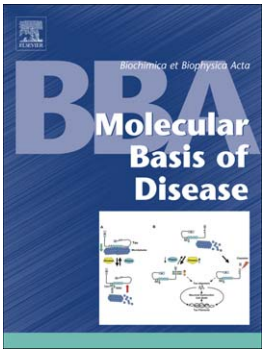

Met signaling in cardiomyocytes is required for normal cardiac function in adult mice

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PII: S0925-4439(13)00273-1
DOI: doi: 10.1016/j.bbadis.2013.08.008
Reference: BBADIS 63785

To appear in: *BBA - Molecular Basis of Disease*

Received date: 27 April 2013
Revised date: 2 August 2013
Accepted date: 20 August 2013

Please cite this article as: María Arechederra, Rita Carmona, María González-Nuñez, Álvaro Gutiérrez-Uzquiza, Paloma Bragado, Ignacio Cruz-González, Elena Cano, Carmen Guerrero, Aránzazu Sánchez, José Miguel López-Novoa, Michael D. Schneider, Flavio Maina, Ramón Muñoz-Chápuli, Almudena Porras, Met signaling in cardiomyocytes is required for normal cardiac function in adult mice, *BBA - Molecular Basis of Disease* (2013), doi: 10.1016/j.bbadis.2013.08.008

Met signaling in cardiomyocytes is required for normal cardiac function in adult mice

María Arechederra*[1], Rita Carmona*[2], María González-Nuñez*[3], Álvaro Gutiérrez-Uzquiza[1a], Paloma Bragado[1b], Ignacio Cruz-González[4], Elena Cano[2], Carmen Guerrero[5], Aránzazu Sánchez[1], José Miguel López-Novoa[3], Michael D. Schneider[6], Flavio Maina[7], Ramón Muñoz-Chápuli[§2], Almudena Porras[§1]

***** Equally contributed; [§] Co-senior investigator

Affiliations:

[1] Departamento de Bioquímica y Biología Molecular II Facultad de Farmacia, Universidad Complutense de Madrid (UCM); Instituto de Investigación Sanitaria del Hospital Clínico San Carlos (IdISSC); Ciudad Universitaria, 28040 Madrid, Spain;

[2]Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

[3]Unidad de Fisiología Renal y Cardiovascular, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain

[4]Departmento de Cardiología, Hospital Universitario, IBSAL, Salamanca, Spain.

[5]Centro de Investigación del Cáncer, IBMCC, IBSAL, Universidad de Salamanca-CSIC, 37007 Salamanca, Spain.

[6]National Heart and Lung Institute, British Heart Foundation Centre of Research Excellence, Imperial College London, Faculty of Medicine, London W12 0NN, United Kingdom

[7] Aix-Marseille Université, IBDML, CNRS UMR 7288, Parc Scientifique de Luminy, Case 907, 13288 Marseille Cedex 09, France

[a] Present address: Department of Pharmacology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6160, USA

[b] Present address: Division of Hematology and Oncology, Department of Medicine and Department of Otolaryngology, Tisch Cancer Institute, Black Family Stem Cell Institute, Mount Sinai, New York, NY 10029, USA.

Corresponding author: Almudena Porras, Departamento de Bioquímica y Biología Molecular II, Facultad de Farmacia, Ciudad Universitaria, 28040 Madrid, Spain, Phone: 34 91 394 1627, Fax: 34 91 394 1779, e-mail: maporras@ucm.es

Abstract

Hepatocyte growth factor (HGF) and its receptor, Met, are key determinants of distinct developmental processes. Although HGF exerts cardio-protective effects in a number of cardiac pathologies, it remains unknown whether HGF/Met signaling is essential for myocardial development and/or physiological function in adulthood. We therefore investigated the requirement of HGF/Met signaling in cardiomyocyte for embryonic and postnatal heart development and function by conditional inactivation of the Met receptor in cardiomyocytes using the Cre-α–MHC mouse line (referred to as *α–MHCMet-KO)*. Although *α–MHCMet-KO* mice showed normal heart development and were viable and fertile, by 6 months of age males developed cardiomyocyte hypertrophy, associated with interstitial fibrosis. A significant upregulation in markers of myocardial damage, such as β-MHC and ANF was also observed. By the age of 9 months, *α–MHCMet-KO* males displayed systolic cardiac dysfunction. Mechanistically, we provide evidence of a severe imbalance in the antioxidant defenses in *α–MHCMet-KO* hearts involving a reduced expression and activity of catalase and superoxide dismutase, with consequent reactive oxygen species accumulation. Similar anomalies were observed in females, although with a slower kinetics. We also found that Met signaling down-regulation leads to an increase in TGF-β production and a decrease in p38MAPK activation, which may contribute to phenotypic alterations displayed in *α–MHCMet-KO* mice. Consistently, we show that HGF acts through p38α to upregulate antioxidant enzymes in cardiomyocytes. Our results highlight that HGF/Met signaling in cardiomyocytes plays a physiological cardio-protective role in adult mice by acting as an endogenous regulator of heart function through oxidative stress control.

Keywords: Hepatocyte growth factor, Met, cardiomyocytes, oxidative stress, heart, p38MAPK

1. Introduction

The hepatocyte growth factor (HGF) receptor, Met, is a tyrosine kinase receptor expressed in a wide variety of cell types. It regulates several cellular functions, including proliferation, scattering, differentiation and survival, either during development or under some pathological conditions [1-3]. Upon HGF binding, Met is autophosphorylated in different tyrosine residues, leading to the recruitment of several SH2-domain containing proteins through the multifunctional docking site located in its carboxy-terminal tail [4]. Both HGF and Met are essential during development as shown by defects in placenta, liver, muscle, and nerves [5-10]. The embryonic lethality of *met* mutants imposes the use of conditional inactivation strategies to explore the HGF/Met functional requirement during embryogenesis and adulthood.

Several studies have been performed to uncover the HGF/Met properties in the heart. It has been shown that HGF protects rats from myocardial infarction [11] and/or ischemia/reperfusion injury [12]. Post-infarction treatment with HGF also improves left ventricular remodeling and heart function in mice [13]. In addition, HGF improves heart functionality and promotes proliferation of myocardial progenitor cells in doxorubicin-induced cardiomyopathy [14]. It also prevents heart fibrosis, remodeling and dysfunction in a hamster model of cardiomyopathy [15]. In other models of cardiac damage, HGF also inhibits fibrosis and additionally stimulates angiogenesis [16]. Together, these findings indicate that HGF/Met axis exerts a cardio-protective role under pathological conditions. I*n vitro* studies have shown that HGF induces the expression of cardiac markers in mesenchymal stem cells [17] and increases cardiac differentiation of embryonic stem cells [18]. In addition, stem cells express HGF, which improves its survival and that of cardiomycytes and favors angiogenesis [16]. Both HGF and Met are expressed in cardiac myocytes during early cardiogenesis [19,20]. In a mouse model of HGF supra-physiological expression, it has been shown that neonatal cardiomyocytes express Met and respond to HGF by increasing proliferation [21]. However, it remains unknown whether endogenous activity levels of HGF/Met is required for heart development and/or physiology after birth.

In this study, we have genetically evaluated the role played by the HGF/Met axis in developing and adult hearts using a conditional knock-out mouse model lacking Met signaling in cardiomyocytes. In particular, we conditionally deleted Met in cardiomyocytes by combining met floxed mice [22] with a transgenic line expressing the Cre recombinase under the control of the α-myosin heavy chain (α-MHC) promoter [23]. The α-MHC promoter is active in mouse embryos throughout the myocardium starting from E8 [24]. Previous reports have shown that by E12.5, α-MHC is highly expressed in atria and to a lesser extent in the ventricles, whereas its expression increases in the ventricles after birth [25]. Therefore, the α-MHC-Cre line allows c-*met* inactivation in cardiomyocytes at early heart developmental stages.

