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S100A4 is upregulated in injured myocardium and promotes growth and survival of cardiac myocytes

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Abstract

Objective: The multifunctional Ca^{2+}-binding protein S100A4 (also known as Mts1 and Fsp1) is involved in fibrosis and tissue remodeling in several diseases including cancer, kidney fibrosis, central nervous system injury, and pulmonary vascular disease. We previously reported that S100A4 mRNA expression was increased in hypertrophic rat hearts and that it has pro-cardiomyogenic effects in embryonic stem cell-derived embryoid bodies. We therefore hypothesized that S100A4 could play a supportive role in the injured heart.

Methods and results: Here we verify by quantitative real-time PCR and immunoblotting that S100A4 mRNA and protein is upregulated in hypertrophic rat and human hearts and show by way of confocal microscopy that S100A4 protein, but not mRNA, appears in cardiac myocytes only in the border zone after an acute ischemic event in rat and human hearts. In normal rat and human hearts, S100A4 expression primarily colocalizes with markers of fibroblasts. In hypertrophy elicited by aortic banding/stenosis or myocardial infarction, this expression is increased. Moreover, invading macrophages and leucocytes stain strongly for S100A4, further increasing cardiac levels of S100A4 protein after injury. Promisingly, recombinant S100A4 protein elicited a robust hypertrophic response and increased the number of viable cells in cardiac myocyte cultures by inhibiting apoptosis. We also found that ERK1/2 activation was necessary for both the hypertrophy and survival effects of S100A4 *in vitro*.

Conclusions: Along with proposed angiogenic and cell motility stimulating effects of S100A4, these findings suggest that S100A4 can act as a novel cardiac growth and survival factor and may have regenerative effects in injured myocardium.

Keywords: S100A4/Mts1; Cardiac hypertrophy; Myocardial infarction; Cardiac myocytes; Apoptosis; Cardioprotection

This article is referred to in the Editorial by Pleger et al. (pages 1–2) in this issue.

1. Introduction

Cardiac hypertrophy is a compensatory mechanism that initially augments global cardiac function after biomechanical stress, while sustained hypertrophy can lead to heart failure and arrhythmia [1]. At the cellular level, a hypertrophic gene program is turned on and cardiac myocytes enlarge in response

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to multiple stimuli including mechanical stretch and a growing number of biologic mediators that regulate growth, differentiation and cell survival [2]. Many of these mediators are peptides and protein growth factors that may be synthesized within the heart in different cells, including cardiac myocytes and fibroblasts, or may originate from inflammatory cells that enter the heart.

We recently found increased expression of S100A4 mRNA in rat models of cardiac hypertrophy [3], and that S100A4 protein is an endogenous factor that stimulates differentiation of embryonic stem cells into cardiac myocytes in a paracrine fashion [4]. We therefore hypothesized that S100A4 could play a supportive role in injured hearts.

S100A4 is an 11 kDa protein of the EF-hand containing, Ca^{2+}-binding S100 family that enhances metastasis of several types of cancer cells [5]. The mechanisms of this effect are not fully clarified, but S100A4 has both intra- and extracellular activities. Inside the cell, S100A4 interacts with cytoskeletal proteins involved in cell motility, such as nonmuscle myosin II (reviewed in [6]). S100A4 also targets and regulates the function of several intracellular signaling proteins, including p53 [7]. As other S100 proteins, S100A4 exists as dimers and does not possess enzymatic activity, but rather regulates the activity of bound protein partners, likely by changing the partners' localization or accessibility. S100A4 is secreted and accordingly has important functions outside the cell [8,9]. Thus, it stimulates neuronal differentiation and survival, angiogenesis, endothelial cell motility and metalloproteinase activity, smooth muscle cell migration and proliferation, and *in vitro* cardiomyogenesis [4,8,10–14]. The molecular mechanisms whereby S100A4 elicits these cellular responses remain elusive. It seems that S100A4 can mediate signals via cell surface receptors [11–14], but a specific S100A4 receptor is still to be identified.

