

## Estrogen receptor alpha up-regulation and redistribution in human heart failure

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**ABSTRACT** Clinical and animal studies suggest that estrogen receptors are involved in the development of myocardial hypertrophy and heart failure. In this study, we investigated whether human myocardial estrogen receptor alpha (ER $\alpha$ ) expression, localization, and association with structural proteins was altered in end stage-failing hearts. We found a 1.8-fold increase in ER $\alpha$  mRNA and protein in end-stage human dilated cardiomyopathy (DCM,  $n=41$ ), as compared with controls ( $n=25$ ). ER $\alpha$  was visualized by confocal immunofluorescence microscopy and localized to the cytoplasm, sarcolemma, intercalated discs and nuclei of cardiomyocytes. Immunofluorescence studies demonstrated colocalization of ER $\alpha$  with  $\beta$ -catenin at the intercalated disc in control hearts and immunoprecipitation studies confirmed complex formation of both proteins. Interestingly, the ER $\alpha$ / $\beta$ -catenin colocalization was lost at the intercalated disc in DCM hearts. Thus, the ER $\alpha$ / $\beta$ -catenin colocalization in the intercalated disc may be of functional relevance and a loss of this association may play a role in the progression of heart failure. The increase of total ER $\alpha$  expression may represent a compensatory process to contribute to the stability of cardiac intercalated discs.—Mahmoodzadeh, S., Eder, S., Nordmeyer, J., Ehler, E., Huber, O., Martus, P., Weiske, J., Pregla, R., Hetzer, R., Regitz-Zagrosek, V. Estrogen receptor alpha up-regulation and redistribution in human heart failure. *FASEB J.* 20, 926–934 (2006)

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SEX DIFFERENCES in the development and progression of myocardial hypertrophy and heart failure (HF) have been described in clinical studies and animal models (1–3). Women with heart failure have a better survival rate than men, and remodeling in male and female hearts appears different (2–4). In an impressive number of transgenic mouse models of cardiomyopathies,

the cardiovascular phenotype is more severe in male animals and can be rescued by the administration of estrogen (5,6). In most models, death from HF occurs earlier in the male than in the female animals. This suggests that estrogen prevents or at least slows down the development of HF in these models.

Estrogen signals through two receptors, estrogen receptor  $\alpha$  and  $\beta$ , which belong to the nuclear hormone receptor superfamily. They are expressed in animal and human hearts (7–9) and are up-regulated in human aortic stenosis (8). In addition to its nuclear localization and its function as a transcription factor, estrogen receptor  $\alpha$  (ER $\alpha$ ) has been claimed to be present and active in the plasma membranes of endothelial cells (10) and to interact with cytoplasmic proteins such as  $\beta$ -catenin and GSK3 $\beta$  (11). Cardiovascular estrogen receptors have been associated with the prevention of apoptosis (19), with the expression of anti-hypertrophic proteins (12) and with the control of cell-cell interaction (13). However, the role of estrogen receptors in myocardial structure and function in normal and diseased human hearts is still largely unknown.

In a recent publication, we described the up-regulation of myocardial ER $\alpha$  in human aortic stenosis and its inverse correlation with calcineurin A $\beta$ , a mediator of myocardial hypertrophy (8). We now examined the intracellular localization and expression levels of ER $\alpha$  in end-stage human heart failure due to dilated cardiomyopathy (DCM). We present novel findings demonstrating a disease-dependent alteration of ER $\alpha$  localization together with an increased expression in DCM hearts.

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TABLE 1. Clinical and hemodynamic data in patients studied

Group	Sex	Age [years]	LVEF $\partial$ [100%]	LVEDD# [mm]	FS** [%]
DCM $\infty$ <i>n</i> = 41	7 female/34 male	43.7 $\pm$ 1.85	27.0 $\pm$ 3.43	73.3 $\pm$ 1.73	10.86 $\pm$ 0.87

$\infty$ DCM, dilated cardiomyopathy;  $\partial$  LVEF, left ventricular ejection fraction; #LVEDD, left ventricular enddiastolic dimension; \*\*FS, fractional shortening;  $\square$ IVS, interventricular septum;  $\Delta$ BMI, body mass index; \*\*\*ACEI, ACE inhibitors; +ARB, angiotensin receptor blocker; *n* = number of patients.

## MATERIALS AND METHODS

### Patients

Myocardial samples were obtained from 66 subjects. 25 were from control subjects (10 females aged 46.9 $\pm$ 4.82 yr and 15 males aged 43.67 $\pm$ 4.53 yr) and 41 were from patients diagnosed with dilated cardiomyopathy (7 females aged 38.12 $\pm$ 8.5 yr and 34 males aged 44.91 $\pm$ 1.56) (Table 1). All control subjects and patients were Caucasian. Patients with DCM had a severely decreased ejection fraction and increased ventricular volumes at normal wall thickness (Table 1). All patients were treated with a diuretic, 90.2% received an angiotensin 1-converting enzyme (ACE)-inhibitor or an angiotensin receptor blocker, 85.4% a beta blocker and 85.4% were treated with digitalis. In addition, histological samples from a 51-yr-old male patient with aortic stenosis (AS), described previously (8) were included. The control group was composed of tissue samples of donor hearts that were rejected for logistic reasons and had normal systolic cardiac function, no history of cardiac disease, and normal postmortem histology.

Tissue samples from patients suffering from dilated cardiomyopathy were obtained at orthotopic heart transplantation. The presence of coronary artery disease, valvular heart disease, or hypertrophic cardiomyopathy was ruled out preoperatively by cardiac catheterization and angiography, and hypertensive heart disease was ruled out by clinical history and findings. Samples from a patient with AS were obtained as described previously (8). All samples were obtained after written informed consent. The study followed the rules of the declaration of Helsinki.

Tissue samples were immediately frozen in liquid N<sub>2</sub> and used for mRNA and protein measurements and immunoprecipitation. For immunofluorescence, tissue samples were fixed in 4% formalin and embedded in paraffin.

### Real-time RT-polymerase chain reaction

RNA was extracted and reverse transcribed after treatment with DNase as described (14). A "hot start" real-time RT-polymerase chain reaction procedure was performed in duplicates with the Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) for ER $\alpha$  using highly specific, paired hybridization probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantitated using SYBR Green (Table 2A, see supplemental data).

### Western blot analysis

Left ventricular myocardial tissue samples of control and DCM hearts were analyzed for ER $\alpha$  and GAPDH protein by Western blot analysis as described before (8).

