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1 **Mineralocorticoid Receptor Overexpression in Embryonic Stem Cell**
2 **Derived Cardiomyocytes Increases Their Beating Frequency**

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26 **Abstract**

27

28 **Aims**

29 Cardiac Mineralocorticoid Receptor (MR) activation triggers adverse cardiovascular
30 events that could be efficiently prevented by mineralocorticoid antagonists. To gain
31 insights into the pathophysiological role of MR function, we established embryonic
32 stem (ES) cell lines from blastocysts of transgenic mice overexpressing the human
33 MR driven by its proximal P1 or distal P2 promoter and presenting with
34 cardiomyopathy, tachycardia and arrhythmia. Cardiomyocyte differentiation allowed
35 us to investigate the molecular mechanisms contributing to MR-mediated cardiac
36 dysfunction.

37

38 **Methods and Results**

39 During cardiac differentiation, wild-type (WT) and recombinant ES cell cultures and
40 excised beating patches expressed endogenous MR along with cardiac gene
41 markers. The two-fold increase in MR protein detected in P1.hMR and P2.hMR
42 cardiomyocytes led to a parallel increase of the spontaneous beating frequency of
43 hMR-overexpressing cardiomyocytes compared to WT. The MR-mediated
44 chronotropic effect was ligand-independent, could be partially repressed by
45 spironolactone and was accompanied by a significant 2- to 4-fold increase in mRNA
46 and protein levels of the pacemaker channel HCN1, generating depolarizing I_f
47 currents, thus revealing a potential new MR target. This was associated with
48 modification in the expression of HCN4, the inward rectifier potassium channel Kir2.1
49 and the L-Type dependent calcium channel Cav1.2.

50

51 **Conclusion**

52 We demonstrate that the amplification of MR signaling in ES-derived cardiomyocytes
53 has a major impact on cardiomyocyte contractile properties through an important
54 remodelling of ion channel expression contributing to arrhythmias. Our results
55 highlight the prominent role of MR function in cardiac physiology and support the
56 benefit of MR antagonists in the management of cardiac dysfunctions.

57

57 **Introduction**

58 The Mineralocorticoid Receptor (MR or NR3C2) is a nuclear receptor exerting
59 various pleiotropic actions on a wide series of target tissues. MR acts as a ligand-
60 dependent transcription factor, and is involved in numerous physiological processes
61 and pathological conditions¹. MR is expressed in many components of the
62 cardiovascular system such as blood vessels², endothelial cells³, cardiomyocytes⁴,
63 vascular smooth muscle cells⁵ and macrophages⁶. The importance of cardiac MR
64 has been strikingly underscored by several direct and indirect evidences. Indeed,
65 RALES and EPHESUS clinical trials have demonstrated the major benefit of
66 mineralocorticoid antagonist (spironolactone and eplerenone) administration on the
67 heart failure patient's survival⁷⁻⁸. MR can be activated both by mineralo- and
68 glucocorticoid hormones but selectivity-conferring mechanisms, mostly dependent
69 upon the cellular context¹, have been described. Most notably in tight epithelia, the
70 large excess of glucocorticoids is metabolized into inactive compounds by the 11
71 beta hydroxysteroid dehydrogenase type 2 (11 β HSD2) enzyme⁹, preventing an illicit
72 occupation of the receptor. Although glucocorticoids are most likely the natural MR
73 ligands in cardiomyocytes, which lack 11 β HSD2, they seem to be unable to fully
74 activate the receptor¹⁰. Indeed, Transgenic mice ectopically expressing 11 β HSD2 in
75 cardiomyocytes exhibit cardiac hypertrophy, fibrosis and heart failure, but no
76 hypertension, this phenotype being reversed by eplerenone¹¹. These findings in an
77 animal model where the receptor is almost exclusively activated by aldosterone
78 underline the deleterious effect of inappropriate MR activation.

79 On the other hand, MR gene inactivation in the mouse leads to early post-natal
80 lethality caused by salt loss¹² which can be rescued by daily NaCl injections followed
81 by high salt diet¹³. MR^{-/-} mice have no cardiovascular abnormalities, notwithstanding

82 the expected activation of the renin-angiotensin system¹⁴, thus excluding a crucial
83 role of MR during cardiac development.

84 In order to better understand MR-dependent pathophysiological processes *in vivo*,
85 our group and others have exploited alternative strategies of MR overexpression in
86 the heart of transgenic mice¹⁵⁻¹⁶. In particular, we have generated a murine model in
87 which the proximal P1 promoter of the human *MR* (hMR) was used to drive the
88 expression of its own cDNA. P1.hMR animals express the transgene in most MR
89 target tissues including kidney, brain and heart. Interestingly, two extensively studied
90 mouse lines exhibited a moderate dilated cardiomyopathy associated with
91 arrhythmia, but without hypertension or cardiac fibrosis¹⁵ in contrast with aldosterone-
92 high salt diet animal models¹⁷. In another mouse model, conditional MR
93 overexpression in the heart triggers cardiac hypertrophy and life-threatening
94 ventricular arrhythmias¹⁶. All these *in vivo* studies underlined a specific role of MR in
95 cardiomyocyte contractile properties. However, it cannot be excluded that transgene
96 expression might induce some adaptive and compensatory mechanisms secondary
97 to various feedback regulatory loops prevailing *in vivo*. The utilization of
98 cardiomyocytes isolated from animal models would thus facilitate analysing the
99 specific MR effects, regardless of the compensatory factors. Along similar lines, it
100 has been also reported that MR/aldosterone have major effects on cardiomyocyte
101 contraction frequency associated with an increased expression of T-type (Cav3.2)
102 and L-type (Cav1.2) calcium channels, consequently augmenting Ca²⁺ currents in
103 isolated rat ventricular myocytes^{18,19}. However, such cell-based systems are quite
104 difficult to obtain in rodents, usually give poor yield, and often lead to highly variable
105 results.

106 To better comprehend MR actions in the heart, we decided to use an alternative
107 approach that consists in deriving cardiomyocytes from transgenic animals which
108 allows a fine tuned control of experimental conditions. A more effective strategy,
109 already validated by several groups (²⁰ for review) is based on the utilization of the
110 embryonic stem (ES) cells that can be indefinitely expanded at the undifferentiated
111 stage and, under appropriate conditions, are able to differentiate into cell types
112 originating from the three germ layers (endoderm, mesoderm, ectoderm) including
113 cardiomyocytes²¹.

114 In this study, we established ES cell lines derived from hMR-overexpressing mice
115 that can undergo highly efficient differentiation into cardiomyocytes. Our cell-based
116 models permit not only to investigate a potential involvement of MR and/or
117 aldosterone during cardiomyocyte development but also to discriminate MR-
118 dependent actions from those induced by various ligands. Herein, we show that hMR
119 overexpression leads to an increase of the beating frequency in ES derived-
120 cardiomyocytes. We demonstrate that this is associated with an increase of the
121 expression of the pacemaker channel HCN1 and with an altered expression of
122 calcium and potassium cardiac ion channels involved in cardiomyocyte contractility.

