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## Accepted Manuscript

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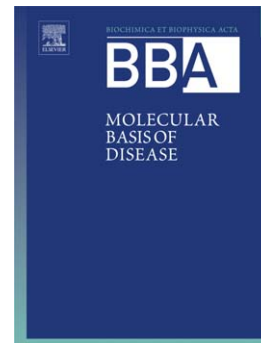
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1 **Altered expression of FHL1, CARP, TSC-22 and P311 provide insights into**  
2 **complex transcriptional regulation in pacing-induced atrial fibrillation**

3

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21 **Abstract**

22 Atrial fibrillation (AF) is the most common progressive disease in patients with  
23 cardiac arrhythmia. AF is accompanied by complex atrial remodeling and changes in  
24 gene expression, but only a limited number of transcriptional regulators have been  
25 identified. Using a low-density cDNA array, we identified 31 genes involved in  
26 transcriptional regulation, signal transduction or structural components, which were  
27 either significantly upregulated or downregulated in porcine atria with fibrillation  
28 (induced by rapid atrial pacing at a rate of 400-600 bpm for 4 weeks that was then  
29 maintained without pacing for 2 weeks). The genes for four and a half LIM domains  
30 protein-1 (*FHL1*), transforming growth factor- $\beta$  (TGF- $\beta$ )-stimulated clone 22 (*TSC-22*),  
31 and cardiac ankyrin repeat protein (*CARP*) were significantly upregulated, and  
32 chromosome 5 open reading frame gene 13 (*P311*) was downregulated in the fibrillating  
33 atria. *FHL1* and *CARP* play important regulatory roles in cardiac remodeling by  
34 transcriptional regulation and myofilament assembly. Induced mRNA expression of  
35 both *FHL1* and *CARP* was also observed when cardiac H9c2 cells were treated with an  
36 adrenergic agonist. Increasing *TSC-22* and marked *P311* deficiency could enhance the  
37 activity of TGF- $\beta$  signaling and the upregulated *TGF- $\beta$ 1* and *- $\beta$ 2* expressions were  
38 identified in the fibrillating atria. These results implicate that observed alterations of  
39 underlying molecular events were involved in the rapid-pacing induced AF, possibly via  
40 activation of the  $\beta$ -adrenergic and TGF- $\beta$  signaling.

41

42 *Keywords:* Atrial fibrillation; Low-density cDNA array; Transcriptional regulator;  
43 Transforming growth factor- $\beta$  signaling.

## 44 1. Introduction

45 Atrial fibrillation (AF), the most common sustained arrhythmia, causes  
46 progressive alterations in atrial electrical, contractile and structural properties, which are  
47 associated with changes in cardiac gene expression [1,2]. Recent studies have focused  
48 on the molecular basis of atrial remodeling using candidate gene and genome-wide  
49 approaches in AF patients [3-5]. Gene expression profiling studies on dedifferentiation  
50 [3], apoptosis [5], fibrosis and thromboembolic events [4] have highlighted the complex  
51 regulation of gene expression during AF formation and maintenance. However, thus  
52 far, transcriptional regulators involved in regulating transcription in AF have been  
53 proposed [6], but attempts to identify the regulators have been lacking. Activation of  
54 transcriptional regulators during AF may coordinately lead to sequential transcriptional  
55 control events that regulate the phenotype of cardiomyocytes in response to AF disease  
56 conditions [6]. In fact, AF is often associated with other cardiovascular diseases such  
57 as hypertension, thyrotoxic heart disease, coronary artery disease, rheumatic valve  
58 disease, and heart failure [7]. Moreover, changes in gene expression in clinical AF may  
59 be the result of AF combined with other underlying heart diseases [4]. Therefore,  
60 functional studies of transcriptional regulators in rapid pacing-induced AF or transgenic  
61 animal AF models [8] may clarify the underlying molecular mechanisms of AF because  
62 the potential confounding effects of other underlying heart diseases have been  
63 eliminated.

64 In the present study, we investigated 84 candidate genes specifically associated  
65 with fibrillating atria of a porcine AF induced by rapid atrial pacing using a low-density  
66 cDNA array. Three genes, four and a half LIM domains protein-1 (*FHL1*), transforming

67 growth factor beta-stimulated clone 22 (*TSC-22*) and cardiac ankyrin repeat protein  
68 (*CARP*), encoding transcriptional regulators, were significantly upregulated, and  
69 chromosome 5 open reading frame gene 13 (*P311*), an anti-fibrotic gene, was  
70 downregulated in the fibrillating atria. Along with confirmation of the selected genes  
71 for differential expression profiles, the further identification of protein expression and  
72 implicated mechanism in the fibrillating atria were subsequently evaluated. The results  
73 provide more insights into the underlying molecular events in pacing-induced AF and  
74 evidence of complex transcriptome changes that may accompany AF development.

75

## 76 **2. Materials and methods**

### 77 *2.1. AF induced by rapid atrial pacing*

78 A porcine model of AF was used as described previously [9]. Eighteen adult  
79 Yorkshire-Landrace strain pigs were used (12 in the AF group and 6 in the sham control  
80 group), with mean body weight  $62 \pm 5$  kg. In this study, six pigs in the AF group were  
81 added besides the specimens of 6 AF and 6 sham control that were sampled in our  
82 previous study [10]. The experimental protocol conformed to the Guide for the Care  
83 and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and was  
84 approved by the Institutional Animal Care and Use Committee of the National Taiwan  
85 University College of Medicine. All pigs were provided by the Animal Technology  
86 Institute in Taiwan (ATIT) and housed at the animal facility in the ATIT. Each animal  
87 was transvenously implanted with either a high-speed atrial pacemaker (Itrel-III;  
88 Medtronic Inc., Minneapolis, MN) for the AF group or an inactive pacemaker for the  
89 sham control group (i.e., sham hearts maintained normal sinus rhythm, SR). The atrial

90 pacing lead (Medtronic) was inserted through the jugular vein and screwed to the right  
91 atrium. The atrial high-speed pacemaker was programmed to a rate of 400-600 beats  
92 per min for 4 weeks in the AF group. After continuous pacing, the atrial pacemaker was  
93 turned off and the animals remained in AF. The animals were sacrificed 2 weeks after  
94 the pacemaker was turned off, and thus the total duration of atrial depolarization was 6  
95 weeks. In the sham control group, the pacemaker remained off for the entire 6 weeks  
96 after implantation.

### 97 *2.2. Tissue processing*

98 The pigs were anaesthetized and sacrificed at the end of the experimental period.  
99 The right atrial appendages (RAA) and left atrial appendages (LAA) were excised and  
100 immediately frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until use for RNA or  
101 protein extraction for later experiments.

### 102 *2.3. RNA isolation*

103 Total RNA was extracted and quantified from the pig LAA and RAA according to  
104 our previous report [11]. In brief, 200 mg of atrial tissue was homogenized on ice by a  
105 rotor-stator-type tissue homogenizer in 1 ml TRIzol reagent (GIBCO BRL,  
106 Gaithersburg, MD). Cellular debris was removed by centrifugation for 10 min at  
107  $12,000 \times g$  at  $4^{\circ}\text{C}$ , and RNA was precipitated by adding equal volumes of isopropanol  
108 and then washing with 75% ethanol. The resultant RNA was further purified with the  
109 RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.  
110 The amount of total RNA was determined spectrophotometrically at 260 nm, and the  
111 integrity was confirmed by analysis on a denaturing agarose gel. The RNA was used in

112 the cDNA microarray and quantitative real-time RT-PCR analysis.

#### 113 *2.4. Specialized AF Chip design and preparation*

114 A total of 84 gene sequences were selected for a low-density cDNA array, named  
115 the AF Chip. Selection of these genes was based on the following three considerations:  
116 the gene had been reported to be associated with a cardiomyopathy [12], the gene was  
117 significantly and differentially expressed in fibrillating tissue of a rapid pacing-induced  
118 AF model in our previous report on a microarray containing 6,032 human cDNA clones  
119 (UniversoChip, AsiaBioinnovations, Newark, CA) [10], and the gene clone was  
120 available from the IMAGE consortium (Open Biosystems, Huntsville, AL). All  
121 symbols and accession numbers of the selected genes used in the AF Chip are shown in  
122 Fig. 1. Additionally, two control cDNAs, glyceraldehyde-3-phosphate dehydrogenase  
123 (*GAPDH*) and  $\beta$ -tubulin, as well as pUC19, were included in the chip. The selected  
124 clones were purchased from IMAGE consortium, and the sequences were verified in our  
125 laboratory. The clones were amplified using PCR with 36 cycles of a denaturing  
126 temperature of 95°C for 30 s, annealing temperature of 55°C for 30 s, and extension  
127 temperature of 72°C for 45 s. The commercial primers for amplification were as  
128 follows: T7 primer (5'-TAA TAC GAC TCA CTA T AG GG-3'), Sp6 primer (5'-CAT  
129 ACG ATT TAG GTG ACA CTA TAG-3'), T3 primer (5'-AAT TA A CCC TCA CTA  
130 AAG-3'), M13 forward primer (5'-GTA AAA CGA CGG CCA G-3'), and M13 reverse  
131 primer (5'-CAG GAA ACA GCT ATG AC-3') (Invitrogen, Carlsbad, CA). After  
132 amplification, the quality and specificity of the PCR products were confirmed by  
133 agarose gel electrophoresis.

134 PCR-amplified DNA products were mixed with dimethyl sulfoxide (1:1, v/v) and



135 then spotted onto amino-coated glass slides (TaKaRa Mirus Bio Inc., Madison, WI)  
136 using a robotics SpotArray 24 (PerkinElmer Life Sciences, Boston, MA). To evaluate  
137 the reliability of the AF Chip, PCR-amplified DNA products were spotted in duplicate  
138 onto a slide. Spotted DNA was crosslinked and denatured according to the  
139 manufacturer's instructions (Takara Mirus Bio Inc., <http://www.takaramirusbio.com>).