### Immunofluorescence

Human myocardial left ventricular samples were fixed in 4% formalin, embedded in paraffin and cut into 5- $\mu$ m sections and mounted on superfrost glass slides. Sections were kept at 60°C overnight, deparaffinized with xylol followed by washing in 100%, 96%, 80%, and 70% ethanol. Heat unmasking of epitopes was done by boiling the samples three times in citrate buffer of pH-6 at 600 W in the microwave. After cooling down to room temperature and 1 h of blocking in BSA, slides were incubated overnight at room temperature with primary antibodies (Table 2B, see supplemental data). After washing and blocking again, sections were incubated with secondary FITC-conjugated anti-rabbit Igs (Dianova, Barcelona, Spain) and Cy3-conjugated anti-mouse Igs (Dianova) both at a dilution of 1:50 for 2 h. Sections were mounted with VectaShield (Linaris) and viewed on a confocal microscope (Zeiss Confocal LSM510 Microscope). Intercalated disc staining for ER $\alpha$  in controls and DCM was evaluated separately by two observers analyzing 10 different areas within each sample studied. Negative controls included sections in which the primary antibodies were omitted, incubated with secondary antibodies only, (Fig. 2L, see also supplemental data, Figure B), or sections in which both primary and secondary antibodies were omitted (supplemental data, Figure A). Further negative controls included use of ER $\alpha$  primary antibody (Ab) after preincubation with its specific blocking peptide (Santa Cruz); no ER $\alpha$  signal was detectable (Fig. 2M).

### Immunoprecipitation

Cell lysates of human heart biopsies were generated by first crushing frozen samples into powder. Samples were then homogenized in 5 volumes of ice-cold buffer A (50 mM Tris pH 6.8, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 300 mM sucrose, 0.25% (v/v) Triton  $\times$ 100 and Complete Protease Inhibitor™ mix (Roche)) with 30–40 strokes in a Dounce homogenizer at 4°C. The homogenates were incubated for 30 min with benzonase (250 U) at 4°C under constant agitation, followed by centrifugation. Protein was quantified by a bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein (5 mg) were used for immunoprecipitation. After centrifugation (20,800 g, 20 min, 4°C) 1.1 ml of the supernatant was precleared by incubation with 50  $\mu$ l protein A-Sepharose for 30 min at 4°C under constant agitation. Protein A beads were subsequently removed by centrifugation (20,800 g, 10 min, 4°C), and 20  $\mu$ l of anti-ER $\alpha$  Ab (MC-20, Santa Cruz Biotechnology, Santa Cruz, CA) were added to 0.5 ml supernatant. After 30 min of incubation at 4°C, 35  $\mu$ l of a 1:1 slurry of protein A-Sepharose beads was added and incubated for 1 h. Protein A-Sepharose beads and lysate alone were used as negative controls for coprecipitation. Protein A-beads were sedimented by centrifugation (1 min, 2,700 g, 4°C), washed 5 times with buffer A and resuspended in 20  $\mu$ l of 2 $\times$  SDS

TABLE 1. (continued)

IVS□ [mm]	BMIΔ [kg/m <sup>2</sup> ]	Heart weight [g]	diuretics [%]	ACEI***/+ARB [%]	Beta blocker [%]	Digitalis [%]
9.26 ± 0.40	24.64 ± 0.74	522.79 ± 16.8	100.00	90.2	85.4	85.4

sample buffer. The proteins were separated by 7.5% SDS-PAGE, and Western blotting with monoclonal anti-β-catenin Ab was performed as described previously (15).

### Statistics

Data are presented as means ± SEM. To obtain normal distribution, mRNA expression data were logarithmically transformed and analyzed by two way ANOVA (gender, diagnosis) with interaction. Protein content was analyzed nonparametrically (Mann-Whitney *U* test). The percentage increase of mRNA and protein in DCM as compared to controls was analyzed by two-way ANOVA. The comparison of the increase between male and female subjects was performed by a test of the interaction term between gender and diagnosis.

Differences in positive ERα signal in intercalated discs were evaluated by two independent observers and were analyzed by Pearson correlation analysis. A two-tailed *P* value ≤ 0.05 was considered to indicate statistical significance. All analyses

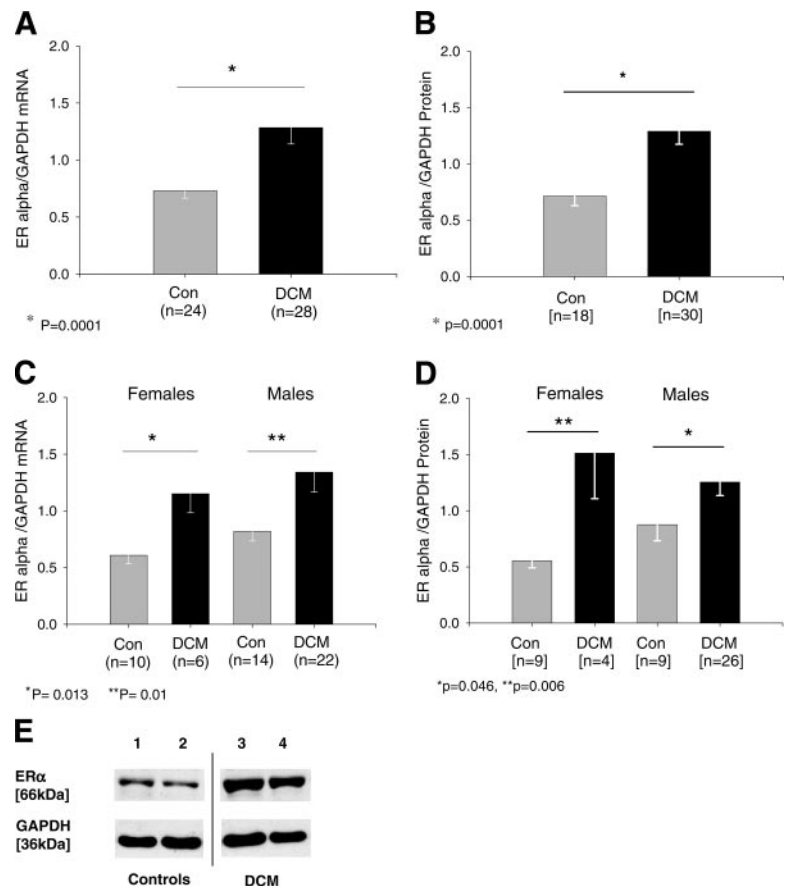
were performed using statistical Packages for the Social Sciences for Windows (release 12.0).