123

123 **Materials and Methods**

124

125 **Derivation and culture of ES cell lines**

126 To generate ES cell lines, P1 or P2.hMR females were crossed with males from the
127 129 strain (Charles River, L'Arbresle, France) and checked daily for vaginal plugs.
128 Mice were cared according to the Guide for the Care and Use of Laboratory Animals
129 published by the US National Institutes of Health (NIH Publication No. 85-23, revised
130 1996). The animal facility was granted approval (N° B94-043-12), with an
131 authorization to experiment on living animals (75-978, ML) given by the French
132 Administration. At day 3.5 *post-coitum*, females were sacrificed, the uterus removed
133 and flushed with culture medium. Blastocysts were picked up with a mouth pipette
134 and plated in a 6 cm Petri dish on a SNL feeder cell layer. After 3 more days each
135 inner cell mass was recovered and plated in a well of a 24-well plate with SNL.
136 Cultures were split 1:1 every 3 days and an ES cell line was successfully derived in
137 approximately 20% of the attempts. Undifferentiated ES cells were cultured and
138 amplified as previously described²². See supplemental methods for details.

139

140 **Cardiac differentiation**

141 Cardiac differentiation was based on the hanging drop method²³. Drops containing
142 400 cells were grown hanging on a Petri dish lid for 3 days in ES cell medium
143 containing 20% fetal calf serum. The embryoid bodies (EB) were then cultured for 2
144 days in the same medium (alternatively the serum was dextran-coated charcoal
145 (DCC) stripped of steroids for hormone experiments) complemented with 1 μ M
146 ascorbic acid and 0.5% DMSO (cardiac differentiation medium). EB were then

147 seeded in gelatinized Petri dishes or culture plate wells and allowed to differentiate 5
148 to 15 days. Spontaneously beating areas were excised with a scalpel blade.

149

150 **RT-PCR**

151 RNA from ES, EB and beating patches were extracted with the Trizol reagent
152 (Invitrogen, Cergy Pontoise, France) according to the manufacturer's instructions.
153 After DNase I treatment, one μ g total RNA was reverse-transcribed with the
154 MultiScribe reverse transcriptase kit (Applied Biosystems, Courtaboeuf, France).
155 Semi-quantitative PCR were performed using the Taq Polymerase kit (Invitrogen)
156 and real-time quantitative PCR with the Power SYBR Green® PCR Master mix
157 (Applied Biosystems). For the latter, a standard curve was obtained with serial
158 dilutions of an amplicon subcloned in pGEMTeasy vector (Promega, Charbonnières,
159 France). Primers are listed in Supplemental Table 1.

160

161 **Immunocytochemistry**

162 Excised beating patches were digested in DMEM with 1 mg/ml collagenase (Sigma)
163 for 30 min with occasional shaking in a microcentrifuge tube, then spun down at 2000
164 rpm for 5 min. Collagenase was removed and patches were digested for 20 min in
165 trypsin-EDTA (invitrogen), spun down, resuspended with cardiac differentiation
166 medium, plated in 0.1% gelatin-coated LabTek (Nunc, Rochester, NY, USA) or
167 coverslips and incubated overnight. See supplemental methods for details.

168

169 **Western blot**

170 Total protein extracts were prepared from wild type and transgenic cardiomyocytes,
171 15 μ g of proteins from cardiomyocyte homogenates were processed for

172 immunoblotting. See supplemental methods for details. Immunoblots were blocked
173 with TBST 0.5% non fat milk and incubated overnight with 1/1000 anti-MR 39 N²⁴ or
174 1/300 anti-HCN1 (AB5884, Millipore) or 1/15,000 anti- α -tubulin (Sigma) as an internal
175 standard. Quantitative analysis was performed using QuantityOne software (Bio-Rad
176 Laboratory, Inc., Hercules, CA).

177

178 **Statistical analysis**

179 Results represent mean \pm SEM with at least 6 independent determinations for each
180 condition. Statistical analyses were performed using a non parametric Mann-Whitney
181 test (Prism4, Graphpad Software, Inc., San Diego, CA).

182

182 **Results**

183 **Generation of WT and hMR-overexpressing ES cell lines**

184 We have previously generated transgenic mouse models using 1.2 kb of the proximal
185 (P1) and 1.8 kb of the distal (P2) hMR promoter to drive the hMR cDNA expression
186 as previously described^{15, 25}. Figure 1A illustrates the schematic representation of the
187 transgenes inserted into P1.hMR and P2.hMR transgenic mouse genome. Both
188 constructs contained an untranslated region as well as part of the human β globin
189 sequences to stabilize the transgene transcripts. As expected, we confirmed by RT-
190 PCR using species-specific primers that the recombinant hMR was expressed in the
191 heart of both transgenic P1.hMR and P2.hMR mouse lines (Fig 1B), demonstrating
192 that these animals were suitable to investigate the functional impact of cardiac MR.
193 P1 and P2.hMR female mice were backcrossed with strain 129 males characterized
194 by a high success rate of ES cell derivation from blastocysts²⁶ (Fig 1C, see
195 Supplemental methods). Several ES cell lines were established and genotyped as
196 wild type (WT), P1.hMR or P2.hMR (Fig 1D). Further experiments were mostly
197 performed on one representative ES cell line of each genotype.

198

199 **MR expression during cardiac differentiation of ES cells**

200 We optimized a protocol of cardiac differentiation based on the hanging drop
201 method²⁰. The different steps of ES cell cardiac differentiation are represented in Fig
202 2A. As shown in the upper panel (ES), undifferentiated ES cells grew as compact
203 clusters on the feeder cell layers. Suspension culture of 400 undifferentiated ES cells
204 per drop led to standardized size embryoid bodies (EB) of approximately 200 μ m in
205 diameter at day 3 (EB, middle upper panel). These spherical-shaped structures
206 contained cells that are able to differentiate into the three germ layers. From day 5,

207 cultures were grown in adherence in gelatinized Petri dishes and from day 7 onward
208 spontaneously beating areas arose, enlarging with time (D18 culture, middle bottom).
209 Beating areas containing a tight network of cardiomyocytes were excised with a
210 scalpel blade around day 16 to 18 (Patch, bottom panel) and were further used for
211 gene expression studies or immunodetection experiments. We defined an index of
212 cardiac differentiation by assessing the percentage of cultures originating from a
213 single EB presenting spontaneously beating cardiomyocytes. Approximately 70% of
214 EB presented beating areas at day 18. No significant variation in the cardiac
215 differentiation efficacy has been found among cell lines (see Fig 4).