#### 140 *2.5. Hybridization and imaging of fluorescently labeled cDNA*

141 Twenty  $\mu\text{g}$  of total RNA extracted from the SR and AF subjects was reverse-  
142 transcribed with an oligo-dT primer to prepare fluorophore-labeled SR and AF cDNA  
143 with Cyanine-3 dUTP (Cy3) and Cyanine-5 dUTP (Cy5) (PerkinElmer Life Science,  
144 Boston, MA), respectively. Fluorophore-labeled cDNA pairs were precipitated together  
145 with ethanol and purified using Microcon YM-30 purification columns (Millipore,  
146 Bedford, MA). The labeled cDNAs were resuspended in the hybridization buffer of  
147 20% formamide, 5 $\times$  SSC, 0.1% SDS and 0.1 mg/ml salmon sperm DNA (Ambion,  
148 Austin, TX), and then denatured by heating at 95 $^{\circ}\text{C}$  for 3 min. The mixture of labeled  
149 cDNA pairs was applied to the AF Chips under a 22-mm<sup>2</sup> cover slip and allowed to  
150 hybridize for 16 h at 55 $^{\circ}\text{C}$  in a hybridization chamber (GeneMachines, San Carlos, CA).  
151 The chips were washed at 45 $^{\circ}\text{C}$  for 10 min in 2 $\times$  SSC, 0.1% SDS, followed by two  
152 washes at room temperature in 1 $\times$  SSC (10 min) and 0.2 $\times$  SSC (15 min). After  
153 hybridization, the slides were scanned with a dual-laser scanner GenePix 4000B at 10-  
154  $\mu\text{m}$  resolution (Axon Instruments Inc., Union, CA). Results of the competitive  
155 hybridization were imaged at different photomultiplier settings to yield a balanced and  
156 applicable signal. Spots with saturated signal intensity or with signal less than  
157 background were excluded from the final data set. The data were converted from image

158 to signal using GenePix Pro 4.1 software (Axon Instruments Inc.) for further statistical  
159 analysis.

## 160 2.6. Data analysis and gene ontology

161 The fluorescence intensity of the local mean background was calculated for each  
162 spot, and we accepted only those cDNA spots with a fluorescence signal intensity more  
163 than the mean local background plus 2 standard deviations (SD) and greater than the  
164 intensity of the negative control. The signals from all the experimental arrays were  
165 normalized based on the *GAPDH* probe sets in which the average signal from *GAPDH*  
166 (as an internal control) was defined as unchanged. On the same AF Chip, the two  
167 intensity values of the duplicate cDNA clone spots were averaged and used to determine  
168 the ratio between the AF and sham control subjects (i.e., the ratio of Cy5/Cy3  
169 intensities). Genes were classified as differentially expressed when the value of the  
170 mean ratios was  $\geq 1.5$  or  $\leq 0.67$ . Functional classification of the differentially expressed  
171 genes was according to the GeneCards web site ([http://www.genecards.org/  
172 index.shtml](http://www.genecards.org/index.shtml)) and the Gene Ontology Consortium (<http://www.geneontology.org>).

## 173 2.7. Quantitative real-time reverse transcription-PCR

174 SYBR Green quantitative real-time reverse transcription-PCR (RT-PCR) was  
175 performed on the genes *FHL1*, *TSC-22*, *CARP*, *P311*, *TGF- $\beta$ 1*, *- $\beta$ 2*, *- $\beta$ 3* and *GAPDH* (as  
176 an internal control) to confirm the results obtained by the AF Chip and *TGF- $\beta$*  isoforms  
177 mRNA expression. The specific forward and reverse primers were designed with  
178 Primer Express software (PE Applied Biosystems, Foster, CA) (Table 1). The cDNA  
179 was synthesized by extension with oligo (dT) primer at 42°C for 60 min in a 25- $\mu$ l

180 reaction containing 5 µg total RNA, 1× First-Strand Buffer (75 mM KCl, 3 mM MgCl<sub>2</sub>,  
181 50 mM Tris-HCl, pH 8.3), 8 mM DTT, 10 µM dNTPs, 20 U RNase inhibitor, and 200 U  
182 Superscript II reverse transcriptase (Invitrogen). For each selected gene, the primer sets  
183 were tested for quality and efficiency to ensure optimal amplification of the samples.  
184 Real-time RT-PCR was performed at 1, 1/4, 1/16, 1/64, 1/128, and 1/512 dilution of the  
185 synthetic cDNAs to define relative fold changes. Real-time RT-PCR reactions  
186 contained 12.5 µl SYBR Green PCR master mix (PE Applied Biosystems), 2 µl forward  
187 primer (10 µM), 2 µl reverse primer (10 µM), 3 µl cDNA, and 5.5 µl distilled water. All  
188 PCR reactions were carried out in triplicate with the following conditions: 2 min at  
189 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at  
190 60°C in a 96-well optical plate (PE Applied Biosystems) in the ABI 7000 Sequence  
191 Detection System (PE Applied Biosystems). A PCR reaction without cDNA was  
192 performed as a template-free negative control. According to the instructions of PE  
193 Applied Biosystems, the expression of each gene was quantified as  $\Delta C_t$  ( $C_t$  of target  
194 gene –  $C_t$  of internal control gene) using *GAPDH* as the control and applying the  
195 formula  $2^{-\Delta C_t}$  to calculate the relative fold changes [13].

## 196 2.8. Protein preparation and western blot analysis

197 Western blotting was performed to determine the protein levels of FHL1 and  
198 CARP in the atrial AF and SR tissues. Frozen atrial tissues (about 200 mg) were  
199 homogenized in 1 ml of ice-cold lysis buffer containing 20 mM Tris-HCl, pH 7.6, 1 mM  
200 dithiothreitol, 200 mM sucrose, 1 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM  
201 sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride and 1% (v/v) Triton X-100.  
202 The homogenates were then centrifuged at 10,000 × g for 30 min at 4°C. Supernatants

203 were collected and protein concentration was measured with the Bio-Rad protein assay  
204 (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (20  $\mu$ g /lane) were  
205 separated on 10% polyacrylamide gels by SDS-PAGE and transferred onto  
206 polyvinylidene fluoride membranes (Millipore) at 50 mA for 90 min in a semi-dry  
207 transfer cell (Bio-Rad). Membranes were blocked for 60 min in TBS (10 mM Tris-HCl,  
208 0.15 M NaCl, pH 7.4) containing 5% nonfat dry milk with gentle shaking. Following  
209 three washes with TBS containing 0.1% (v/v) Tween-20 for 5 min, the membrane was  
210 incubated with anti-human FHL1 (1:5000), anti-human CARP (1:5000), or anti-human  
211  $\beta$ -tubulin (1:5000; all three antibodies were from Santa Cruz Biotechnology, Santa  
212 Cruz, CA), and detected using horseradish peroxidase-conjugated anti-goat IgG and  
213 ECL western blotting detection reagents (Amersham Pharmacia Biotech, Little  
214 Chalfont, UK) according to the manufacturer's instructions. The images were scanned,  
215 and densities of each band were analyzed by Scion image software (National Institutes  
216 of Health, Bethesda, MD). The relative protein expression of FHL1 and CARP was  
217 normalized to  $\beta$ -tubulin expression.

### 218 *2.9. Histological examination*

219 Fresh tissues of atrial appendages were cut into 3-5 mm slices and fixed in 4%  
220 (v/v) formalin buffer containing 0.1 M sodium phosphate (pH 7.5) before paraffin  
221 embedding. Tissue blocks were treated into 6  $\mu$ m sections and deparaffinized for  
222 Hematoxylin-eosin and Masson's trichrome blue staining.

### 223 *2.10. Immunohistochemical assay*

224 Immunohistochemical assay was performed on 6  $\mu$ m sections that were rehydrated

225 and blocked with 10% (v/v) normal goat serum (Santa Cruz Biotechnology).  
226 Subsequently, the sections were incubated overnight at 4°C with anti-FHL1 or -CARP  
227 specific antibodies (Santa Cruz Biotechnology) in Tris-buffered saline (10 mM Tris-  
228 HCl, pH 8.0, 150 mM NaCl), followed by incubations with biotinylated secondary  
229 antibodies that were detected with streptavidin– horseradish peroxidase conjugates  
230 (Vectastain Elite ABC kit; Vector Laboratory, Burlingame, CA). Peroxidase activity  
231 was visualized with diaminobenzidine and hydrogen peroxide. For detection, a control  
232 was done by omitting the primary antibody to determine the nonspecific binding.  
233 Nuclei were counterstained with hematoxylin.

#### 234 *2.11. Cell culture and treatment*

235 H9c2 cardiomyocytes (ATCC; CRL1446) were plated at 60% confluence in  
236 Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS),  
237 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (growth-promoting  
238 medium). The medium was switched to DMEM containing 1% FCS for 48 h  
239 (differentiation-promoting medium) when the H9c2 cells had grown to 70-80%  
240 confluence. The cells were then treated with 1 µM angiotensin II (Ang II) (Sigma, St.  
241 Louis, MO), 10 µM isoproterenol (Sigma), 0.2 mM H<sub>2</sub>O<sub>2</sub> (Merck, Darmstadt,  
242 Germany), or the same volume of Dulbecco's PBS (DPBS; control treatment). The  
243 treated cells were harvested 12 h after treatment, and total RNA was isolated as  
244 described above.

#### 245 *2.12. Semi-quantitative RT-PCR*

246 Unique PCR primers specific for *FHL1* (5'-AGG GGA GGA CTT CTA CTG TGT

247 G-3' and 5'-CCA GAT TCA CGG AGC ATT TT-3'), *CARP* (5'-AAA TCA GTG CCC  
248 GAG ACA AG-3' and 5'-ATT CAA CCT CAC CGC ATC A-3') and *GAPDH* (5'-GGT  
249 GAT GCT GGT GCT GAG TA-3' and 5'-TTC AGC TCT GGG ATG ACC TT-3') were  
250 designed using PRIMER3 software (available online at [http://frodo.wi.mit.edu/cgi-bin/  
251 primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and the nucleic acid sequence database from the National  
252 Center for Biotechnology Information (NCBI). Total RNA from H9c2 cells was  
253 prepared as described above. The cDNA was synthesized by Superscript II reverse  
254 transcriptase (Invitrogen) from 5 µg of total RNA. The PCR reaction contained 3 µl  
255 cDNA, 2 µl forward and reverse primers (10 µM), 5 µl 10× PCR buffer, 2 µl 10 mM  
256 dNTPs, 1 µl of 5 U/µl Taq polymerase (Promega, Madison, WI) and 37 µl distilled  
257 water in a total volume of 50 µl. DNA was amplified by an initial incubation at 94°C  
258 for 3 min followed by 25-30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a  
259 final extension at 72°C for 6 min. The PCR products were separated by electrophoresis  
260 in a 2% agarose gel, visualized by ethidium bromide staining, and the band intensity  
261 was quantified using an image analyzer (Kodak DC290 Digital camera System;  
262 Eastman Kodak, Rochester, NY). The relative amount of mRNA was measured using  
263 densitometric analysis by Scion image (National Institutes of Health) and normalized to  
264 the level of *GAPDH* mRNA.