Using the cardiomyocyte conditional Met knockout, we have found that Met signaling in cardiomyocytes appears to be dispensable for heart development. However, it is required to protect cardiomyocytes from oxidative stress, thus preventing cardiomyocyte hypertrophy, heart fibrosis, and

heart dysfunction in adult mice. Together, these studies unveil that HGF/Met signaling in cardiomyocytes is a key regulator for proper heart function.

2. Materials and methods

2.1 Generation of cardiomyocyte *c-met* knock-out mice and genotyping

The animals used in our research program were handled in compliance with the institutional and European Union guidelines for animal care and welfare. All mouse lines were maintained and bred at the UCM facility, allowed food and water *ad libitum* and routinely screened for pathogens in accordance with FELASA (Federation of European Laboratory Animal Science Associations) procedures.

c-*met* flox/flox (wt) mouse line (in a CD1 genetic background), which carries c-*met* alleles with exon 16 flanked by loxP sites was obtained from Dr. S. Thorgeirsson [22]. This mouse was crossed with α-MHC-Cre transgenic mouse in CD1 background (obtained from Dr. M. D. Schneider [23]) to generate α-MHC-Cre/+: c-*met* flox/+ mice, which were then crossed with c-*met* flox/flox mice. Cre-mediated excision led to a null allele for c-*met*, which encodes an inactive Met receptor only in cardiomyocytes. Mice genotyping was performed by PCR analysis (supplementary detailed methods).

RT-qPCR analysis

After the isolation of total RNA, DNA and proteins using AllPrep DNA/RNA/Protein Mini Kit, RNA was reverse transcribed using SuperScript III RT kit (Quiagen, 18080) to generate cDNA. Then, Real Time PCR was performed using specific primers (see supplemental methods). Quantification was performed calculating RQ ($2^{-\Delta\Delta Ct}$), so referring the values to control mice (supplemental methods). An Invitrogen custom RNA profiling by RT-qPCR was performed for the analysis of anti- and pro-fibrotic genes and those involved in reactive oxygen species (ROS) metabolism (supplemental methods).

2.2 Western-blot analysis

Western-blot analysis was carried out as previously described [26]. Proteins were separated by electrophoresis using Anderson or SDS-page gels, and transferred to nitrocellulose membranes that were probed with the following antibodies: catalase (Sigma, C0979), superoxide dismutase-2 (SOD-2) (Upstate Biotech, 06-984), β-actin (Sigma A5441), c-Met (Santa Cruz Biotechnology sc-260), cytochrome c (BD 556433), porin (Sigma V2139), phospho-p38MAPK (Cell Signaling, 9211), p38α (Santa Cruz Biotechnology sc-535).

2.3 Histology

Hearts were processed for histology using conventional techniques involving paraformaldehyde fixation and paraffin embedding. The degree of interstitial fibrosis was assessed by image analysis on Mallory's trichrome stained tissue sections (see supplemental methods). Laminin immunostaining of heart sections was done to delineate the cardiomyocyte profiles, measuring their areas with the ImageJ analysis package. Apoptotic cardiomyocytes were detected by using anti-desmin (Sigma, D1033) and anti-active caspase-3 antibodies (Promega, G74819).

2.4 ROS detection and quantification

Reactive oxygen species (ROS) accumulation in hearts was detected by staining of frozen sections with 1μM 2-hydroethidine (in PBS). The sections were then washed with PBS, mounted and analyzed by fluorescence microscopy. The number of stained nuclei was counted on digitalized images using ImageJ software. Alternatively, in hearts from 9 old mice, lipid peroxidation was determined by immunolocalization of 4-OH-Nonenal (Alpha Diagnostics, HNE11-S) in paraffin sections. Stained areas were assessed on digitalized images using ImageJ software.

Carbonylated proteins were quantified as an indirect measurement of ROS [27]. Heart extracts were treated with 2,4-Dinitrophenylhydrazine or maintained untreated (blank). Then, proteins were precipitated with TCA and absorbance at 360 nm was determined. The concentration of carbonylated proteins was calculated upon correction with the blanks and referred to protein content (supplemental methods).

2.5 Measurement of catalase, SOD, and cytochrome c oxidase activities

Catalase activity was measured by quantification of peroxide decomposition spectrophotometrically at 240 nm. Superoxide dismutase (SOD) activity was quantified using a Kit (BioVision) (supplemental methods). Cytochrome c oxidase activity was determined by quantification of cytochrome c oxidation spectrophotometrically at 550nm.

2.6 Electrocardiographic measurements in conscious mice using radiotelemetry

Telemetric electrocardiogram (ECG) recordings in conscious mice were carried out using an implantable telemetry system with ECG transmitters (ETA-F10) (Data Sciences International, St Paul, MN, USA). Recordings were taken every 3 h, 5 min/each, over a 24 h period and analyzed using ECG-auto software supplied by Data Systems (see extensive description in supplemental methods).

2.7 Echocardiographic assessment of cardiac function and structure

Transthoracic echocardiography was performed on lightly anesthetized mice using a Vivid 7 (GE Medical Systems) cardiac ultrasound machine equipped with a 10-14-MHz transducer il3L (GE Medical Systems), as previously described [28]. Measurements of chamber dimensions and cardiac function were made offline (EchoPac Software, GE Medical Systems). Structural and functional heart parameters were calculated as previously described [28] (see extensive description in supplemental methods).

2.8 Assays in cardiomyocytes derived cell lines

Cardiomyocyte cell lines (wt and p38α-/-) were generated from E9.5 immortomouse embryos expressing a temperature-sensitive large T antigen (LTAg) [29]. Before the experiments, cells were transferred to 37°C in the absence of IFN-γ to abolish LTAg expression. Neonatal cardiomyocyte cell lines were obtained by immortalization of neonatal mice (P4) cardiomyocytes by transfection with LTAg. For the experiments, all the cell lines were maintained in a low serum medium (2%) and

treated with the Met inhibitor SU11274 (5μM) or TGF-β (1ng/mL) in the presence or absence of HGF (30ng/mL, 16h pretreatment) or N-acetyl cysteine (2.5mM) for 1-4 days as indicated.

2.9 Statistical analysis

Data are represented as means ±SEM. The comparisons were made between two experimental groups. An unpaired Student's t-test was used and alternatively, ANOVA test was carried out for comparisons of more than two experimental groups. Differences between groups were considered significant when $p<0.05$.

3. Results

3.1 Conditional inactivation of Met in cardiomyocytes does not overtly affect embryonic and postnatal heart development, but leads to cardiac anomalies in adult mice

In order to study the role played in vivo by Met in cardiomyocytes and to bypass embryonic lethality, we generated a mouse model with a selective inactivation of Met signaling in cardiomyocytes. To do it, we intercrossed the c-*met* floxed mouse, which carries c-*met* alleles with exon 16 flanked by loxP sites (c-$met^{fl/fl}$) [22] with a transgenic mouse bearing the Cre recombinase under the α-MHC promoter [23]. The resulting *α–MHCCre:*$met^{fl/fl}$ conditional knockout will be herein referred to as *α–MHCMet-KO*. Because some strains of transgenic *α–MHCCre* mice expressing very high levels of Cre-recombinase in cardiomyocytes have been described to present heart anomalies [30], we checked the hearts from our *α–MHCCre* mice. They were similar to c-$met^{fl/fl}$ (considered as controls) in all experiments performed (supplementary Fig. 1). So, both were indistinctly used as controls.

Efficiency of the c-*met* deletion was determined by PCR and western-blot analysis. A deletion-specific fragment (650bp) was amplified by PCR using DNA from E15.5 *α–MHCMet-KO* embryonic hearts. Consistently, a sharp decrease in c-Met protein levels was also found (Fig. 1a), confirming the efficient deletion of the Met floxed exon 16, as previously shown in other tissue-specific Cre lines [22, 31-32]. The Met deletion was further confirmed in adult hearts (data not shown). Quantification revealed a Met deletion of around 70% and 90% in embryonic (E15.5) and adult *α–MHCMet-KO* hearts, respectively (data not shown). The remaining Met expression corresponds most likely to that present in non-cardiomyocyte cells. No significant morphological differences were found between control and *α–MHC-Met-KO* mutant hearts, either during embryonic development or after birth (Fig. 1b and c). These data indicate that Met signaling in cardiomyocytes is dispensable for heart development.