216 The temporal expression pattern of early and late cardiac marker genes was
217 analyzed concomitantly with recombinant (hMR) and endogenous MR (mMR) by RT-
218 PCR. Fig 2B presents data obtained from day 0 to 7 cultures and Fig 2C shows data
219 from day 18 excised patches. Transgene expression in the recombinant cells was
220 detected at all stages of cardiac differentiation and, importantly, a strong signal was
221 found in excised patches. Of note, the progressive appearance of early (NKx2.5) and
222 late (α MHC) cardiac marker gene expression was observed along differentiation as
223 early as day 4 and 7, in all cell lines while late markers such as α MHC and Troponin
224 T transcripts were expressed in day 18 excised patches. Quantification of mMR
225 mRNA levels by qPCR showed an approximately ten-fold expression increase in day
226 18 cardiomyocytes than in earlier differentiation stages (D0 undifferentiated ES cells
227 or D7 EB) (Fig 2D).

228 We next analyzed MR expression at the protein level in cardiac differentiation. We
229 used an anti-MR antibody recognizing both the endogenous murine MR and
230 recombinant hMR²⁴. Western blot analyses of day 12 cardiomyocyte cultures
231 revealed an approximately two-fold increase in MR expression in the P1.hMR

232 cardiomyocytes as compared to the WT cells (Fig 3A and 3B). Confocal microscopy
233 imaging confirmed the coexpression of MR and α -sarcomeric actin in the
234 cardiomyocytes of each genotype detected by double immunolabelling (Fig 3C). Note
235 the nucleocytoplasmic distribution of MR and the stronger signal in P1.hMR and
236 P2.hMR cardiomyocytes. Thus, these ES cell-derived cardiomyocytes provided an
237 effective cell-based system to investigate the functional consequences of hMR
238 overexpression in cardiomyocytes.

239

240 **MR overexpression causes an increase in cardiomyocyte beating frequency**

241 We first tested a potential effect of MR overexpression and aldosterone exposure on
242 cardiac differentiation efficiency (See Fig 4 and Supplemental Fig S1). Since there
243 was no modification of the differentiation index compared to control conditions, we
244 excluded a major impact of MR signaling in early cardiac development. This
245 assumption seems to be supported by the lack of morphological alterations in the
246 cardiovascular system of MR KO newborn mice¹².

247 In order to better understand the role of MR in cardiac function, we examined the
248 influence of hMR overexpression on cardiomyocyte contractile properties (Fig 5). The
249 beating rate of day 14-18 cultures was assessed by video capture. We found a highly
250 significant increase of the beating frequency in transgenic P1.hMR and P2.hMR
251 cardiomyocytes compared to WT (Fig 5A, WT: 1.09 ± 0.2 Hz $n=33$, P1.hMR: 1.8 ± 0.3
252 Hz $n=12$, P2.hMR: 1.7 ± 0.5 Hz, $n=31$, $P < 0.005$, and see Supplemental videos). This
253 increase in the spontaneous beating frequency of hMR-overexpressing
254 cardiomyocytes has been reproduced in several differentiation experiments and has
255 been confirmed in two different ES cell lines with the same genotype. Owing to the
256 chronotropic effect of β -adrenergic stimulation, we observed a significant increase in

257 the beating frequency of both WT and transgenic cardiomyocytes upon isoproterenol
258 exposure (Fig 5B). A 2.2-fold induction of isoproterenol-induced stimulation was
259 found in the WT cardiomyocytes while the amplitude of catecholamine-stimulated
260 beating frequency was only 1.4-fold in P1.hMR and P2.hMR cardiomyocytes,
261 suggesting that amplification of MR activation might somehow compromise
262 cardiomyocyte β -adrenergic signaling. Under these experimental conditions, the
263 adrenergic-stimulated beating frequency remained significantly higher in hMR-
264 overexpressing cardiomyocytes than in WT ES derived cells, providing additional
265 support for a primary role of MR on cardiomyocyte contractile properties.

266 An important issue was whether the increase in the hMR-driven beating frequency
267 depended upon the presence of the ligand. To test this hypothesis, ES cells were
268 submitted to the cardiac differentiation process using DCC serum, in the presence or
269 absence of 10 nM aldosterone for 24 h before video capture. As shown in Fig 5C,
270 beating rate analysis indicated that, hMR overexpression in cardiomyocytes caused a
271 striking chronotropic effect (WT=0.25 \pm 0.075 Hz, P1.hMR=1.0 \pm 0.25 Hz, $P < 0.05$),
272 even in steroid-free medium. On the other hand, aldosterone treatment induced a
273 significant increase of the beating frequency of WT cardiomyocytes
274 (WT+Aldo=0.91 \pm 0.5 Hz) compared to untreated cells but was unable to accelerate
275 further the already higher spontaneous contraction of P1.hMR cardiomyocytes.
276 Interestingly, the beating frequency of P1.hMR cardiomyocytes differentiated in DCC
277 medium could be reduced with 100 nM spironolactone (Fig 5D), suggesting that MR
278 blockade might reverse the MR-mediated positive chronotropic effect. Taken
279 together, these data show that MR overexpression *per se* is a potent regulator of
280 cardiomyocyte chronotropy, and that MR-induced increase of cardiomyocyte
281 contractility is at least partially independent of the ligand.

282

283 **hMR overexpression alters cardiomyocyte ion channel expression**

284 Cardiomyocyte contractions are tightly regulated by many ion channels and pumps²⁷.
285 We therefore investigated the expression of several key regulators of cardiac
286 contractility to decipher the underlying mechanisms associated with the positive
287 chronotropic phenotype of MR-overexpressing cardiomyocytes. The
288 hyperpolarization-activated cyclic nucleotide gated potassium channels (HCN) were
289 especially good candidates since they participate to the occurrence of the pacemaker
290 currents (I_f) initiating the depolarization process²⁸. We showed that the HCN1
291 channel mRNA level almost doubled in excised beating patches of P1.hMR
292 cardiomyocytes compared to WT controls (Fig 6A). Western blot analysis confirmed
293 a 4-fold increase in HCN1 channel expression in hMR-overexpressing
294 cardiomyocytes (Fig 6B), providing evidence for a direct relationship between the
295 expression of this pacemaker channel and the MR-increased cardiomyocyte beating
296 rate.

297 We next analyzed the relative abundance of other calcium and potassium channels
298 (Fig 7). Unexpectedly, we found that mRNA levels of another pacemaker channel,
299 HCN4, were repressed by 75% in hMR-overexpressing cardiomyocytes (Fig 7A),
300 suggesting a counter-regulatory mechanism to dampen the depolarization process
301 due to HCN1 up-regulation. Interestingly, we also demonstrated a 2-fold increase of
302 the inward rectifier potassium ion channel Kir2.1 expression in P1.hMR
303 cardiomyocytes (Fig 7B), which was accompanied by a parallel increase of the L-
304 Type voltage dependent calcium channel Cav1.2 mRNA levels (Fig 7C). Collectively,
305 hMR overexpression in ES cell-derived cardiomyocytes leads to a major alteration of
306 the expression of several ion channels, all involved in cardiomyocyte contractility.

307 These results give a rationale for the faster contraction frequency observed in hMR-
308 overexpressing cardiomyocytes strongly supporting the notion that MR signaling is a
309 pivotal regulator of cardiomyocyte function.