### RESULTS

#### Female and male patients with DCM show an increase in ERα expression

ERα expression was up-regulated in heart tissue of patients with dilated cardiomyopathy. ERα mRNA content was increased 1.8-fold (*P*=0.001) in DCM in comparison to controls (Fig. 1A). ERα protein was also increased 1.8-fold (*P*=0.0001) in DCM (Fig. 1B). ERα expression levels did not show sex differences in control (Fig. 1C–D), but due to a slightly lower basal and higher cardiomyopathic expression in females the percentage rise of ERα protein was significantly higher in

**Figure 1.** ERα up-regulation in patients with DCM. A and B) ERα mRNA and protein content were significantly increased in patients with DCM (*P*=0.001, *P*=0.0001 resp.). C, D) ERα mRNA and protein content in control hearts and their increase in patients with DCM were comparable in females and males. E) Representative Western blot analyses: 66 kDa ERα protein, detected by MC-20 Ab (Santa Cruz) was up-regulated in left ventricular myocardium of patients with DCM (3: female, 4: male) compared to controls (1: female, 2: male). ERα protein levels were normalized to GAPDH.



female DCM than in male DCM ( $P=0.013$ , interaction term of ANOVA).

No effect on ER $\alpha$  expression was seen with treatment of Diuretics, an ACE-inhibitor/angiotensin receptor blocker, beta blocker or digitalis. Our study included pre- and postmenopausal women (10 premenopausal/7 postmenopausal). We did not detect significant differences between pre- and postmenopausal women according to use or nonuse of heart failure medications. Hormone treatment or oral anticoagulation was not used in this cohort. Furthermore the control and DCM pre- and postmenopausal women did not show significant differences in their expression of ER $\alpha$  at both mRNA and protein level.

### **ER $\alpha$ localization and distribution are altered in the myocardium of patients with DCM**

ER $\alpha$  was detected by immunofluorescence microscopy at intercalated discs and adjacent to the sarcolemma of cardiac myocytes (Fig. 2A–F) and in nuclei of myocytes (Fig. 2C and Fig. 3A, see also supplemental data, Figure C) and nonmyocytes (Fig. 2G–K).

Fibroblasts and endothelial cells are both stained by vimentin in the myocardium. ER $\alpha$  was seen in nuclei of vimentin-positive cells throughout the myocardium and in vimentin-positive cells lining blood vessels, most likely the endothelial layer of blood vessels (Fig. 2G–K). The ER $\alpha$  signal never colocalized with vimentin. Larger vessels tended to show an ER $\alpha$  signal where vascular smooth muscle cells are localized (Fig. 2K). Cardiomyocytes in control hearts showed ER $\alpha$  localization in sarcolemma, cytoplasm and nucleus (Fig. 3A). In addition, in 12 out of 14 control hearts the ER $\alpha$  signal was found at the intercalated discs. In cardiomyopathic hearts ER $\alpha$  was still localized in the cytoplasm and nuclei of cardiomyocytes, but ER $\alpha$  localization in the intercalated disc was significantly decreased in the majority of DCM patients (9 out of 13,  $P<0.001$ ) (Fig. 3B,C).

Staining for ER $\alpha$  was specific based on negative controls that included either the omission of primary Ab (Fig. 2L, supplemental data Figure B), or omission of both primary and secondary antibodies (supplemental data Figure A) or the addition of a specific blocking peptide for the ER $\alpha$  Ab (Fig. 2M). Nonspecific staining was found in the perinuclear areas of myocytes (autofluorescence of fluorescent pigment lipofuscin, Fig. 2, L–M, supplemental data, Figures A and B) and in elastic fibers (data not shown).

### **Localization of ER $\alpha$ in relation to other myocardial proteins**

To identify the subcellular localizations of ER $\alpha$  in the human heart we performed colocalization studies with multiple markers against myocardial regulatory and structural proteins.

Vinculin is a myocardial structural protein that localizes to the sarcolemma and intercalated discs. We found a colocalization of the ER $\alpha$  signal with vinculin

in human control hearts, as shown by the merged images (supplemental data Figures D–F), suggesting localization of ER $\alpha$  to these structures.  $\beta$ -catenin is part of an adhesion complex in intercalated discs of cardiomyocytes. In control hearts, we observed the colocalization of ER $\alpha$  with  $\beta$ -catenin at the intercalated disc (Fig. 4A–C). Interestingly the ER $\alpha$  was no longer localized to the intercalated discs in hearts with dilated cardiomyopathy or was strongly reduced there, whereas  $\beta$ -catenin was still clearly detectable (Fig. 4D–F). Consequently, the ER $\alpha$ / $\beta$ -catenin colocalization at intercalated discs was not detected in DCM. In addition, immunofluorescence staining in myocardial biopsies of patients with aortic stenosis (AS) was consistent with our observation in patients with DCM. Figure 4, G–I showed representatively the immunofluorescence experiment in a 5- $\mu$ m paraffin section of the heart biopsy of a male AS-patient. ER $\alpha$  signal was also absent or strongly reduced in most of the intercalated discs in patients with AS. The  $\beta$ -catenin staining pattern alone was almost identical in the control hearts and in hearts of patients with dilated cardiomyopathy and aortic stenosis (Fig. 4B, E, H). To explore the specificity of the ER $\alpha$ / $\beta$ -catenin colocalization at the intercalated disc, we stained for other intercalated disc proteins like the gap junction protein connexin 43 (Cx43). We did not observe a colocalization of ER $\alpha$  with Cx43 (Fig. 5A–C).

Having detected a striated pattern of the ER $\alpha$  signal, alternating with troponin T (Fig. 3A), we further investigated whether ER $\alpha$  was associated with myofibrillar contractile structures. Costaining for myosin heavy chain and ER $\alpha$ , however, did not reveal any colocalization (data not shown). ER $\alpha$  has also been discussed to be present at the caveolae of plasma membranes of myocytes (16, 17). In double-staining experiments for ER $\alpha$  and caveolin-3 as a marker for caveolae in human hearts, we could not show a colocalization signal that supported this finding (Fig. 2D–F).