Conditional *α–MHCMet-KO* mutants were born following a mendelian ratio. They displayed a normal lifespan and weight curve (not shown), thus indicating that other tissues had been preserved from Met inactivation. Therefore, the *α–MHCMet-KO* mutant mice represent a unique genetic setting to investigate Met signaling requirement for heart function. This issue was assessed by performing a series of morphological, molecular, and functional studies on 3, 6, and 9 months old *α–MHCMet-KO* mice. It is well know that the gender may influence the molecular process underlying heart function, as shown by a number of studies in mice [33-35]. We therefore first focused our analyses on *α–MHCMet-KO* males. Whereas morphological analysis of the heart did not reveal significant abnormalities in 3 months old *α–MHCMet*-KO males, we observed structural abnormalities in 6 months old *α–MHCMet-KO* mutant hearts (Fig. 2a, b, d). In particular, a significant increase in the cross-sectional area of cardiomyocytes was observed in *α–MHCMet-KO* mutant hearts from 6 and 9 months old males compared to controls (Fig. 2b, c and supplementary Fig. 2a). In addition, myocardial fibrosis, diffuse in some places and more prominent in defined areas, with cardiomyocyte disorganization and strong collagen deposition, was also found in the heart of 6 and 9 months old *α–MHCMet-KO* males as compared to controls (Fig. 2d, e and supplementary Fig. 2b and c). As

fibrosis can be a physiological response to cardiomyocyte death, we analyzed the presence of active caspase-3 in heart sections. We found an increase in the percentage of active caspase 3 positive cells, mainly on highly fibrotic areas of hearts from *α–MHCMet-KO* males as compared to controls (Fig. 2f).

We next asked whether these morphological defects observed in *α–MHCMet-KO* males were parallel to heart dysfunction markers. In particular, we performed RT-qPCR analyses to assess the levels of β-MHC and atrial natriuretic factor (ANF) mRNAs, known to be induced during hypertrophy [36-37]. No differences were observed in 3 months-old mutants (Fig. 2g). In contrast, we found increased mRNAs expression levels of ANF, and β-MHC in hearts of 6 months old *α–MHCMet-KO* males as compared to controls (Fig. 2g and supplementary Fig. 1a). Moreover, RT-qPCR expression analyses of pro-fibrotic genes such as TGF-β2 and TGF-β3, or fibrotic markers such as α-smooth muscle actin (Acta-2) revealed significantly increased levels in *α–MHCMet-KO* males as compared to controls (Fig. 2h).

We next assessed whether these morphological and molecular alterations impacted the heart electrical function by performing electrocardiography (ECG) analyses. Typical non-sedated mice ECG shows a J wave just after QRS complex, an almost isoelectric SP interval and a P wave [38]. This typical ECG was observed in control mice (Fig. 3a). However, we found specific alterations in 6 months-old *α–MHCMet-KO* males. Anomalies consisted in the reduction or disappearance of the J wave [38] and a negative deflection of the S-P interval (-0.21 ± 0.02 mV, versus -0.09 ± 0.02 mV in control mice, $p < 0.01$), in contrast with the isoelectric SP-interval observed in control animals [38] (Fig. 3a). Although visual inspection suggested also an increased P wave in the *α–MHCMet-KO* compared with control male mice, a detailed measurement of the wave amplitude (0.14 ±0.02 mv in *α–MHCMet-KO* and 0.12 ± 0.02mV in control mice ($p > 0.1$)) showed no statistically significant differences (Fig. 3a). However, data from echocardiographic analyses demonstrated no significant differences in cardiac output or left ventricular mass in *α–MHCMet-KO* males (not shown). In contrast, echocardiographic analyses of 9 months-old *α–MHCMet-KO* males revealed signs of systolic dysfunction characterized by a significantly higher left ventricular systolic diameter and volume compared to controls. Shortening fraction and ejection fraction were also significantly decreased (Fig. 3b and c). The LV posterior wall thickness/LV end systolic dimension ratio was slightly lower in 9 months *α–MHCMet-KO* males (0.56 ± 0.03) than in controls (0.66 ± 0.03, $p < 0.05$), which suggests the existence of a left ventricular dilation.

Morphological, molecular, and functional studies were also performed in females. Increased cardiomyocyte size and extensive areas of fibrosis were detected in 9 months-old *α–MHCMet-KO* females (Supplementary Fig. 3a-c). Echocardiographic analysis revealed no significant differences between *α–MHCMet-KO* and controls, although a similar tendency as that found in mutant males was observed (data not shown). Together, these studies showed that HGF/Met signaling in cardiomyocytes is dispensable during development and in young mice, whereas it is required for proper heart function in adult mice. Moreover, they indicate that males are more susceptible to an altered HGF/Met signaling than females.

3.2 Loss-of-function of Met signaling disrupts the antioxidant defenses in cardiomyocytes, leading to an increase in ROS accumulation and altered mitochondria in adult mice heart

The oxidative stress increases during aging, leading to cardiac alterations, including hypertrophy and heart dysfunction [39]. As HGF/Met signaling regulates the antioxidant defenses in different cells types by preventing ROS accumulation [40-42], we investigated whether heart defects observed in adult *α–MHCMet-KO* males were linked to an imbalance between pro- and anti-oxidant mechanisms. We found that protein levels of the antioxidant enzymes, catalase and SOD-2, were reduced in the heart of 6 and 9 months old *α–MHCMet-KO* males as compared to controls (Fig. 4a and supplementary Fig. 1b). Consistently, catalase and SOD-2 activities were also decreased in *α–MHCMet-KO* males (Fig. 4b). We also found that reactive oxygen species were accumulated in cardiomyocytes of *α–MHCMet-KO* males as shown by 2-hydroethidine staining and 4-OH-nonenal immunolocalization in tissue sections from 6 and 9 months old mice, respectively (Fig. 4c and d). In addition, carbonylated protein levels increased in 6 months-old mutant males (Fig.4e), as a readout of proteins oxidation by ROS.

Because ROS accumulation can either be a consequence of an altered mitochondrial function and/or lead to mitochondrial damage, we next investigated whether *α–MHCMet-KO* 6 months-old male hearts presented alterations in mitochondria. We found that the mitochondrial proteins, porin and cytochrome c, were significantly decreased in mutants as compared to controls (Fig. 4f). Consistently, cytochrome c oxidase activity was also reduced in *α–MHCMet-KO* hearts (Fig. 4g). In contrast, we did not observe any alteration in anti-oxidant enzymes, ROS accumulation, carbonylated proteins or mitochondrial proteins in 6 months-old *α–MHCMet-KO* females, consistent with normal morphology and function, as previously assessed (Supplementary Fig.3). However, in 9 months-old mutant female hearts anti-oxidant enzymes levels significantly decreased and ROS accumulation was detected (Supplementary Fig. 4c), according to the presence of fibrosis and the increased cardiomyocytes size (Supplementary Fig. 3). Altogether, these findings strongly indicate that HGF/Met signalling is required for normal mitochondrial function and redox homeostasis in adult cardiomyocytes.