310

310 **Discussion**

311 In the present work, we have successfully developed new cellular models to study
312 cardiac MR function by means of ES cell-derived cardiomyocytes. We show that
313 hMR overexpression during cardiac differentiation leads to a sharp increase of
314 spontaneous cardiomyocyte beating frequency associated with the increase of the
315 pacemaker channel HCN1 expression as well as that of the L-type voltage
316 dependent calcium channel Cav1.2 and the inward-rectifier potassium ion channel
317 Kir2.1.

318 Myocardial contraction originates from the sino-atrial node and Purkinje fibers, where
319 HCN pacemaker channels initiate the spontaneous depolarization of cardiomyocytes
320 by the I_f current followed by the action of T-type and L-type calcium currents²⁹.
321 Cardiomyocyte differentiation from ES cells may generate cells that exhibit these
322 electrical properties along with that of the atrium³⁰, thus providing interesting cell-
323 based models to investigate the implication of key factors involved in cardiomyocyte
324 functions.

325 Although MR activation and aldosterone action in the heart seem to play critical roles
326 in the pathogenesis of several cardiac diseases, the precise molecular events
327 leading to cardiac hypertrophy, fibrosis and arrhythmia remain elusive and both the
328 relative contribution of MR and the exact nature of the endogenous activating ligands
329 are far from being well understood³¹. Several recent studies provided interesting new
330 insights in this field. Aldosterone exposure was shown to drastically increase
331 contraction frequency of isolated rat ventricular myocytes, associated with an
332 increase of the expression of L-type channel Cav1.2 and T-type channel Cav3.2¹⁸⁻¹⁹.
333 This result paralleled the data obtained with our ES cell-derived cardiomyocytes.
334 Similarly, aldosterone treatment was reported to accelerate the spontaneous beating

335 rate of neonatal rat ventricular cardiomyocytes by increasing If currents due to
336 enhanced expression of HCN4 channel at both mRNA and protein level³².

337 Our work strongly supports a central role of MR in cardiomyocyte contractibility and
338 ion channel expression. This should be discussed in view of previous studies
339 demonstrating that the electrical remodeling of Ca²⁺ and K⁺ currents and the
340 modification of channel expression in an experimental myocardial infarction rat model
341 occur prior to cardiomyocyte hypertrophy and are prevented by MR antagonists³³. As
342 a whole, our data show that MR overexpression in ES cell-derived cardiomyocytes
343 partially mimics aldosterone effects, inducing a positive chronotropic effect
344 associated with alterations of the expression of several ion channels, however our
345 results differ from previous reports under many aspects.

346 Interestingly, the increased beating frequency associated with MR overexpression
347 was also observed using DCC medium as early as day 3 of differentiation,
348 suggesting that cardiac MR is at least partially activated in a ligand-independent
349 manner. Under such experimental conditions, we showed that spironolactone
350 reduced spontaneous contraction of MR-overexpressing cardiomyocytes, providing
351 additional supports for the beneficial effect of anti-mineralocorticoid compounds on
352 MR-related cardiac arrhythmias. This finding is reminiscent of a recent proposal of a
353 ligand-independent activation of MR by Rac1 GTPase, responsible for deleterious
354 renal consequences, linking activation of MR signaling in podocytes, renal failure and
355 proteinuria³⁴. In any case, the exact mechanisms involved in the cardiac MR-
356 mediated activation remain to be further investigated, most notably because of major
357 pharmacological perspectives.

358 ES cell-derived cardiomyocyte models likely differ from the neonatal ventricular
359 cardiomyocytes exploited by other groups, accounting for some differences between

360 our work and other studies. Murine ES cell cardiac differentiation lasts approximately
361 two and half weeks and we hypothesize that ES cell-derived cardiomyocytes might
362 represent an earlier stage of differentiation than isolated neonatal ventricular
363 myocytes. Indeed, a recent study reported the temporal expression pattern of ion
364 channels involved in contractility during ES cell cardiac differentiation³⁵. It was shown
365 that the pacemaker cells maintained the expression of HCN channels during cardiac
366 differentiation while a ventricular-like phenotype was associated with a slight increase
367 in Kir2.1 rectifier potassium channel expression. We propose that our model of ES
368 cell-derived cardiomyocytes might correspond to an intermediate differentiation stage
369 exhibiting a pacemaker cell phenotype.

370 One of our main findings is that hMR overexpression in ES-derived cardiomyocytes
371 leads to the up-regulation of HCN1 pacemaker channel expression while the HCN4
372 channel expression is down-regulated. This could represent a compensatory
373 mechanism due to HCN1 up-regulation. It has also been proposed that in murine ES
374 cell-derived cardiomyocytes, HCN1 is a fast component while HCN4 is a slow
375 component of the If current³⁶. This could potentially explain the resulting positive
376 chronotropic effect we observed. Of note, the conditional deletion of HCN4 in adult
377 mouse revealed that HCN4 is not directly involved in heart rate acceleration but
378 rather provides a depolarization reserve³⁷, excluding a pivotal role of HCN4 in
379 autonomous cardiomyocyte contractility.

380 However, as expected, we could not find any difference in HCN1 expression in the
381 heart of the parental WT and P1.hMR adult mice (data not shown) since HCN1 is
382 predominantly, if not exclusively, expressed in the sinus node and because it is well
383 established that the atrial HCN1 expression gradually diminished in the postnatal

384 period³⁸. This finding strengthens the advantage of our *in vitro* ES system to unravel
385 MR-mediated ionic channel remodeling during cardiomyocyte differentiation.

386 HCN channels are cyclic nucleotide-gated channels whose activity is dependent
387 upon the intracellular cAMP concentrations that are regulated by the beta-adrenergic
388 signaling³⁹. Interestingly, the heart beating rate of embryos with a HCN4 mutant that
389 is unable to bind cAMP was not accelerated upon adrenergic stimulation, providing a
390 functional linking between pacemaker channel HCN4 and catecholamine
391 responses⁴⁰. The down-regulation of HCN4 in hMR-overexpressing cardiomyocytes
392 is associated with a decrease of β 1-adrenergic receptor expression (Supplemental
393 Fig S2). We believe that both molecular events may account for the attenuated
394 responses to isoproterenol stimulation.

395 The mechanisms by which MR regulates the expression of HCN1, as well as other
396 Cav1.2 and Kir2.1 ion channels remain to be defined. However, we identified several
397 corticosteroid response element half-sites within the 0.9 kb of mouse HCN1 promoter
398 (MatInspector online software, Genomatix), suggesting the possibility of a direct
399 transcriptional control of ion channel genes by MR that remains to be studied.

400 Beside the obvious role of HCN1 channel in the control of cardiomyocyte beating
401 frequency, we cannot exclude the involvement of other molecular mechanisms in the
402 positive chronotropic effect of MR overexpression such as modifications in the cardiac
403 calcium handling through alteration of the intrinsic functional properties of ryanodine
404 receptor as recently proposed⁴¹.

