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# Angiotensin-(1-7) receptor Mas is an essential modulator of extracellular matrix protein expression in the heart

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ABSTRACT

In this study we investigated the effects of genetic deletion of the Angiotensin-(1-7) receptor Mas or the Angiotensin II receptor $AT_2$ on the expression of specific extracellular matrix (ECM) proteins in atria, right ventricles and atrioventricular (AV) valves of neonatal and adult mice. Quantification of collagen types I, III and VI and fibronectin was performed using immunofluorescence-labeling and confocal microscopy. Picrosirius red staining was used for the histological assessment of the overall collagen distribution pattern. ECM proteins, metalloproteinases (MMP), ERK1/2 and p38 levels were quantified by western blot analysis. Gelatin zymography was used to evaluate the activity of MMP-2 and MMP-9. We observed that the relative levels of collagen types I and III and fibronectin are significantly higher in both the right ventricle and AV valves of neonatal $Mas^{-/-}$ mouse hearts (e.g., collagen type I: $85.28 \pm 6.66$ vs $43.50 \pm 4.41$ arbitrary units in the right ventricles of $Mas^{+/+}$ mice). Conversely, the level of collagen type VI was lower in the right ventricle and AV valves of $Mas^{-/-}$ mice. Adult $Mas^{-/-}$ mouse hearts presented similar patterns as observed in neonates. No significant differences in ECM protein level were detected in atria. Likewise, no changes in ECM levels were observed in $AT_2$ knockout mouse hearts. Although deletion of Mas induced a significant reduction in the level of the active form of MMP-2 in neonate hearts and a reduction of both MMP-2 and MMP-9 in adult $Mas^{-/-}$ mice, no significant differences were observed in MMP enzymatic activities when compared to controls. The levels of the active, phosphorylated forms of ERK1/2 and p38 were higher in hearts of both neonatal and adult $Mas^{-/-}$ mice. These observations suggest that Mas is involved in the selective expression of specific ECM proteins within both the ventricular myocardium and AV valves. The changes in the ECM profile may alter the connective tissue framework and contribute to the decreased cardiac performance observed in $Mas^{-/-}$ mice.



## 1. Introduction

The extracellular matrix (ECM) has been described as a supportive scaffold which is important in both formation and maintenance of tissues. In the heart, ECM forms an elaborate, stress-tolerant network, interconnecting myocytes to each other and myocytes to capillaries within the ventricular wall [1]. Interactions between cells and the surrounding ECM play critical roles in a number of cellular processes, including migration, proliferation, differentiation and survival. The interstitial network within the myocardium is composed predominantly of fibrillar collagen types I and III [2]. Cardiac fibrillar collagen provides structural scaffolding for cardiomyocytes and coronary vessels and imparts cardiac tissue with physical properties that include stiffness and resistance to deformation [2,3]. It is now clear that the ECM is a dynamic structure whose organization and composition are known to modulate various cellular processes. Events that alter the molecular composition of the ECM, or the structural organization of ECM components, can induce profound changes in cellular functions [4]. Excessive deposition of collagen is thought to contribute to abnormal stiffness and function of the ventricular myocardium [5]. In many cases these changes are associated with activation of humoral systems such as the renin–angiotensin system (RAS) [6].

Components of the circulating and local RAS are closely involved in the development of myocardial fibrosis in hypertensive heart

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disease and chronic heart failure. The classical effector of this system, the octapeptide Angiotensin (Ang) II, exerts its effects through specific Ang II receptor isoforms, $AT_1$ and $AT_2$. Ang II binds $AT_1$ receptor and stimulates synthesis and deposition of collagen in a dose-dependent manner and suppresses the activity of matrix metalloproteinase (MMP) 1, an enzyme which plays an important role in interstitial collagen degradation [7]. On the other hand, accumulating lines of evidence support the broad view that Ang II can bind to the $AT_2$ receptor and induce growth suppression [8]. Conversely, Ang-(1-7), a biologically active member of the RAS and an endogenous ligand for the G protein-coupled Mas receptor [9], has been suggested to act as an antiproliferative [10–14] and anti-fibrotic peptide [13,14]. Ang-(1-7) inhibits growth of cardiomyocytes through Mas-mediated events, which include ERK1/2 activities [13]. In addition, Ang-(1-7) and its analog AVE 0991 have been shown to attenuate the development of heart failure after myocardial infarction, a finding that suggests a role for this peptide in cardiac remodeling [15,16]. In keeping with these data, AVE 0991 also prevented isoproterenol-induced cardiac remodeling [17]. These effects are apparently independent of changes in blood pressure since Grobe and colleagues [18,19] have demonstrated that the anti-fibrotic and anti-hypertrophic actions of Ang-(1-7) are still observed in Ang II-infused [19] or in DOCA-salt hypertensive rats [18]. Overexpression of the main Ang-(1-7)-forming enzyme, angiotensin-converting enzyme 2 (ACE2), in a rat model of myocardial infarction protected the infarcted myocardium against pathological remodeling and cardiac systolic dysfunction [20]. The anti-fibrotic and anti-

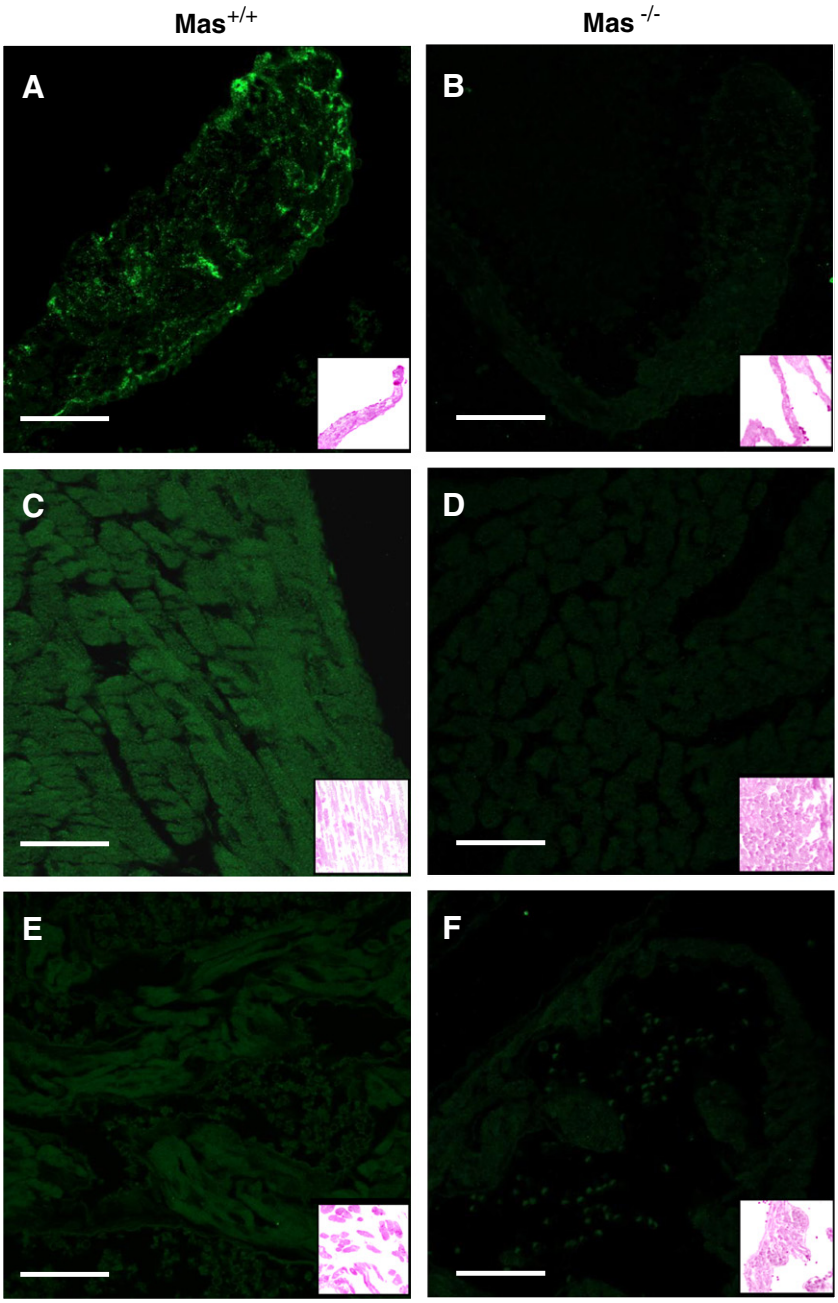


**Fig. 1.** Immunofluorescent localization of Mas in hearts. Mas is present in tricuspid valve (A), right ventricle (C) and atria (E) of adult $Mas^{+/+}$ mouse hearts but absent in the tricuspid valve (B), right ventricle (D) and atria (F) of adult $Mas^{-/-}$ mouse hearts. No immunostaining was detected in any of the samples when the primary antibody was omitted from the incubation procedure (not shown). Insets are low magnification views of the tissue areas analyzed. Representative figures of three different animals. Bar = 50 μm.