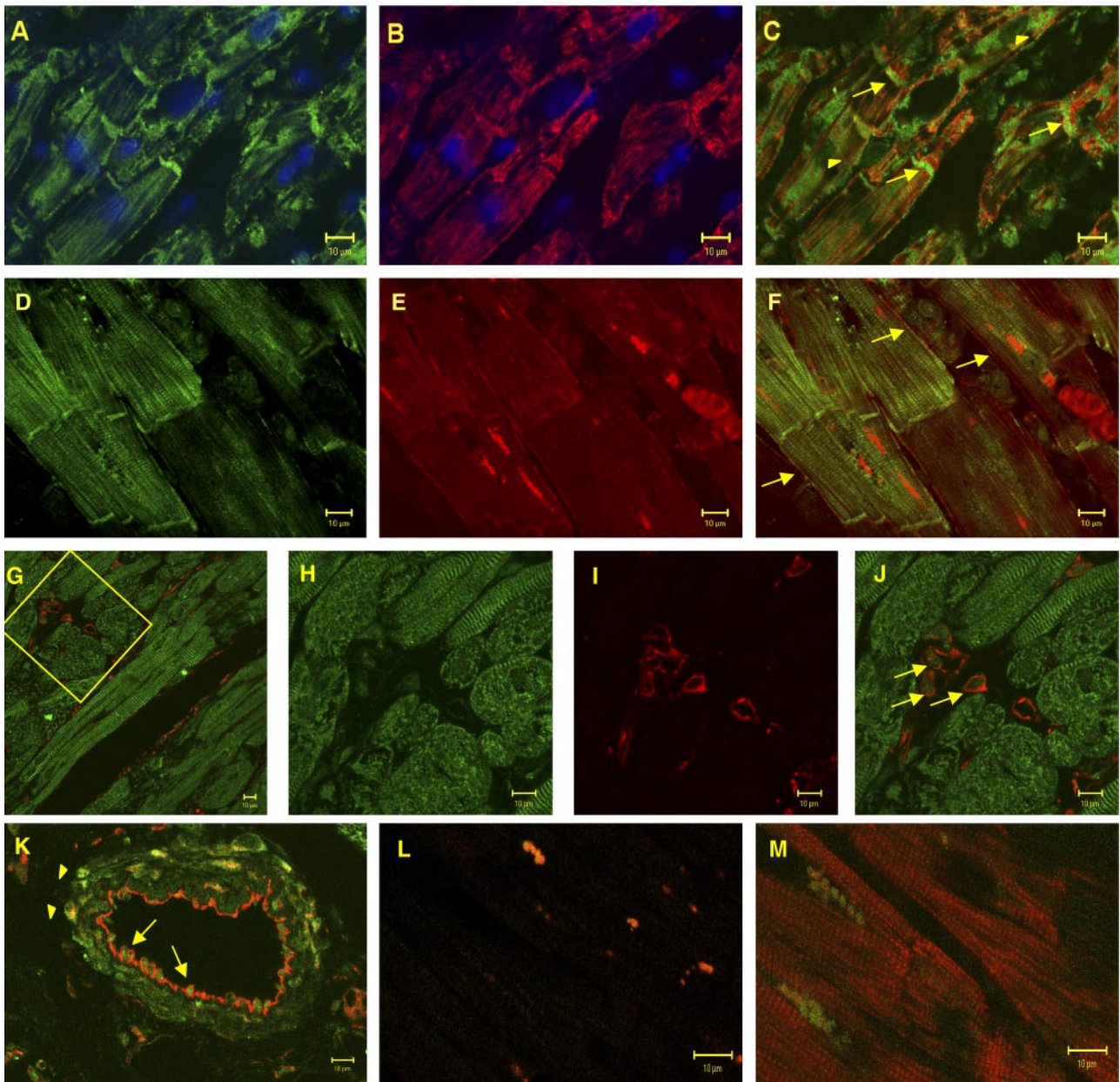
### **ER $\alpha$ and $\beta$ -catenin interact in the human heart**

On the basis of our observations that ER $\alpha$  and  $\beta$ -catenin colocalized at intercalated discs in human heart samples, we next examined whether an interaction of ER $\alpha$  and  $\beta$ -catenin could be found. Coimmunoprecipitation experiments with anti-ER $\alpha$  Ab were performed with lysates prepared from human control hearts. Indeed,  $\beta$ -catenin was detectable in immunoprecipitates of anti-ER $\alpha$  Ab, indicating that the two proteins formed a complex in vivo (Fig. 6, lane 1). In control experiments without the Ab no binding of  $\beta$ -catenin was found (Fig. 6, lane 2).

## **DISCUSSION**

In this study, we show for the first time that the myocardial ER $\alpha$  expression and distribution pattern are significantly altered in human DCM. We found an up-regulation of ER $\alpha$  mRNA in human dilated cardio-



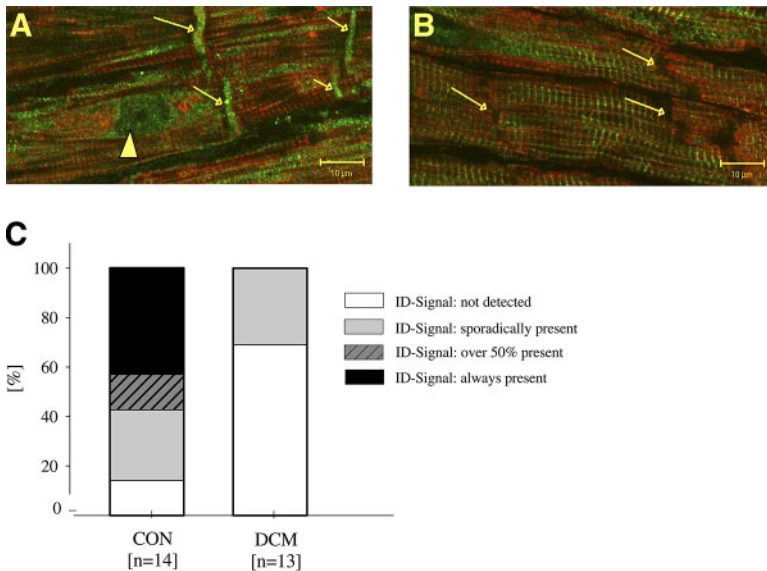


**Figure 2.** A–M) Detection of ER $\alpha$  in 5- $\mu$ m paraffin sections of the left ventricle of the control human hearts by immunofluorescence staining and confocal laser-scanning microscopy. A–C) immunofluorescent costaining for ER $\alpha$ , troponin T and 4',6'-diamidino-2-phenylidole (DAPI) in a female control heart. The blue staining represents DAPI-stained nuclei. A) ER $\alpha$  (FITC Green), B) troponin T (Cy3-red), C) merged image of A and B; arrows show the localization of ER $\alpha$  in the intercalated discs, and triangles indicate the localization of ER $\alpha$  in some nuclei of myocytes. In this image, for better illustration of the localization of ER $\alpha$  in the nuclei, we switched off the blue channel (DAPI). D–F) immunofluorescent costaining for ER $\alpha$  and caveolin-3 in a male control heart. D) ER $\alpha$  (FITC Green), E) Caveolin-3 (Cy3-red), F) the merged image of D and E; arrows show the localization of ER $\alpha$  at the sarcolemma. G illustrates ER $\alpha$  (FITC Green) and vimentin (Cy3-red) in a female control heart. The rectangular area represents the localization of ER $\alpha$  in the fibroblasts. H–I) Higher-magnification images of the rectangle area in (2G). H) ER $\alpha$  (FITC Green), I, Vimentin (Cy3-red). J) Merged image of H and I. K) Costaining of ER $\alpha$  (FITC Green) and vimentin (Cy3-red) in a vessel of a male control heart. Arrows indicate the ER $\alpha$  positive endothelial cells, and arrowheads the ER $\alpha$ -positive smooth muscle cells; L and M are negative controls. L) Myocardial section treated only with secondary antibodies after omitting primary antibodies. Autofluorescence (lipofuscin) in red. M) myocardial section costained with antibodies directed against troponin T (Cy3-red) and ER $\alpha$  (FITC Green) incubated with its blocking peptide before use. No nonspecific binding of primary or secondary Ab was detected for ER $\alpha$ . All scale bars represent 10  $\mu$ m.

