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Abstract

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

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

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

The Mineralocorticoid Receptor (MR or NR3C2) is a nuclear receptor exerting various pleiotropic actions on a wide series of target tissues. MR acts as a ligand-dependent transcription factor, and is involved in numerous physiological processes and pathological conditions\(^1\). MR is expressed in many components of the cardiovascular system such as blood vessels\(^2\), endothelial cells\(^3\), cardiomyocytes\(^4\), vascular smooth muscle cells\(^5\) and macrophages\(^6\). The importance of cardiac MR has been strikingly underscored by several direct and indirect evidences. Indeed, RALES and EPHESUS clinical trials have demonstrated the major benefit of mineralocorticoid antagonist (spironolactone and eplerenone) administration on the heart failure patient’s survival\(^7\)\(^-\)\(^8\). MR can be activated both by mineralo- and glucocorticoid hormones but selectivity-conferring mechanisms, mostly dependent upon the cellular context\(^1\), have been described. Most notably in tight epithelia, the large excess of glucocorticoids is metabolized into inactive compounds by the 11 beta hydroxysteroid dehydrogenase type 2 (11βHSD2) enzyme\(^9\), preventing an illicit occupation of the receptor. Although glucocorticoids are most likely the natural MR ligands in cardiomyocytes, which lack 11βHSD2, they seem to be unable to fully activate the receptor\(^10\). Indeed, Transgenic mice ectopically expressing 11βHSD2 in cardiomyocytes exhibit cardiac hypertrophy, fibrosis and heart failure, but no hypertension, this phenotype being reversed by eplerenone\(^11\). These findings in an animal model where the receptor is almost exclusively activated by aldosterone underline the deleterious effect of inappropriate MR activation.

On the other hand, MR gene inactivation in the mouse leads to early post-natal lethality caused by salt loss\(^12\) which can be rescued by daily NaCl injections followed by high salt diet\(^13\). MR\(^{-/-}\) mice have no cardiovascular abnormalities, notwithstanding...
the expected activation of the renin-angiotensin system, thus excluding a crucial role of MR during cardiac development.

In order to better understand MR-dependent pathophysiological processes in vivo, our group and others have exploited alternative strategies of MR overexpression in the heart of transgenic mice. In particular, we have generated a murine model in which the proximal P1 promoter of the human MR (hMR) was used to drive the expression of its own cDNA. P1.hMR animals express the transgene in most MR target tissues including kidney, brain and heart. Interestingly, two extensively studied mouse lines exhibited a moderate dilated cardiomyopathy associated with arrhythmia, but without hypertension or cardiac fibrosis in contrast with aldosterone-high salt diet animal models. In another mouse model, conditional MR overexpression in the heart triggers cardiac hypertrophy and life-threatening ventricular arrhythmias. All these in vivo studies underlined a specific role of MR in cardiomyocyte contractile properties. However, it cannot be excluded that transgene expression might induce some adaptive and compensatory mechanisms secondary to various feedback regulatory loops prevailing in vivo. The utilization of cardiomyocytes isolated from animal models would thus facilitate analysing the specific MR effects, regardless of the compensatory factors. Along similar lines, it has been also reported that MR/aldosterone have major effects on cardiomyocyte contraction frequency associated with an increased expression of T-type (Cav3.2) and L-type (Cav1.2) calcium channels, consequently augmenting Ca\(^{2+}\) currents in isolated rat ventricular myocytes. However, such cell-based systems are quite difficult to obtain in rodents, usually give poor yield, and often lead to highly variable results.
To better comprehend MR actions in the heart, we decided to use an alternative approach that consists in deriving cardiomyocytes from transgenic animals which allows a fine tuned control of experimental conditions. A more effective strategy, already validated by several groups (20 for review) is based on the utilization of the embryonic stem (ES) cells that can be indefinitely expanded at the undifferentiated stage and, under appropriate conditions, are able to differentiate into cell types originating from the three germ layers (endoderm, mesoderm, ectoderm) including cardiomyocytes21.

