17β-Estradiol-induced interaction of ERα with NPPA regulates gene expression in cardiomyocytes

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Aims
17β-oestradiol (E2) and its receptors (ERα and ERβ) are important regulators of physiological and pathological processes in the cardiovascular system. ER act in concert with other regulatory factors mediating oestrogenic effects. However, the underlying mechanisms modulating ER transcriptional activity are not fully elucidated. To gain better understanding of E2-induced ERα action in the human heart, we aimed to identify and functionally analyse interaction partners of ERα.

Methods and results
Using yeast two-hybrid assays with a human heart cDNA library, we identified atrial natriuretic peptide precursor A (NPPA), a well-known cardiac hypertrophy marker, as a novel ERα interaction partner interacting in an E2-dependent manner. Mutation analyses and immunofluorescence data indicated that the LXXLL motif within NPPA is necessary for its E2-induced interaction with ERα, its action as a corepressor of ERα, and its translocation into the nucleus of human and rat cardiomyocytes. Expression analysis and chromatin immunoprecipitation assays in a human left ventricular cardiomyocyte cell line, AC16, showed that NPPA interacts with E2/ERα, suppressing the transcriptional activity of ERα on E2-target genes, such as NPPA, connexin43, α-actinin-2, nuclear factor of activated T-cells, and collagens I and III.

Conclusion
We characterize for the first time an E2-regulated interaction of NPPA with ERα in cardiomyocytes, that may be crucial in physiological and/or pathological cardiac processes, thereby representing a potential therapeutic target.

Keywords
Oestrogen receptor-α • Oestrogen • Atrial natriuretic peptide precursor A • Cardiomyocyte • Gene expression

1. Introduction
17β-oestradiol (oestrogen, E2) influences a number of physiological and pathological processes in mammals, including the cardiovascular system.1–4 However, the underlying molecular mechanisms are still not fully understood. E2 mediates its effects predominantly via oestrogen receptor α (ERα) and β (ERβ), which are members of the nuclear receptor superfamily.5 The ER act as ligand-induced transcription factors and regulate the expression of E2-target genes (genomic effects).5

The ER are expressed in human and rodent hearts,7,8 and regulate upon E2 activation the expression of relevant E2-target genes, such as connexin 43 (Cx43), matrix metalloproteinases, and atrial natriuretic peptide (ANP), which play a role in the pathogenesis of myocardial diseases.7,9

It is generally accepted that ER require a number of co-regulatory proteins whose cell-specific expression may explain some of the distinct cellular actions of E2.11 The transcriptional activity of ER can be enhanced or suppressed by regulatory proteins called co-activators and co-repressors, respectively. Most of these co-regulators interact with ER through their highly conserved LXXLL motif, where L is leucine and X is any amino acid.12,13 So far, only few ER co-regulators have been described in the human heart. To gain a better understanding of E2-mediated ER action in the human heart, we focused in this work on ERα and aimed to characterize and functionally analyse novel interaction partners of ERα. We identified atrial natriuretic...
peptide precursor A (NPPA), the precursor of ANP, a well-known marker of myocardial hypertrophy (MH) and heart failure (HF), as a novel co-repressor of ERα action in the human heart. Our findings suggest that the interaction of ERα and NPPA contributes, at least partly, to the E2-mediated regulation of cardiomyocyte gene expression and function.

2. Methods

2.1 Study approval

The use of human cardiac tissues was approved by the ethical committee of Charité University Hospital and followed the principles of the Declaration of Helsinki. All animal experiments [isolation of cardiac myocytes from 1 to 3-day-old Sprague-Dawley rat hearts (Ref. T0333/08) and left ventricular (LV) tissue from ERα-deficient (ERαKO) mice (Ref. O0243/05)] were approved by the State Agency for Health and Social Affairs (LaGeSo, Berlin, Germany) and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of health (NIH Publication No. 85–23, revised 1996).