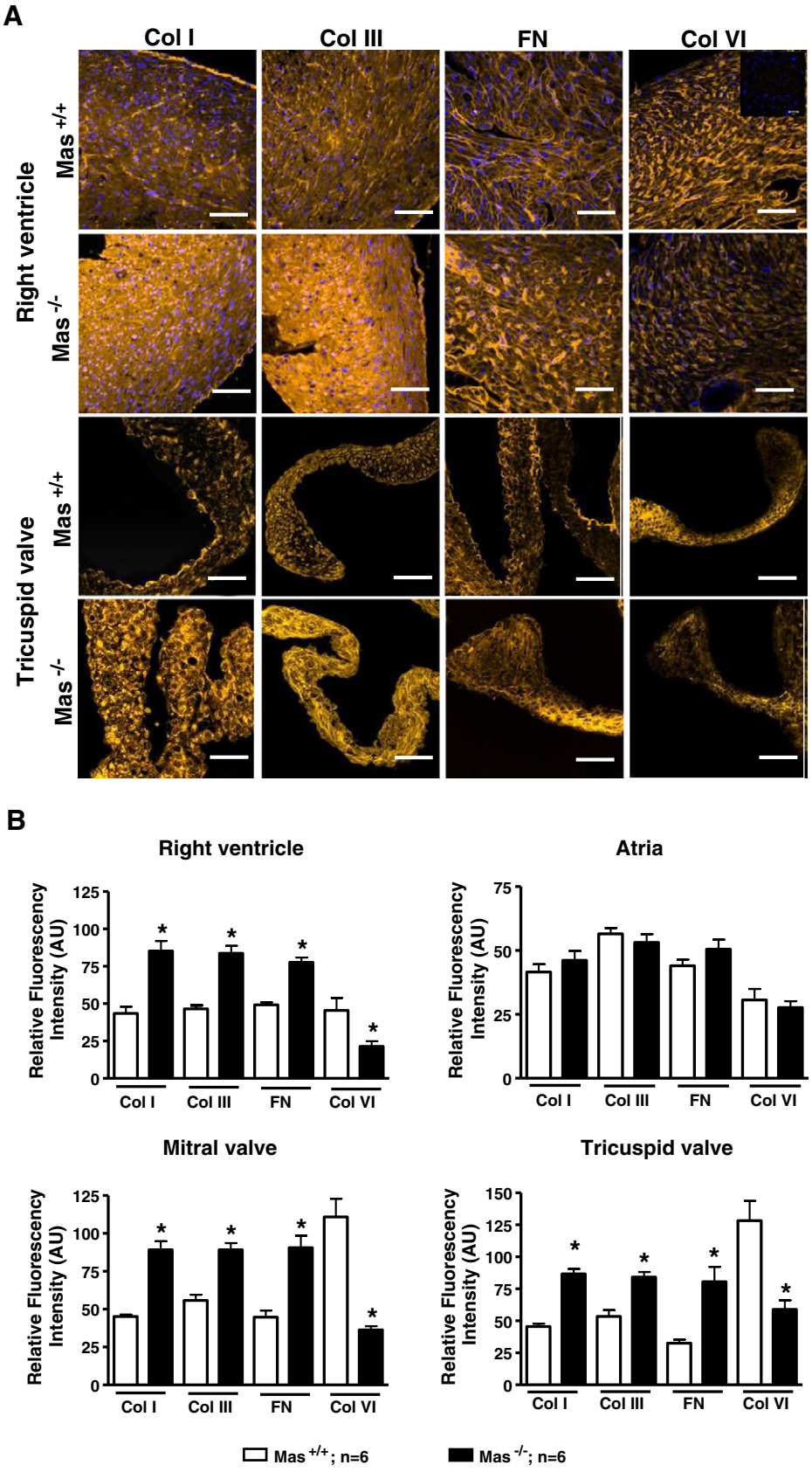


**Fig. 2.** Immunofluorescent localization of collagen types I, III and VI and fibronectin in the right ventricle and tricuspid valve of neonatal Mas$^{+/+}$ and Mas$^{-/-}$ mouse hearts (A). The inset shows the level of immunostaining obtained when the primary antibody was omitted from the incubation procedure. Quantification of ECM proteins in the right ventricle, AV valves and atria of neonatal Mas$^{+/+}$ and Mas$^{-/-}$ mice (B). Values are expressed as arbitrary units (AU). Data are shown as mean ± SEM. *$p<0.05$. Bar = 50 μm.

hypertrophic actions of ACE2/Ang-(1-7) were also observed in animals (rats and mice) with pulmonary hypertension induced by monocrotaline [21,22] and in rats with pulmonary fibrosis caused by bleomycin treatment [23]. Recently, we have shown that deletion of the Ang-(1-7) receptor Mas markedly decreases cardiac function in adult mice in part due to changes in collagen levels to a pro-fibrotic profile, i.e. an increase of collagen types I and III and fibronectin and a decrease of collagen type VI in left ventricles [24]. However, it is unknown whether these alterations in ECM extend to other regions of hearts from neonatal and adult mice, such as the right ventricle, atria and atrioventricular (AV) valves. Thus, in the current study we evaluated the presence of Mas in the right ventricle, atria and AV valves of neonatal and adult mice and checked if its deletion leads to alterations in ECM deposition in these regions. In addition, we investigated the effects of deletion of the $AT_2$ receptor on the expression of ECM proteins in adult mouse hearts, since in certain circumstances $AT_2$ receptors appear to be involved in the Ang-(1-7) effects.

## 2. Material and methods

### 2.1. Mas knockout mice and $AT_2$ knockout mice

Male wild-type ($Mas^{+/+}$, $AT_2^{+/y}$) and Mas and $AT_2$ knockout ($Mas^{-/-}$, $AT_2^{-/y}$) C57BL/6 mice (adult mice: 8–12 weeks old; neonate mice: 1 day old) were obtained from the transgenic animal facilities of the Laboratory of Hypertension, Federal University of Minas Gerais, Brazil. All animal procedures were performed in accordance with institutional guidelines (Federal University of Minas Gerais, Brazil). Genotypes were confirmed by PCR analysis.

### 2.2. Immunostaining and confocal microscopy

Immunofluorescence-labeling and quantitative confocal microscopy were used to investigate the distribution and quantity of Mas, collagen types I, III and VI and fibronectin present in $Mas^{+/+}$, $Mas^{-/-}$, $AT_2^{+/y}$ and $AT_2^{-/y}$ mice hearts. Hearts were collected from $Mas^{+/+}$ and $Mas^{-/-}$ neonatal (n = 6) and adult (n = 6) male mice and from $AT_2^{+/y}$ and $AT_2^{-/y}$ adult (n = 4) male mice, washed in phosphate-buffered saline (PBS) and cryofixed in a −80 °C solution of 80% methanol and 20% dimethyl sulfoxide. After 5–7 days of freeze-substitution, samples were embedded in paraffin following standard methods [24]. Five micrometer thick sections were mounted on slides, deparaffinized, rehydrated and then incubated in blocking solution (1% BSA and 0.1% Tween 20 in PBS) at room temperature for 1 h. Sections were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-human Mas (1:100, Abcam, Cambridge, MA), rabbit anti-human collagen type I (1:400, Rockland Immunochemicals Inc., Gilbertsville, PA), rabbit anti-human collagen type III (1:400, Rockland Immunochemicals Inc., Gilbertsville, PA), rabbit anti-human collagen type VI (1:600, Research Diagnostics Inc., Acton, MA) or rabbit anti-human fibronectin (1:800, Rockland Immunochemicals Inc., Gilbertsville, PA). After 4–5 rinses in PBS, donkey anti-rabbit IgG conjugated with Cy3 (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h in the dark at room temperature. Following washes with PBS, sections were mounted and viewed with a laser scanning confocal microscope (Zeiss 510Meta). Optimal confocal settings (aperture, gain and laser power) were determined at the beginning of each imaging session and then held constant during the analysis of all the samples. Nuclei were labeled with 4′6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Carlsbad,