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

Derivation and culture of ES cell lines
To generate ES cell lines, P1 or P2.hMR females were crossed with males from the 129 strain (Charles River, L'Arbresle, France) and checked daily for vaginal plugs. Mice were cared according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal facility was granted approval (N° B94-043-12), with an authorization to experiment on living animals (75-978, ML) given by the French Administration. At day 3.5 post-coitum, females were sacrificed, the uterus removed and flushed with culture medium. Blastocysts were picked up with a mouth pipette and plated in a 6 cm Petri dish on a SNL feeder cell layer. After 3 more days each inner cell mass was recovered and plated in a well of a 24-well plate with SNL. Cultures were split 1:1 every 3 days and an ES cell line was successfully derived in approximately 20% of the attempts. Undifferentiated ES cells were cultured and amplified as previously described\(^22\). See supplemental methods for details.

Cardiac differentiation
Cardiac differentiation was based on the hanging drop method\(^23\). Drops containing 400 cells were grown hanging on a Petri dish lid for 3 days in ES cell medium containing 20% fetal calf serum. The embryoid bodies (EB) were then cultured for 2 days in the same medium (alternatively the serum was dextran-coated charcoal (DCC) stripped of steroids for hormone experiments) complemented with 1 \(\mu\)M ascorbic acid and 0.5% DMSO (cardiac differentiation medium). EB were then
seeded in gelatinized Petri dishes or culture plate wells and allowed to differentiate 5 to 15 days. Spontaneously beating areas were excised with a scalpel blade.

**RT-PCR**

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

**Immunocytochemistry**

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

**Western blot**

Total protein extracts were prepared from wild type and transgenic cardiomyocytes, 15 \( \mu \)g of proteins from cardiomyocyte homogenates were processed for
immunoblotting. See supplemental methods for details. Immunoblots were blocked with TBST 0.5% non fat milk and incubated overnight with 1/1000 anti-MR 39 N$^{24}$ or 1/300 anti-HCN1 (AB5884, Millipore) or 1/15,000 anti-α-tubulin (Sigma) as an internal standard. Quantitative analysis was performed using QuantityOne software (Bio-Rad Laboratory, Inc., Hercules, CA).

**Statistical analysis**

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

Generation of WT and hMR-overexpressing ES cell lines

We have previously generated transgenic mouse models using 1.2 kb of the proximal (P1) and 1.8 kb of the distal (P2) hMR promoter to drive the hMR cDNA expression as previously described\textsuperscript{15, 25}. Figure 1A illustrates the schematic representation of the transgenes inserted into P1.hMR and P2.hMR transgenic mouse genome. Both constructs contained an untranslated region as well as part of the human β-globin sequences to stabilize the transgene transcripts. As expected, we confirmed by RT-PCR using species-specific primers that the recombinant hMR was expressed in the heart of both transgenic P1.hMR and P2.hMR mouse lines (Fig 1B), demonstrating that these animals were suitable to investigate the functional impact of cardiac MR.

P1 and P2.hMR female mice were backcrossed with strain 129 males characterized by a high success rate of ES cell derivation from blastocysts\textsuperscript{26} (Fig 1C, see Supplemental methods). Several ES cell lines were established and genotyped as wild type (WT), P1.hMR or P2.hMR (Fig 1D). Further experiments were mostly performed on one representative ES cell line of each genotype.

MR expression during cardiac differentiation of ES cells

We optimized a protocol of cardiac differentiation based on the hanging drop method\textsuperscript{20}. The different steps of ES cell cardiac differentiation are represented in Fig 2A. As shown in the upper panel (ES), undifferentiated ES cells grew as compact clusters on the feeder cell layers. Suspension culture of 400 undifferentiated ES cells per drop led to standardized size embryoid bodies (EB) of approximately 200 µm in diameter at day 3 (EB, middle upper panel). These spherical-shaped structures contained cells that are able to differentiate into the three germ layers. From day 5,
cultures were grown in adherence in gelatinized Petri dishes and from day 7 onward spontaneously beating areas arose, enlarging with time (D18 culture, middle bottom). Beating areas containing a tight network of cardiomyocytes were excised with a scalpel blade around day 16 to 18 (Patch, bottom panel) and were further used for gene expression studies or immunodetection experiments. We defined an index of cardiac differentiation by assessing the percentage of cultures originating from a single EB presenting spontaneously beating cardiomyocytes. Approximately 70% of EB presented beating areas at day 18. No significant variation in the cardiac differentiation efficacy has been found among cell lines (see Fig 4).