Here, we show that S100A4 was upregulated in two rat models of cardiac hypertrophy on both RNA and protein levels. The S100A4 protein colocalized with markers of a variety of interstitial cells in rat as well as human hearts, extending the clinical importance of our findings. Quantification of immunohistochemical data revealed an increased S100A4 expression in inflammatory cells of hypertrophic human hearts. Intriguingly, in both species, cardiac myocytes were completely devoid of S100A4 before injury; however, after acute ischemia, myocytes in infarct border zones showed marked S100A4 protein staining without expressing S100A4 mRNA. We also studied the direct effects of recombinant S100A4 protein on cultured cardiac myocytes, and indeed observed hypertrophic growth and survival promoting activity through a signaling pathway that required ERK1/2 activation. These data suggest that S100A4 is a novel cardioprotective factor that is upregulated after injury.

2. Materials and methods

2.1. Experimental animals

Methods of aortic banding (AB) and induction of myocardial infarction (MI) by coronary artery occlusion in rats, is described in [3]. The mortality rate of operated animals was ~30% within the first 24 h; hereafter ~16% died before analysis. The investigation conforms 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).

2.2. Quantitative real-time PCR

Total RNA was isolated *de novo* (for each time-point: MI, $n=15$; AB, $n=8$; sham, $n=6$) with TriReagent (#TR118, Molecular Research Center) after homogenization with a Mixer Mill 300MM (Retsch, Germany). RNA quality was analyzed as previously described [15]. First-strand cDNA was synthesized from 1 μg RNA using random hexamer primers and Omniscript Reverse Transcription kit (#205111, Qiagen). Q-PCR analyses were performed with the RotorGene 3000 (Corbett Research, Australia) and the Quantitect SYBR Green PCR kit (Qiagen) as described [16]. All mRNAs were quantified twice in separate analyses. The primers used were GAPDH: 5′-GTCGGTGTGAACGGATTTG-3′, 5′-CTTGCCGTGGGTAGAGTCAT-3′; S100A4: 5′-AGCTACTGACCAGGGAGCTG-3′, 5′-TGCAGGACAGGAAGACACAG-3′. Results were normalized to GAPDH within the same sample.

2.3. Western blotting of S100A4 in rat hearts

Frozen left ventricles were homogenized and following procedures were performed as previously described [17] with use of polyclonal anti-S100A4 antibody [14]. Protein bands were quantified by densitometry (NIH Image 1.62).

2.4. Tissue sampling

Rat tissues were sampled from frozen left ventricles of rats subjected to AB or MI and sham operated controls. Human left ventricular (LV) samples were collected from 4 explanted hearts of patients undergoing orthotopic heart transplantation due to end-stage heart disease (ischemic cardiomyopathy; ICM). Myomectomy samples from the LV subvalvular septum were obtained from 4 patients with aortic stenosis (AS). Clinical data is presented in Supplementary Table 1. LV myocardium from 3 donor hearts that for technical reasons were not used for transplantation served as control tissues. Tissue samples were mounted in Tissue Tec and immediately frozen in liquid nitrogen. This investigation conforms with the principles outlined in the Declaration of Helsinki.

2.5. Immunolabeling and confocal microscopy

Two to three samples were analyzed per subject. Frozen sections (10 μm) were fixed 10 min with 4% paraformaldehyde, followed by staining procedures with primary and secondary antibodies (listed in Supplementary Table 2). The samples were examined by confocal microscopy (Leica TCS SP2) and images were processed using Imaris® (Bitplane, Switzerland) as previously described [18]. Quantification of

fluorescence intensity (FI) was performed as described [18,19]. Specifically, from each optical field (250×250 μm), 5 confocal slides were obtained at 1 μm intervals. The area of positive S100A4 labeling was defined as the number of pixels with S100A4 signal intensity exceeding the threshold of 20 on the 0–255 FI scale. The quantity of S100A4 per myocardial area was expressed as the percent of myocyte surface area occupied by S100A4 positive label. For each heart, 10–15 optical fields were analyzed. All sections were immunolabeled simultaneously using identical dilutions of antibodies.