3.3 Met acts on p38 MAPK to increase antioxidant enzymes levels and counteracts TGF-β effect in cardiomyocytes

We next aimed at uncovering the molecular mechanism by which HGF/Met signaling in cardiomyocytes ensures proper levels of antioxidant enzymes. As it has been shown that p38α MAPK regulates the expression of SOD-2 and catalase in mouse embryonic fibroblasts (MEFs) [43], we determined P-p38MAPK levels, as a readout of p38 activity in control and *α–MHCMet-KO* hearts. Notably, we found that P-p38MAPK levels were significantly decreased in 6 and 9 months-old mice *α–MHCMet-KO* hearts (Fig. 5a and Supplementary Fig. 4g). We therefore studied whether p38α was required for HGF/Met-mediated regulation of antioxidant enzymes in cardiomyocytes through the use of pharmacological approaches in genetically modified embryonic cardiomyocyte cell lines generated from wt or p38α deficient mice (p38α-/-). We found decreased catalase and SOD-2 protein

levels in p38α-/- as compared to wt cardiomyocytes (Fig. 5b). Met inhibition with SU11274, detected by the decrease in Met phosphorylation (Fig. 5b), reduced the levels of catalase, SOD-2 and P-p38 MAPK in wt cells, but not in p38α-/- cells (Fig. 5b). These findings were further confirmed using neonatal cardiomyocytes (data not shown). Moreover, HGF stimulation increased the levels of catalase, SOD-2 and P-p38 MAPK proteins in wt, but not in p38$\alpha^{-/-}$ cardiomyocytes (Fig. 5b).These findings indicate that HGF/Met acts through p38 MAPK to increase catalase and SOD-2 levels. According to this, an increase in the levels of carbonylated proteins was detected in wt cardiomyocytes treated with SU11274 (Fig. 5c). HGF treatment (alone) slightly decreased them. As expected, HGF had no effect when used in combination with SU11274. Consistently, higher basal levels of carbonylated proteins were found in p38$\alpha^{-/-}$ cardiomyocytes as compared to those of wt cells, which were further increased upon SU11274 treatment. No changes were observed upon treatment with HGF (either alone or in combination with SU11274).

The increased levels of TGF-β observed in *α–MHCMet-KO* hearts (Fig. 2h) prompted us to investigate whether the levels of antioxidant enzymes were influenced by TGF-β. We found that TGF-β led to a decrease in catalase and SOD-2 protein levels, mainly in wt cardiomyocytes (Fig. 5d). Intriguingly, HGF counteracted the decrease in catalase and SOD-2 protein levels mediated by TGF-β in wt cardiomyocytes (Fig. 5d). Accordingly, HGF activated p38α, both in the absence and presence of TGF-β (Fig. 5d). In agreement with all these changes in SOD-2 and catalase expression, TGF-β increased the levels of carbonylated proteins in wt cardiomyocytes (Fig. 5e). HGF slightly decreased them, but this effect was not statistically significant. In p38α-/- cardiomyocytes, TGF-β did not further increase its intrinsic high basal levels of carbonylated proteins and HGF had no effect on them.

Taken together, these findings indicate that HGF/Met acts through p38α MAPK to increase the levels of antioxidant enzymes in cardiomyocytes and to avoid ROS accumulation, thus partially counteracting the effects elicited by TGF-β.

3.4 Increased cardiomyocyte size, occurring in the absence of Met signaling, is prevented by antioxidant treatment

As alteration of Met signaling in cardiomyocytes leads to decreased antioxidant enzymes levels, it is tempting to speculate that accumulation of ROS in *α–MHCMet-KO* hearts could be responsible for the increased size of cardiomyocytes. This would be coherent with previous studies showing that ROS accumulation is responsible for cardiomyocyte hypertrophy as it was observed in cardiomyocytes isolated from Sirt3-deficient mice [44]. We therefore explored this hypothesis using wild-type neonatal cardiomyocyte cell lines. Remarkably, we found that Met inhibition led to an increase in cell size in a time-dependent manner (Fig. 6a, b). Treatment of cardiomyocytes with the antioxidant N-acetyl-cysteine partially prevented the increase in the size of cells exposed to the Met inhibitor, SU11274 (Fig. 6a, b). These findings support that ROS accumulation might account for the hypertrophy of cardiomyocytes occurring in the absence of HGF/Met signaling. Additionally, the fact that Met inhibition itself leads to an increase in cardiomyocyte cell size suggests the existence of an autocrine signaling loop.

4. Discussion

Genetically modified mouse models have unveiled several genes involved in heart development and in regulation of adult heart physiology. However, key players for adult heart function and homeostasis remain to be uncovered. Their identification would improve our understanding of heart dysfunction, uncover new diagnostic markers and be instrumental for therapy and/or regenerative interventions. HGF/Met is an attractive signal as administration of exogenous HGF has a cardio-protective role in models of heart damage [11-13]. By exploring *in vivo* the role played by Met in heart during embryogenesis and in adulthood through selective inactivation of the *met* gene in cardiomyocytes, we demonstrated that while it is not essential for heart development, Met is required for heart homeostasis in adult mice. Intriguingly, Met becomes progressively required during aging. Whereas 3 months-old *α–MHCMet-KO* mutant hearts are normal, hearts from 6 months old males show an increase in cardiomyocyte size and myocardial fibrosis. Molecular markers of cardiac damage, such as ANF or β-MHC are up-regulated in *α–MHCMet-KO* males. This is accompanied by abnormal ECG, characterized by a marked depression of the S-P segment, a feature reported in rats with isoproterenol-induced cardiac hypertrophy [45]. As age increases, lack of HGF/Met signaling impacts heart function, so 9 months-old *α–MHCMet-KO* males suffer left ventricle systolic dysfunction. In addition, taking into account that, although the LV posterior wall thickness/LV end systolic dimension ratio is slightly lower in 9 months-old *α–MHCMet-KO* than in wt males, the ventricular mass/body weight ratio is similar, which suggests the existence of heart dilation rather than hypertrophy. Heart dysfunction of *α–MHCMet-KO* mutants is paralleled by a significant reduction in protein levels and enzymatic activities of catalase and SOD-2 in the heart of *α-MHCMet-KO* mutants, accompanied by ROS accumulation. These alterations were found in both male and females. The delayed dysfunction observed in *α–MHCMet-KO* females is most likely due to the existence of estrogen-induced cardio-protective mechanisms, which have been extensively studied [33-35] and which might partially compensate HGF/Met requirement for heart function in young females.

The cardio-protective role of Met during aging and in the absence of experimentally induced damage is in agreement with the HGF/Met properties reported in experimental models of human pathologies such as myocardial infarction [11, 13], ischemia/reperfusion injury [12], or other models of cardiomyopathy, where fibrosis is produced [15]. However, in all these studies it was not possible to clarify in which cell types HGF/Met signaling was required, as HGF can activate Met in most of the cell types present in the heart. Thus, some of the HGF effects previously described could be mediated by Met activation in non-cardiomyocyte cell types, such as fibroblasts, coronary endothelium or vascular smooth muscle. It is also important to highlight that HGF is a potent pro-angiogenic factor and its effect promoting neovascularization would be cardioprotective *per se* [16, 46]. The inhibition of Met signaling in myocardium, leaving intact the angiogenic roles of HGF on the coronary endothelium, allows excluding that the observed phenotype can be due to a vascular defect. Thus, our genetic mouse model determines *in vivo* a cell autonomous requirement of HGF/Met signaling in cardiomyocytes for their maintenance and function. Although our results clearly suggest a direct effect

of HGF on mature cardiomyocytes, we cannot formally exclude that HGF signaling may also influence the expansion of cardiomyocyte progenitors as they express α–MHC [47]. In fact, the improved heart functionality induced by HGF treatment in doxorubicin-induced cardiomyopathy [14] or the regeneration produced in an infarcted myocardium [48-49], could be explained by enhanced myocardial progenitor cells proliferation and/or migration. If the effect on cardiomyocyte progenitors is confirmed, HGF/Met signaling would be not only cardioprotective, but also involved in cardiac homeostasis.