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

We next analyzed MR expression at the protein level in cardiac differentiation. We used an anti-MR antibody recognizing both the endogenous murine MR and recombinant hMR. Western blot analyses of day 12 cardiomyocyte cultures revealed an approximately two-fold increase in MR expression in the P1.hMR
cardiomyocytes as compared to the WT cells (Fig 3A and 3B). Confocal microscopy imaging confirmed the coexpression of MR and α-sarcomeric actin in the cardiomyocytes of each genotype detected by double immunolabelling (Fig 3C). Note the nucleocytoplasmic distribution of MR and the stronger signal in P1.hMR and P2.hMR cardiomyocytes. Thus, these ES cell-derived cardiomyocytes provided an effective cell-based system to investigate the functional consequences of hMR overexpression in cardiomyocytes.

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

We first tested a potential effect of MR overexpression and aldosterone exposure on cardiac differentiation efficiency (See Fig 4 and Supplemental Fig S1). Since there was no modification of the differentiation index compared to control conditions, we excluded a major impact of MR signaling in early cardiac development. This assumption seems to be supported by the lack of morphological alterations in the cardiovascular system of MR KO newborn mice. In order to better understand the role of MR in cardiac function, we examined the influence of hMR overexpression on cardiomyocyte contractile properties (Fig 5). The beating rate of day 14-18 cultures was assessed by video capture. We found a highly significant increase of the beating frequency in transgenic P1.hMR and P2.hMR cardiomyocytes compared to WT (Fig 5A, 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, P<0.005, and see Supplemental videos). This increase in the spontaneous beating frequency of hMR-overexpressing cardiomyocytes has been reproduced in several differentiation experiments and has been confirmed in two different ES cell lines with the same genotype. Owing to the chronotropic effect of β-adrenergic stimulation, we observed a significant increase in
the beating frequency of both WT and transgenic cardiomyocytes upon isoproterenol exposure (Fig 5B). A 2.2-fold induction of isoproterenol-induced stimulation was found in the WT cardiomyocytes while the amplitude of catecholamine–stimulated beating frequency was only 1.4-fold in P1.hMR and P2.hMR cardiomyocytes, suggesting that amplification of MR activation might somehow compromise cardiomyocyte β-adrenergic signaling. Under these experimental conditions, the adrenergic-stimulated beating frequency remained significantly higher in hMR-overexpressing cardiomyocytes than in WT ES derived cells, providing additional support for a primary role of MR on cardiomyocyte contractile properties.

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

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

We next analyzed the relative abundance of other calcium and potassium channels (Fig 7). Unexpectedly, we found that mRNA levels of another pacemaker channel, HCN4, were repressed by 75% in hMR-overexpressing cardiomyocytes (Fig 7A), suggesting a counter-regulatory mechanism to dampen the depolarization process due to HCN1 up-regulation. Interestingly, we also demonstrated a 2-fold increase of the inward rectifier potassium ion channel Kir2.1 expression in P1.hMR cardiomyocytes (Fig 7B), which was accompanied by a parallel increase of the L-Type voltage dependent calcium channel Cav1.2 mRNA levels (Fig 7C). Collectively, hMR overexpression in ES cell-derived cardiomyocytes leads to a major alteration of the expression of several ion channels, all involved in cardiomyocyte contractility.
These results give a rationale for the faster contraction frequency observed in hMR-overexpressing cardiomyocytes strongly supporting the notion that MR signaling is a pivotal regulator of cardiomyocyte function.
Discussion