2.6. In situ hybridization

First strand cDNA was synthesized from total human myocardial tissue RNA. Reverse transcription reactions were performed using SuperscriptII (Invitrogen) and oligod$T_{(15)}$ (Promega), followed by PCR reaction with 0.2 pmol of each primer targeting S100A4 (forward: 5′-GTACGTGTTGATCCTGACTGCTGTCATGG-3′; reverse: 5′-TCATTTCTTCCTGGGCTGCTTATCTGGG-3′) and 5U Taq polymerase (Eppendorf). The PCR amplified sequences were purified, cloned into the pCRII-TOPO vector (Invitrogen), and sequenced. Probes were synthesized on TOPO-S100A4 linearized with KpnI plasmid using T7 RNA polymerase (Promega). In situ hybridization was prepared in paraffin embedded human heart slides. Briefly, tissue samples were hydrated with decreasing concentrations of ethanol, fixed with 4% PFA, followed by digestion with proteinase K and overnight hybridization with sense or antisense S100A4 probes at 65 °C. Staining was performed with alkaline phosphatase-conjugated anti-digoxygenin antibody (1:2000; Roche) and visualized by Vector Substrate detection Kit (Vector Laboratories).

2.7. Neonatal rat ventricular cardiac myocytes

Ventricular cardiac myocytes were prepared from 1–3 days old Wistar rats and cultured by a protocol previously published to yield high quality cultures [20–22]. Cardiac myocytes were plated at 50,000–100,000 cells/cm^2 depending on assay and left untreated 48 h. L-glutamine was included in all medium added later than 48h of culturing. ANP content of conditioned medium was analyzed by an ANP EIA kit (EK-005-24, Phoenix Pharmaceuticals Inc).

2.8. Immunocytochemistry

Cardiac myocytes were cultured in 2-well chamber slides and stimulated with 0.1–100 μg/ml S100A4 or 5% fetal bovine serum as indicated. Isolation of recombinant His-tagged human S100A4 protein was performed as previously described [14]. In antibody inhibition experiments, S100A4 protein and antibody were pre-incubated. Cells were fixed after 5 days (cell size) or 30 min (S100A4 uptake) with 4% paraformaldehyde, and stained with TRITC-labeled phalloidin and Sytox Green nucleic acid stain (#S7020, Molecular Probes). Cells were additionally labelled with monoclonal anti-His antibody (1:100; #2366, Cell Signaling Technology) and Alexa Fluor 633-conjugated anti-mouse IgG antibody (1:100; A21050, Molecular Probes) for S100A4 uptake analysis. Images were acquired by confocal microscopy. Computer software (MetaMorph, Universal Imaging Corporation) was used to quantify cell size. At least 50 cells were quantified for each treatment in each experiment.

2.9. Cell counting and PCNA Western blotting

Cardiac myocytes were stimulated with or without 50 μg/ml S100A4 or 5% serum. Where the MEK1/2 inhibitor U0126 (#U112A, Promega) was used, this was added 30 min prior to stimulation. Cells were counted in a haemocytometer before and after stimulation for 2 days; at least 200 cells were counted in each of duplicate samples. To determine levels of proliferating cell nuclear antigen (PCNA), cell lysates were subjected to Western blotting and immunolabeled with anti-PCNA antibody (1:2500; #610664, BD Biosciences) as previously described [22]. Specific PCNA bands were quantified by densitometry.

2.10. Apoptosis assays

Cardiac myocytes were plated in the presence of 5% serum to minimize "basal" apoptosis, and medium was changed after 48 h. 72 h after seeding, cells were incubated 24 h in medium without serum and with/without S100A4 (50% μg/ml). Where indicated, doxorubicin was added 1 h after serum deprivation/S100A4 stimulation. Quantification of DNA fragmentation was done using the Cell death detection ELISA kit (#1774425, Roche). To determine levels of cleaved caspase 3, cell lysates were subjected to Western blotting and immunolabeled with an antibody that recognizes cleaved as well as uncleaved caspase 3 (#9662, Cell Signaling Technology), and densities of cleaved caspase 3 bands were quantified.

2.11. [6-^{3}H]-thymidine incorporation

New medium with 0.2 μl [6-^{3}H]-thymidine and different agonists were added to cardiac myocytes 48 h after plating. After 24 h incubation cells were fixed in 10% TCA, 30 min at 4 °C. Cells were washed in ethanol (0 °C) before lysis in 400 μl lysisbuffer (0.1 M NaOH, 1% SDS) 20 min at room temperature. Lysates were counted in scintillation counter.

2.12. ERK1/2-phosphorylation

Cardiac myocytes were serum starved 24 h prior to experiments and treated with S100A4 (50 μg/ml) or angiotensin II (AngII; 100nM) for indicated times. U0126 was added 30 min before agonist. Cell lysates were subjected to Western blotting, immunolabeled with anti-ERK1/2 and anti-pERK1/2 (#9107 and #9101, Cell Signaling Technology), and analyzed as described [20,21].