### 265 2.13. Statistical analysis

266 Experimental results are expressed as means ± SD. Data from the AF Chip, real-  
267 time RT-PCR, semi-quantitative RT-PCR and western blots were analyzed using the  
268 Student's *t* test. Differences with  $P < 0.05$  were considered significant.

269

## 270 3. Results

### 271 3.1 Quality assessment of the AF Chip hybridization

272 In our previous study using a high-density cDNA microarray, many genes  
273 demonstrated marked expression changes in the fibrillating atria of a rapid pacing-  
274 induced AF model [10]. However, the genes involved in signal transduction and  
275 transcriptional regulation during AF have not been evaluated in detail. Therefore, we  
276 constructed a low-density cDNA array, the AF Chip, which included genes in these  
277 functional pathways and others to investigate changes in gene expression in atrial  
278 tissues during AF. The chip was fabricated with 84 AF or heart diseases-related genes  
279 mentioned in published papers, including our own previous report [10], to further  
280 analyze their relationship with AF.

281 To validate the reproducibility and quality of the AF Chip, we analyzed correlation  
282 factors from the intensity values of each gene in duplicated spots and replicated  
283 hybridizations. The scatter-plot analysis of the raw signal intensities of duplicated spots  
284 in a representative hybridization (Fig. 2A) fit a linear line with correlation factors of  
285 0.985 and 0.981 in the Cy5 (in red; Fig. 2B) and Cy3 (in green; Fig. 2C) channels,  
286 respectively. In the AF Chip experiments, two independent hybridizations were  
287 performed for each sample, and the mean intensity values for each gene from one chip  
288 were plotted against those from the second chip. After normalization, the correlation  
289 factors of the 12 replicate hybridization data with Cy3 and Cy5 normalized intensities  
290 ranged from 0.921 to 0.993 (average of 0.964 in the Cy3 channel and 0.947 in the Cy5  
291 channel). The results demonstrate that the AF Chip data were reproducible. Moreover,  
292 these high correlation factors indicate that the AF Chip was consistent and reliable.

## 293 3.2 Genes with altered expression in AF

294 Following competitive hybridization using labeled cDNAs from individual RAA  
295 tissues of AF and SR, the ratio of expression of each gene (Cy5 signal intensity in AF,  
296 and Cy3 signal intensity in SR) was averaged and evaluated by the Student's *t* test. The  
297 31 genes with significantly altered mRNA expression in rapid-pacing induced AF were  
298 shown in Table 2. These genes were classified into seven functional categories  
299 according to the molecular and biological function of their encoded protein. The  
300 categories include transcription (4 genes), structural components (8), metabolism (4),  
301 signal transduction (3), cell proliferation (2), and others (10, including ESTs), among  
302 which, two genes encoding proteins associated with the myofibrillar apparatus, myosin  
303 regulatory light chain-2 (*MLC-2V*) and cysteine and glycine-rich protein 1 (*CSRPI*),  
304 showed a 4.4- and 2.6-fold increase in AF, respectively. Several genes encoding  
305 components of the extracellular matrix were differentially expressed in AF, like  
306 increased expression of spondin 1 (*SPONI*), fibronectin 1 (*FNI*), cadherin 2 (*CDH2*)  
307 and a disintegrin and metalloprotease domain 10 (*ADAM10*), and decreased expression  
308 of cartilage-associated protein (*CRTAP*) and matrix metalloproteinase 11 (*MMP-11*).  
309 Genes involved in signal transduction pathways, including calmodulin 2 (*CALM2*),  
310 beta-2 adrenergic receptor ( $\beta_2AR$ ) and insulin-like growth factor binding protein 5  
311 (*IGFBP5*), showed upregulation. Among the unclassified genes, the marked  
312 downregulation of *P311* in fibrillating atria is particularly interesting because of its  
313 putative anti-fibrotic function. There were four genes, *FHL1*, *CARP*, *TSC-22* and  
314 *TCEB1*, which encode transcriptional cofactors or modulators [14-17], that showed  
315 increased expression of between 1.6- and 3.2-fold ( $P < 0.01$ ) (Table 2). They may  
316 contribute to the altered transcription processes involved in the complex remodeling



317 during AF, but, to the best of our knowledge, the transcriptional regulators associated  
318 with AF have not been well explored.

### 319 3.3 Validation of altered gene expression by quantitative real-time RT-PCR

320 Quantitative real-time RT-PCR was performed to validate the mRNA expression  
321 results of the AF Chip. We selected four differentially expressed genes *FHL1*, *TSC-22*,  
322 *CARP*, and *P311*. As mentioned above, *P311* was of particular interest because of its  
323 putative anti-fibrotic function, and *FHL1*, *TSC-22* and *CARP* were chosen for further  
324 study because these genes play regulatory roles in transcription and structural alterations  
325 in cardiomyocytes [15-17]. Total RNA extracted from the RAA and LAA of AF and SR  
326 model pigs was used in real-time RT-PCR, and the relative mRNA expression level of  
327 each gene was normalized to that of *GAPDH* mRNA. The relative *FHL1*, *TSC-22* and  
328 *CARP* mRNA expression levels in RAA with AF were significantly upregulated 5.1-,  
329 4.0- and 3.4-fold, respectively, as compared with those in the SR RAA, and the relative  
330 *P311* mRNA in the AF RAA was significantly downregulated by 5.6-fold compared  
331 with the SR RAA. Therefore, the trend of mRNA expression changes as determined by  
332 real-time RT-PCR was in good agreement with that determined by the AF Chip (Fig. 3).

333 Fig. 4 shows the comparisons of relative mRNA expression determined by real  
334 time RT-PCR of the four selected genes in the LAA and RAA of AF subjects.  
335 Expression of *FHL1*, *TSC-22* and *CARP* was significantly increased, and the levels  
336 were similar in the right and left atria during AF. Interestingly, the fold decrease in  
337 *P311* mRNA expression in the LAA with AF was approximately twice that of *P311*  
338 mRNA expression in the RAA.

339 *3.4 Increased distributions of FHL1 and CARP in the atria with AF*

340 To determine whether the increased *FHL1* and *CARP* mRNA levels also resulted  
341 in an increase in protein levels in pacing-induced AF atria, we performed a western  
342 blotting analysis. As shown in Fig. 5, a 2.9-fold and 1.6-fold average increase in *FHL1*  
343 and *CARP*, respectively, was observed in the fibrillating atria. These *FHL1* and *CARP*  
344 protein expression data are consistent with the mRNA expression data of the cDNA  
345 array and quantitative real-time RT-PCR.

346 For confirming the increased expression of both *FHL1* and *CARP* in rapid-pacing  
347 induced AF upon quantitative real-time RT-PCR and Western blot analysis, we  
348 investigated the intracellular distribution of *FHL1* and *CARP* in porcine atrial  
349 appendage tissue with SR and AF using immunohistochemical assay (Fig. 6). By the  
350 immunohistochemical assay, *FHL1* and *CARP* proteins were detectable in almost all  
351 myofibrils in atrial myocytes and increased positive immunoreaction coincides with  
352 muscle striated and filamentous forms in the atrial myocytes in AF. Furthermore, the  
353 both of anti-*FHL1* and -*CARP* antibodies detected more diffuse cytoplasmic staining  
354 with slightly increased nuclear localization in the fibrillating atria (Fig. 6B and 6D).

355 *3.5 Histological findings in the rapid-pacing induced AF*

356 Histological studies were performed to identify the potential pathological substrate  
357 underlying conduction abnormalities in the sustained AF. Representatively histological  
358 sections from the atria with AF and SR were stained with Hematoxylin-eosin and  
359 Masson's trichrome blue. Evidences provided by the Masson's trichrome blue showed  
360 that endocardial fibrosis and extracellular matrix proteins markedly accumulated in

361 interstices between cardiomyocytes in the AF atria (Fig. 7).

### 362 *3.6 Selective increase of TGF- $\beta$ isoform expression in the fibrillating atria*

363 Because increased expression of *TSC-22* gene and decreased expression of *P311*  
364 gene in atrium with AF may be associated with increased TGF- $\beta$  signal activity, we  
365 examined levels of expression of *TGF- $\beta$*  isoforms. By real-time RT-PCR analysis,  
366 expression of *TGF- $\beta$ 2* RNA levels were markedly increased 6.0-fold in RAA and 8.8-  
367 fold in LAA during AF, and *TGF- $\beta$ 1* mRNA were slightly increased respectively by 2.0-  
368 fold and 2.9-fold, however, *TGF- $\beta$ 3* mRNA levels were no significant change (Fig. 8).  
369 These results indicated that a selective increase of expression of *TGF- $\beta$ 1* and - $\beta$ 2  
370 isoforms was occurred in the pacing-induced AF.

### 371 *3.7 Effects of Ang II, isoproterenol, and H<sub>2</sub>O<sub>2</sub> on FHL1 and CARP mRNA expression in* 372 *rat cardiac H9c2 cells*

373 Several studies have indicated that the Ang II system, adrenergic signal  
374 transduction and oxidative stress are associated with the cellular signaling mechanisms  
375 in AF [6,18]. To determine whether proarrhythmogenic substrates alter the expression  
376 of *FHL1* or *CARP* in cardiac myocytes, H9c2 cells were treated with Ang II, the  
377 adrenergic agonist isoproterenol, or H<sub>2</sub>O<sub>2</sub>, and *FHL1* and *CARP* transcript levels were  
378 measured by semi-quantitative RT-PCR. The cells were pre-treated with differentiation-  
379 promoting medium (containing 1% FCS) for 48 h and then treated with 1  $\mu$ M Ang II, 10  
380  $\mu$ M isoproterenol, or 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 h. *FHL1* and *CARP* expression significantly  
381 increased 3.1- and 3.3-fold, respectively, in the H9c2 cells following treatment with  
382 isoproterenol, *CARP* expression slightly increased with Ang II treatment, but the two

383 genes expression did not increase in response to H<sub>2</sub>O<sub>2</sub> (Fig. 9).