The herein described property of HGF/Met signaling to protect cardiomyocytes from ROS-induced damage is in agreement with previous studies performed under non-physiological conditions. For example, HGF protects cultured adult rat cardiomyocytes from apoptosis induced by oxidative stress upon serum deprivation, treatment with daunorubicin, or hydrogen peroxide [50]. A role for HGF preventing heart fibrosis has been previously described in the cardiomyopathic Syrian hamster [15]. In this pathological model, HGF down-regulation in endothelial and interstitial cells triggers TGF-β1 up-regulation and interstitial fibrosis, whereas HGF treatment decreases TGF-β1 expression and suppressed fibrosis. We show here that under physiological conditions, HGF/Met signaling in cardiomyocytes is also essential to prevent fibrosis in adult heart. Furthermore, we demonstrate a negative correlation between Met activity and levels of pro-fibrotic factors such as TGF-β2 and TGF-β3. We also provide evidence that TGF-β treatment leads to down-regulation of antioxidant enzymes in cultured cardiomyocytes, thus linking fibrosis and oxidative stress.

The accumulation of ROS found in *α-MHCMet-KO* mutant hearts could be responsible for their dysfunction, based on the well-known link between oxidative stress and heart failure [51]. Consistently, the oxidation of several proteins might account for a reduced contractile heart capacity. For example, proteins regulating the release and uptake of Ca by the endoplasmic reticulum, such as SERCA, or proteins involved in the regulation of myofilaments can be inactivated through oxidation, which leads to a reduced contractile force [51-52]. In addition to the impact on contractile capacity, ROS accumulation can induce cardiomyocyte cell death [51]. The ability of HGF/Met to counteract SOD dysfunction in a neurodegenerative animal model of amyotrophic lateral sclerosis [53] and to regulate antioxidant defenses [40-42] has been reported. For example, *in vivo* hepatocyte-specific Met deletion unbalances ROS regulation and sensitizes cells to Fas-mediated apoptosis [40]. HGF treatment also leads to increased mitochondrial activity and mass in glioblastoma cells [54]. The link between increased ROS levels and mitochondrial dysfunction in defective hearts is supported by other studies [51-52, 55-56]. Therefore, it is tempting to speculate that mitochondrial functional alterations occurring in Met deficient cardiomyocytes could cause inappropriate antioxidant capability, leading to increased oxidative stress. It is also possible that the reduced contractile function observed in the *α–MHCMet-KO* mice could be a consequence of the decrease in the number of mitochondria and/or their functionality [52] as they are critical to provide ATP for contraction.

The effects of Met inactivation in cardiomyocytes partially resemble heart defects in Sirt3-deficient mice [44]. These mice present heart fibrosis and ROS accumulation as a consequence of the decrease in catalase and SOD-2 expression in cardiomyocytes, but they also show cardiac hypertrophy [44]. The capability of Sirt3 and Met to prevent cardiac damage by suppressing ROS accumulation is

in agreement with the lethality of SOD-2 deficient mice a few days after birth due to dilated cardiomyopathy, despite normal heart development [57]. It is also important to highlight that the contractile defect observed in the inducible HGF transgenic (tg) mice shares certain similarities to the one induced in *α–MHCMet-KO* mice [21]. Because in these HGF tg mice, the endogenous Met receptor is downregulated, this might partially resemble our mouse model lacking a functional Met receptor in cardiomyocytes. However, different from the HGF tg mouse, HGF would not be over-expressed in our *α–MHCMet-KO* mouse model.

Mechanistically, we provide *in vivo* and *in vitro* evidences supporting a role for p38 MAPK in the antioxidant effect of HGF/Met in cardiomyocytes. HGF increases SOD-2 and catalase expression and prevents their down-regulation by TGF-β when p38α MAPK signaling is intact. These findings are consistent with the previously reported role of p38α MAPK as a mediator of SOD-2 and catalase expression in response to oxidative stress in MEFs [43]. These findings, together with recent studies on p38MAPK requirement for the survival effect of HGF in embryonic and cancer cells [58-59], unveil unexpected requirements of the p38MAPK pathway in cell regulation by receptor tyrosine kinases.

Our studies demonstrate a cell autonomous requirement of HGF/Met signaling in cardiomyocytes to ensure heart function in adult mice by preventing oxidative stress generation and ROS-induced damage. The control of TGF-β production and p38α MAPK activity by the HGF/Met axis appears to be essential for the maintenance of heart homeostasis. Indeed, p38α MAPK appears to be an important mediator of HGF/Met actions, as it is required for the up-regulation of antioxidant enzymes. Notably, HGF not only regulates the production of TGF-β in the heart, but also partially counteracts its effect on cardiomyocytes.

Our findings open a new perspective to further explore HGF/Met as putative diagnostic markers of individuals subjective to heart defects and therapeutic intervention to treat cases of idiopathic cardiomyopathies.

Acknowledgments

We thank Dr. S. Thorgeirsson for providing us with conditional c-met knock-out (Met flox/flox) mice, Dr. S. Ramos for her help in carbonylated proteins determination, Dr. A. Silva for his contribution to initial experiments, Rebeca Rodríguez and David Navas for technical assistance and Dr. T. Crepaldi for reading of the manuscript and helpful discussions. We also thank Ana Vázquez, Daniel Caballero and Neibla Priego for their help in some experiments. This work was supported by grants: (AFM)-13683 from Association Française contre les myopathies, France, FIS-PI070071 and SAF-2010-20198-C02-01 from Ministry of Science and Innovation, Spain, and grants from Comunidad de Madrid/Universidad Complutense de Madrid: CAM/UCM 920384 and UCM-BSCH 920384, Spain to A.P.; BFU2011-25304 from Ministry of Science and Innovation, Spain, RD06/0010/0015 (TerCel network, ISCIII), P11-CTS-7564 (Junta de Andalucía) to R M-Ch; FRM (Fondation pour la recherche médicale), Fondation Bettencourt-Schueller, and Association Française contre les myopathies to F.M.; SAF2010-15881 from Ministry of Science and Innovation, Spain, and RD012/0021 (RedinRen network, ISCIII) GR100 (Junta de Castilla y León) to JM L-N. The cardiovascular phenotyping unit of the University of Salamanca, including the telemetry equipment, has been acquired with the support of the European Regional Development Funds (FEDER).

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Figure legends

Fig. 1 Conditional deletion of c-met in cardiomyocytes allows normal development and function of the heart in mice. **a:** Left panel: Representative PCR analysis showing a deletion (del) specific fragment (650bp) from the Cre-recombined allele detected in E15.5 embryonic hearts from α-MHCMet-KO mice after c-met exon 16 deletion and the floxed (flx) fragment (380bp) present in met flox/flox hearts without Cre recombination (named as $Met^{fl/fl}$ or control) (n=5). Right panel: Western blot analysis of Met protein levels in hearts from E15.5 embryos. **b and c:** Heart sections from wt and α-MHCMet-KO E15.5 embryos (b) and P2 newborn mice (c) stained with hematoxylin/eosin (scale bars: 250μM for b top panel and c, and 50μM for b low panel) (n=10).

Fig. 2 Met inactivation in cardiomyocytes leads to cardiomyocyte hypertrophy, fibrosis and apoptosis in adult mice. **a:** Heart sections from control and α-MHCMet-KO males (3 and 6 months old) stained with hematoxylin/eosin (scale bar: 100μm) (n=5). **b:** Representative laminin immunostaining of heart sections from 6 months (mo) old males (control and α-MHCMet-KO). **c:** Histogram showing the mean values ±SEM of the transverse cardiomyocyte area in 6 and 9 months old male hearts. Differences between control and α-MHCMet-KO are significant (**p<0.01, n=5). **d:** Mallory's trichrome staining of collagen. Fibrotic areas (in blue) are present in 6 months old α-MHCMet-KO male hearts (scale bar: 200μm). **e:** Histogram showing the mean values ±SEM of the fibrotic cardiac surface in 6 and 9 months old male hearts (control and α-MHCMet-KO). **p<0.01, control as compared with α-MHCMet-KO (n=5). **f:** Immunolocalization of active caspase-3 in 6 months old male hearts (left) and histrogram showing the mean values ±SEM of the percentage of active caspase 3 positive cells (right) (n=4). Positive cells (arrows) can be observed in the fibrotic areas of α-MHCMet-KO hearts (scale bar: 25μm). Some of them show remaining desmin immunoreactivity (in green), suggesting they are apoptotic cardiomyocytes. Note the depletion of desmin in cardiomyocytes from the fibrotic areas. **g**: Quantification of ANF and β-MHC mRNAs expression by RT-qPCR in hearts from 3 and 6 months old males, comparing α-MHCMet-KO with controls of the same age. RQ values ±SEM are shown in the histogram (n=5). A significant increase (***p<0.001) is produced in 6 months old MHCMet-KO males. **h**: Significant increased (*p<0.05) expression of TGF-β2, TGF-β3 and Acta2 mRNAs in 6 months old mutant males as compared with controls detected by RT-qPCR analysis (mean RQ values ±SEM are shown, n=3).