2.13. Statistical analysis

Data is expressed as means ±SE or +SE. Statistical analysis of *in vivo* data was performed using factorial ANOVA followed by a Tukey post hoc analysis of mean differences, while analysis of *in vitro* data was done with either paired student's *t*-test, indicated in figures by lines connecting compared values, or one-sample *t*-tests comparing with the value 1. $P<0.05$ was accepted as significant and is indicated with an asterisk.

3. Results

3.1. Expression levels of S100A4 are increased in hypertrophic hearts

We previously reported in a microarray study of gene expression profiles in four different rat models of cardiac hypertrophy that S100A4 is part of a single common gene program underlying hypertrophic remodeling [3]. Accordingly, S100A4 expression was increased in hypertrophic left ventricles regardless of the underlying experimental cause of hypertrophy [3]. In a more detailed analysis, we used Q-PCR and focused on the AB and MI models. This approach revealed S100A4 mRNA expression levels in hypertrophic hearts ranging from 2.5-fold to 5.6-fold compared to the levels in hearts from corresponding sham-operated rats (Fig. 1A). Western blotting experiments showed that the protein level of cardiac S100A4 expression was similarly elevated to 1.8–5.9-fold of sham in animals with hypertrophy elicited by either surgical procedure (Fig. 1B). Additionally, when correlating S100A4 RNA or protein levels to extent of hypertrophy of all hypertrophic hearts, significant positive correlations were obtained (Spearman $r=0.2297$ or 0.4153, respectively; $P<0.05$ for both; data not shown).

3.2. S100A4 protein is localized to areas of fibrosis and inflammation as well as blood vessel cells and only found in myocytes after injury

We next determined S100A4 localization in healthy and hypertrophic hearts. Others have described specific fibroblast localization of S100A4 [23], but also localization to endothelial cells, smooth muscle cells, various blood cells and injured cardiac myocytes has been reported [24–31]. Accordingly, we costained rat myocardial sections with anti-S100A4 antibodies, TRITC-conjugated phalloidin to visualize f-actin in cardiac myocytes, and markers of fibroblast-like cells (vimentin), macrophages (CD68), endothelial cells (CD31), smooth muscle cells and myofibroblasts (α-SM actin), or neutrophiles (CD45; Fig. 2 and Supplementary Fig. 1A–C). In sections from sham operated rats, S100A4 colocalized with all applied markers, except phalloidin, suggesting that S100A4 is absent from cardiac myocytes. In sections from MI and AB hearts, we observed a markedly stronger and more widespread S100A4 staining. Nonetheless, the colocalization patterns were similar to those in sham operated animals. However, surprisingly, infarct border zones showed strong S100A4 staining in cardiac myocytes. The increased S100A4 protein staining was quantified by measuring fluorescence intensity in sections from single MI and AB hearts, showing approximately 4-fold higher levels in AB and MI hearts as compared to control hearts (Supplementary Fig. 1D, E). These data suggest that the increased S100A4 RNA and protein levels are caused by the large invasion of CD45 and CD68 positive cells, as well as increased numbers of fibroblast-like, endothelial, and smooth muscle cells, that are found in cardiac hypertrophy due to ongoing inflammation, fibrosis, and angiogenesis.

3.3. S100A4 in human hearts

To address the clinical relevance of these immunohistochemical findings, we extended the study to hearts from humans with normal cardiac physiology and from patients

A

Models	Weeks	Hypertrophy	S100A4 m RNA
Aortic banding	6	1.6 ± 0.09 *	5.1 ± 1.3 *
	12	1.5 ± 0.10 *	2.5 ± 0.8
	30	1.9 ± 0.16 *	5.6 ± 1.0 *
Myocardial infarction	3	1.3 ± 0.03 *	4.4 ± 0.7 *
	9	1.7 ± 0.05 *	4.8 ± 1.6 *