405 In conclusion, we demonstrate that amplification of MR activation in ES-derived
406 cardiomyocytes leads to chronotropic responses associated with cardiac ion channel
407 alterations contributing to arrhythmias. Our results underscore the pivotal role of MR
408 in the homeostasis of cardiac contractibility and provide further support for the benefit

409 of mineralocorticoid antagonist treatments in the management of cardiac
410 dysfunctions⁴².

411

411 **Figure legends**

412

413 **Figure 1**

414 **Generation of ES cell lines.**

415 A) Schematic representation of P1.hMR and P2.hMR transgenes. The *HindIII-AvaI* (-
416 969, +239) fragment of P1 promoter and the *SspI-SspI* (-1682, +123) fragment of P2
417 promoter have been used to target the expression of hMR cDNA in transgenic
418 mice¹⁵. B) Endogenous (mMR) and recombinant MR (hMR) mRNA expression in the
419 heart of WT, P1 and P2.hMR mice were detected by RT-PCR with species specific
420 primer set. Amplification of the β -actin was used as an internal control. C) The ES cell
421 lines were derived from blastocysts recovered 3.5 days post coitum from P1 or
422 P2.hMR females crossed with the 129 strain males. D) Genotyping of various ES cell
423 lines using the rapsn as an internal genomic PCR control.

424

425 **Figure 2**

426 **Characterization of ES cell lines during cardiac differentiation.**

427 A) ES cell differentiation: Top panel, (ES), undifferentiated ES cells; Middle top panel,
428 (EB), D3 embryoid bodies; Middle bottom panel, D18 culture; Bottom panel, excised
429 spontaneously beating patch. Scale bar = 50 μ m. B and C) RT-PCR of hMR, mMR
430 and cardiac marker genes (B) on Day 0, 4, 7 cultures and (C) on D18 excised
431 beating patches; α MHC: α myosin heavy chain; Troponin T. D) Relative
432 expression of endogenous mouse MR expression at D0 and D7 cultures and D18
433 excised beating patches measured by quantitative real-time PCR. Results are
434 expressed in amol mMR/ fmol 18S transcripts, and are means \pm SEM of triplicates
435 from WT, P1.hMR and P2.hMR differentiation.

436

437 **Figure 3**

438 **hMR overexpression in ES cell-derived cardiomyocytes.**

439 A) Western blot on WT and P1.hMR cardiomyocyte lysates with anti-MR 39N
440 antibody and α -tubulin used as an internal loading control. A specific band for MR is
441 detected at ~130 kDa. B) MR protein levels were quantified and normalized to those
442 of α -tubulin using QuantityOne software (BioRad). Results are means \pm SEM of 6
443 independent determinations and are expressed relative to MR expression measured
444 in WT, arbitrarily set at 1. Statistical significance: *, $P < 0.05$. C) Confocal imaging of
445 immunofluorescence experiments with α -sarcomeric actin antibody (green, left
446 panels) and MR antibody (red, middle panels), double labeling (merged, right
447 panels), in WT, P1.hMR, P2.hMR enzymatically dissociated beating patches. X40
448 magnification.

449

450 **Figure 4**

451 **Index of cardiac differentiation is not modified by MR overexpression.**

452 Cardiac differentiation index of WT and hMR-overexpressing (hMR) cultures, means
453 \pm SEM of 4 and 5 experiments, respectively. Data represent the percentage of
454 cardiac differentiation that contains beating areas in a 24-well plate over time (day 0,
455 7, 10, 12, 18). Each differentiation arose from a single EB.

456

457 **Figure 5**

458 **hMR overexpression increases cardiomyocyte beating frequency.**

459 Beatings are recorded by video capture for more than 30 s between days 14 and 18
460 (see Supplemental Videos). A) WT, P1 and P2 cardiomyocyte differentiation cultures.

461 (WT: 1.09 ± 0.2 Hz, n=33; P1.hMR: 1.8 ± 0.3 Hz, n=12, P2.hMR: 1.7 ± 0.5 Hz, n=31;
462 ***, $P < 0.005$). B) Effect of β -adrenergic stimulation. Cardiomyocytes were exposed
463 to 1 μ M isoproterenol (Iso) for 15 min. **, $P < 0.01$. C) Effect of 10 nM aldosterone
464 treatment (Aldo) or vehicle on the beating frequency of WT and P1.hMR
465 cardiomyocytes grown in steroid-stripped medium; **, $P < 0.01$; *, $P < 0.05$. D) Effect of
466 100 nM spironolactone treatment (spiro) or vehicle on WT and P1.hMR
467 cardiomyocytes in steroid stripped medium. *, $P < 0.05$. **, $P < 0.01$.

468

469 **Figure 6**

470 **Pacemaker channel HCN1 expression is increased in P1.hMR cardiomyocytes.**

471 A) Relative expression of HCN1 transcripts in excised beating patches of WT and
472 P1.hMR Day 16 cardiomyocytes was determined by qPCR. Results, normalized by
473 the amplification of 18S RNA, are means \pm SEM of 3 experiments performed in
474 triplicate and are expressed relative to the WT value arbitrary set at 1. **, $P < 0.01$. B)
475 Western blot analysis of HCN1 expression in WT and P1.hMR day 12
476 cardiomyocytes with HCN1 antibody, α -Tubulin was used as a loading control.
477 Results are means \pm SEM of 6 independent determinations and are expressed
478 relative to HCN1 expression in WT, arbitrarily set at 1. *, $P < 0.05$.

479

480 **Figure 7**

481 **Altered expression of ion channels in hMR-overexpressing cardiomyocytes.**

482 Relative expression of ion channel transcripts in WT and P1.hMR Day 16
483 cardiomyocytes was determined by qPCR. Results normalized by the amplification of
484 18S RNA are means \pm SEM of 2 or 3 experiments performed in triplicate and are
485 expressed relative to the WT value arbitrarily set at 1. A) Relative HCN4 expression,

486 ***, $P < 0.005$. B) Relative Kir2.1 expression, *, $P < 0.05$. C) Relative Cav1.2
487 expression, **, $P < 0.01$.