2.2 Cell culture and treatment

For in vitro investigations, we used a human cardiomyocyte-like cell line, AC16 cells, derived from adult female LV heart tissue, and neonatal rat cardiac myocytes (NNRCM) isolated from 1 to 3-day-old Sprague-Dawley rats. For detailed cell culture conditions see Supplementary material online, Methods. Prior E2 treatment, cells were starved with 2.5% charcoal stripped FBS (cs FBS) in phenol-red free media, subsequently incubated with the physiological concentration of E2 (10⁻⁸ mol/L; Sigma) or ethanol as vehicle for 24 h at 37°C in 5% CO₂. Treatment with ICI 182,780, an ER-inhibitor (10⁻⁵ mol/L; Tocris), was performed 1 h before E2 treatment.

2.3 Plasmid construction

Constructs were generated by PCR and standard cloning techniques. Detailed description of how the vectors were constructed and the list of primers can be found under Supplementary material online, Table S1.

2.4 Yeast two-hybrid screen and β-galactosidase assay

Yeast two-hybrid (Y2H) screening was performed using the BD Matchmaker Two-Hybrid System 2 according to the manufacturer's instructions (CLONTECH). Full-length human (h)ERα-pB1M16 and hERα-EF-pB1M16 constructs were used as baits to screen 3.5 × 10⁸ clones from a human heart BD Matchmaker TM cDNA library in Saccharomyces cerevisiae strain L40. Two independent large screens were carried out on selection plates lacking tryptophan, histidine, leucine, and myce cerevisiae strain L40. Two independent large screens were carried out to eliminate spurious interactions according to manufacturer's instructions. For co-transfection experiments, cells were co-transfected with pSG-hERα vector (0.3 µg HEGO-vector (designated as ERα-vector), kind gift of P. Chambon, France) and different amounts (0.3 or 0.15 µg) of either NPPA-WT-pcDNA3.1 (NPPA-WT) or NPPA-MUT-pcDNA3.1 (NPPA-MUT) or corresponding empty vectors (pcDNA3.1). After transfection, cells were grown overnight in medium used for treatment experiments. Following treatment with E2, vehicle or ICI 182,780, cell extracts were prepared and Firefly and Renilla luciferase activities were measured in a multilabel counter Victor3™ (Perkin Elmer) using the Dual-Glow™-Luciferase Assay system (Promega) according to the manufacturer’s instructions. Variations in transfection efficiency were normalized to Renilla luciferase activity. All transfections were carried out in triplicates and performed independently at least three times. Results are expressed as mean ± SEM.

2.5 Transient transfections and reporter assays

The transient transfection/dual-luciferase reporter assays were carried out as previously described. Briefly, AC16 cells were plated on 12-well plates (6 × 10⁵ cells/well). At a confluence of ~60%, cells were transfected for 6 h with the promoter-luciferase reporter constructs (0.8 µg) and the internal reference Renilla luciferase reporter plasmid phRL-TK vector (10 ng, Promega) using FuGENE®-HD Transfection Reagent, according to the manufacturer’s instructions. For co-transfection experiments, cells were co-transfected with pSG-hERα vector (0.3 µg HEGO-vector (designated as ERα-vector), kind gift of P. Chambon, France) and different amounts (0.3 or 0.15 µg) of either NPPA-WT-pcDNA3.1 (NPPA-WT) or NPPA-MUT-pcDNA3.1 (NPPA-MUT) or corresponding empty vectors (pcDNA3.1). After transfection, cells were grown overnight in medium used for treatment experiments. Following treatment with E2, vehicle or ICI 182,780, cell extracts were prepared and Firefly and Renilla luciferase activities were measured in a multilabel counter Victor3™ (Perkin Elmer) using the Dual-Glow™-Luciferase Assay system (Promega) according to the manufacturer’s instructions. Variations in transfection efficiency were normalized to Renilla luciferase activity. All transfections were carried out in triplicates and performed independently at least three times. Results are expressed as mean ± SEM.

