regulation ■ genomics ■ sex-specific.

APPENDIX

For supplemental methods and tables, please see the online version of this paper.