B

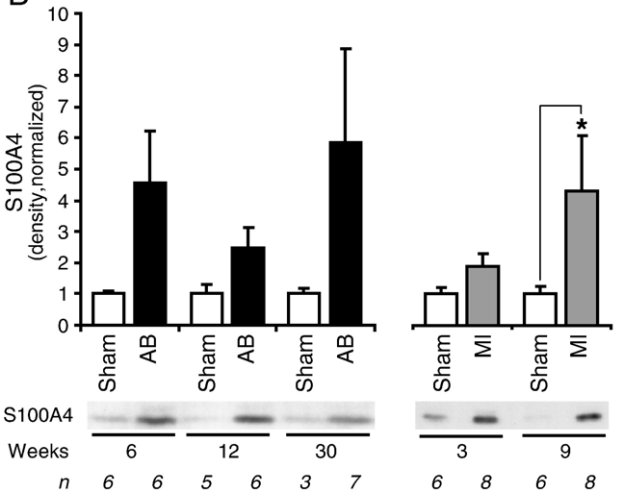

Fig. 1. Expression of S100A4 is increased in hypertrophic rat hearts. Rats subjected to AB or MI were sacrificed after indicated number of weeks. The extent of hypertrophy is presented as mean heart weight to body weight ratio relative to sham of individual groups. S100A4 mRNA and protein levels in left ventricles were analyzed by A, Q-PCR, normalized to GAPDH levels, and by B, densitometric immunoblotting, respectively. Expression levels were normalized to matching shams. In B, average values of analyzed samples are presented along with representative blots of single samples. Though S100A4 protein levels were markedly increased in both models and at all time points, only levels in MI animals 9 weeks after surgery reached significance (*, $P<0.05$).