2.6 Gene expression analysis

Total RNA isolation and quantitative real-time polymerase chain reaction (qRT–PCR) was conducted as previously described. Primer sequences used for amplification are listed in Supplementary material online, Table S1.

2.7 Immunoblot and co-immunoprecipitation analyses

For Western blot analysis, AC16 cell extracts or whole extracts of LV tissues isolated from female ERαKO mice (n = 7) or wild-type littermates (n = 6) were separated by SDS–polyacrylamide gel electrophoresis as previously described. Western blot analysis was performed using rabbit polyclonal antibodies against NPPA (FL-153, Santa Cruz) and Cx43 (ab11370, Abcam), and for normalization rabbit polyclonal antibody against actin ([I-19]-R; Santa Cruz). Specific bands were visualized using the ECL™ Plus detection kit (GE Healthcare). Band intensities were quantified with the Imagej software (Version 1.37, Wayne Rasband, NIH). Co-immunoprecipitation (Co-IP) analyses were performed as described before. Briefly, proteins derived from human atrial biopsies of five female patients were isolated and pre-cleared by adding protein G sepharose beads (PGS, Amersham) washed with HNTG-buffer (20 mmol/L Hapes (pH 7.5), 50 mmol/L NaCl, 10% Glycerol, and 0.1% Triton-X-100), for 2 h with gentle shaking at 4°C. 500 µg pre-cleared protein lysates were incubated with different amount of an anti-ERα polyclonal antibody (MC-20, Santa Cruz) diluted in HNTG-buffer plus 1 mmol/L NAF, 1 mmol/L PMSF, 1× Mini complete (Protease Inhibitor Cocktail, Roche) and 1 mmol/L Na3VO4 for 2 h at 4°C, followed by an incubation with blocked PGS (with 1% BSA) overnight with gentle shaking at 4°C. After centrifugation, pellets containing antibody/antigen/PGS-complexes were washed four times with HNTG-buffer, and finally resuspend in HNTG-buffer and 1 x reducing sample buffer (Roht). After incubation at 95°C for 5 min and short centrifugation, the proteins were subjected to SDS–PAGE, which were then analysed by immunoblotting using an anti-NPPA polyclonal antibody (FL-153, Santa Cruz). For the reverse-CoIP reactions, the anti-NPPA polyclonal antibody was used and the immunoblot was incubated with the ERα antibody. As positive control served input (50 µg), and as negative controls served samples without antibody addition and protein lysate derived from LV myocardial biopsies.
2.8 Immunofluorescence and confocal laser microscopy
AC16 cells or NNRCMs were grown on eight-chamber culture slides (BD Bioscience) at a density of 2 × 10^5 cells/chamber. For co-localization studies, AC16 cells were either left non-transfected, or co-transfected with pFLAG-CMV4-ERα and NPPA-WT-pcDNA3.1 or NPPA-MUT-pcDNA3.1 vectors. The cells were treated with E2 (10^{-8} mol/L) or vehicle (ethanol) for 24 h, or pre-treated with ICI 182 780 (10^{-5} mol/L) 1 h before E2 treatment. Cells were fixed, permeabilized, blocked, and stained as previously described. Following antibodies were used: anti-ERα (C-542; ENZO Life Sciences), anti-NPPA (FL-153), anti-Flag (M2, Sigma), and anti-His (H-15, Santa Cruz). For detailed description of immunofluorescence (IF), see Supplementary material online.