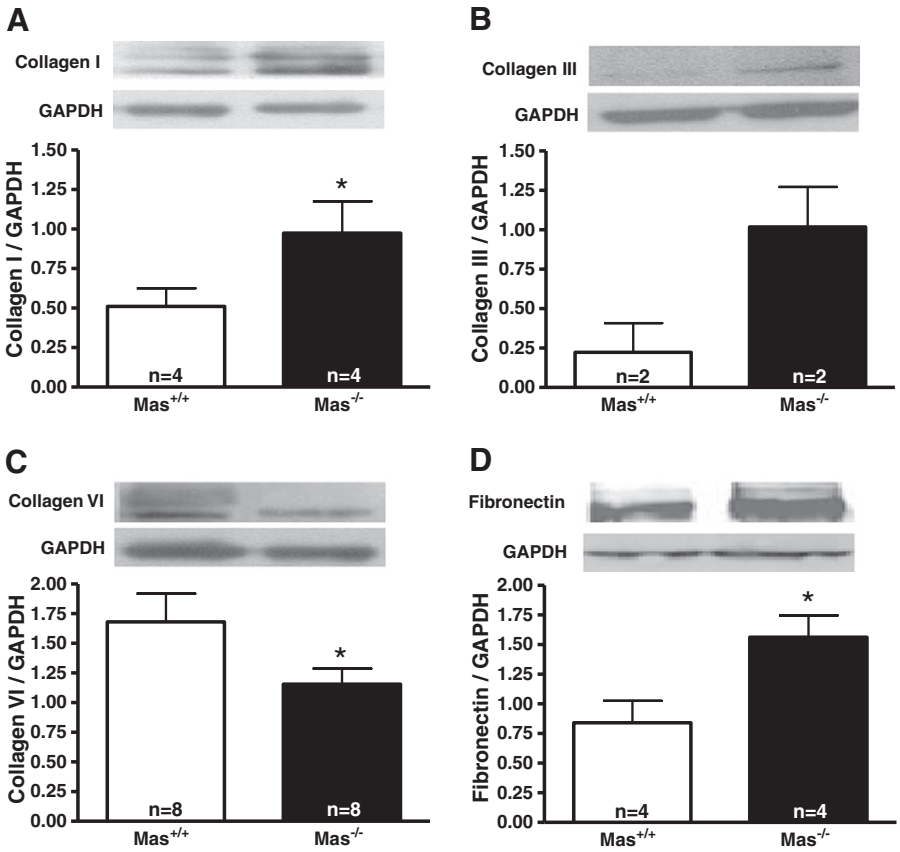


**Fig. 3.** Western blotting analysis of the levels of collagen types I (A), III (B) and VI (C) and fibronectin (D) in hearts of neonatal $Mas^{+/+}$ and $Mas^{-/-}$ mice. Densitometry values were normalized using GAPDH. Data are shown as the SEM. $^*p<0.05$.

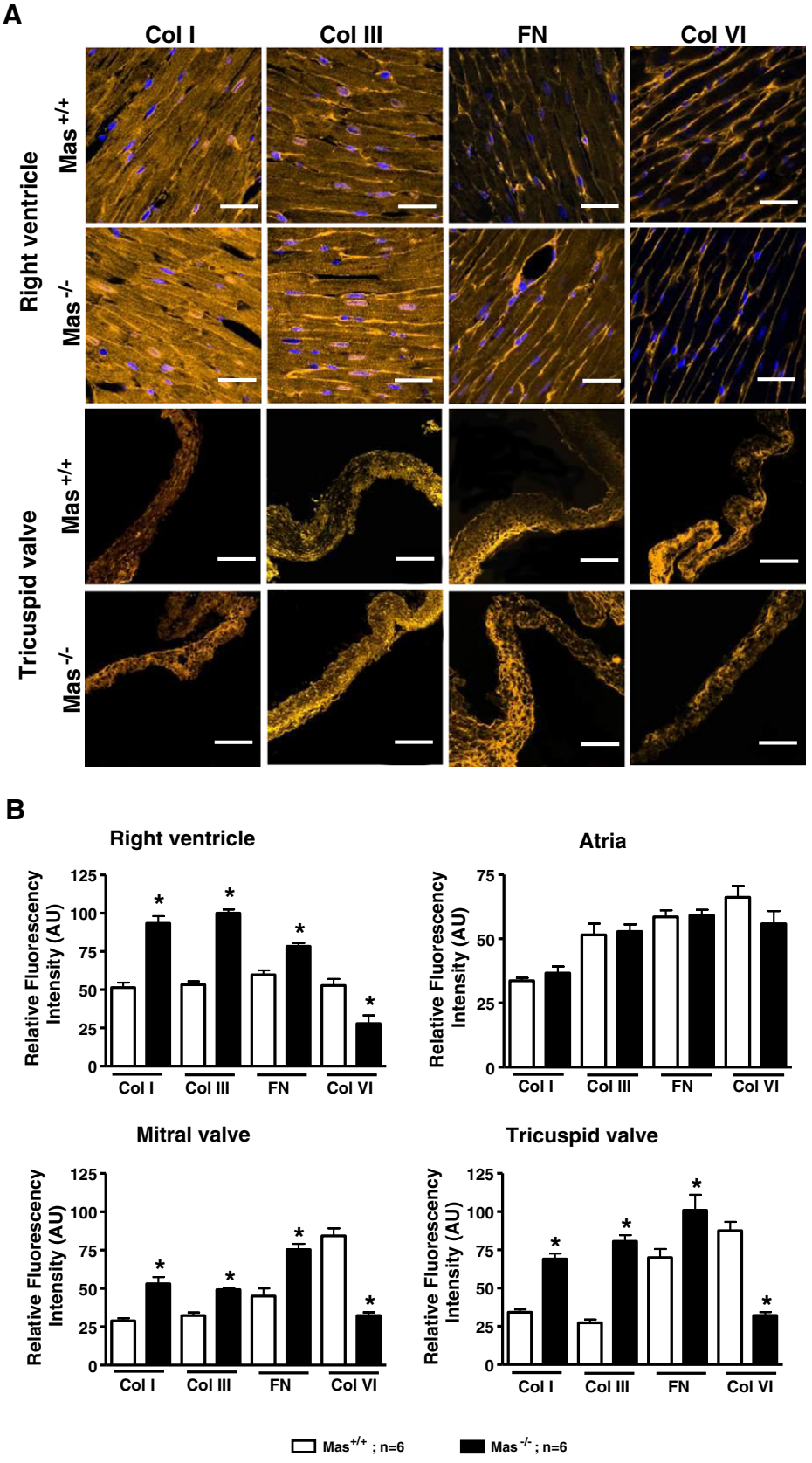


**Fig. 4.** Immunofluorescent localization of collagen types I, III and VI and fibronectin in the right ventricle and tricuspid valve of adult $Mas^{+/+}$ and $Mas^{-/-}$ mouse hearts (A). Quantification of ECM proteins in the right ventricle, AV valves and atria of adult $Mas^{+/+}$ and $Mas^{-/-}$ mice (B). Values are expressed as arbitrary units (AU). Data are shown as mean ± SEM. $^{*}p<0.05$. Bar = 50 μm.

CA). For quantitative analysis of collagens I, III and VI and fibronectin, we used the ImageTool 2.0 image analysis program (http://ddsdx.uthscsa.edu/dig/itdesc.html) to measure the fluorescence intensity in images randomly selected from the right ventricle, AV valves and atria of $Mas^{+/+}$ and $Mas^{-/-}$ mice and from the right and left ventricles of $AT_2^{+/y}$ and $AT_2^{-/y}$ mice. Images were captured at 12 bit and analyzed in the gray scale range of 0 to 255. Fluorescence intensity was measured as an average of the area (i.e. the sum of gray values of all pixels divided by the number of pixels in the area) and values recorded as arbitrary units (AU). Background fluorescence was measured and subtracted from the region of interest.