Fig. 3 Conditional deletion of c-met in cardiomyocytes leads to heart dysfunction in 9 months-old mice. **a**: Representative electrocardiogram (ECG) of an α-MHCMet-KO compared with a control male (6 months old). The figure shows a negative deflection in the S-P segment (a) and a lower J wave (b). **b**: Echocardiography analysis in 9 months-old males. The following parameters are shown: IVS, interventricular septum thickness; LVPW, left-ventricular posterior wall thickness; LVDd, left-ventricular diastolic dimension; LVDs, left-ventricular systolic dimension; LVDEV, left-ventricular end-diastolic volume; LVESV, left-ventricular end-systolic volume; LVM, left ventricular mass. All data are expressed as mean ±SEM. *p<0.05, control as compared with α-MHCMet-KO. **c**: Representative echocardiographic images of an α-MHCMet-KO compared with a control male.

Fig. 4 Changes in anti-oxidant enzymes, ROS levels and mitochondrial proteins in adult α-MHCMet-KO male hearts. **a:** Representative Western-blot analysis of catalase and superoxide dismutase-2 (SOD-2) protein levels in α-MHCMet-KO and control hearts from 6 and 9 months old males normalized with β-actin (left). The histrogram shows the mean values ±SEM of the densitometry of the blots from different experiments (n=4): catalase and SOD-2 versus β-actin. *p<0.05, control as compared with mutants (right). **b**: Catalase and SOD-2 activities from 6 months old males is shown as the percentage of the activities of control hearts (mean values ±SEM), ***p<0.001, control as compared with α-MHCMet-KO (n=4). **c:** Representative sections showing accumulation of ROS (detected by 2-hydroethidine staining) in cardiomyocytes from 6 months old mutant males. **d**: Histograms show the mean values ±SEM (n=3) of the number of 2-hydroethidine stained nuclei by microscopy field in cardiac tissue sections from 6 months old males (left) and the percentage of 4-OH-nonenal immunoreactive surface in hearts from 9 months old males (right). *p<0.05 and **p<0.01, control as compared with α-MHCMet-KO. **e**: Histograms represent the fold increase of carbonylated protein levels (mean values ±SEM, n=4) present in heart extracts from α-MHCMet-KO males referred to controls (6 months old). ***p<0.001, α-MHCMet-KO as compared with control mice. **f**: Representative cytochrome c (Cyt c) and porin western-blots (left) and densitometry of blots (porin and cyt c versus β-actin) (right) from 6 months old male hearts (n=4). *p<0.05 and **p<0.01, control as compared with α-MHCMet-KO. **g**: Cyt c oxidase activity is significantly decreased (***p<0.001) in 6 months old α-MHCMet-KO as compared with control males. Histograms represent the percentage of the activities in the mutants as compared to controls (mean values ±SEM, n=4)

Fig. 5 HGF/Met signaling increases antioxidant enzymes in cardiomyocytes acting through p38α MAPK. Effect on TGF-β mediated down-regulation and ROS accumulation. **a**: Representative Western-blot analysis of P-p38MAPK and total p38 in hearts from 6 and 9 months-old males (left) and densitometric analyses of the blots (referred to β-actin, n=4). **b**: Upper panel, representative Western-blot analysis of catalase, SOD-2, P-Met, P-p38MAPK and total p38 protein levels in wt and p38α-/- cardiomyocytes maintained untreated or treated with the Met inhibitor, SU11274 (SU) (3 days) and/or HGF (16h pre-incubation), as indicated. Lower panel, histograms show the mean values ±SEM (n=3) of the densitometric analyses of catalase and SOD-2 blots (referred to β-actin). *p<0.05, as compared with wt control cells; +p<0.05, as compared with HGF treated wt cells. **c**: Levels of carbonylated proteins in wt and p38α-/- cardiomyocytes treated as indicated in b section. The histogram shows the fold increase referred to control values from wt cells (mean values ±SEM (n=3). **p<0.01 and ***p<0.001, as compared with wt control. **d:** Upper panel, representative Western-blot analysis of catalase, superoxide dismutase-2, P-p38MAPK and total p38 protein levels in wt and p38α-/- cardiomyocytes maintained in the absence or presence of TGF-β (3 days) and/or HGF (16h pre-incubation), as indicated. Lower panel, histograms showing the mean values ±SEM (n=3) of the densitometric analyses of catalase and SOD-2 blots (referred to β-actin). *p<0.05, as compared with wt control. **e**: Levels of carbonylated proteins in wt and p38α-/- cardiomyocytes treated as indicated in d section. The histogram shows the fold increase referred to control values from wt cells (mean values ±SEM (n=3)). **p<0.01 and ***p<0.001, as compared with wt control.

Fig. 6 Treatment with antioxidants partially prevents the increase in cardiomyocyte cell size induced by Met inhibition. Immortalized neonatal wt cardiomyocytes were maintained in the absence or presence of the Met inhibitor SU11274 (SU) and/or N-acetyl cysteine (NAC) for 1 or 4 days as indicated. **a:** Representative phase-contrast microscopy images after 1 day of treatment (scale bar: 25μm). **b:** Quantification of cardiomyocytes size after treatment, expressed as arbitrary units (***$p<0.001$, $n=3$).