2.9 Chromatin immunoprecipitation assay
AC16 cells (6 × 10^5) were cultured as described in Supplementary material online. Methods, and treated with E2 (10^{-8} mol/L) or vehicle (ethanol) for 1 h. Cellular DNA and protein interactions were cross-linked for 10 min with formaldehyde (Thermo Scientific) at a final concentration of 1%, followed by the addition of 125 mmol/L glycine for 5 min. Chromatin immunoprecipitation assay (ChIP) experiments were conducted according to a standard protocol of the Transcription Factor ChIP Kit (Diagenode). The following antibodies were used for ChIP experiments: a ChIP-grade rabbit polyclonal anti-ERα (HC-20, Santa Cruz) antibody and a normal rabbit IgG antibody (Cell Signaling Technology, Inc.) as a negative control. DNA was analysed using the Applied biosystems 7300 system and SYBR green master mix. According to manufacturer’s protocol, a volume of chromatin was processed without antibody and used as total input control for qRT-PCR. As positive controls for E2 treatment, primers directed against human c-Myc (cellular myeloma oncogene) and TFF1 (trefoil factor 1) promoters were used. ChIP primer sequences used in this study are listed in Supplementary material online, Table S1. The experiments were performed in duplicates and repeated independently at least three times. Enrichment of specific promoter regions after immunoprecipitation was calculated as a percentage of the total input

3. Results

3.1 Identification of atrial natriuretic peptide precursor A as a hERα-interacting protein
To identify novel proteins interacting with hERα, we screened a human heart cDNA library using the Y2H. From two independent Y2H screens, 25 individual clones were found to contain the full coding sequence of NPPA, which interacts with hERα in the presence of E2. 1:1 retransformation experiments and β-Gal assays showed that NPPA interacts with full-length hERα (ERα-FL) and the EF domain of hERα (ERα-EF) in an E2-dependent manner (Figure 1A).

3.2 NPPA interacts via its LXXLL motif with hERα
To refine the binding site on NPPA, we performed 1:1 retransformation experiments and β-Gal assays using ERα-FL-pBSTM116 and ERα-EF-pBSTM116 as bait constructs and NPPA-WT-pACT2 or NPPA-MUT-pACT2 with mutations within the LXXLL motif, as prey constructs. Although NPPA-WT exhibited an interaction with both ERα-FL and ERα-EF in the presence of E2, NPPA-MUT was only able to interact with ERα-FL but not with ERα-EF (Figure 1B). These findings imply that at least two independent regions in the NPPA protein are involved in the interaction with ERα and suggest that the LXXLL motif within the NPPA selectively interacts with EF domain of ERα in the presence of E2.

3.3 ERα interacts with NPPA in the human atrium
To ascertain the ERα–NPPA interaction in a mammalian system, endogenous ERα protein from the human atrium was immunoprecipitated with an ERα antibody. Subsequent western blotting with an anti-NPPA antibody indicated that endogenous NPPA had coprecipitated with ERα (Figure 1C). Reverse immunoprecipitation with NPPA antibody and western blotting with an anti-ERα antibody confirmed the interaction of NPPA and ERα in the human atrium (Figure 1D). These results confirm the obtained data from the Y2H assays and the interaction of ERα with NPPA in the human atrium.

3.4 E2 increases the nuclear translocation and co-localization of NPPA and ERα in cardiomyocytes
To determine the subcellular localization and co-localization of NPPA and ERα in cardiomyocytes, we performed IF staining of AC16 cells, using antibodies against ERα and NPPA. In contrast to vehicle-treated AC16 cells, E2 treatment led to an increased amount of ERα and NPPA in the nuclei and cytoplasm of AC16 cells, where they co-localized (Figure 2A). E2 also triggered the translocation of both ERα and NPPA into nuclei of NNRCM, confirming the E2-induced nuclear translocation of these proteins in another model (Figure 2B). Further IF experiments showed that as a consequence of the inhibition of activation of ERα by ICI 182 780, NPPA remained mostly in the cytoplasm and was not shuttled into the nuclei of AC16 cells (Figure 2C). These data indicate that the activation of ERα by E2 is on one hand a prerequisite for the interaction of ERα with NPPA and on other hand necessary for the nuclear localization/shuttling of NPPA in AC16 cells.