### 2.3. Histological analysis

Picrosirius red staining was used for histological analysis of total collagen content in tricuspid valves of neonatal and adult $Mas^{-/-}$ and $Mas^{+/+}$ mice and right ventricles of adult $Mas^{-/-}$ and $Mas^{+/+}$ mice (n = 5 per group). Samples were prepared as described above, sectioned at a thickness of 5 μm, stained with picrosirius red (direct red 80) [25] and then analyzed at 10× magnification using polarizing filters adapted onto a conventional light microscope.

### 2.4. Western blotting

Right ventricles from adult mice (n = 2–4) and whole hearts from neonate mice (n = 2–8) were collected and homogenized in lysis buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mmol/L PMSF, 1 mmol/L pepstatin A, 1 mmol/L leupeptin, and 1 mmol/L aprotinin. Forty micrograms of protein from each sample were separated by electrophoresis on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation in 5% milk and 0.1% Tween 20 in Tris-buffered saline. Membranes were probed with specific primary antibodies: anti-collagen type I (1:500, Rockland Immunochemicals Inc., Gilbertsville, PA), anti-collagen type III (1:3000, Rockland Immunochemicals Inc., Gilbertsville, PA), anti-collagen type VI (1:500, Research Diagnostics Inc., Acton, MA), anti-fibronectin (1:5000, Rockland Immunochemicals Inc., Gilbertsville, PA), anti-p38MAPK (1:1000, Cell Signaling Technology Inc., Danvers, MA), anti-ERK1/2 (1:1000, Cell Signaling Technology Inc., Danvers, MA), anti-MMP-2 (1:500, Chemicon International, Billerica, MA), anti-MMP-9 (1:500, Chemicon International, Billerica, MA) or anti-GAPDH (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with enzyme-labeled secondary antibodies. The membrane was incubated in chemiluminescent substrate then exposed to film. Immunoreactive bands were quantified by densitometry using GAPDH, total p38 or total ERK1/2 as a normalization control.

**Table 1**
Echocardiographic analysis of $Mas^{+/+}$ and $Mas^{-/-}$ mice.

| Parameter | $Mas^{+/+}$ (n = 5) | $Mas^{-/-}$ (n = 5) |
|---|---|---|
| Cardiac output (mL/min) | 18.76 ± 1.30 | 16.80 ± 1.05 |
| LV internal dimension at diastole (mm) | 3.72 ± 0.14 | 4.20 ± 0.06* |
| LV internal dimension at systole (mm) | 2.46 ± 0.14 | 3.32 ± 0.03* |
| LV ejection fraction (%) | 63.48 ± 2.82 | 42.90 ± 1.26* |
| LV fractional shortening (%) | 34.07 ± 2.06 | 20.93 ± 0.74* |
| LV systolic volume (μL) | 37.37 ± 2.70 | 33.85 ± 2.05 |
| End diastolic LV volume (μL) | 59.45 ± 5.04 | 78.72 ± 2.80* |
| End systolic LV volume (μL) | 22.08 ± 2.97 | 44.87 ± 1.24* |
| Heart rate (bpm) | 492.60 ± 25.70 | 487.80 ± 20.70 |
| RV fractional area change (FAC) (%) | 57.58 ± 1.20 | 38.69 ± 2.30* |

Data are reported as mean ± SEM. Statistical analyses were performed using Student's *t* test. LV: left ventricle; RV: right ventricle. *$p < 0.05$.

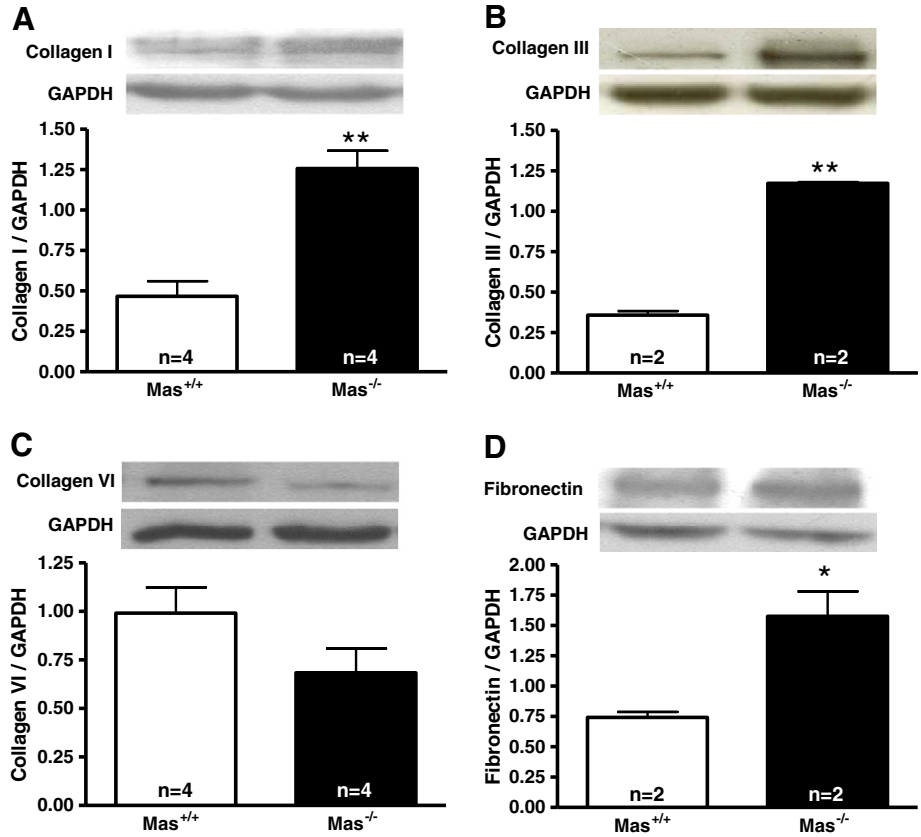


**Fig. 5.** Western blotting analysis of the levels of collagen types I (A), III (B) and VI (C) and fibronectin (D) in hearts of adult $Mas^{+/+}$ and $Mas^{-/-}$ mice. Densitometry values were normalized using GAPDH. Data are shown as the SEM. *$p < 0.05$ and **$p < 0.001$.

### 2.5. Gelatin zymography

Right ventricles from adult mice (n = 3) and whole hearts from neonate mice (n = 3–8) were homogenized in 50 mmol/L Tris–HCl pH 7.4, 0.2 mol/L NaCl, 0.1% Triton, 10 mmol/L $CaCl_2$ and 1% Protease Inhibitor Cocktail (PI860, Sigma Chemical Co., St. Louis, MO) using an ultrasonic cell disruptor. The homogenates were incubated for 2 h at 4 °C and centrifuged at 2080 *g* for 20 min at 4 °C. The supernatants were removed and the pellets were suspended once again in the same solution as described above, heated to 60 °C for 5 min and centrifuged at 2080 *g* for 20 min at 4 °C. Protein was quantified using the Bradford dye binding assay kit (BioAgency, São Paulo, SP, Brazil). Twenty micrograms of protein extract obtained as above were electrophoresed on a 10% SDS polyacrylamide gel containing 0.1% gelatin (used as protein substrate) at 4 °C under non-reducing conditions. After electrophoresis, the gel was washed twice, and then gently shaken under 2.5% Triton X-100 for 30 min at room temperature to remove SDS. The gel was incubated overnight in a 50 mmol/L Tris–HCl solution pH 7.4, containing 0.01 mol/L $CaCl_2$, 0.1 mol/L NaCl and 0.03% sodium azide at 37 °C. The gel was then stained with Coomassie Brilliant Blue (0.5% dye in 20% methanol and 10% acetic acid) for 1 h. Unstained bands indicating gelatinolytic activity were seen after slight destaining with 30% ethanol and 10% acetic acid. Quantitative assessment of band intensity was done by densitometry using the ImageJ image processing program (http://rsb.info.nih.gov/ij/). All experiments were done in quadruplicate.