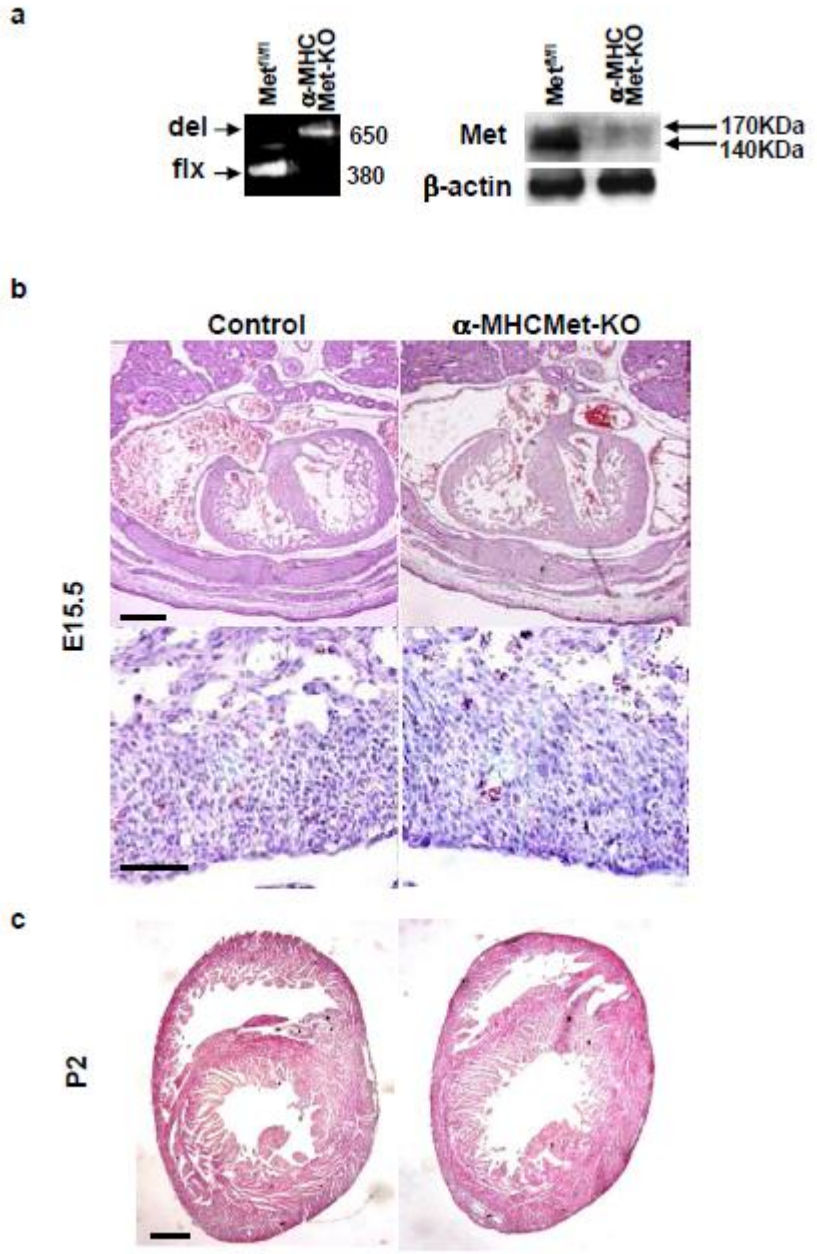

a
Met^fl/fl
α-MHC Met-KO
del
flx
650
380
Met
β-actin
170KDa
140KDa
b
Control
α-MHCMet-KO
E15.5
c
P2

Fig. 1

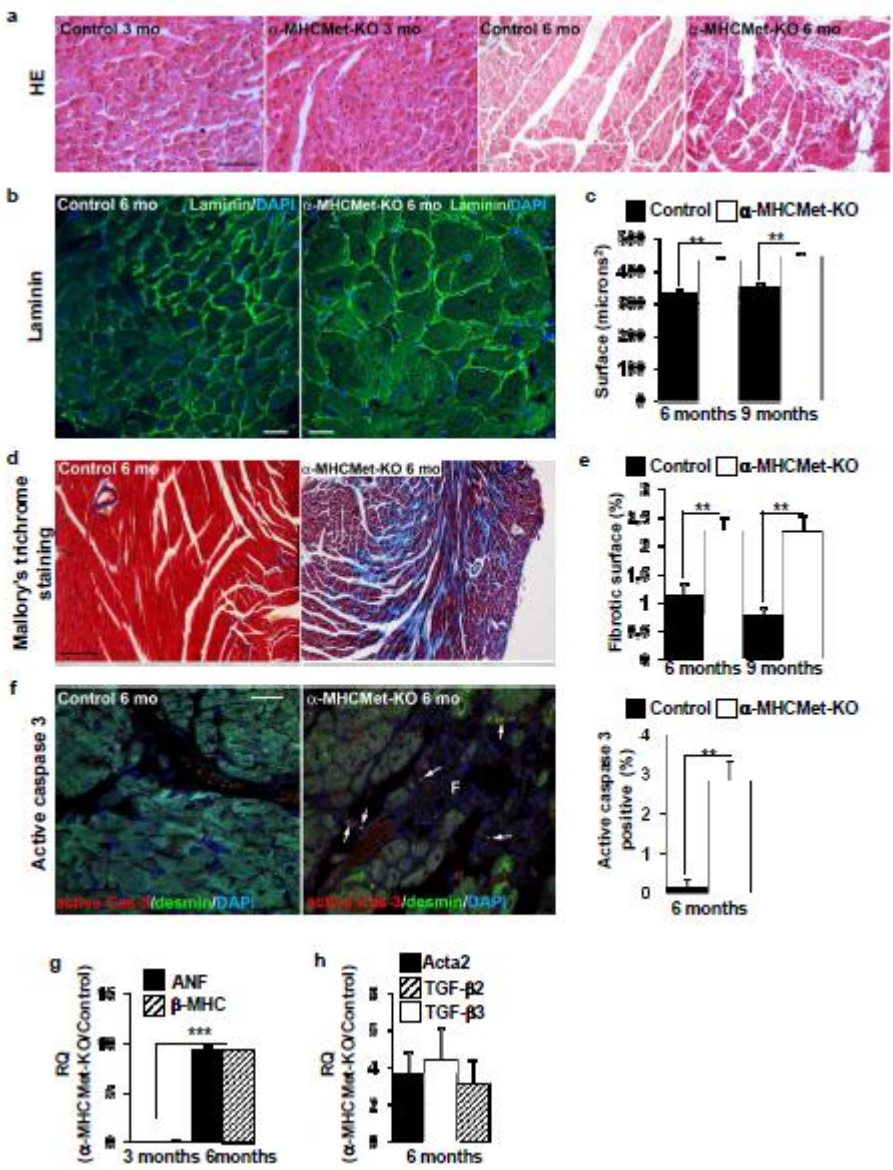
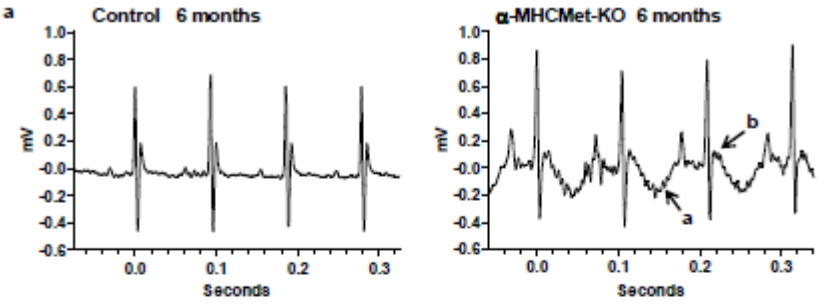


Fig. 2

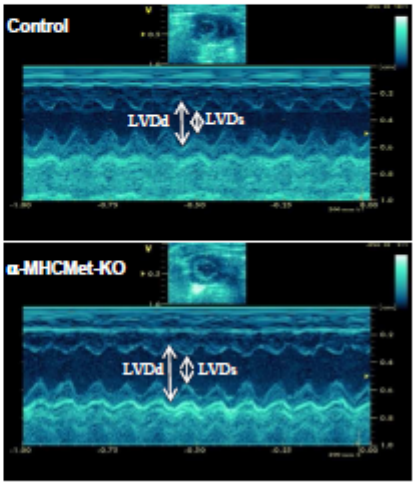
a



b

Echocardiographic Analysis	Control males	α–MHCMet-KO males
n	4	4
Age (months)	9	9
Body weight (g)	53.73 ± 4.67	60.0 ± 6.11
Heart rate (bpm)	680 ± 29.23	644 ± 28.18
IVS (mm)	0.90 ± 0.05	1.0 ± 0.00
LVPW (mm)	1.03 ± 0.06	1.03 ± 0.07
LVDd (mm)	3.15 ± 0.18	3.23 ± 0.12
LVDs (mm)	**1.55 ± 0.07**	**1.77 ± 0.03***
LVEDV (µl)	39.89 ± 5.76	42.19 ± 3.72
LVESV (µl)	**6.67 ± 0.83**	**9.28 ± 0.44***
Stroke volumen (µl)	33.22 ± 5.08	32.91 ± 3.29
Cardiac output (ml/min)	22.59 ± 3.64	21.38 ± 2.95
Shortening fraction (%)	**59.28 ± 1.62**	**44.33 ± 1.76***
Ejection fraction (%)	**92.65 ± 0.94**	**81.90 ± 1.63***
LVM (mg)	84.01 ± 9.83	94.17 ± 6.08
LVM/BW (mg/g)	1.56 ± 0.09	1.75 ± 0.08