3.5 The LXXLL motif in NPPA is required for its E2-induced translocation into nuclei of cardiomyocytes
To test whether the LXXLL motif in NPPA plays a role in the E2-ERα-induced nuclear translocation of NPPA, AC16 cells co-transfected with pFLAG-CMV4-ERα and NPPA-WT or NPPA-MUT constructs were treated with E2 or vehicle. IF analyses using antibodies against ERα or Flag and NPPA or His demonstrated, similar to the endogenous proteins, a nuclear translocation and extensive co-localization of transiently expressed ERα and NPPA proteins in the presence of E2 (Figure 2D). However, in cells expressing ERα/Flag and NPPA-MUT/His, even in the presence of E2, we predominately observed a cytoplasmic localization with a perinuclear distribution of NPPA-MUT protein (Figure 2E). These data indicate that the LXXLL motif in NPPA protein is important for the interaction of ERα with NPPA and for the translocation of NPPA into nuclei of cardiomyocytes, since mutated NPPA is not able to be shuttled into the nucleus.
3.6 E2 enhances the NPPA promoter activity and NPPA gene expression in the presence of ERα

Next, we focused on functional aspects of this interaction. Based on our own and others results, we hypothesized that E2 induces the transcriptional activity of the NPPA promoter via ERα. To prove this hypothesis, a human NPPA promoter luciferase reporter construct (pGL2-1200-prom) was co-transfected with ERα vector into AC16 cells and subsequently treated with E2; ICI 182 780; E2 + ICI 182 780; or vehicle. E2 treatment of cells expressing both ERα and pGL2-1200-prom plasmids resulted in a significant increase in relative luciferase reporter activity (Figure 3A). Pre-treatment with ICI 182 780 significantly reversed the effect of E2, confirming that ERα is necessary for mediating the enhancing effect of E2 on NPPA promoter activity. Treatment with ICI 182 780 alone had no significant effects. Relative luciferase activities did not change in response to E2 in AC16 cells transfected with a NPPA-promoter construct alone. In further experiments, we demonstrated that the expression of NPPA at mRNA and protein levels is significantly increased in the presence of E2 (Figure 3B and C).

3.7 E2/ERα affects NPPA levels in the heart

To confirm that ERα affects the basal NPPA expression in the heart, we measured the expression of NPPA in the hearts of female ERα KO mice. They showed significantly lower amount of NPPA in comparison with female wild-type littermates (Figure 3D). Thus, the stimulatory effect of E2 on NPPA expression may have been lost in female ERαKO mice due to the lack of ERα. In agreement with our previous results, these data show that ERα is a key mediator for the E2-mediated cardiac effects on NPPA expression.

3.8 NPPA inhibits E2/ERα-induced NPPA promoter activity through LXXLL motif binding of NPPA and ERα

Since our data showed that NPPA interacts with hERα through the LXXLL motif, we hypothesized that NPPA acts as a cofactor of
Figure 2 Localization of ERα and NPPA in cardiomyocytes. (A) Non-transfected AC16 cells and (B) NNRCM were treated with vehicle or E2 (10⁻⁸ mol/L) for 24 h and used for immunofluorescence staining. Images are shown at indicated magnification with a confocal microscope. E2-treatment increases the NPPA and ERα signals in the cytoplasm and nuclei of AC16 cells in comparison with vehicle-treated cells, where they co-localize (yellow colour). (C) Inhibition of ERα activation by ICI 182 780 prevents ERα–NPPA interaction and their translocation into the nuclei of AC16 cells. Green signal: ERα (FITC); red signal: NPPA (Cy3); blue signal: DAPI. (D and E): Analysis of ERα, NPPA-WT, and NPPA-MUT localization in transfected AC16 cells by IF using antibodies against ERα or Flag (green signal) and NPPA or His (red signal). After E2 treatment, (D) NPPA-WT and ERα signals increase in the nuclei of AC16 cells, while (E) NPPA-MUT remains mainly in the perinuclear region.
ERα on its own transcriptional activity. Luciferase reporter assays in AC16 cells showed that in the presence of E2/ERα, NPPA-WT dose-dependently repressed E2/ERα-induced NPPA promoter activity (Figure 4A). These findings indicate that NPPA acts as a corepressor for ERα in the regulation of its own promoter activity in cardiomyocytes. Interestingly, co-transfection of the cells with increasing amount of NPPA-MUT did not show any changes in the E2/ERα-induced NPPA promoter activity (Figure 4B). These data confirm that the LXXLL motif in NPPA is required for the ligand-dependent ERα/NPPA interaction. Furthermore, this observation reveals that the LXXLL motif of NPPA is necessary for its action as a corepressor to inhibit the E2/ERα-induced NPPA promoter activity.