### 2.6. Echocardiographic analysis

Cardiac morphology and function in adult $Mas^{+/+}$ and $Mas^{-/-}$ mice (9–10 weeks old, n = 5) were assessed noninvasively using a high-frequency, high-resolution echocardiographic system consisting of a VEVO 2100 ultrasound machine equipped with a 30–40 MHz bifrequencial transducer (Visual Sonics, Toronto, Canada). The mice were anesthetized with 5% isoflurane during 1 min for induction. Anesthesia was sustained via a nose cone with 1.25% isoflurane. The anterior chest was shaved and the mice were placed in supine position on an imaging stage equipped with built-in electrocardiographic electrodes for continuous heart rate monitoring and a heater to maintain the body temperature at 37 °C. High-resolution images were obtained in the right and left parasternal long and short axes and apical orientations. Standard B-mode images of the heart and pulsed Doppler images of the mitral and tricuspid inflow were acquired. Left ventricular (LV) dimensions and wall thickness were measured at the level of the papillary muscles in the left and right parasternal short axis during the end-systole and end-diastole. LV ejection fraction (EF), fractional shortening (FS) and mass were measured. All the measurements and calculations were done in accordance with the American Society of Echocardiography. The following M-mode measurements were performed: LV internal dimensions at diastole and systole (LVIDD and LVIDS, respectively), LV posterior wall dimensions at diastole and systole (LVPWD and LVPWS, respectively) and interventricular septal dimensions at

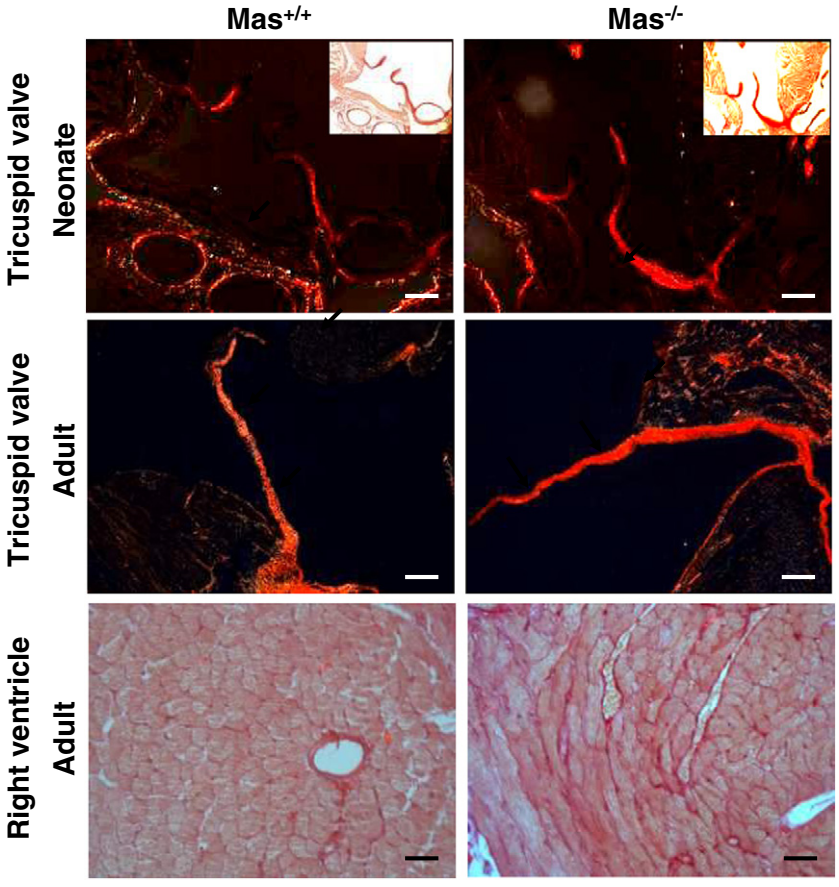


**Fig. 6.** Sections of tricuspid valves from neonatal and adult $Mas^{-/-}$ and $Mas^{+/+}$ mice and right ventricles of adult $Mas^{-/-}$ and $Mas^{+/+}$ mice stained with picrosirius red and analyzed at 10× magnification using polarizing filters adapted to a conventional light microscope. Based on observations with conventional microscopy, the total collagen content was significantly higher in AV valves (insets) of neonatal $Mas^{-/-}$ mouse hearts and in right ventricles of adult $Mas^{-/-}$ mouse hearts. An increased birefringence with polarization microscopy is indicative of an increase in fibrillar collagen. An increase was evident in the AV valves of neonatal and adult $Mas^{-/-}$ mice. No increase in birefringence was evident in sections of the ventricular wall (data not shown), suggesting that the increased amount of collagen in the ventricle is present in the microfibrillar form. Representative figures of five different animals. Bar = 50 μm.

diastole and systole (IVSDD and IVSDS, respectively). Based on these parameters, end diastolic and end systolic LV volumes (EDLVV and ESLVV, respectively), FS, EF, stroke volume (SV) and cardiac output (CO) were calculated. Also, in the right parasternal short axis view, bidimensional images from the right ventricle were acquired for fractional area change (FAC) calculation.

### 2.7. Statistical analysis

Data are reported as mean ± SEM. Statistical analyses were performed using the Mann Whitney test or Student's *t* test (echocardiography). p values of 0.05 or less were considered significant.

## 3. Results

First, we evaluated the presence of Mas in the different cardiac chambers of mice. Immunofluorescence-labeling and confocal microscopy demonstrated that, in addition to left ventricles [24], Mas is expressed in the tricuspid valve, right ventricle and atria of adult $Mas^{+/+}$ mice (Fig. 1A, C and E). As expected, no expression of Mas was observed in hearts of $Mas^{-/-}$ adult mice (Fig. 1B, D and F).

Fig. 2A shows immunostaining profile of several ECM proteins in the right ventricles and tricuspid valves of $Mas^{+/+}$ and $Mas^{-/-}$ neonatal mice. Quantitative analysis of the level of immunofluorescent staining revealed that hearts from $Mas^{-/-}$ neonatal mice present significantly higher levels of collagen types I and III and fibronectin in right ventricles and tricuspid valves (Fig. 2B). In contrast, the level of collagen type VI in $Mas^{-/-}$ neonatal mice was lower. A similar pattern was observed in mitral valves of neonatal $Mas^{-/-}$ mice (Fig. 2B). However, no significant changes were viewed in the expression of collagen types I, III and VI and fibronectin in atria of $Mas^{-/-}$ neonatal mice (Fig. 2B). To confirm the protein expression profile of ECM proteins in hearts of $Mas^{-/-}$ neonatal mice, western blotting analysis was performed. In agreement with the immunofluorescence-labeling findings, we found that the levels of collagen types I and III and fibronectin were higher while the level of collagen type VI was lower in hearts of $Mas^{-/-}$ neonatal mice (Fig. 3).

A similar pattern of ECM protein expression was observed in hearts of adult $Mas^{-/-}$ mice. Indeed, Mas deficient adult mice presented significantly higher levels of collagen types I and III and fibronectin and a lower level of collagen type VI in the right ventricle and tricuspid valves (Fig. 4A and B). These changes were also detected in mitral valves of adult $Mas^{-/-}$ mice (Fig. 4B). Nevertheless, no significant changes in the expression of these proteins were observed in atria of adult $Mas^{-/-}$ mice (Fig. 4B). Importantly, these alterations were confirmed by western blotting analysis (Fig. 5). Of note, the fibrosis observed in $Mas^{-/-}$ mice was accompanied by a significant reduction in the right ventricular function, i.e. these mice presented a reduced fractional area change (FAC) of the right ventricle. Also, in keeping with a recent study [24], $Mas^{-/-}$ mice showed an impaired left ventricular function (Table 1).