488

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666

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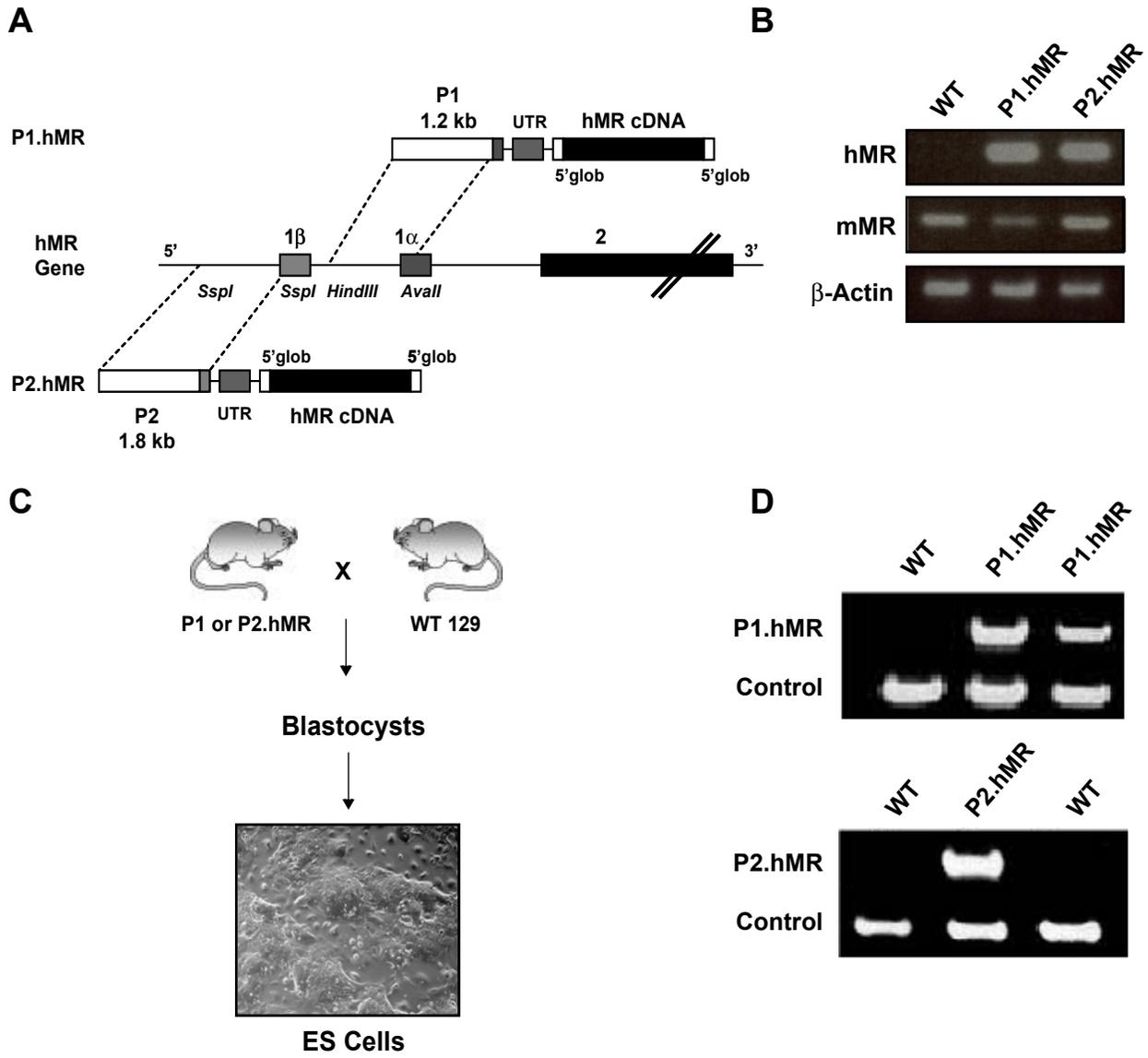
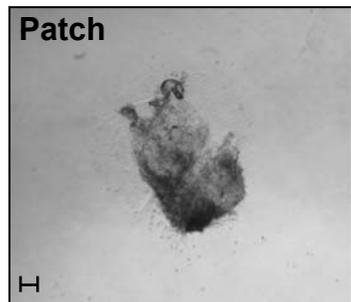
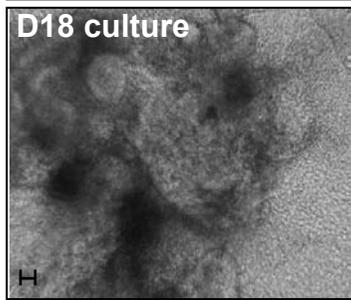
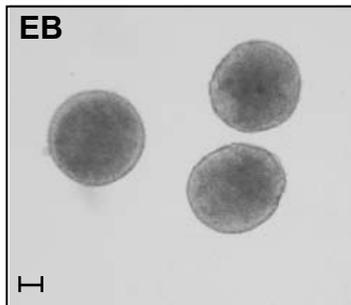
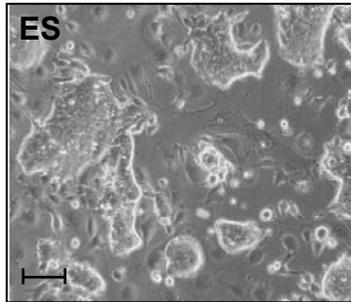
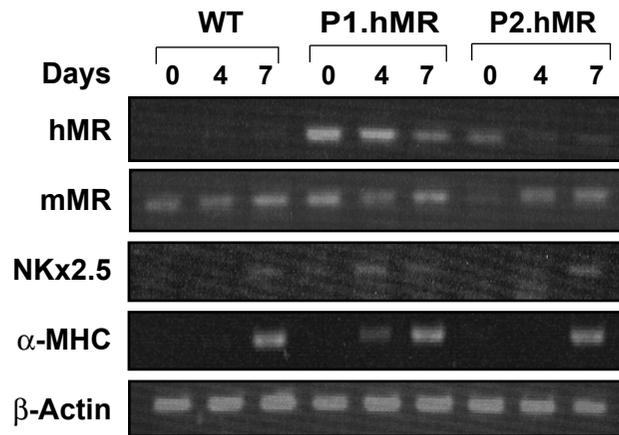
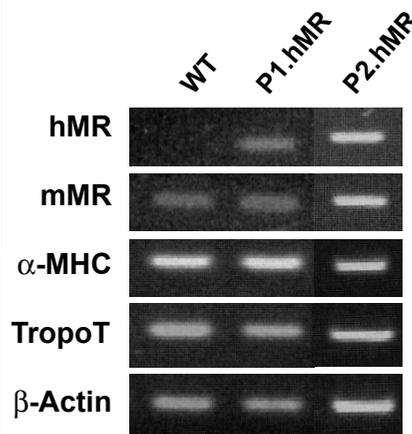
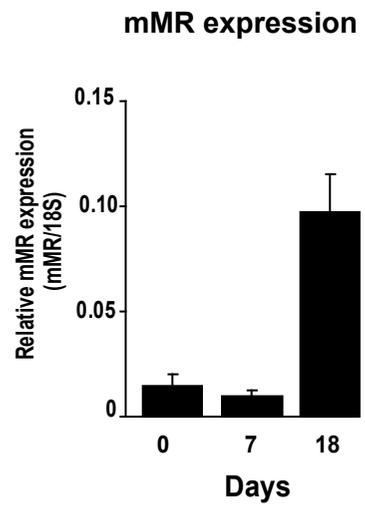