384

#### 385 **4. Discussion**

386 AF is a progressive and self-sustaining disease. The processes include electrical,  
387 contractile, neurohormonal, macroanatomical and ultrastructural changes, and these  
388 may lead to the vulnerability of AF over time. Many factors such as ion channels,  
389 proteins influencing calcium homeostasis, connexins, autonomic innervation, fibrosis,  
390 and cytokines may be involved in the molecular mechanism of AF [1,2]. In the present  
391 study, a porcine model with sustained AF in which structural changes in the atrium  
392 [9,10] are induced by 4 weeks of rapid atrial depolarization was used to obtain more  
393 insight to molecular processes of atrial remodeling. Genes involved in transcriptional  
394 regulation, ion transport, signal transduction, metabolism, cell proliferation,  
395 extracellular matrix and structural proteins, and a few unclassified genes were used to  
396 study the differential expression in AF via a low-density cDNA array, named AF Chip  
397 (Fig. 1). To evaluate the threshold in differential expression of mRNA in our AF Chip,  
398 mock hybridization of two differentially labeled cDNAs to three replicates was first  
399 performed. More than 98% of the visible signals revealed Cy5/Cy3 ratios between 0.67  
400 and 1.50 (data not shown). These ratios were used as cut-off values to identify  
401 significant differential expression throughout our experiments, in addition, the  
402 consistent and reliable AF Chip processes were also confirmed (Fig. 2). Results from  
403 the AF Chip analysis revealed that 31 mRNAs out of 84 clones were differentially  
404 expressed in atrial tissues (ratios  $\geq 1.50$  or  $\leq 0.67$ ;  $P < 0.05$ ) in the rapid pacing-induced  
405 AF model (Table 2). Most observation of the 31 genes included those involved in

406 activation of signal transduction systems, transcriptional regulation and structural  
407 alterations characteristic of AF. These alterations in expression by AF Chip assay might  
408 be the focal or varied changes within the atrial tissue induced by AF, however, a locally  
409 pronounced change might affect whole atrial function gradually like as an  
410 arrhythmogenic substrate in AF.

411 Confirmatory real-time RT-PCR supported our AF Chip findings and emphasized  
412 the marked differential expression of the transcriptional regulator genes *FHL1*, *CARP*  
413 and *TSC-22* and an anti-fibrotic gene, *P311*, in the LAA and RAA tissues with AF (Fig.  
414 3 and 4). Furthermore, FHL1 and CARP protein levels were also confirmed by western  
415 blotting (Fig. 5).

416 The LIM domain of FHL1 is located at the amino terminus, which is associated  
417 with GATA1-type zinc fingers [19], and the role of FHL1 in transcriptional regulation  
418 was proposed [16]. Besides the function in transcriptional regulation, FHL1 has also  
419 shown integrin-dependent localization in the nucleus, cytoskeleton, focal adhesions and  
420 stress fibers in cardiomyocytes [20]. The CARP protein, a nuclear transcriptional co-  
421 factor that negatively regulates cardiac gene expression [15], was upregulated in human  
422 heart failure and animal models of cardiac hypertrophy [21,22]. Witt et al. [23]  
423 demonstrated that CARP variably localizes in the sarcoplasm and the nucleus in adult  
424 skeletal muscle cells. There was documented that CARP overexpression induces  
425 contractile dysfunction in an engineered heart tissue [24].

426 Upon the information of molecular functions for FHL1 and CARP, they are  
427 proposed as the critical roles in the transcriptional regulation, myofibrillar assembly and  
428 even communication between sarcoplasm and nucleus in the cardiomyocytes [20,24].  
429 In the present study, increased expressions and distributions of FHL1 and CARP in AF

430 atrial myocytes were detected, revealing that they may play a role associated partially  
431 with structures of the striated sarcomeres and protein filaments in AF atrial myofibrils  
432 (Fig. 6). Together these functional studies, the increased distributions of FHL1 and  
433 CARP might effect on atrial gene expression or contractility in the fibrillating atria by  
434 modulating transcription and myofilament assembly.

435 Factors that may contribute to progressive stability of AF with changed cellular  
436 signaling in a prolonged time course, also termed “arrhythmogenic substrates”, have  
437 been discussed [18]. Among which, abnormal neurohormonal stimuli, such as  
438 angiotensin II (Ang II) and norepinephrine, may play a critical role in the sustained AF.  
439 Recent reports demonstrated activation of the renin angiotensin system (RAS) and  
440 RAS-dependent signaling pathways by AF in atria in humans [25] and in a dog model  
441 [26]. Ang II is an important modulator of cardiac and cardiomyocyte contractility. Ang  
442 II has been shown to exacerbate contractile dysfunction in experimental models of  
443 pressure-overload cardiac hypertrophy [27] and pacing- or infarction-induced heart  
444 failure [28,29]. In addition to RAS, sympathetic hyperinnervation, nerve sprouting, and  
445 a heterogeneous increase in atrial sympathetic stimulation have also been demonstrated  
446 in a canine model of sustained AF produced by prolonged right atrial pacing [30]. By  
447 isoproterenol infusion, Doshi et al. [31] demonstrated that the rapid automatic activity  
448 trigger the onset of AF, which occurs only in the atrium harvested from dogs with long-  
449 term pacing-induced AF but not in normal atrium. These findings underline the well-  
450 known profibrillatory effect resulting from a local excess of catecholamines [32]. The  
451 most important pattern of signal transduction linking the sympathetic nervous system to  
452 the intracellular effector in cardiomyocytes is mediated through the  $\beta$ 1- and  $\beta$ 2-  
453 adrenoceptors. The  $\beta$ -adrenergic receptors are stimulated by the catecholamines, which

454 initiates a cascade of intracellular events that leads to an increase in conduction velocity  
455 and shortening of the refractory period.

456 Direct effect of oxidative stress on atrial remodeling in a pacing-induced AF was  
457 also demonstrated by Carnes et al. [33]. Mihm et al. [34] found that increased oxidative  
458 stress during AF could contribute to contractile dysfunction. Most effects of mentioned  
459 above, neurohormonal stimuli and oxidative stress, can be provoked by the rapid atrial  
460 pacing and appear to be of importance for molecular changes in fibrillating atrium and  
461 effect on atrial contractile dysfunction [26,30,33].

462 We speculated that the *FHL1* and *CARP* expression may be affected by  
463 neurohormonal stimuli and/or oxidative stress, since the two proteins represent the roles  
464 as the transcription regulators, myofilament components and were upregulated in this  
465 AF mode with rapid atrial depolarization for 6 weeks. Therefore, we were undertaken  
466 to investigate whether the expressions of *FHL1* and *CARP* in cardiomyocytes *in vitro*  
467 are directly regulated due to these proarrhythmogenic stimulations which were  
468 implicated in the atrial remodeling during AF. We found that the  $\beta$ -adrenergic agonist  
469 isoproterenol, but not Ang II or  $H_2O_2$ , significantly increased mRNA expression of both  
470 *FHL1* and *CARP* in H9c2 cells (Fig. 9). The rapid upregulation of *FHL1* and *CARP*  
471 expression in H9c2 cells stimulated by isoproterenol showed that regulation of the two  
472 genes may be mediated via the  $\beta$ AR signal transduction pathway. In the fibrillating  
473 atria, there was also evidence that the upregulated  $\beta_2AR$  mRNA was observed by cDNA  
474 array analysis (Table 2); these results implicate that upregulation of *FHL1* and *CARP* in  
475 the fibrillating atria might be associated with the activation of  $\beta$ AR signal pathway. In  
476 the study performed by Gaussin et al. [35], the expression of *FHL1* was dramatically  
477 upregulated in different mouse models of overexpressing  $\beta_1AR$ ,  $\beta_2AR$  or PKA,

478 indicating that *FHL1* expression is upregulated by the  $\beta$ AR-PKA pathway. In addition,  
479 induced expression of CARP in cardiomyocytes by activation of PKA and CaMK was  
480 also obtained [24]. These studies strongly support our suggestions that the  $\beta$ AR signal  
481 pathway might be activated to upregulate two important regulators, *FHL1* and *CARP*,  
482 and effect on the changes of transcriptional regulation and atrial contractile in AF.

483 The increased expression of *CARP* induced by Ang II in H9c2 cells has also been  
484 detected (Fig. 9B). Ang II affects the expression of a wide range of signaling pathways  
485 [6]. Therefore, we would not exclude the possibility that upregulated expression of  
486 *CARP* in the pacing-induced AF model might be partially effected by activation of RAS  
487 signal pathway. However, the results of the *in vitro* experiment demonstrate that *FHL1*  
488 and *CARP* expression were not elevated in the H<sub>2</sub>O<sub>2</sub>-treated H9c2 cells, suggesting that  
489 changes in *FHL1* and *CARP* expression may be not associated with modulation of gene  
490 expression via oxidative damage in the fibrillating atria. These results of *in vitro*  
491 experiments provide a reference value to ongoing studies of the underlying molecular  
492 mechanism and even for novel insight in strategy of AF treatment. Nevertheless, with  
493 only limited studies in cardiac H9c2 cells in this regard, the results of changed *FHL1*  
494 and *CARP* expressions associated with cell signaling and transcription regulation in AF  
495 may be fundamental and deserve future investigations.