3.9 E2 increases the recruitment of ERα to the NPPA promoter

We next investigated whether ERα binds directly on the NPPA promoter to regulate NPPA gene transcription using the ChIP assay. It is known that ERα binds to a specific DNA sequence, oestrogen response element (ERE), located in the regulatory regions of NPPA.
oestrogen-responsive genes. Database analysis revealed the presence of one putative full ERE (at site –993 bp: R1) and one ERE half-site (at site –881: R2) within the 1200 bp of the NPPA promoter sequence. As shown in Figure 5, in AC16 cells exposed to E2 (10^{-8} mol/L), the binding of ERα to both ERE on the NPPA promoter was significantly increased compared with vehicle-treated cells (3.3- and 1.75-fold induction, respectively). Since ERα was not recruited neither to a region of NPPA promoter with no ERE consensus sequence (between –299 and –196: R5) nor showed an association with rabbit IgG used as negative control, the recruitment of ERα was specific to the characterized regions with ERE within the NPPA promoter. However, in cells overexpressing NPPA, anti-ERα antibody failed to detect any recruitment of ERα to the target promoters NPPA, TTF1, and cMYC (Figure 5). Collectively, these results clearly demonstrate that ERα is transiently recruited to the NPPA promoter in the presence of E2, and NPPA is a bona fide transcriptional repressor of ERα and inhibits the recruitment of E2/ERα to the promoters of multiple E2-responsive genes.

3.10 Functional significance of ERα and NPPA interaction in cardiomyocytes

Since we showed that the E2-induced nuclear ERα/NPPA interaction regulates the expression of the NPPA gene, we asked whether NPPA also affects the regulation of some other E2-responsive genes and/or M/H/F marker genes. In AC16 cells transfected with ERα vector, E2 significantly increased the expression levels of Cx43 (GJA1) and alpha actinin-2 (ACTN2), and decreased the expression of NFAT (nuclear factor of activated T-cells), type-1 collagen (Col I; COL1A1), type-3 collagen (Col III; COL3A1) (Figure 6A–F). However, co-transfection with NPPA-WT vector led to an opposite effect. Cells overexpressing NPPA-WT showed upon E2 treatment a marked reduction in the expression of Cx43 and ACTN2, and an induction of NFAT, Col I, and Col III, whereas an overexpression of NPPA-MUT did not alter the E2/ERα transcriptional activity for these genes (Figure 6A–D). However, the expression of brain natriuretic peptide, transforming growth factor beta-1, and sarcoplasmic reticulum Ca^{2+} ATPase were not affected in AC16 cells (Supplementary material online, Figure S1). In essence, these observations indicate that NPPA acts as a novel corepressor of ERα transcriptional activity for multiple E2-responsive genes in cardiomyocytes which may have a role in human cardiovascular pathophysiology.