myopathy, which led to elevated receptor expression in female and male patients. In myocytes from control, but not from DCM hearts, ER $\alpha$  was localized to the intercalated disc and colocalized with  $\beta$ -catenin. In DCM hearts, the localization of ER $\alpha$  to the intercalated

disc and colocalization with  $\beta$ -catenin was lost. Colocalization with ER $\alpha$  was also found for the sarcolemmal marker vinculin, but not for caveolin, troponin T, or myosin heavy chain.

In addition to their role as transcription factors,



**Figure 3.** ER $\alpha$  distribution patterns differ in hearts of patients with DCM compared with the control hearts. A) Section of the left ventricle of a control male human heart and a section of the left ventricle of the heart of a male patient with DCM (B), which are costained for ER $\alpha$  (FITC Green), and troponin T (Cy3-red). Arrows show the position of the intercalated discs, and the triangle shows the localization of ER $\alpha$  in the nucleus of a myocyte. All scale bars represent 10  $\mu$ m. C) Different distribution patterns of ER $\alpha$  signal in intercalated discs (ID) of controls and DCM patients. In DCM patients, the ER $\alpha$  signal is significantly decreased ( $P < 0.001$ ).

myocardial ER also interact with cytoplasmic proteins and activate signaling pathways (18, 19). Most of their myocardial effects, however, are still unknown. The increase in ER $\alpha$  expression in cardiomyopathic hearts found here is similar to the changes we found in human aortic stenosis (i.e., in a purely hypertrophic state) (8). In both diseases, ER $\alpha$  was expressed at a comparable concentration in women and men and the increase in ER $\alpha$  expression in heart failure occurred in both sexes, but may have been more pronounced in females. Given that estrogens reduce cardiomyocyte apoptosis by ER-dependent mechanisms (19), up-regulation of ER $\alpha$  in failing left ventricles of patients with DCM may represent a compensatory mechanism.

The subcellular distribution of ER in human hearts may offer a clue for understanding its function. Our immunofluorescence experiments showed that in cardiomyocytes of control hearts, ER $\alpha$  was localized to intercalated discs, adjacent to the sarcolemma, and in some, but not all nuclei. Furthermore, a positive ER $\alpha$  signal was found in nuclei of fibroblasts, endothelial cells, and smooth muscle cells. The absence of the ER $\alpha$  signal in some nuclei of cardiomyocytes could be explained with the differential state of the cells and regulatory signals. It is known that nuclear receptors shuttle between the cytoplasm and the nucleus in the absence of ligands. On binding to the ligand or to specific proteins, they translocate to the nucleus where, acting as a transcription factor, they initiate transcription (20). Grohe et al. (21) have already shown the translocation of ER $\alpha$  from the cytoplasm to the nucleus after stimulation with estrogen. Moreover, it has been suggested that the posttranslational modification of the nuclear receptor itself or its coregulatory proteins influence cellular localization of the receptor in the cells (22). These findings may explain in part the differential ER $\alpha$  signal in some nuclei of the cardiomyocytes.

Membrane association of ER $\alpha$  has been suggested before and is supported by the colocalization with

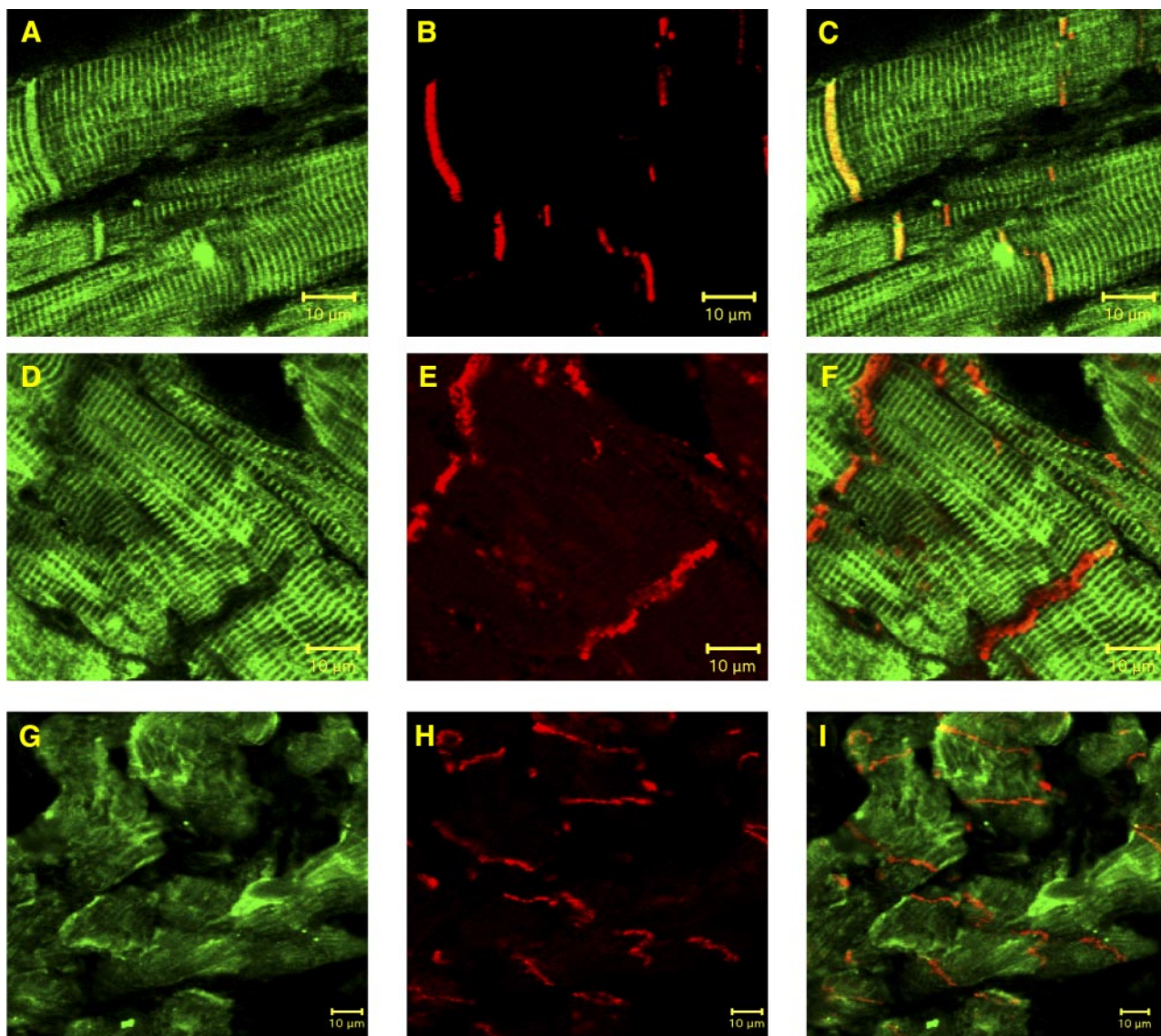
vinculin, a sarcolemmal marker, that we describe here (23). However, the lack of a colocalization with the caveolar marker, caveolin, does not support the tempting hypothesis of an interaction with caveolar proteins such as eNOS. Because colocalization with eNOS was not tested separately, we cannot exclude such an interaction.