496 Calcium overload in AF was suggested a critical factor in the electrophysiological  
497 remodeling process and altered contraction and signaling in cardiomyocytes [36,37]. It  
498 is commonly believed that even minutes of rapid atrial rates can result in intracellular  
499 calcium overload, which decreases the L-type calcium current ( $I_{CaL}$ ) [38]. In the present  
500 study, the mRNA expression levels of genes for L-type voltage-dependent calcium  
501 channel  $\alpha$ -1C and-1S subunits (CACNA1C and CACNA1S), involved in



502 influencing calcium homeostasis, were also evaluated in our microarray analysis (Fig.  
503 1). However, no significantly different was found between the AF and sham control  
504 groups. Our results seem to disagree with the published literature suggesting a decrease  
505 in L-type  $\text{Ca}^{2+}$  channel density that contributes to the reduction of  $I_{\text{CaL}}$  [36].  
506 Nevertheless, studies on gene expression of the L-type  $\text{Ca}^{2+}$  channel, so far, have  
507 reported different results in AF. Schotten et al. [39] reported that there is no change in  
508 L-type  $\text{Ca}^{2+}$  channel density in persistent AF patients. An ultrastructural calcium  
509 distribution in goat atria with 1–16 weeks of burst-pacing-induced AF was performed  
510 [40]. Ausma et al. [40] showed that  $\text{Ca}^{2+}$  overload persists for 2 weeks, after which the  
511 cardiomyocytes apparently adapt to a new  $\text{Ca}^{2+}$  homeostasis and reach normal level, and  
512 then  $\text{Ca}^{2+}$  levels are below normal level after 16 weeks of AF. This study implicated  
513 that  $\text{Ca}^{2+}$  overload induced by over-drive pacing is reversible. Accordingly, we  
514 conceived that the new  $\text{Ca}^{2+}$  homeostasis and even recovery expression of calcium  
515 channel might be taken place in our AF model, which was induced by atrial rapid  
516 pacing for 4 weeks and then maintained AF without pacing for 2 weeks. Although the  
517 clear mechanism of reversible downregulation of  $I_{\text{CaL}}$  has not yet been uncovered, the  
518 calcium channel expression might be unchanged or have be recovery in our pacing-  
519 induced AF and play in part roles for the new  $\text{Ca}^{2+}$  homeostasis. Interestingly, the  
520 mRNA expression of calmodulin 2 (*CALM 2*) was increased by 1.7-fold in our AF  
521 model (Table 2). CALM is a critical  $\text{Ca}^{2+}$  sensor and regulatory protein and the  
522 formation of  $\text{Ca}^{2+}$ / CALM complex selectively activates specific downstream signaling  
523 pathways in response to local changes in calcium concentration [41]. We suggest that  
524 the increased CALM 2 in AF might act as a critical  $\text{Ca}^{2+}$  sensor for the modulatory  
525 effects on calcium channels function or downstream signaling and therefore to

526 overcome the detrimental  $\text{Ca}^{2+}$  overload. However, much additional work in this  
527 regard, such as the respective roles of functional ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Cl}^-$ ) channels and  
528 the precise signaling of  $\text{Ca}^{2+}$ /CaML, need to be performed to truly understand the  
529 changes and effects of  $\text{Ca}^{2+}$  homeostasis in AF.

530 A number of cardiovascular diseases are associated with extracellular matrix  
531 homeostasis [42,43]. The occurrence of atrial fibrosis in the development of AF has  
532 been documented [44-46] and also found in our AF model (Fig. 7). Interesting, an  
533 unclassified gene, *P311*, was markedly downregulated in the fibrillating atria. Recent  
534 studies indicate that P311 blocks TGF- $\beta$  autoinduction and downregulates the  
535 expression of genes encoding extracellular matrix proteins in cardiac myofibroblasts,  
536 suggesting that P311 exerts anti-fibrotic effects by inhibiting TGF- $\beta$  signaling [47].  
537 Furthermore, TSC-22 is a TGF- $\beta$ -inducible gene and represent a transcriptional  
538 regulator that enhances the activity of TGF- $\beta$  signaling by binding to and modulating  
539 the transcriptional activity of Smad3 and Smad4 [48]. The expression of *TSC-22* was  
540 significantly induced by 4.0-fold in our AF model, implicating the enhanced activation  
541 of TGF- $\beta$  signaling in the fibrillating atria. In concordance with our supposition, we  
542 identified the upregulated expressions of *TGF- $\beta$*  isoforms in the AF model by real-time  
543 RT-PCR analysis. The expression of *TGF- $\beta$ 2* RNA levels were markedly increased in  
544 atrial tissues with AF and *TGF- $\beta$ 1* RNA were slightly increased; however, *TGF- $\beta$ 3*  
545 RNA levels were no significant change (Fig. 8). Interestingly, the differently  
546 upregulated levels of *TGF- $\beta$ 2* between RAA and LAA with AF seemingly showed the  
547 parallel with the decreased expression of *P311* in atrial tissues (Fig. 4 and 8),  
548 implicating that effects of *TGF- $\beta$ 2* expression in AF may at least partially result from

549 loss of P311 blocked TGF- $\beta$  autoinduction. Recent studies have demonstrated an  
550 upregulation of *TGF- $\beta$ 1* expression in human AF patients by microarray analysis,  
551 indicating activation of *TGF- $\beta$ 1* signaling is involved in the process of development  
552 fibrosis in AF [3,4]. Beside, the studies on transgenic mice have also shown that  
553 increased cardiac expression of TGF- $\beta$ 1 leads to atrial fibrosis [49] and increased  
554 vulnerability to AF [50]. The upregulation of *TGF- $\beta$ 1* and *- $\beta$ 2* identified in the rapid-  
555 pacing induced AF model may therefore promote the activation of fibroblasts and atrial  
556 fibrosis. These results suggest that the dramatic *P311* downregulation and *TSC-22*  
557 upregulation may play a significant critical role and contribute to the development of  
558 fibrosis during AF while the increased *TGF- $\beta$ 1* and *- $\beta$ 2* expression were also obtained.

559 In conclusion, use of a low-density cDNA array of 84 selected genes identified  
560 four genes, *FHL1*, *CARP*, *TSC-22* and *P311*, as differentially expressed in the rapid-  
561 pacing induced AF model in pig. The observed alterations in AF were proven by further  
562 identification of protein expression and possible mechanism in the fibrillating atria.  
563 These data implicated that the changes in transcriptional regulator expressions,  
564 myofilament assembly and genes responsible for TGF- $\beta$  signaling might play a  
565 significant functional role in AF-induced the structural alterations, such as atrial  
566 contractile dysfunction and interstitial fibrosis, and provide future avenues for  
567 investigation of the underlying mechanisms of AF pathogenesis.

568

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746 **Figure legends**

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748 Fig. 1. Design of the AF Chip. The 84 selected cDNA clones are members of the  
749 following functional classes: extracellular matrix, structural components, signal  
750 transduction, metabolism, ion transporters, cell proliferation, transcription regulation,  
751 EST sequences, and others. Two housekeeping genes,  $\beta$ -tubulin and *GAPDH*, were  
752 spotted onto the slides for signal normalization and positive controls. To evaluate the  
753 reliability of the experimental hybridization, a blank and a negative control (pUC19)  
754 were also included in the chip.

755

756 Fig. 2. Quality assessment of the AF Chip. A representative result from the AF Chip is  
757 shown (A). AF Chip hybridization comparing mRNA isolated from AF and SR  
758 (control) subjects. Message RNA from SR tissue was used to prepare cDNA labeled  
759 with Cy3, and mRNA extracted from AF tissue was used to prepare cDNA labeled with  
760 Cy5. The two cDNA probes were mixed and simultaneously hybridized to the AF Chip.  
761 Red indicates genes whose mRNAs were more abundant in AF, and green indicates  
762 genes whose mRNAs were more abundant in SR. Yellow spots represent genes whose  
763 expression did not vary substantially between the two samples. Scatter-plot analyses of  
764 spot intensities between duplicate spots with the Cy5 (B) and Cy3 (C) channels.

765

766 Fig. 3. Verification of altered gene expression by quantitative real-time RT-PCR.  
767 Comparison of results obtained with the AF Chip and quantitative real-time RT-PCR for  
768 mRNA levels of *FHL1*, *TSC-22*, *CARP* and *P311* during AF. Real-time RT-PCR and AF

769 Chip hybridizations were performed as described in the Materials and methods.  
770 Relative to the levels in the RAA with SR, expression of *FHL1*, *TSC-22* and *CARP*  
771 mRNAs was increased by 5.1-, 4.0- and 3.4-fold, respectively, in the parallel AF  
772 subjects. *P311* mRNA expression was decreased by 5.6-fold in AF. Bar graphs with  
773 error bars represent the means  $\pm$  SD (n = 12 for real-time RT-PCR and chip analysis).

774

775 Fig. 4. Comparisons of mRNA expression in the RAA and LAA with AF. The cDNAs  
776 were prepared from the RAA and LAA of AF and SR groups and then subjected to real-  
777 time PCR with gene-specific primers. The ratio of the abundance of each transcript to  
778 that of the *GAPDH* transcript was calculated, and the amount of mRNA expression in  
779 the AF group was expressed as a relative change standardized to the control group. Bar  
780 graphs with error bars represent the means  $\pm$  SD (n = 6 in the SR group; n = 12 in the  
781 LAA and RAA of the AF group). \* $P < 0.05$  vs. RAA.

782

783 Fig. 5. Western blot analysis of FHL1 and CARP protein expression in SR and AF atria.  
784 Tissue homogenates (20  $\mu$ g protein/lane) from RAA with AF or SR were separated on  
785 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membrane.  
786 Immunoblots were incubated with antibodies specific for FHL1, CARP or  $\beta$ -tubulin  
787 (A). The relative protein expression of FHL1 (B) and CARP (C) was normalized to  $\beta$ -  
788 tubulin. The histograms show the means  $\pm$  SD (n = 6 in the SR group; n = 12 in the AF  
789 group). \* $P < 0.05$  and \*\* $P < 0.01$ , vs. SR.