4. Discussion

In the present study, we show a novel mechanism of E2 action in cardiomyocytes. We demonstrate for the first time that E2-activated ERα physically interacts with NPPA via its LXXLL motif in the cytoplasm and shuttles it into the nucleus, where both regulate the expression of multiple E2/ERα-target genes. E2/ERα bind to the promoter of the NPPA gene and induce its expression. In line with these data, myocardial NPPA expression is low in ERαKO mice. Next, we discovered a negative feedback loop: NPPA acts as a corepressor of ERα for its own gene regulation. E2-induced ERα–NPPA interaction reduces NPPA promoter activity dose-dependently in human cardiomyocytes by the inhibition of E2/ERα binding to an NPPA promoter. Additionally, NPPA also suppresses the transcriptional activity of E2/ERα on multiple genes, such as Cx43, ACTN2, NFAT, Col I, and Col III, which have important roles in cardiovascular physiology and pathophysiology in AC16 cells. Due to the fact that NPPA/ANP limit cardiac hypertrophy and that E2 has cardiac hypertrophy-reducing effects, which are thought to be partly mediated via increased ANP expression, we suggest that the E2-induced ERα–NPPA interaction represents a novel mechanism involved in the regulation of M/H and HF.

In the healthy heart, the expression of the NPPA gene is relatively high in both foetal atria and ventricles, but, after birth, the expression
is mostly restricted to the atria.\textsuperscript{22} However, under pathological conditions, NPPA is re-expressed and up-regulated in the adult ventricles.\textsuperscript{23–25} The human NPPA gene encodes initially for a preprohormone of 151 amino acids (aa). After cleavage of N-terminal signal sequence (25aa), the resulting pro-hormone of 126aa (pro-ANP) is stored in atrial secretory granules.\textsuperscript{26} Upon secretion, pro-ANP is processed into its N-terminal pro-ANP (1–98aa) and its biologically active ANP (99–126aa).\textsuperscript{27} During pathological MH and HF, both pro-ANP and ANP are constitutively secreted from the ventricular myocardium, which makes their plasma levels a good prognostic indicator of cardiac disease.\textsuperscript{28,29} Several studies indicate that ANP possess an anti-hypertrophic effect in the heart and on cardiomyocytes in culture.\textsuperscript{9,19,30}

\section*{4.1 The LXXLL motif in NPPA is necessary for the function of E2-induced interaction of ERα and NPPA}

The LXXLL motif is a characteristic binding site within many cofactors of nuclear receptors, as demonstrated to interact with nuclear receptors in a ligand-dependent manner.\textsuperscript{13} Our analyses reveal that the LXXLL motif in NPPA is necessary and sufficient to mediate the binding of NPPA to ERα in the presence of E2 and for its translocation into nuclei of AC16 cells. Deletion of the LXXLL motif results in substantial retention of NPPA fusion protein in the cytoplasm, even in the presence of E2 and ERα (Figure 2E). Similar results have also been reported elsewhere for the contribution of the LXXLL motif within cofactors of nuclear receptors on nuclear localization/trafficking.\textsuperscript{31,32} Calderone et al.\textsuperscript{33} also observed the translocation of NPPA into the nuclei of scar myofibroblasts. In line, Shan et al.\textsuperscript{34} showed the physical interaction of NPPA with the apoptosis signal-regulating kinase 1, a kinase involved in the pathogenesis of HF, in HeLa cells. Closer observation of presented IF images also indicates the partial localization of NPPA in the nuclei of HeLa cells. These results along with our results provide evidence for the fact that either a portion of synthesized NPPA is not transported into the storage vesicles, or there are other processes by which the pro-ANP can be translocated from the vesicles into the nuclei of the cells. The latter possibility seems more likely. Several studies showed that ERα is localized in the membrane of intracellular vesicles in hippocampal neurons, pituitary cells, and astrocytes and E2 increases the amount and the mobilization of these ERα-immunoreactive vesicles.\textsuperscript{35–37} Further studies should elucidate whether ERα is also localized on the NPPA-containing vesicles in cardiomyocytes and whether upon E2 treatment, ERα is able to mobilize these vesicles towards the nucleus.