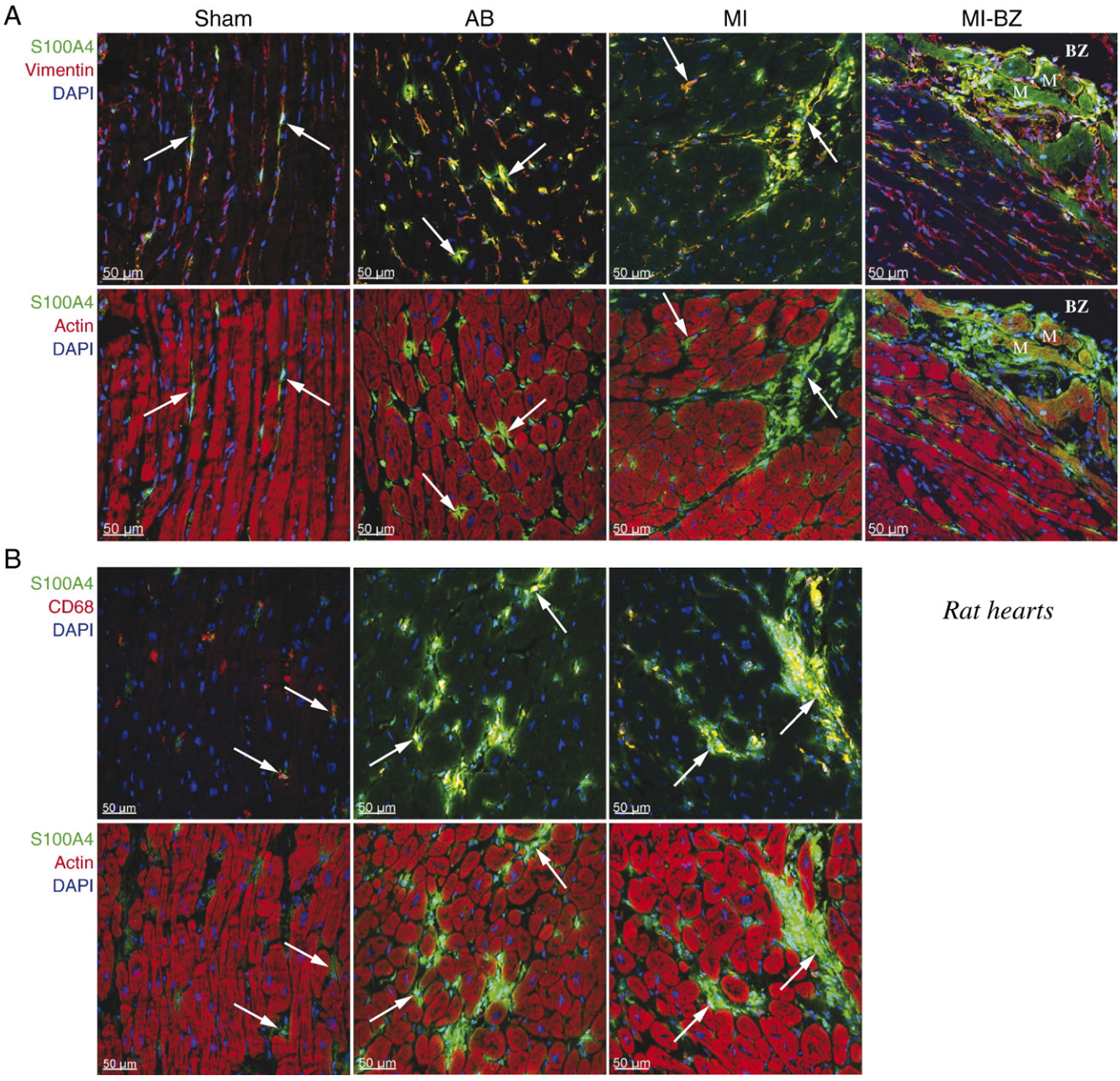

Fig. 2. S100A4 protein colocalizes with vimentin and CD68 positive cells of normal and hypertrophied rat hearts, and with cardiac myocytes of the infarct border zone. Immunohistochemical staining of heart samples from sham operated, AB, and MI rats with antibodies against S100A4 (green) and A, vimentin or B, CD68 (both red). Analogous S100A4 and f-actin (red) costains are shown below each slide. Colocalization of S100A4 and relevant markers appear yellow. Arrows indicate fibroblast-like cells in A, and CD68-macrophages positive for S100A4 in B. M, myocytes; BZ, infarct border zone.

suffering from ICM and AS. These diseases are closely mimicked by our rat models of MI and AB, respectively. Correspondingly, the colocalization patterns of S100A4 in human hearts closely reproduced the rat data, and quantitative immunoconfocal microscopy revealed an increase in S100A4 protein staining levels in patient hearts to more than 3-fold the levels in normal hearts (Fig. 3 and Supplementary Fig. 2). In addition, the overall S100A4 staining in infarct border zones was almost twice as high as that in infarct remote zones. At least some of this increased expression could be explained by a similar pattern of increased numbers of S100A4 staining CD45- and CD68-positive cells (Fig. 3C and D and Supplementary Fig. 2). Notably, S100A4 staining of cardiac myocytes again appeared exclusively in the infarct border zone of human hearts as evidenced by both phalloidin and anti-α-actinin costaining (Fig. 3E). To determine whether this localization was due to S100A4 expression or uptake we performed in situ hybridization analysis targeting S100A4 mRNA. Various interstitial cells stained positive for S100A4 mRNA, whereas all cardiac myocytes were devoid