790

791 Fig. 6. Immunohistochemical assay of FHL1 and CARP protein in the atrial appendage

792 tissues with SR (A and C) and AF (B and D). Increased FHL1 and CARP are detectable  
793 in almost all myofibrils in the atrial myocytes and positive immunoreaction coincides  
794 with muscle striation and protein filaments in the muscle fibers in AF. In fibrillating  
795 atria, immunostaining of FHL1 and CARP showed more abundant and disorganized (B  
796 and D). (Scale bar represents 50  $\mu\text{m}$ )

797

798 Fig. 7. Representative photomicrographs of atrial appendage tissue showing fibrosis in  
799 rapid-pacing induced AF. Histological sections from the atrial appendage tissue with  
800 SR (A) and AF (B) were stained by Masson's trichrome blue. The blue color  
801 characteristic of collagen after Masson's trichrome staining was obvious in interstices  
802 between cardiomyocytes in the AF atria. Sections from sham control, when stained  
803 similarly reflect the extracellular matrix marked accumulation in the rapid-pacing  
804 induced fibrillating atria. (Scale bar represents 100  $\mu\text{m}$ )

805

806 Fig. 8. Three *TGF- $\beta$*  isoforms mRNA levels in right and left atrial appendages with AF  
807 compared with SR were determined by real-time RT-PCR. Differently expressed  
808 changes of three *TGF- $\beta$*  isoforms mRNA were observed in AF, and upregulation of  
809 *TGF- $\beta$  1* and *- $\beta$  2* expression in RAA and LAA with AF were found. Expression of  
810 *TGF- $\beta$  2* mRNA levels were markedly increased 6.0-fold in RAA and 8.8-fold in LAA  
811 during AF, and the changes of *TGF- $\beta$  1* mRNA in RAA and LAA were increased  
812 respectively by 2.0-fold and 2.9-fold, however, *TGF- $\beta$  3* mRNA levels were no  
813 significant change, when compared with SR. (n = 6 in the SR group; n = 12 in the AF  
814 group). \*  $P < 0.05$  vs. SR, \*\*  $P < 0.05$  vs. SR and  $\dagger$  indicates  $P < 0.05$  vs. RAA.

815

816 Fig. 9. Effects of Ang II, isoproterenol, or H<sub>2</sub>O<sub>2</sub> on the transcription levels of *FHL1* and  
817 *CARP* in cardiac H9c2 cells. H9c2 cells grown to 80% confluency were treated with 1  
818 μM Ang II, 10 μM isoproterenol (Isop), 200 μM H<sub>2</sub>O<sub>2</sub> or PBS (as a control) for 12 h  
819 and then RNA was isolated for semi-quantitative RT-PCR. The fold changes in mRNA  
820 expression of *FHL1* (A) and *CARP* (B) were calculated based on *GAPDH* as a  
821 covariate. Data represent the means ± SD of three independent experiments. \**P* < 0.05,  
822 and \*\**P* < 0.01, vs. control.

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837 **Table 1**

838 Primers for quantitative real-time RT-PCR.

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Gene name (Gene symbol/Accession no.)	Primer <sup>a</sup>	Sequence (5' → 3')	Amplified length (bp)
Four and a half LIM domains 1 (FHL1/ NM_214375)	Forward	CTG CGT GGA TTG CTA CAA GA	115
	Reverse	GTG CCA GGA TTG TCC TTC AT	
Transforming growth factor beta-stimulated clone 22 (TSC-22/ NM_006022)	Forward	CCA TGA AGG TTG TTT TGC T	130
	Reverse	ACC TCC TCA GAC AGC CAA T	
Cardiac ankyrin repeat protein (CARP/ NM_213922)	Forward	CTT CCC GTA GGT AGC TCT TA	122
	Reverse	GCA ACA ATC ATC CCC TCT G	
Chromosome 5 open reading frame 13 (P311/ NM_004772)	Forward	GCG AGG TAG CTC TGA TGG A	123
	Reverse	GCC ACA CTG AAG ACA CAA GG	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH/AF017079)	Forward	AGA AGA CTG TGG ATG GCC C	110
	Reverse	ATG ACC TTG CCC ACA GCC T	
Transforming Growth Factor $\beta$ 1 (TGF $\beta$ 1/ NM_214015)	Forward	GGC CGT ACT GGC TCT TTA CA	138
	Reverse	TAG ATT TGG TTG CCG CTT TC	
Transforming Growth Factor $\beta$ 2 (TGF $\beta$ 2/ L08375)	Forward	ATG GCA CCT CCA CAT ATA CCA	101
	Reverse	GGG CAA CAA CAT TAG CAG GA	
Transforming Growth Factor $\beta$ 3 (TGF $\beta$ 3/ NM_214198)	Forward	AAG AAG GAA CAC AGC CCT CA	116
	Reverse	GCG GAA GCA GTA GTT GGT GT	

840 <sup>a</sup> Primer sequences for each target gene were selected to minimize the formation of self-

841 complementarity and hairpins using the software Primer Express (PE Applied

842 Biosystems).

843

844 **Table 2**

845 Genes with significantly altered expression in the right atrial appendages with sustained  
 846 AF as assayed by the AF Chip.

Accession no.	Function/Gene name	Symbol	Ratio (Cy5/Cy3)	SD	P value
<b>Transcription-related genes</b>					
AA725097	Four and a half LIM domains 1	FHL1	3.17 ↑	1.27	0.001
NM_006022	Transforming growth factor TSC-22	TSC-22	3.09 ↑	1.71	0.011
AA521128	Transcription elongation factor B (SIII)	TCEB1	2.49 ↑	1.10	0.001
X83703	Cardiac ankyrin repeat protein	CARP	1.61 ↑	0.45	0.001
<b>Structural components and extracellular matrix-related genes</b>					
S69022	Myosin regulatory light polypeptide 2	MYL2	4.35 ↑	1.70	0.001
BE388159	Cysteine and glycine-rich protein 1	CSRP1	2.57 ↑	1.81	0.012
AI809596	Spondin 1	SPON1	2.05 ↑	1.13	0.017
X02761	Fibronectin 1	FN1	1.94 ↑	0.72	0.001
M34064	Cadherin 2	CDH2	1.76 ↑	0.77	0.005
AA043347	A disintegrin and metalloprotease domain 10	ADAM10	1.50 ↑	0.77	0.044
AA451883	Cartilage associated protein	CRTAP	0.63 ↓	0.49	0.025
AI189375	Matrix metalloproteinase 11	MMP11	0.48 ↓	0.38	0.001
<b>Signal transduction-related genes</b>					
D45887	Calmodulin 2	CALM2	1.71 ↑	0.66	0.004
U02390	Beta-2 adrenergic receptor	B2AR	1.64 ↑	0.73	0.030
AW157548	Insulin-like growth factor binding protein 5	IGFBP5	1.55 ↑	0.72	0.022
<b>Cell proliferation-related genes</b>					
Y12084	Cysteine-rich, angiogenic inducer, 61	CYR61	1.50 ↑	0.53	0.007
XM_001393	Natural killer-enhancing factor A	PRDX1	0.63 ↓	0.35	0.006
<b>Metabolism-related genes</b>					
NM_001825	Creatine kinase	CKMT2	1.59 ↑	0.65	0.009
AI494124	Selenoprotein P, plasma, 1	SEPP1	0.69 ↓	0.36	0.015
AI207602	Hemoglobin, gamma G	HBG2	0.57 ↓	0.46	0.008
AW249010	Heat shock 70kD protein 10	HSPA8	0.53 ↓	0.35	0.001
<b>Genes with other functions and EST sequences</b>					
NM_004854	HNK-1 sulfotransferase	CHST10	2.08 ↑	1.10	0.008
AA375284	Poly (rC)-binding protein 2	PCBP2	1.64 ↑	0.49	0.001
AI275225	MAX-like bHLHZIP protein	MLX	0.64 ↓	0.19	0.000
Z48042	Membrane surface marker 1	M11S1	0.60 ↓	0.40	0.005
AI815757	Ribosomal protein L35	RPL35	0.48 ↓	0.38	0.001
NM_004772	Chromosome 5 open reading frame 13	P311	0.40 ↓	0.17	0.000
AA431300	EST6		2.24 ↑	1.28	0.009
N92589	EST4		2.12 ↑	1.42	0.019
N93201	EST5		2.00 ↑	1.35	0.033
AA195902	EST14		0.66 ↓	0.31	0.011

847

848 The signal intensities of the competitive hybridization were quantified with a two-

849 channel (Cy3 and Cy5) fluorescence scanner. Analysis of the fluorescence intensities  
850 and expression ratios were performed using GenePix Pro 4.1 software. Data represent  
851 the mean and SD of the Cy5/Cy3 intensity ratios ( $n = 12$ ). Those signal intensities more  
852 than 2 SDs from the background intensity and greater than the negative sham control  
853 intensity were considered as acceptable signals. This table lists results for which the  
854 differential expression was  $\geq 1.5$ -fold, which was considered as significant ( $P < 0.05$ ).

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

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	1	2	3	4	5	6	7	8	9	10	11	12
A	GAPDH AJ986320	Blank	pUC19	TUBB BC029529	Blank	TNNT2 BE393657	AGRT1 NM_009585	CALM D45887	LYN AA939217	CALM D45887	CAP2 U02390	ARF4 AA307503
B	EST1 AA923015	EST2 R09547	EST3 N44535	EST4 N92589	EST5 N93201	EST6 AA431300	EST7 AI051950	EST8 AI090186	PRKAR1A AA015682	IGFBP5 AW157548	PTGER4 L28175	TNFSF10 BE350219
C	EST9 AI188760	EST10 AI261936	EST11 AI380932	EST12 AI479494	EST13 AW051824	EST14 AA195902	EST15 AA922329	EST16 AW772770	EDN1 NM_001955	$\beta$ 2AR M15169	ACE M26658	ACE M26658
D	CACNA1C AJ224873	CACNA1S AI417964	CACNA1S AI417964	KCNK3 AI193606	GJB3 AW276400	GJA1 X52947	HSPA8 AW249010	PRDX1 X67951	CCND1 M73554	STATS3 AJ012463	BTEB1 D31716	TSC-22 NM_006022
E	MMP11 AI189375	ADAM10 AA043347	ADAM15 U41767	FN1 X02761	CKMT2 NM_001825	UCHL1 AI928978	LPL NM_000237	CYR61 Y12084	CAPNS1 BE397929	ZNF177 U37263	CARP X83703	MITF Z29678
F	CDH2 M34064	CHST10 NM_004854	CLDN1 AI300819	SPON1 AI809596	YWHAZ BE315169	HBG2 AI207602	SEPP1 AI494124	PRDX1 X67951	FHL1 AA725097	GTF2E2 S67861	BAZ2B AB032255	TCEB1 AA521128
G	CRTAP AA451883	PLS3 NM_005032	SPTBN1 M96803	ACTR1A AI120727	SERPIN2 Y00630	PCBP2 AA375284	CAV1 AI878826	MI1S1 Z48042	CYFIP2 AB032994	U5-116KD D21163	CSRP1 BE388159	RPL35 AI815757
H	ACTG1 BE314833	ACTN1 X15804	MYL2 S69022	TUBB BC029529	MMP2 BQ674774	CAST U38525	AHR AA844153	HUMGT 198A AI275225	C5orf13 NM_004772	GAPDH AI986320	Blank	pUC19