In cultured neonatal rat cardiomyocytes, previous studies have shown that ER $\alpha$  exhibited a striated pattern (24). In our study, we found an alternating pattern with troponin T and confirmed the striated pattern of ER $\alpha$  expression. The significance of this ER $\alpha$  localization has yet to be determined.

Intercalated discs are essential sites for normal propagation of electrical stimuli in cardiac myocytes, and they undergo significant changes in failing hearts. Cardiac remodelling in heart failure also affects intercalated disc structure (25). The components of intercalated discs include gap junctions, desmosomes, and adherens junctions. Gap junctions regulate electrical coupling, while desmosomes and adherens junctions integrate intermediate filaments and anchor myofibrils to the sarcolemma. They provide critical end-to-end linkage between adjacent cells (26), which cooperatively maintain normal cell-cell interactions between myocytes.

Gap junctions in the adult myocardium consist of a large extent of connexin 43 (Cx43), which is mainly localized at the intercalated discs (27). In cardiomyocytes of normal myocardium Cx43 is mainly present in its phosphorylated state. After myocardial injury, Cx43 is dephosphorylated and translocated from gap junctions into the cytoplasm (28). In rat cardiomyocytes, estrogen acting via estrogen receptors increased the phosphorylation state of Cx43 at the gap junction (29). This could be a major function of ER in the intercalated disc. Because in our study, Cx43 and ER $\alpha$  are both lost from the intercalated disc in DCM (Fig. 5A-F), it may be speculated that ER $\alpha$  contributes to the phosphorylation of Cx43 in normal human hearts as in rat





**Figure 4.** Colocalization studies of ER $\alpha$  with  $\beta$ -catenin in control, DCM and AS hearts. *A–C*) Same section of a control female heart stained for ER $\alpha$  (FITC Green) (*A*) and  $\beta$ -catenin (Cy3-red) (*B*). *C*) merged image of *A* and *B*. *D–F*) same section of a DCM male heart stained for ER $\alpha$  (FITC Green) (*D*) and  $\beta$ -catenin (Cy3-red) (*E*). *F*) Merged image of *D* and *E*. *G–I*) Same section of the heart of a male AS patient stained for ER $\alpha$  (FITC Green) (*G*) and  $\beta$ -catenin (Cy3-red) (*H*). *I*) Merged image of *G* and *H*. All scale bars represent 10  $\mu$ m.

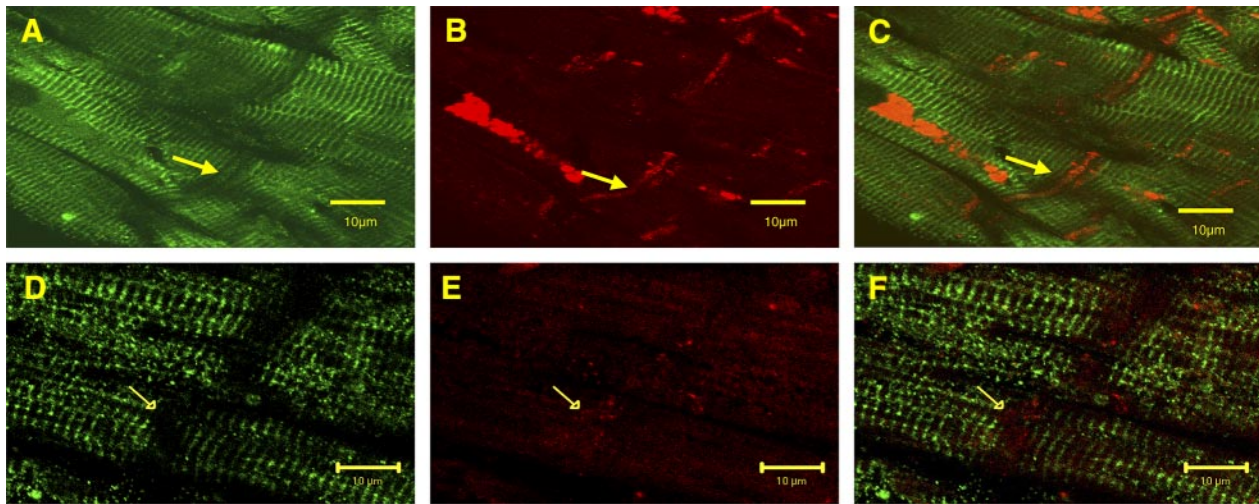
myocytes, and this function is lost in DCM. The fact that no colocalization of ER $\alpha$  and Cx43 was detected by immunofluorescence does not exclude such an interaction if other proteins were involved.

$\beta$ -catenin is a membrane-associated protein located predominantly at the intercalated disc.  $\beta$ -catenin is involved in the formation of gap junctions (30) and in rat cardiomyocytes, it has been shown that an association of  $\beta$ -catenin,  $\alpha$ -catenin, ZO-1 (Zonula Occludens-1 protein) and Cx43 is required for the development of gap junctions (30).