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

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

Although MR activation and aldosterone action in the heart seem to play critical roles in the pathogenesis of several cardiac diseases, the precise molecular events leading to cardiac hypertrophy, fibrosis and arrhythmia remain elusive and both the relative contribution of MR and the exact nature of the endogenous activating ligands are far from being well understood. Several recent studies provided interesting new insights in this field. Aldosterone exposure was shown to drastically increase contraction frequency of isolated rat ventricular myocytes, associated with an increase of the expression of L-type channel Cav1.2 and T-type channel Cav3.2. This result paralleled the data obtained with our ES cell-derived cardiomyocytes. Similarly, aldosterone treatment was reported to accelerate the spontaneous beating
rate of neonatal rat ventricular cardiomyocytes by increasing If currents due to enhanced expression of HCN4 channel at both mRNA and protein level$^{32}$. Our work strongly supports a central role of MR in cardiomyocyte contractibility and ion channel expression. This should be discussed in view of previous studies demonstrating that the electrical remodeling of Ca$^{2+}$ and K$^+$ currents and the modification of channel expression in an experimental myocardial infarction rat model occur prior to cardiomyocyte hypertrophy and are prevented by MR antagonists$^{33}$. As a whole, our data show that MR overexpression in ES cell-derived cardiomyocytes partially mimics aldosterone effects, inducing a positive chronotropic effect associated with alterations of the expression of several ion channels, however our results differ from previous reports under many aspects. Interestingly, the increased beating frequency associated with MR overexpression was also observed using DCC medium as early as day 3 of differentiation, suggesting that cardiac MR is at least partially activated in a ligand-independent manner. Under such experimental conditions, we showed that spironolactone reduced spontaneous contraction of MR-overexpressing cardiomyocytes, providing additional supports for the beneficial effect of anti-mineralocorticoid compounds on MR-related cardiac arrhythmias. This finding is reminiscent of a recent proposal of a ligand-independent activation of MR by Rac1 GTPase, responsible for deleterious renal consequences, linking activation of MR signaling in podocytes, renal failure and proteinuria$^{34}$. In any case, the exact mechanisms involved in the cardiac MR-mediated activation remain to be further investigated, most notably because of major pharmacological perspectives. ES cell-derived cardiomyocyte models likely differ from the neonatal ventricular cardiomyocytes exploited by other groups, accounting for some differences between
our work and other studies. Murine ES cell cardiac differentiation lasts approximately two and half weeks and we hypothesize that ES cell-derived cardiomyocytes might represent an earlier stage of differentiation than isolated neonatal ventricular myocytes. Indeed, a recent study reported the temporal expression pattern of ion channels involved in contractility during ES cell cardiac differentiation. It was shown that the pacemaker cells maintained the expression of HCN channels during cardiac differentiation while a ventricular-like phenotype was associated with a slight increase in Kir2.1 rectifier potassium channel expression. We propose that our model of ES cell-derived cardiomyocytes might correspond to an intermediate differentiation stage exhibiting a pacemaker cell phenotype.

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

However, as expected, we could not find any difference in HCN1 expression in the heart of the parental WT and P1.hMR adult mice (data not shown) since HCN1 is predominantly, if not exclusively, expressed in the sinus node and because it is well established that the atrial HCN1 expression gradually diminished in the postnatal
period. This finding strengthens the advantage of our in vitro ES system to unravel MR-mediated ionic channel remodeling during cardiomyocyte differentiation.

HCN channels are cyclic nucleotide-gated channels whose activity is dependent upon the intracellular cAMP concentrations that are regulated by the beta-adrenergic signaling. Interestingly, the heart beating rate of embryos with a HCN4 mutant that is unable to bind cAMP was not accelerated upon adrenergic stimulation, providing a functional linking between pacemaker channel HCN4 and catecholamine responses. The down-regulation of HCN4 in hMR-overexpressing cardiomyocytes is associated with a decrease of β1-adrenergic receptor expression (Supplemental Fig S2). We believe that both molecular events may account for the attenuated responses to isoproterenol stimulation.