	Positive and negative control		Signal transduction related genes
	Blank		Transcription related genes
	EST clones		Metabolism related genes
	Connexin and ion transporter related genes		Cell proliferation related genes
	Structural component and extracellular matrix related genes		Other genes

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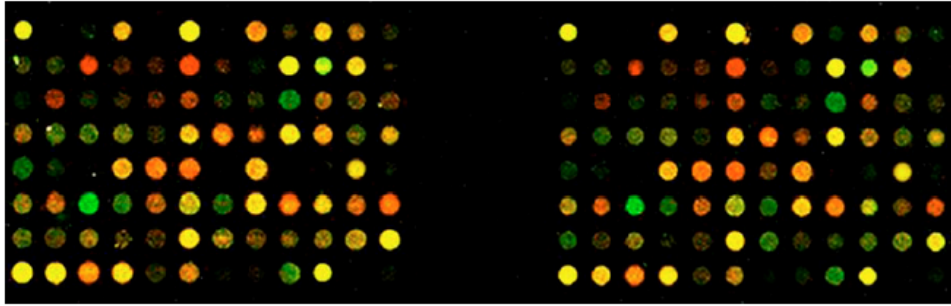
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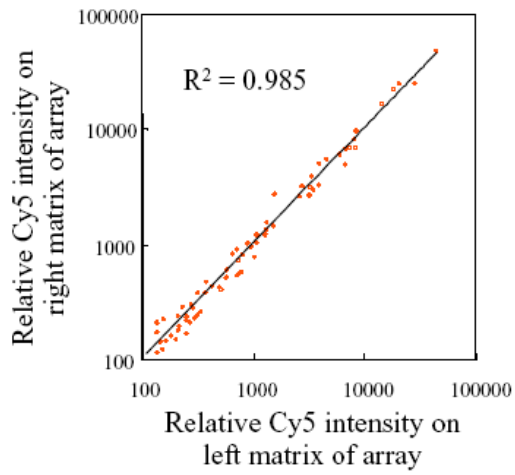
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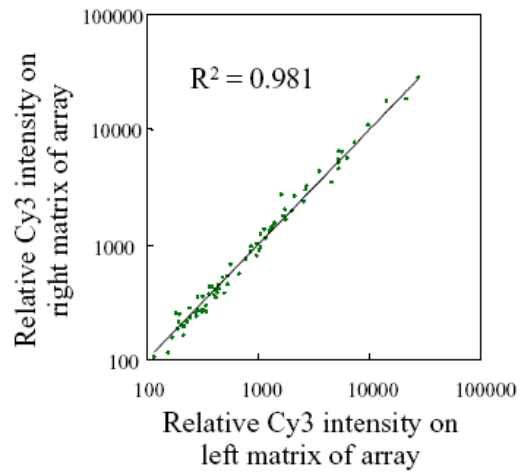
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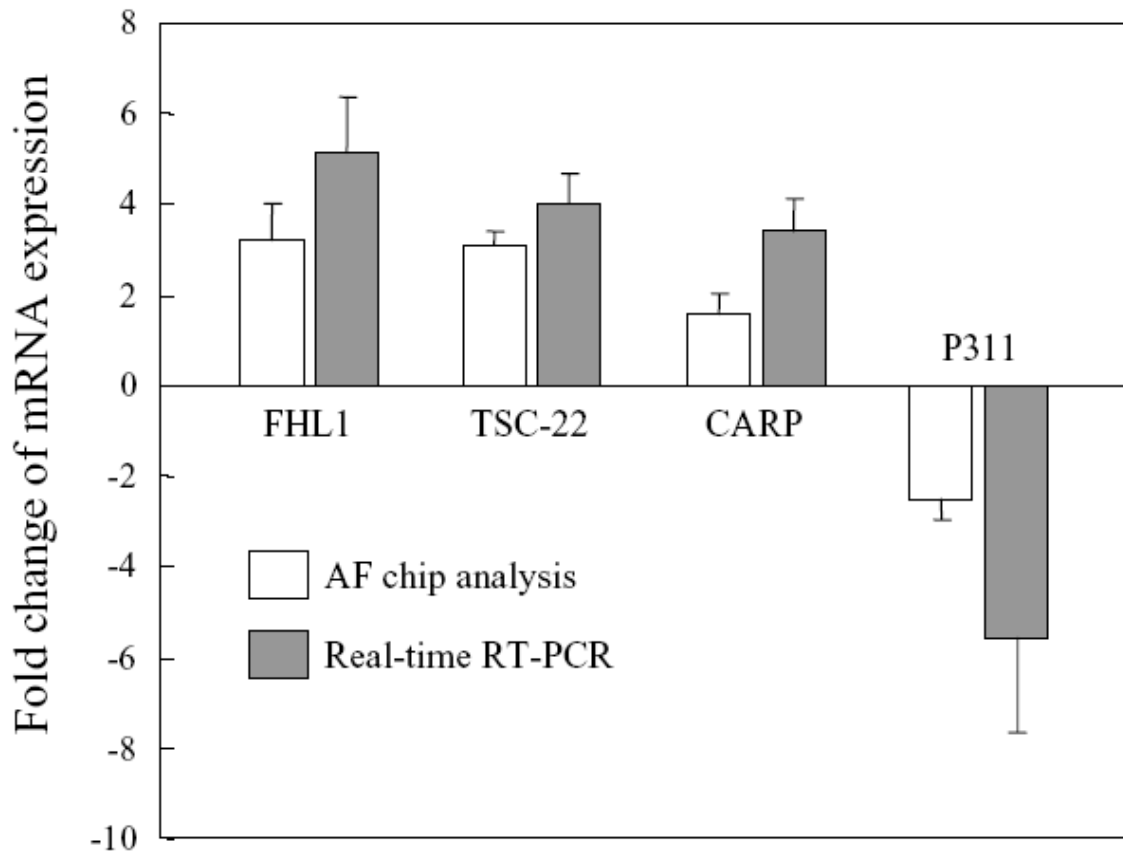
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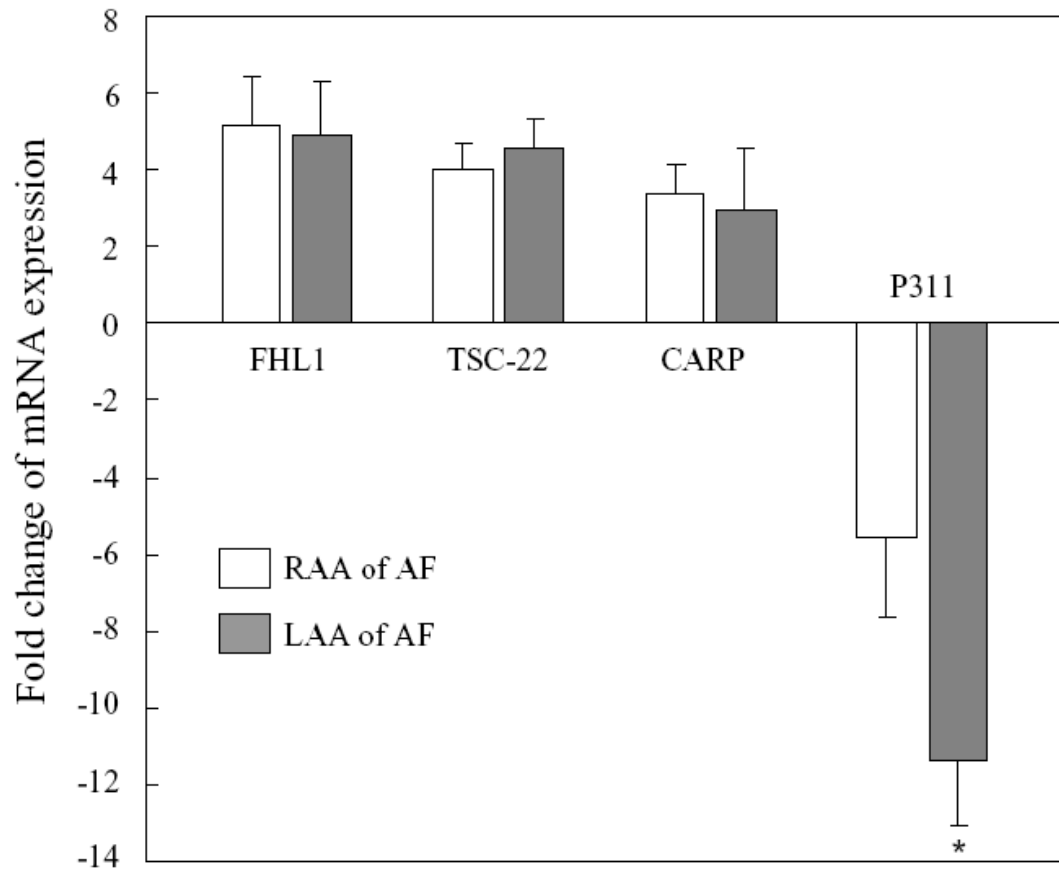
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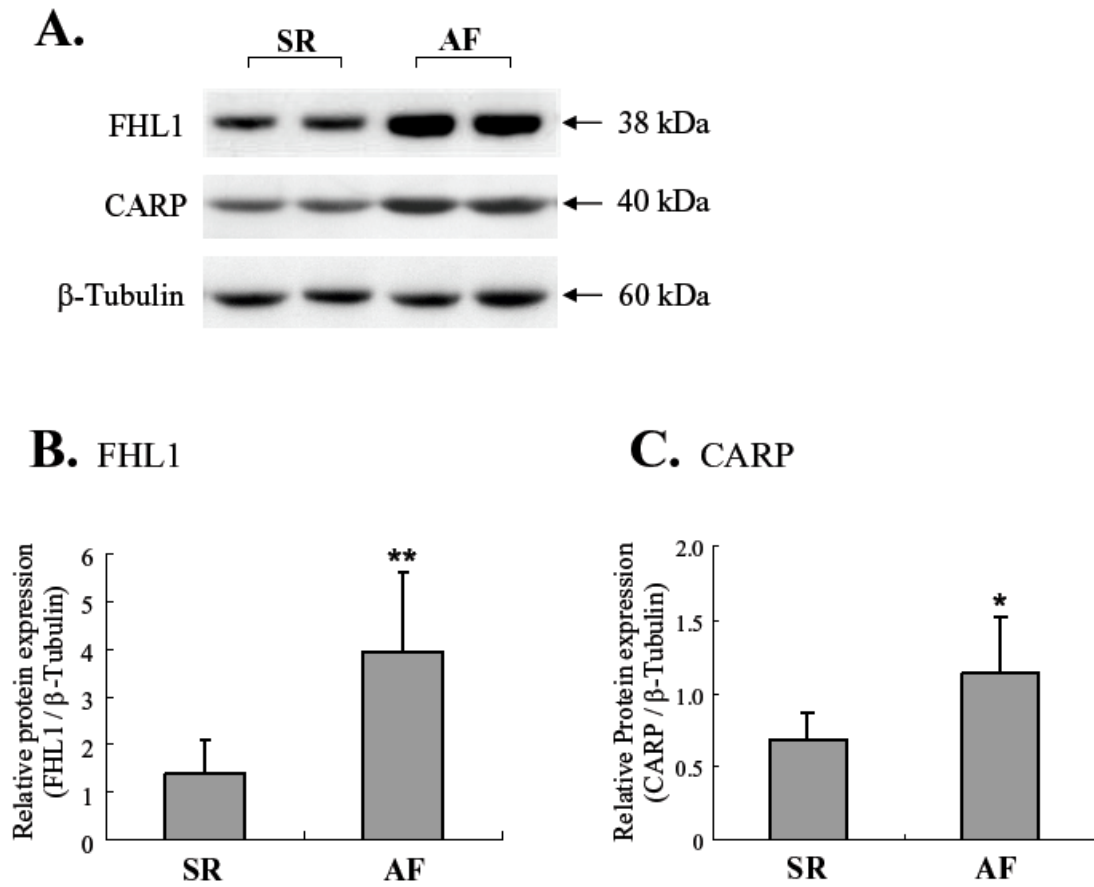
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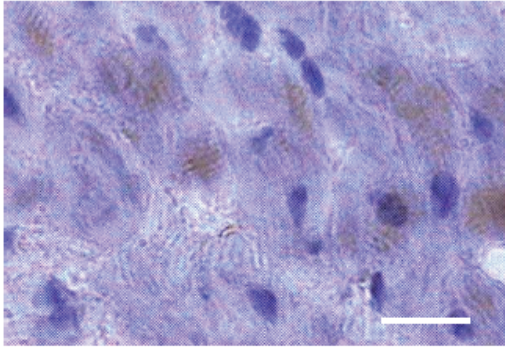
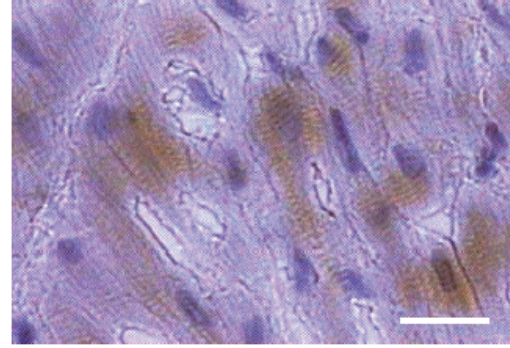
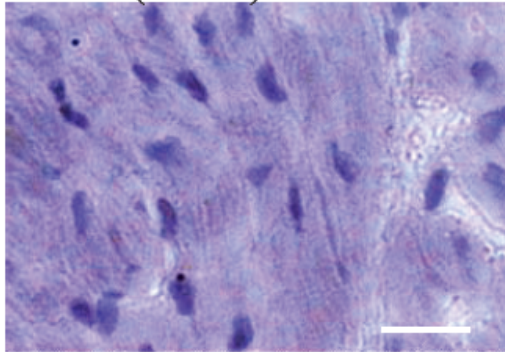
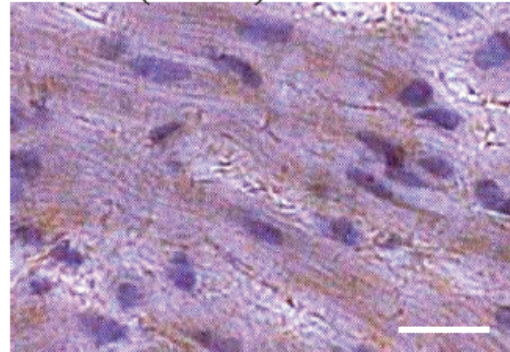
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**A. SR (FHL1)****B. AF (FHL1)****C. SR (CARP)****D. AF (CARP)**