$\beta$ -catenin also plays an important role in the regulation of cell-cell adhesion, as well as in intracellular signaling (11, 31). Our immunofluorescence studies demonstrated colocalization of ER $\alpha$  and  $\beta$ -catenin at the intercalated disc in normal human hearts, which was lost in DCM due to the loss of ER $\alpha$  from the

intercalated discs. Additional immunofluorescence experiments provided further evidence that ER $\alpha$  was not detectable or strongly reduced at the intercalated discs in the hearts of patients with AS. This is in agreement with the fact that the up-regulation of ER $\alpha$  is also similar in AS and DCM (8). Thus, loss of ER $\alpha$ / $\beta$ -catenin interaction at the intercalated disc and up-regulation of ER $\alpha$  may represent molecular features of myocardial hypertrophy and heart failure.

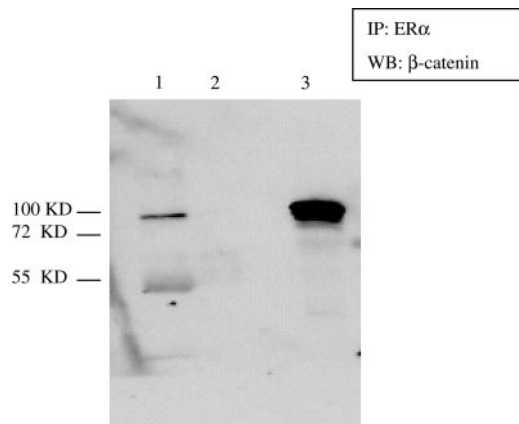
Although the disruption of the ER $\alpha$ / $\beta$ -catenin interaction in failing myocytes is a novel and intriguing finding, the significance of the loss of this association at intercalated discs remains to be determined, and a potential indirect interaction with connexin 43 phosphorylation and function should be studied. Groten et al. (13) showed that in endothelial cells E2 transiently disconnects the adherens junction complex from the



**Figure 5.** A–F) Detection of ER $\alpha$  and connexin 43 in 5- $\mu$ m paraffin sections of the left ventricle of human heart by immunofluorescence staining and confocal laser-scanning microscopy. A–C) Same section of a control male heart. D–F) Same section of the heart of a female patient with DCM, stained for ER $\alpha$  (FITC Green) (A, D); connexin 43 (Cy3-red) (B, E). C, F) Merged images of ER $\alpha$  (FITC Green) and connexin 43 in control and DCM hearts, respectively. Arrows indicate intercalated discs. All scale bars represent 10  $\mu$ m.

cytoskeleton in an ER-dependent manner, suggesting that ER are involved in cell-cell interactions.

Our IP data in healthy human hearts indicate that an *in vivo* complex and interaction between  $\beta$ -Catenin and ER $\alpha$  proteins exists. An interaction between ER $\alpha$  and  $\beta$ -catenin has been reported in several other cell types,



**Figure 6.** Immunoprecipitation analysis of ER $\alpha$ /  $\beta$ -catenin interactions in the human heart. Lysates from human control heart were incubated with an anti-ER $\alpha$  Ab (lane 1), and subsequently, protein complexes precipitated with protein A-Sepharose beads. Protein A-Sepharose beads and lysate alone were used as negative controls for coprecipitation (lane 2). The immunocomplexes were subjected to immunoblotting as described in Material and Methods and incubated with the anti- $\beta$ -catenin monoclonal antibody to assess the interaction of ER $\alpha$  with  $\beta$ -catenin. Lysates containing neither Ab nor protein A-Sepharose were used as a positive control (lane 3) to indicate the specificity of the  $\beta$ -catenin Ab and the presence of the  $\beta$ -catenin protein in lysates. The anti- $\beta$ -catenin Ab recognizes  $\beta$ -catenin as a single band at the expected MW of 92 kDa in the whole cell lysates (lanes 1 and 3). This figure is the representative result of three separate experiments, yielding similar results.

such as human colon cancer cells (32) and rat hippocampus (11), where ER $\alpha$ ,  $\beta$ -catenin, and GSK3 $\beta$  interacted.

Thus we speculate that there is a multimolecular complex in human cardiac myocytes, with ER $\alpha$ ,  $\beta$ -catenin, and Cx43, which may regulate cell-to-cell adhesion and the structure of the junctions at the intercalated discs. The increase of ER $\alpha$  expression in patients with DCM may indicate a compensatory process to contribute to the stability of intercalated discs.

Working with human heart samples is always limited by the fact that not all the desired samples are available in sufficient quantity to do all the desirable studies. The effect of sexual hormones could not be assessed in detail, as not enough samples from pre- and postmenopausal women with and without hormone treatment were available.

In summary, our work showed that expression and localization of ER $\alpha$  are altered in heart failure. The colocalization of ER $\alpha$  with  $\beta$ -catenin in the healthy human heart at the intercalated disc and its loss from the intercalated disc in parallel with Cx43 in the failing human heart may suggest that ER $\alpha$  contributes to intercalated discs' stability and the ER $\alpha$  up-regulation in hypertrophy and heart failure takes place to compensate for this loss of function. FJ