\section*{4.2 Genomic effects of E2-induced interaction of ERα and NPPA in cardiomyocytes}

Our data show significant elevation of NPPA promoter activity and the expression of NPPA at mRNA and protein levels in response to
Figure 6 The effect of E2-induced interaction of NPPA with E2/ERα on transcription of E2-responsive genes. AC16 cells were transiently transfected with ERα or co-transfected with ERα and NPPA-WT or NPPA-MUT vectors. After 24 h, cells were exposed to E2 or vehicle for 6 or 24 h. The Cx43 mRNA expression was measured by qRT–PCR 6 h after E2 treatment (A), and the Cx43 protein level by western blotting 24 h after E2 treatment (B). The mRNA expression of NFAT (C), ACTN2 (D) 24 h after E2 treatment and those of Col I (E) and Col III (F) 6 h after E2 treatment were measured by qRT–PCR. Data represent mean ± SEM (n = 4, each in duplicate). *P ≤ 0.05.
E2/ERα. The E2-induced recruitment of ERα to the NPPA promoter indicates one of the possible mechanisms how E2-activated ERα can regulate the NPPA gene expression in cardiomyocytes. In addition, we show for the first time that NPPA acts as a corepressor of ERα, and thus controls the transcription of its own gene. Surprisingly, the repressive effects of NPPA are significantly inhibited by mutation in its LXXLL motif, indicating that the LXXLL motif is important for this interaction. We propose that the interaction of ERα with NPPA causes a conformational change of ERα, so that the binding of ERα on the NPPA promoter is prevented leading to a reduced transcriptional activity of ERα (for proposed model see Supplementary material online, Figure S2). In agreement with these findings, an increasing level of NPPA prevents the E2/ERα-induced expression of Cx43 and ACTN2, whose dysregulation plays a role in the pathogenesis of MH. Scott et al. 38 reported that the hearts of mice lacking natriuretic peptide receptor-1 (Nprf−/−) exhibit significantly increased NPPA mRNA and protein expression. Concomitantly, the expression of Cx43 mRNA and protein was down-regulated in these mice. The authors suggested that the decreased expression of Cx43 in this hypertrophy model is associated with the development of cardiac disease. These results are in accordance with our data, which show that an increased amount of NPPA has an inhibitory effect on the expression of Cx43. Additionally, an increasing level of NPPA prevents the inhibitory effect of E2/ERα on the expression of E2-target genes such as NFAT, Col I, and Col III, 39,40 whose dysregulation also plays a role in the pathogenesis of MH. However, in NPPA-MUT overexpressing cells, the expression of these genes is not at the similar levels (excluding Cx43), as they are in cells transfected only with ERα in the presence of E2. This finding confirms that the data obtained from β-galactosidase assays which imply that at least two independent regions in the NPPA protein molecule are involved in the interaction with E2/ERα. However, based on our IF and promoter analysis, we suggest that the LXXLL motif within the NPPA is most important for recognition and contributes to the stability of the complex. Taken together: the interaction of ERα/NPPA mediates the genomic action of E2 on several E2-responsive MH/HF-associated gene(s) in cardiomyocytes, and may adjust the magnitude of the transcriptional response to different physiological and pathophysiological requirements.

4.3 Summary
We have identified a novel ERα-interacting protein, i.e. NPPA, in the human heart, which represses E2-dependently ERα transcriptional activity to regulate the expression of several E2-responsive MH/HF-associated genes. We suggest that E2 and ERα interfere with mechanisms that regulate physiological and pathophysiological processes in cardiomyocytes. Nevertheless, it is conceivable that NPPA might also interact with ERβ to regulate the expression of the same or other sets of genes in cardiomyocytes, which, however, was not the focus of this study and requires further investigation. Therefore, more intense research of the cellular mechanisms in which E2 and ER are involved could substantially contribute to a better understanding of the effects of E2 and ER in the heart, and may elucidate ways to selectively enhance beneficial effects on the cardiovascular system. In this regard, NPPA could represent a therapeutic target to selectively regulate the progression of cardiovascular diseases.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: M.M.D. is the holder of the patent on AC16 cells and the other authors have declared that no competing interests exist.

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