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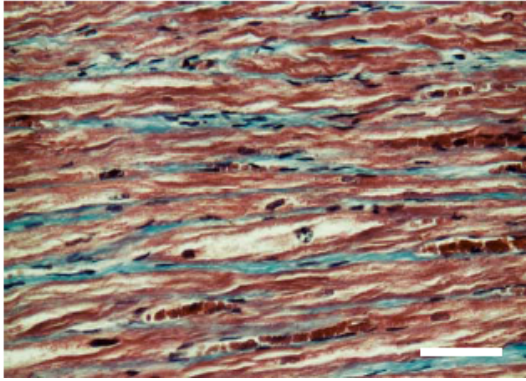
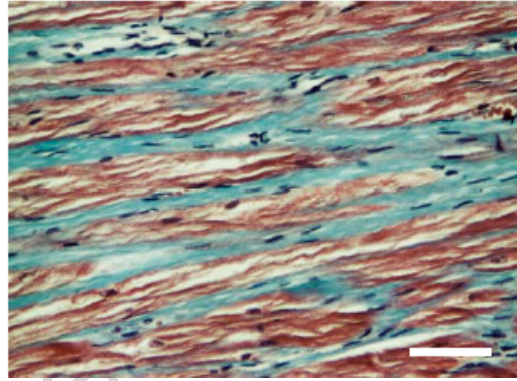
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**A. SR****B. AF**

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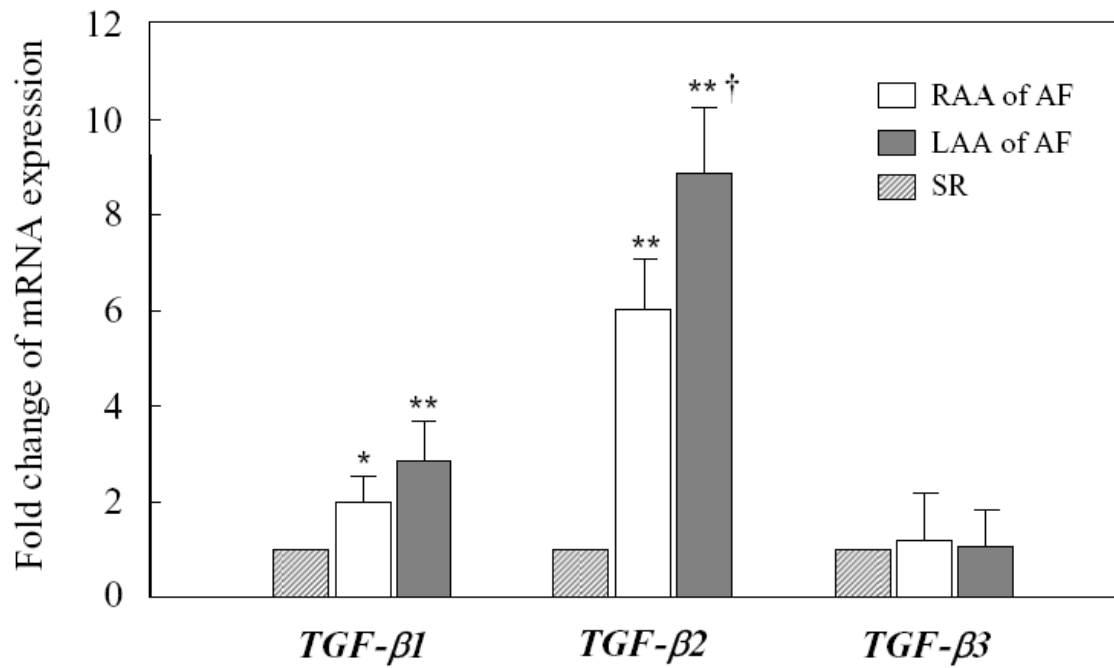
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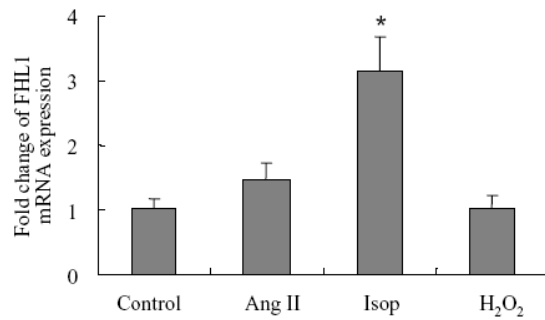
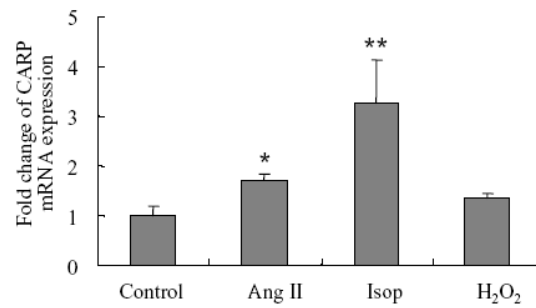
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**A.** FHL1**B.** CARP

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