Picrosirius red staining revealed that total collagen content present in tricuspid valves of neonatal and adult $Mas^{-/-}$ mice, as well as in right ventricles of adult $Mas^{-/-}$ mice appears to be significantly higher when compared with $Mas^{+/+}$ mice (Fig. 6). An increased birefringence pattern observed when polarization microscopy was used indicates that more fibrous collagen was present. Such an increase was evident in tricuspid valves of neonatal and adult $Mas^{-/-}$ mice (Fig. 6). Although sections of the right ventricular wall were stained more intensely with picrosirius red when they were observed with conventional microscopy (Fig. 6), no significant increases in polarization were evident (data not shown). Together, these results suggest

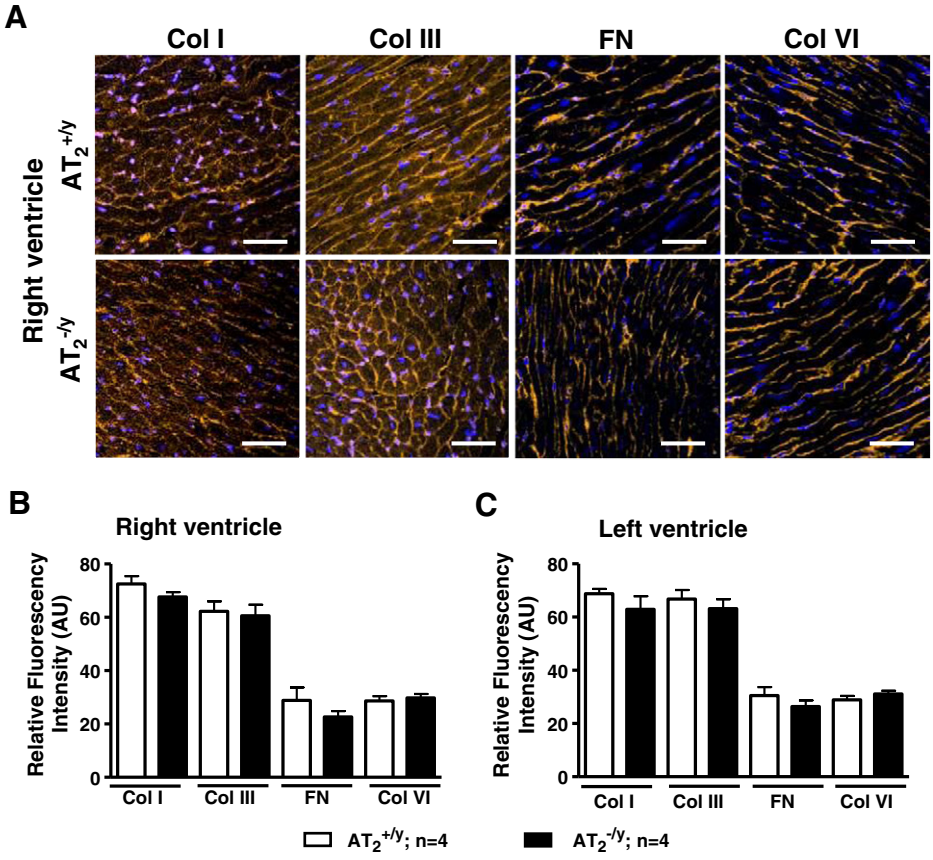


**Fig. 7.** Immunofluorescent localization of collagen types I, III and VI and fibronectin in the right ventricle of adult $AT_2^{+/y}$ and $AT_2^{-/y}$ mice hearts (A). Quantification of ECM proteins in the right (B) and left ventricles (C) of adult $AT_2^{+/y}$ and $AT_2^{-/y}$ mice. Values are expressed as arbitrary units (AU). Data is shown as mean ± SEM. Bar = 50 μm.

that the increased amount of collagen observed with conventional picrosirius red staining, as well as with immunostaining for collagen types I and III, is present as microfibrillar material.

Because $AT_2$ receptors have been suggested to be involved in some Ang-(1-7) effects, we evaluated the expression of ECM proteins in hearts of $AT_2$ deficient mice. Fig. 7A shows the immunostaining profile of the levels of collagen types I, III and VI and fibronectin in right ventricles of $AT_2^{+/y}$ and $AT_2^{-/y}$ adult mice. Quantitative analysis of the level of immunofluorescent staining demonstrated that right (Fig. 7B) and left (Fig. 7C) ventricles from $AT_2^{-/y}$ mice present no significant alterations in the expression of these proteins as compared with $AT_2^{+/y}$ mice.

It is well-known that MMPs have a major role in ECM degradation. Thus, our next objective was to investigate if MMPs were involved in the deposition of ECM proteins in hearts of $Mas^{-/-}$ mice. Deficiency of Mas induced a significant reduction in the level of the active form of MMP-2 in neonate hearts (Fig. 8A). In contrast, no significant alteration was observed in the level of the active form of MMP-9 in hearts of neonate $Mas^{-/-}$ mice (Fig. 8B). In adult hearts, western blotting analysis detected the presence of a single MMP-2 band in the gel whose density was lower in $Mas^{-/-}$ mice (Fig. 8C). Furthermore, the level of the active form of MMP-9 was significantly lower in Mas deficient mice (Fig. 8D).

As observed in Fig. 9A, gelatinolytic activity of the active form of MMP-2 (62 kDa) and its precursor form (68 kDa) were detected only in neonate hearts. Although the active form of MMP-9 (82 kDa) was detected on zymographs in both neonate and adult hearts (Fig. 9A), no significant differences were observed in metalloproteinase activity between the groups (Fig. 9B–E).

In order to investigate the potential involvement of protein kinases in ECM deposition, the levels of phosphorylated ERK1/2 and p38 protein expression were evaluated in hearts of neonatal and adult $Mas^{-/-}$ mice. We observed that the levels of p-ERK1/2 and p-p38 were higher in hearts of both neonatal and adult $Mas^{-/-}$ mice when compared with control animals (Fig. 10).

## 4. Discussion

We have previously described that genetic deletion of Mas leads to a marked impairment of cardiac function [24]. Systolic tension, $+dT/dt$ and $-dT/dt$ were significantly lower in isolated hearts of Mas-deficient mice. Echocardiographic measurements revealed a lower fractional shortening, posterior wall thickness in systole and a higher left ventricular end-systolic dimension. In addition, $Mas^{-/-}$ mice presented a higher coronary perfusion pressure compared with $Mas^{+/+}$ mice. These alterations appear to be caused, at least partially, by severe alterations in collagen protein expression in left ventricles [24]. In the current study, we extended the observation that Mas is a key modulator of several ECM proteins in mouse hearts. Neonatal and adult Mas knockout mice showed significantly higher levels of collagen types I and III and fibronectin in both right ventricle and AV valves. In contrast, the level of collagen type VI in these tissues was lower in $Mas^{-/-}$ mice. In agreement with our data, many studies have demonstrated that Ang-(1-7) induces antiproliferative and anti-fibrotic effects by acting through the Mas receptor [12–14]. Interestingly, the alterations in cardiac expression of ECM proteins were not observed in the atria of neonatal or adult $Mas^{-/-}$ mice,