c



9 months

Fig. 3

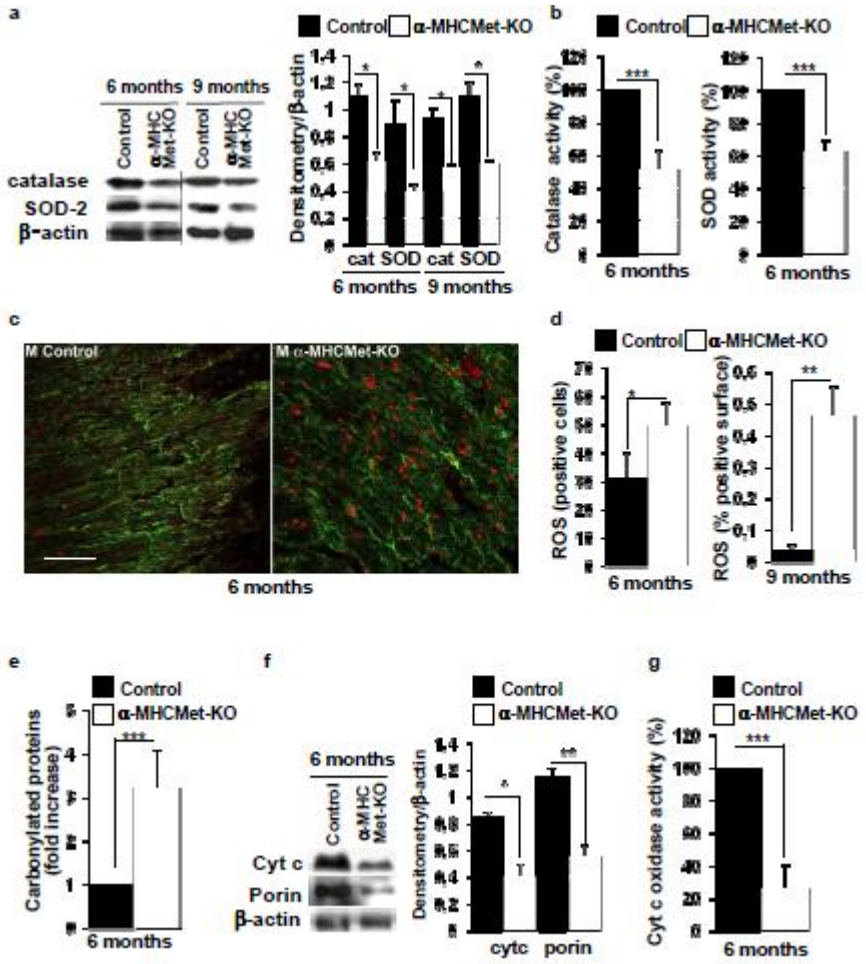


Fig. 4

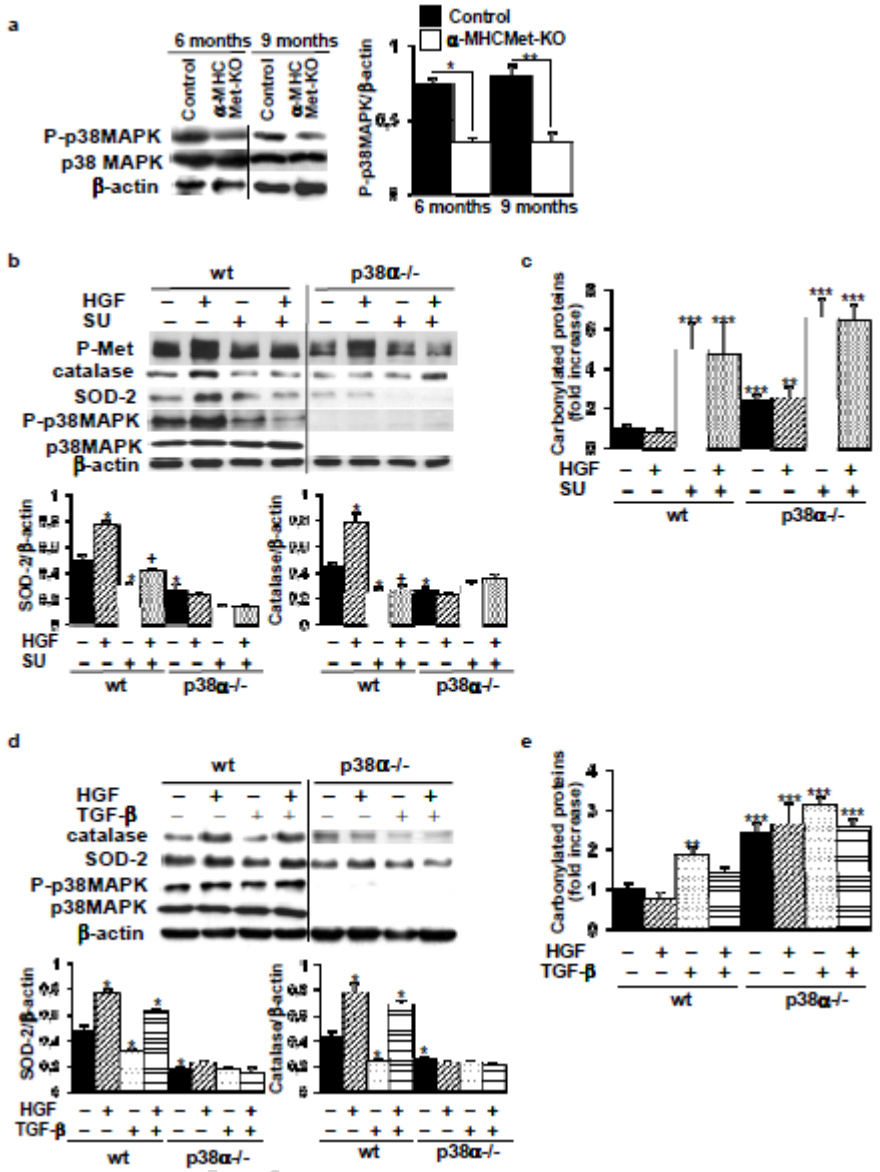


Fig. 5

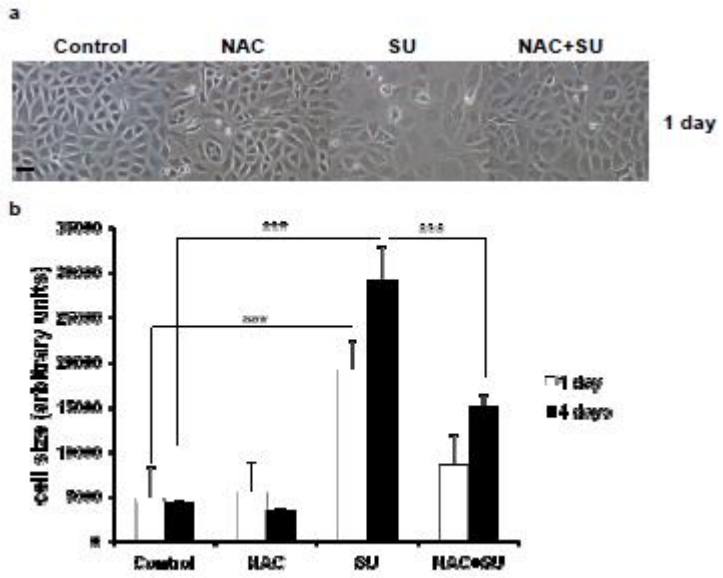


Fig. 6

Highlights

-In vivo genetic inactivation of Met in cardiomyocytes leads to heart dysfunction
-Met signaling prevents reactive oxygen species accumulation in cardiomyocytes
-HGF/Met acting through p38α MAPK up-regulates antioxidant enzymes in cardiomyocytes