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

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

In conclusion, we demonstrate that amplification of MR activation in ES-derived cardiomyocytes leads to chronotropic responses associated with cardiac ion channel alterations contributing to arrhythmias. Our results underscore the pivotal role of MR in the homeostasis of cardiac contractility and provide further support for the benefit...
of mineralocorticoid antagonist treatments in the management of cardiac dysfunctions."
Figure legends

Figure 1

Generation of ES cell lines.

A) Schematic representation of P1.hMR and P2.hMR transgenes. The HindIII-Aval II (−969, +239) fragment of P1 promoter and the SspI-SspI (−1682, +123) fragment of P2 promoter have been used to target the expression of hMR cDNA in transgenic mice\textsuperscript{15}. B) Endogenous (mMR) and recombinant MR (hMR) mRNA expression in the heart of WT, P1 and P2.hMR mice were detected by RT-PCR with species specific primer set. Amplification of the β-actin was used as an internal control. C) The ES cell lines were derived from blastocysts recovered 3.5 days post coitum from P1 or P2.hMR females crossed with the 129 strain males. D) Genotyping of various ES cell lines using the rapsn as an internal genomic PCR control.

Figure 2

Characterization of ES cell lines during cardiac differentiation.

A) ES cell differentiation: Top panel, (ES), undifferentiated ES cells; Middle top panel, (EB), D3 embryoid bodies; Middle bottom panel, D18 culture; Bottom panel, excised spontaneously beating patch. Scale bar = 50\(\mu\)m. B and C) RT-PCR of hMR, mMR and cardiac marker genes (B) on Day 0, 4, 7 cultures and (C) on D18 excised beating patches; αMHC: α myosin heavy chain; TropoT: troponin T. D) Relative expression of endogenous mouse MR expression at D0 and D7 cultures and D18 excised beating patches measured by quantitative real-time PCR. Results are expressed in amol mMR/ fmol 18S transcripts, and are means ± SEM of triplicates from WT, P1.hMR and P2.hMR differentiation.
Figure 3

hMR overexpression in ES cell-derived cardiomyocytes.

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

Figure 4

Index of cardiac differentiation is not modified by MR overexpression.

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

Figure 5

hMR overexpression increases cardiomyocyte beating frequency.

Beatings are recorded by video capture for more than 30 s between days 14 and 18 (see Supplemental Videos). A) WT, P1 and P2 cardiomyocyte differentiation cultures.
(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; ***; P<0.005). B) Effect of β-adrenergic stimulation. Cardiomyocytes were exposed to 1 µM isoproterenol (Iso) for 15 min. **, P<0.01. C) Effect of 10 nM aldosterone treatment (Aldo) or vehicle on the beating frequency of WT and P1.hMR cardiomyocytes grown in steroid-stripped medium; **, P<0.01; *, P<0.05. D) Effect of 100 nM spironolactone treatment (spiro) or vehicle on WT and P1.hMR cardiomyocytes in steroid stripped medium. *, P<0.05. **, P<0.01.

Figure 6

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

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

Figure 7

Altered expression of ion channels in hMR-overexpressing cardiomyocytes.

Relative expression of ion channel transcripts in WT and P1.hMR Day 16 cardiomyocytes was determined by qPCR. Results normalized by the amplification of 18S RNA are means ± SEM of 2 or 3 experiments performed in triplicate and are expressed relative to the WT value arbitrarily set at 1. A) Relative HCN4 expression,
***, \( P<0.005 \). B) Relative Kir2.1 expression, *, \( P<0.05 \). C) Relative Cav1.2 expression, **, \( P<0.01 \).
References


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Conflict of interest: none declared.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Supplemental Figure S1
Supplemental Figure S2

**Relative \( \beta_1 \)-AdR expression**

- WT
- P1.hMR

\[ \frac{\beta_1 \text{-AdR expression} (\beta_1 \text{-AdR/18S})}{\text{WT}} \]

![Bar Graph]

**Supplemental Figure S2**

- WT
- P1.hMR

\[ \text{Relative } \beta_1 \text{-AdR expression} \]

**Supplemental Figure S2**

- WT
- P1.hMR

\[ \frac{\beta_1 \text{-AdR expression} (\beta_1 \text{-AdR/18S})}{\text{WT}} \]