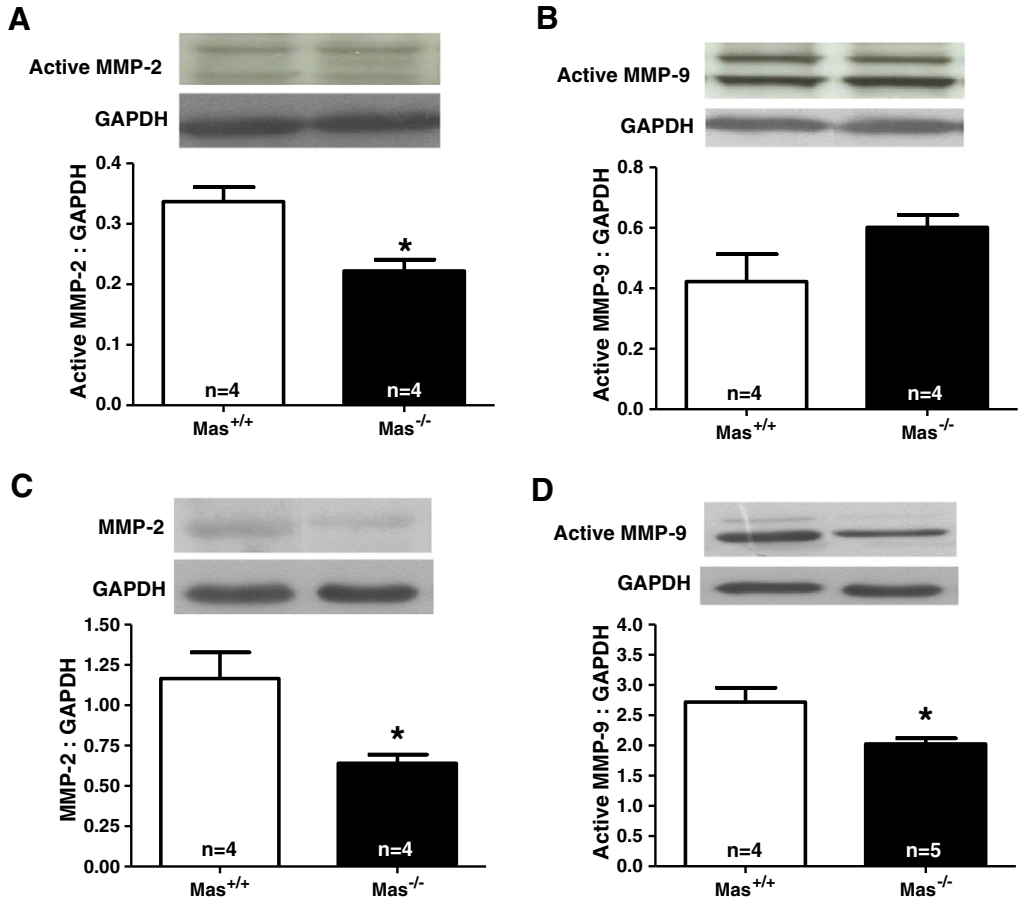


**Fig. 8.** Western blotting analysis of the levels of MMP-2 and MMP-9 in neonatal hearts and right ventricles of adult $Mas^{+/+}$ and $Mas^{-/-}$ mice. Active form of MMP-2 in neonate heart (A). Active form of MMP-9 in neonate heart (B). A single band was detected in the immunoblotting for MMP-2 in adult right ventricles (C). Active form of MMP-9 in adult right ventricles (D). Data are shown as the SEM. $^*p<0.05$.

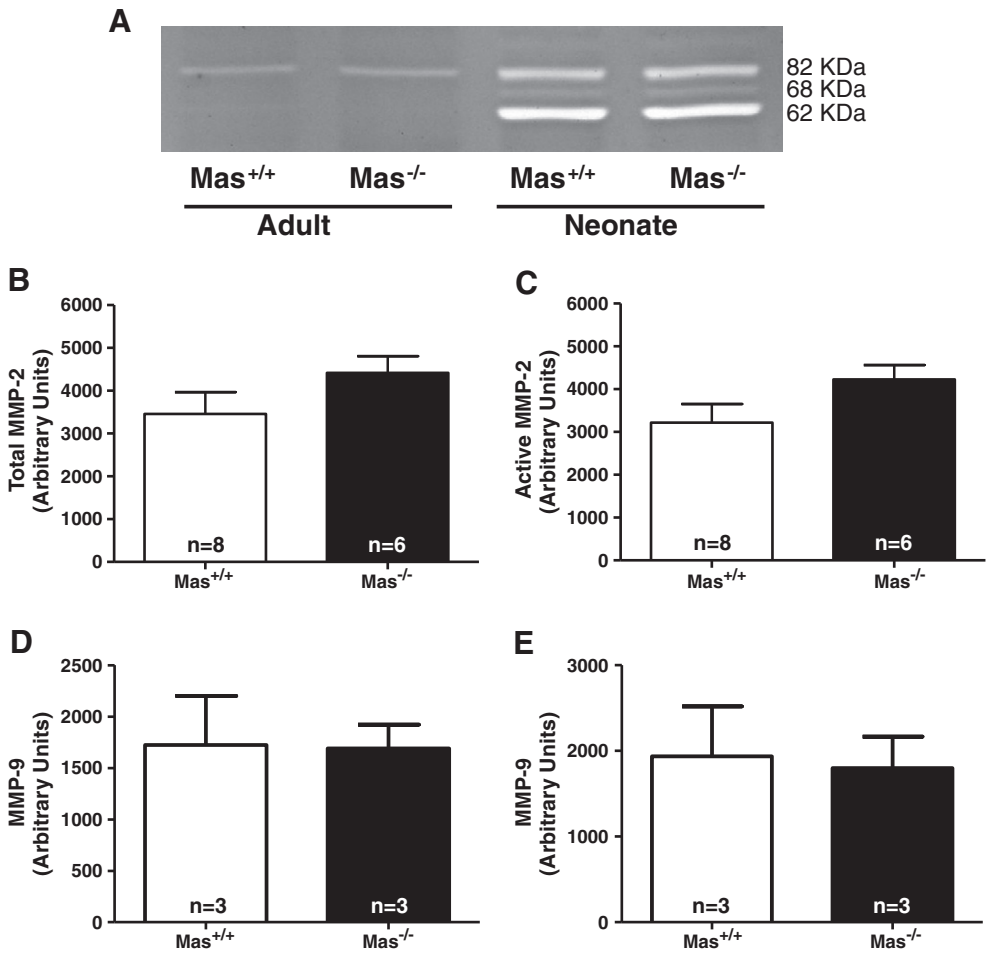


**Fig. 9.** Representative gelatin zymogram of the active form of MMP-2 (62 kDa), precursor form of MMP-2 (68 kDa) and active form of MMP-9 (82 kDa) (A). Quantification of the gelatinolytic activity of the total MMP-2 in neonatal mice (B), active form of MMP-2 in neonatal mice (C), active form of MMP-9 in neonatal mice (D) and active form of MMP-9 in right ventricles of adult mice (E). MMP-2 activity was not detected in right ventricles of adult mice. Data are shown as the SEM. (n = 3–8).

indicating that the modulation of ECM protein expression by Mas is restricted to specific heart regions.

Previous biochemical and molecular studies have been limited to the description of the expression of Mas in the whole heart [24,26]. Here, we showed by immunofluorescence-labeling the precise distribution of Mas in different areas of the heart. Mas was presented in the right ventricle, AV valves and atria. Thus, the lack of effects of genetic deletion of Mas in atria could not be attributed to the absence of this receptor in these chambers.

Surprisingly, in contrast to collagen types I and III, the levels of collagen type VI were decreased in ventricles and AV valves of neonatal and adult $Mas^{-/-}$ mice, indicating that Mas selectively regulates the expression of specific ECM proteins in mouse hearts. Collagen type VI forms a microfibrillar extracellular network that is thought to function as an elastic bridge between cell surfaces or basement membranes and the structural ECM scaffold. It is possible that collagen type VI is important for maintaining an appropriate relationship between cells and ECM in cardiac structures exposed to variable states of wall stress. Heart structures must acutely regulate tensile support in response to alterations in the cardiac cycle [27]. Decreased amounts of collagen type VI may therefore adversely affect cardiac function.

Of note, neonatal mice presented a similar pattern of ECM protein expression as observed in adult mice. These results might suggest that the primary cause of the structural and functional disturbances seen in hearts of adult mice is due to changes in cardiac expression of ECM proteins and not due to chronic adaptative alterations. Indeed, C57BL6 $Mas^{-/-}$ mice presented normal blood pressure as assessed by intra-arterial catheter measurements [24,28]. Furthermore, it was found that $Mas^{-/-}$ mice are healthy, grow normally, display no difference in drinking behavior and show no obvious developmental abnormalities [29]. However, whether this possibility is true remains to be elucidated and warrants further investigations. This includes, but is not limited to, evaluation of shift in elastic versus fibrous ECM components, changes that may occur in heart function from neonatal to adulthood and associations between heart function and expression of specific ECM components.

In certain circumstances, and in some tissues, $AT_2$ receptors appear to be involved in the Ang-(1-7) effects [30]. Furthermore, physical interaction between Mas and $AT_2$ in selected tissues such as the heart has been suggested as a putative mechanism for Ang-(1-7) actions [31]. In order to investigate the participation of $AT_2$ receptors in the anti-trophic and anti-fibrotic actions of the Ang-(1-7), we compared the effects of genetic deletion of Mas and $AT_2$ receptors on the ECM protein expression in the heart. In contrast to the marked fibrotic pattern observed in $Mas^{-/-}$ mice, no significant alterations in the levels of collagen types I, III and VI and fibronectin were observed in $AT_2^{-/y}$ mice. In agreement with this data, Ichihara et al. [32] also failed to demonstrate any significant difference in the collagen deposition between $AT_2^{-/y}$ and $AT_2^{+/y}$ mice in normal conditions. These findings suggest that the modulation of ECM proteins by Ang-(1-7) is $AT_2$-indepentent. Of note, it has been recently demonstrated that the anti-hypertrophic effect of Ang-(1-7) in cardiomyocytes is also independent of $AT_2$ receptors [33].