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## REFERENCES

- CIBISII (1999) The cardiac insufficiency bisoprolol study II (CIBIS-II): a randomised trial. *Lancet* **353**, 9–13
- Levy, D., Kenchaiah, S., Larson, M. G., Benjamin, E. J., Kupka, M. J., Ho, K. K., Murabito, J. M., and Vasan, R. S. (2002) Long-term trends in the incidence of and survival with heart failure. *N. Engl. J. Med.* **347**, 1397–1402
- Guerra, S., Leri, A., Wang, X., Finato, N., Di Loreto, C., Beltrami, C. A., Kajstura, J., and Anversa, P. (1999) Myocyte death in the failing human heart is gender dependent. *Circ. Res.* **85**, 856–866
- Carroll, J. D., Carroll, E. P., Feldman, T., Ward, D. M., Lang, R. M., McGaughey, D., and Karp, R. B. (1992) Sex-associated differences in left ventricular function in aortic stenosis of the elderly. *Circulation* **86**, 1099–1107
- Du, X. J. (2004) Gender modulates cardiac phenotype development in genetically modified mice. *Cardiovasc. Res.* **63**, 510–519
- Leinwand, L. A. (2003) Sex is a potent modifier of the cardiovascular system. *J. Clin. Invest.* **112**, 302–307
- Mendelsohn, M. E., and Karas, R. H. (1999) The protective effects of estrogen on the cardiovascular system. *N. Engl. J. Med.* **340**, 1801–1811
- Nordmeyer, J., Eder, S., Mahmoodzadeh, S., Martus, P., Fielitz, J., Bass, J., Bethke, N., Zurbrugg, H. R., Pregla, R., Hetzer, R., and Regitz-Zagrosek, V. (2004) Upregulation of myocardial estrogen receptors in human aortic stenosis. *Circulation* **110**, 3270–3275
- Grohe, C., Kahlert, S., Lobbart, K., and Vetter, H. (1998) Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis. *J. Endocrinol.* **156**, R1–7
- Razandi, M., Pedram, A., Merchanthaler, I., Greene, G. L., and Levin, E. R. (2004) Plasma membrane estrogen receptors exist and functions as dimers. *Mol. Endocrinol.* **18**, 2854–2865
- Cardona-Gomez, P., Perez, M., Avila, J., Garcia-Segura, L. M., and Wandosell, F. (2004) Estradiol inhibits GSK3 and regulates interaction of estrogen receptors, GSK3, and beta-catenin in the hippocampus. *Mol. Cell Neurosci.* **25**, 363–373
- Babiker, F. A., De Windt, L. J., van Eickels, M., Thijssen, V., Bronsauer, R. J. P., Grohe, C., van Bilsen, M., and Doevendans, P. A. (2004) 17beta-Estradiol antagonizes cardiomyocyte hypertrophy by autocrine/paracrine stimulation of a guanylyl cyclase A receptor-cyclic guanosine monophosphate-dependent protein kinase pathway. *Circulation* **109**, 269–276
- Groten, T., Pierce, A. A., Huen, A. C., and Schnaper, H. W. (2005) 17 beta-estradiol transiently disrupts adherens junctions in endothelial cells. *FASEB J.* **19**, 1368–1370
- Fielitz, J., Hein, S., Mitrovic, V., Pregla, R., Zurbrugg, H. R., Warnecke, C., Schaper, J., Fleck, E., and Regitz-Zagrosek, V. (2001) Activation of the cardiac renin-angiotensin system and increased myocardial collagen expression in human aortic valve disease. *J. Am. Coll. Cardiol.* **37**, 1443–1449
- Weiske, J., Schoneberg, T., Schroder, W., Hatzfeld, M., Tauber, R., and Huber, O. (2001) The fate of desmosomal proteins in apoptotic cells. *J. Biol. Chem.* **276**, 41,175–41,181
- Chambliss, K. L., Yuhanna, I. S., Mineo, C., Liu, P., German, Z., Sherman, T. S., Mendelsohn, M. E., Anderson, R. G., and Shaul, P. W. (2000) Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ. Res.* **87**, E44–E52
- Lu, Q., Pallas, D. C., Surks, H. K., Baur, W. E., Mendelsohn, M. E., and Karas, R. H. (2004) Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 17,126–17,131
- Arnold, S. F., Melamed, M., Vorobjikina, D. P., Notides, A. C., and Sasson, S. (1997) Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. *Mol. Endocrinol.* **11**, 48–53
- Patten, R. D., Pourati, I., Aronovitz, M. J., Baur, J., Celestin, F., Chen, X., Michael, A., Haq, S., Nuedling, S., Grohe, C., Force, T., Mendelsohn, M. E., and Karas, R. H. (2004) 17beta-estradiol reduces cardiomyocyte apoptosis in vivo and in vitro via activation of phospho-inositide-3 kinase/Akt signaling. *Circ. Res.* **95**, 692–699
- Nadal, A., Diaz, M., and Valverde, M. A. (2001) The estrogen trinity: membrane, cytosolic, and nuclear effects. *News Physiol. Sci.* **16**, 251–255
- Grohe, C., Kahlert, S., Lobbart, K., Stimpel, M., Karas, R. H., Vetter, H., and Neyses, L. (1997) Cardiac myocytes and fibroblasts contain functional estrogen receptors. *FEBS Lett.* **416**, 107–112
- Perissi, V., and Rosenfeld, M. G. (2005) Controlling nuclear receptors: the circular logic of cofactor cycles. *Nat Rev Mol. Cell Biol.* **6**, 542–554
- Zhang, Z., Maier, B., Santen, R. J., and Song, R. X. (2002) Membrane association of estrogen receptor alpha mediates estrogen effect on MAPK activation. *Biochem. Biophys. Res. Commun.* **294**, 926–933
- Nuedling, S., Kahlert, S., Lobbart, K., Doevendans, P. A., Meyer, R., Vetter, H., and Grohe, C. (1999) 17 Beta-estradiol stimulates expression of endothelial and inducible NO synthase in rat myocardium in-vitro and in-vivo. *Cardiovasc. Res.* **43**, 666–674
- Perriard, J. C., Hirschy, A., and Ehler, E. (2003) Dilated cardiomyopathy: a disease of the intercalated disc? *Trends Cardiovasc. Med.* **13**, 30–38
- Ehler, E., Horowitz, R., Zuppinger, C., Price, R. L., Perriard, E., Leu, M., Caroni, P., Sussman, M., Eppenberger, H. M., and Perriard, J. C. (2001) Alterations at the intercalated disk associated with the absence of muscle LIM protein. *J. Cell Biol.* **153**, 763–772
- Beardslee, M. A., Laing, J. G., Beyer, E. C., and Saffitz, J. E. (1998) Rapid turnover of connexin43 in the adult rat heart. *Circ. Res.* **83**, 629–635
- Beardslee, M. A., Lerner, D. L., Tadros, P. N., Laing, J. G., Beyer, E. C., Yamada, K. A., Kleber, A. G., Schuessler, R. B., and Saffitz, J. E. (2000) Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. *Circ. Res.* **87**, 656–662
- Chung, T. H., Wang, S. M., and Wu, J. C. (2004) 17beta-estradiol reduces the effect of metabolic inhibition on gap junction intercellular communication in rat cardiomyocytes via the estrogen receptor. *J. Mol. Cell Cardiol.* **37**, 1013–1022
- Wu, J. C., Tsai, R. Y., and Chung, T. H. (2003) Role of catenins in the development of gap junctions in rat cardiomyocytes. *J. Cell. Biochem.* **88**, 823–835
- Hertig, C. M., Eppenberger-Eberhardt, M., Koch, S., and Eppenberger, H. M. (1996) N-cadherin in adult rat cardiomyocytes in culture. I. Functional role of N-cadherin and impairment of cell-cell contact by a truncated N-cadherin mutant. *J. Cell Sci.* **109** (Pt 1), 1–10
- Kouzmenko, A. P., Takeyama, K., Ito, S., Furutani, T., Sawatsubashi, S., Maki, A., Suzuki, E., Kawasaki, Y., Akiyama, T., Tabata, T., and Kato, S. (2004) Wnt/beta-catenin and estrogen signaling converge in vivo. *J. Biol. Chem.* **279**, 40,255–40,258

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