Figure 1

A**B****C****D****Figure 2**

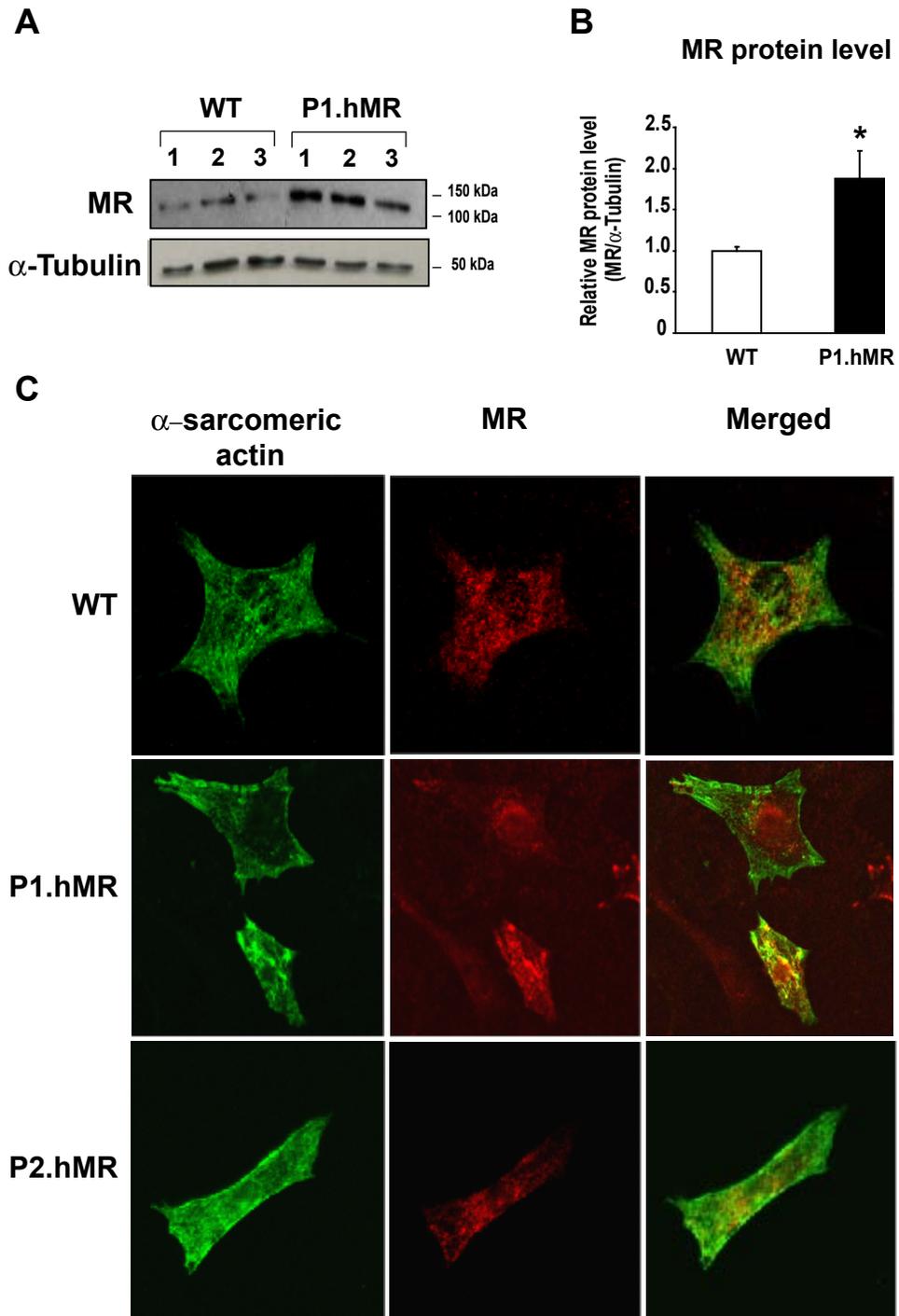


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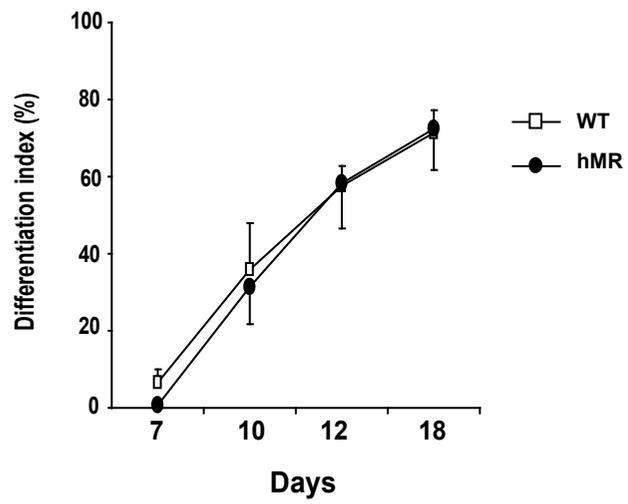


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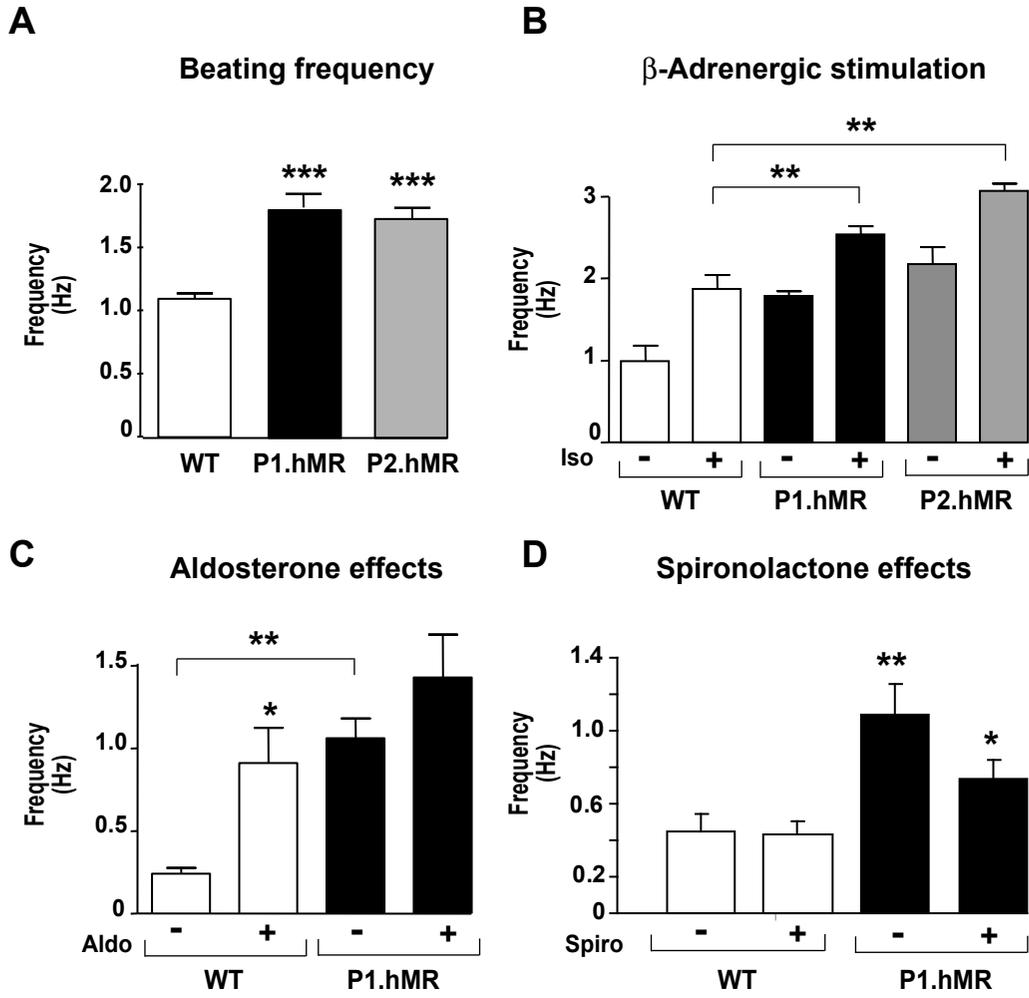