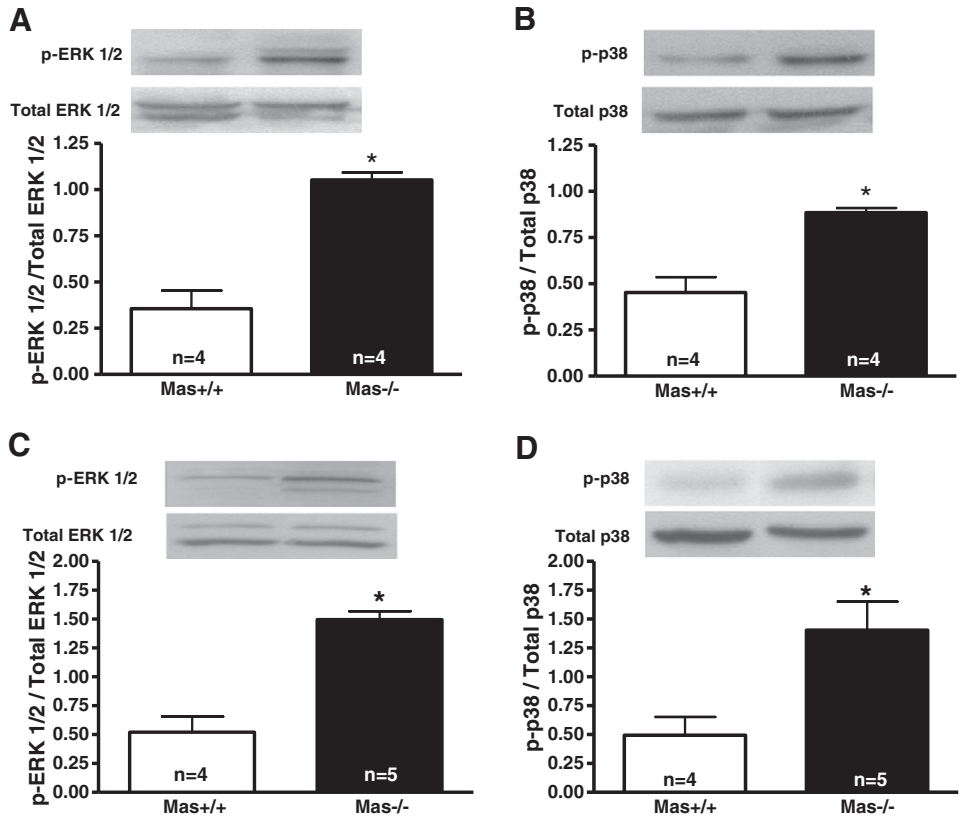


**Fig. 10.** Western blotting analysis of the levels of active, phosphorylated p38 p-p38 and ERK1/2 p-ERK1/2 in neonatal hearts (A, B) and in the right ventricles of adult Mas$^{+/+}$ and Mas$^{-/-}$ mice (C, D). Data are shown as the SEM. *$p<0.05$.

The composition of the extracellular matrix, a complex network of structural proteins including collagen types I and III, provides architectural support for the muscle cells and plays an important role in myocardial function [34]. Collagen type VI is a major microfibrillar component of extracellular matrices and is predicted to play a key role in the maintenance of tissue integrity by providing a structural link between different components of connective tissues, basement membranes, and cells binding to collagen type I, collagen type III and other matrix components. A number of studies have shown changes in the accumulation, composition, or organization of these interstitial collagens, including types I, III, and VI, during cardiac development and disease. The accumulation of myocardial collagen lead to interstitial and perivascular fibrosis which has been correlated with left ventricular early diastolic and systolic dysfunction [6,35,36]. MMP-2 and MMP-9 are expressed by a multitude of cell types including cardiac myocytes and fibroblasts. It was reported that both enzymes are highly upregulated in hypertrophic and failing hearts and they have been implicated in the progression of ventricular dilatation and the development of heart failure. MMP-2 and MMP-9 degrade various types of collagen, fibronectin, and others proteins that are accumulating in the damaged myocardium undergoing fibrosis [37]. Thus, we selected these two metalloproteinases to correlate them to the alterations in the ECM proteins evaluated in our study. In addition, the deposition of ECM proteins in the heart depends on the balance between the synthesis of these proteins and their degradation by metalloproteinases [38]. There is little data available concerning the effects of Ang-(1-7) and its receptor Mas on the biosynthesis of ECM proteins or on the activity of metalloproteinases. We observed that the level of the active form of MMP-2 in hearts of neonatal Mas$^{-/-}$ mice is lower than in Mas$^{+/+}$ mice while the active form of MMP-9 is not altered. Also, the level of MMP-2 and of the active form of MMP-9 were significantly lower in adult Mas$^{-/-}$ hearts. Altogether, these findings suggest that the pro-fibrotic profile observed in Mas$^{-/-}$ mice might be related to alterations in the expression of metalloproteinases. However, it is important to note that although we were able to detect the presence of MMP-2 and MMP-9 in the heart, the changes observed in the levels of these MMPs were not accompanied by modifications in their activities. Indeed, both western blot and gelatin zymography can be used to determine the presence of MMPs in the tissue. However, the western blot shows the presence of MMPs while zymography determines the activity of MMPs.

The lack of effects observed in the MMP activities in Mas$^{-/-}$ mice might be explained by a limitation of the technique. Zymography uses gelatin as a substrate which is composed of denatured collagen. In addition to their activities in collagen degradation, MMP-2 and MMP-9 act in other components of the ECM, such as elastin, fibronectin and laminin [39]. MMP-2 also acts on decorin which is an important component of the ECM [40]. Thus, the divergent results observed between western blot and zymography may be related to the actions of MMP-2 and MMP-9 on other substrates than collagen. Moreover, low concentration of MMPs in our samples might contribute to the lack of effects observed in the MMP activities. Similarly, the discrepancy observed between ECM development and the changes in MMP-9 protein expression when passing from neonatal to adult life may be related to the actions of MMP-9 in other substrates than the proteins evaluated in our study. In fact, MMP-9 may act in other components of the ECM and has high substrate affinity for basement membrane proteins [41–44].

A variety of signal transduction pathways have been shown to be involved in the regulation of ECM deposition/degradation, including p38 and ERK1/2 MAP kinase pathways [45–47]. In the present study, we demonstrated that the active, phosphorylated forms of p38 and ERK1/2 are increased in neonatal and adult Mas$^{-/-}$ mice hearts. These data are in agreement with previous studies demonstrating that Ang-(1-7) is able to inhibit MAP kinase phosphorylation in different tissues [48–51]. These findings suggest that the Ang-(1-7)/Mas axis could be involved in the regulation of the synthesis and/or

degradation of collagen and non-collagen proteins by acting in the regulation of the MAP kinase activity. One may argue that, because Ang-(1-7) at pharmacological concentrations can bind and, eventually, activate $AT_1$ receptors [52], this peptide may change the $AT_1$-mediated stimulation of the ERK/MAPK pathway in the absence of Mas. However, this possibility is unlikely since a recent study has reported that the plasma levels of Ang II and Ang I and the plasma renin activity are similar in both $Mas^{+/+}$ and $Mas^{-/-}$ mice [53]. Although these data do not exclude this hypothesis, they are an indicative that the stimulation of ERK/MAPK observed in our study is not due to the action of Ang-(1-7) on $AT_1$ receptors. Further experiments are obviously needed to confirm this possibility.

## 5. Conclusions

In summary, our data suggest that Mas is involved in the selective regulation of the expression of specific ECM proteins within both ventricular myocardium and AV valves. The profile observed may contribute to the decreased cardiac performance viewed in $Mas^{-/-}$ mice.

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