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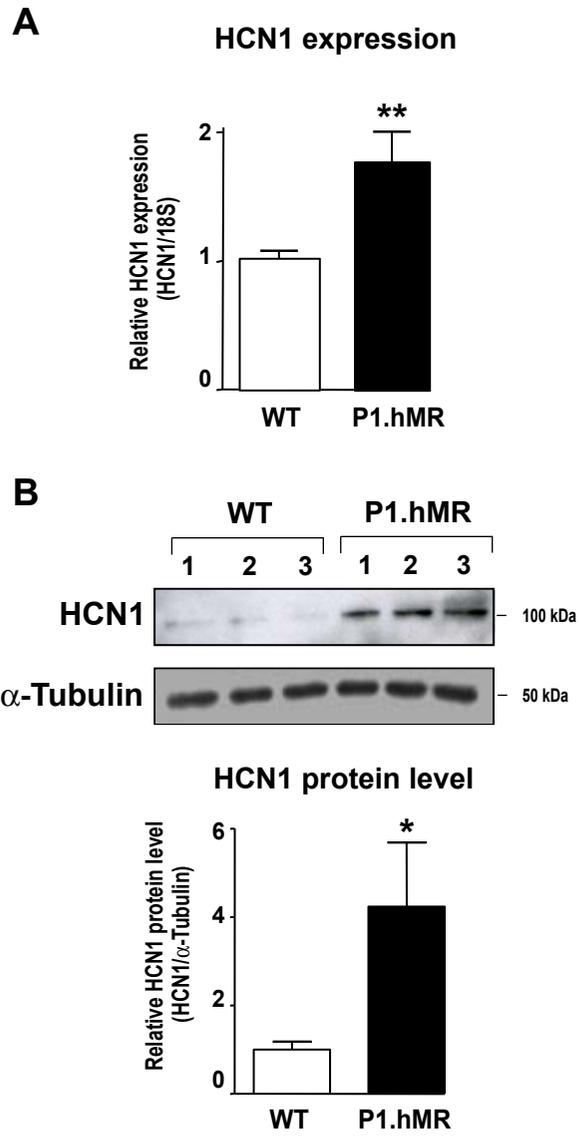


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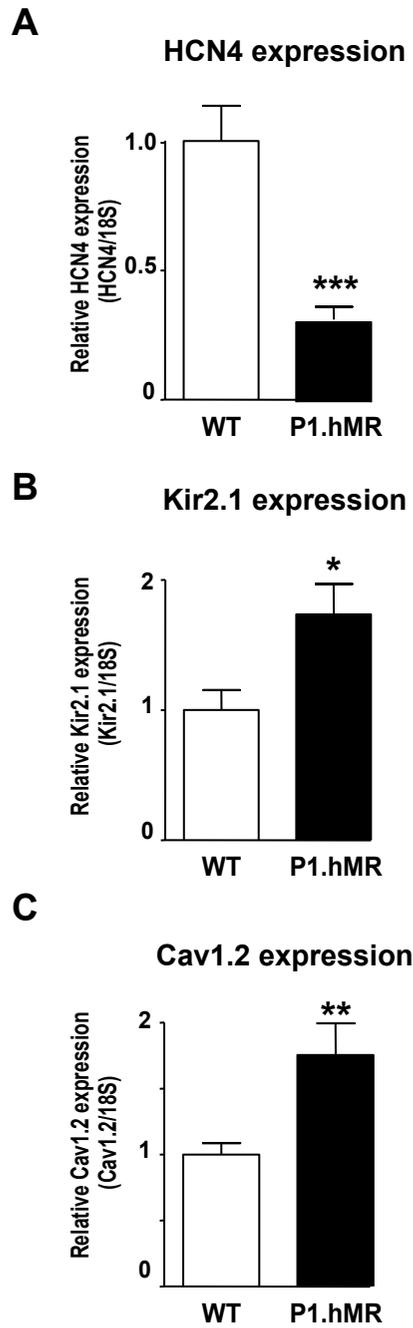
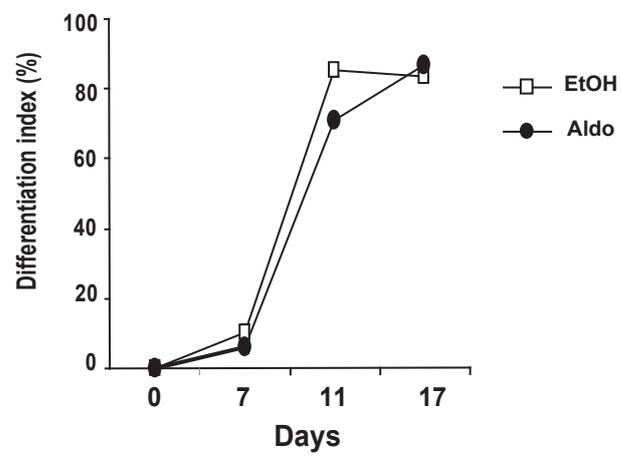
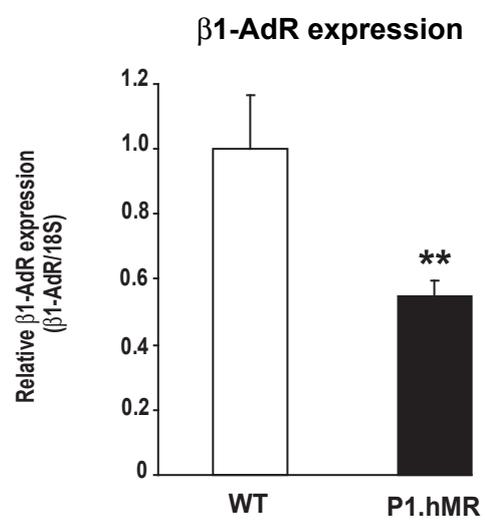


Figure 7



Supplemental Figure S1



Supplemental Figure S2