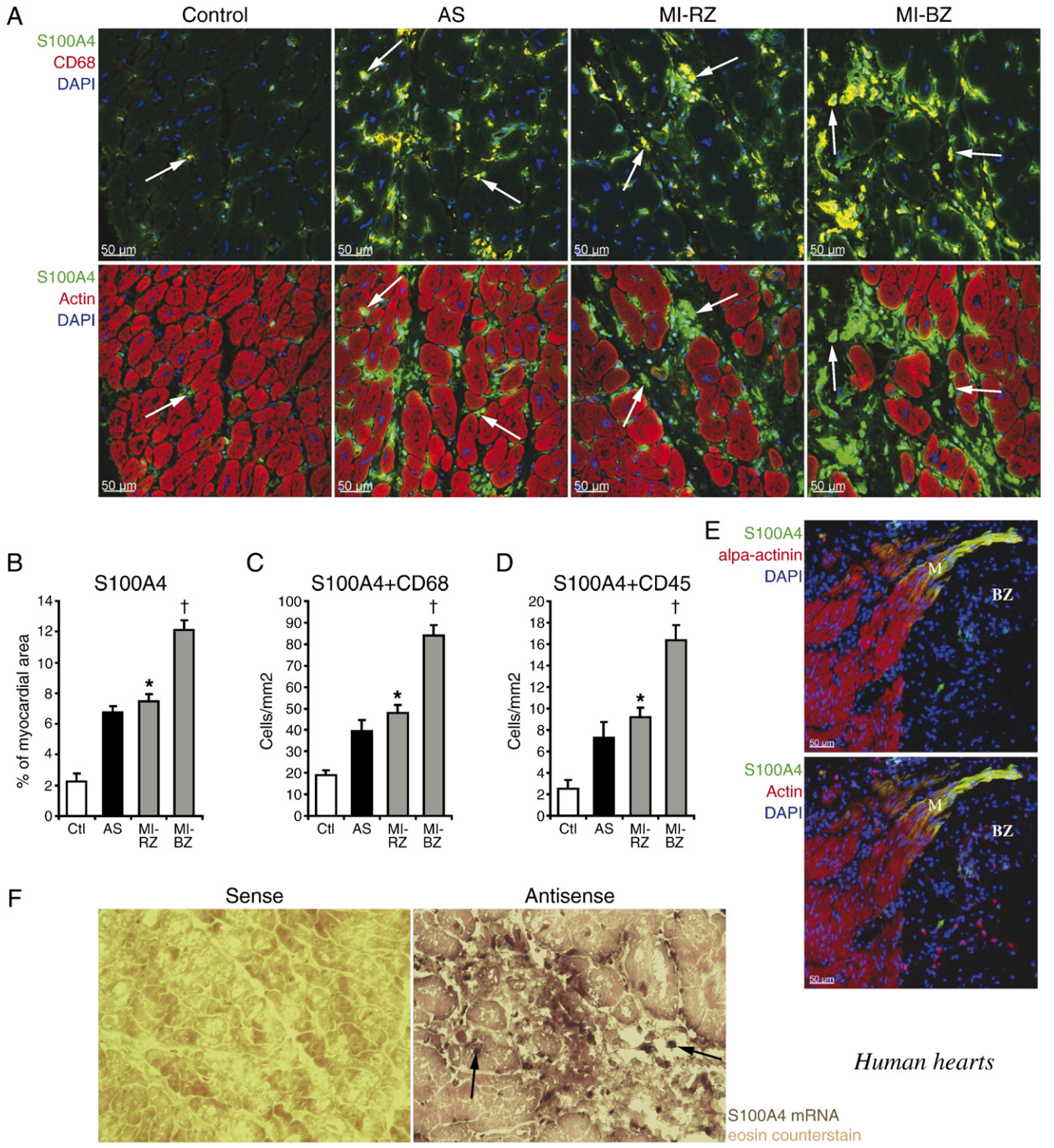

Fig. 3. S100A4 protein is increased in hearts of human patients suffering from aortic stenosis and ischemic cardiomyopathy. A, Immunohistochemical staining of a normal human heart (Control) and hearts from patients suffering from AS or ICM, with antibodies against S100A4 (green) and CD68 (red). Both remote zone (MI-RZ) and border zone (MI-BZ) areas are shown from the infarcted heart. Analogous S100A4 and f-actin (red) costains are shown below each slide. Colocalization of S100A4 to CD68-macrophages appears yellow (arrows). B, Quantitative analysis of immunohistochemical S100A4 staining in controls, in patients with AS, and in patients with ICM in the remote (MI-RZ) and border zone (MI-BZ) myocardium. The numbers of C, CD68-macrophages and D, CD45-leukocytes positive for S100A4 were counted in same samples. E, Immunohistochemical staining of the border zone of a heart from a patient suffering from ICM, with antibodies against S100A4 (green) and α-actinin (red). Analogous S100A4 and f-actin (red) costain is shown below. M, myocytes; BZ, infarct border zone. F, Sections of infarct border zones of human hearts were processed for in situ hybridization with antisense or sense riboprobes and eosin counterstained. Bound probes show dark brown staining. Arrows indicate S100A4 mRNA positive interstitial cells.

of S100A4 mRNA even in the infarct border zones (Fig. 3F). These cardiac myocytes thus uptake, or bind, S100A4 protein originating from other cells strongly suggesting a paracrine behavior of cardiac S100A4 protein.

3.4. Recombinant S100A4 protein promotes hypertrophy and survival of cultured cardiac myocytes

To assess the possible paracrine role of S100A4 on cardiac myocytes we turned to *in vitro* studies of neonatal rat cardiac myocytes. Notably, extracellularly applied S100A4 protein could be detected in intracellular vesicle-like structures and nuclei of cells treated 30 min with purified recombinant S100A4 (Fig. 4A). Treatment with S100A4 protein markedly stimulated a hypertrophic morphology of cardiac myocytes and increased cell size in a concentration dependent manner (Fig. 4B and C). The effect was abolished by pre-incubating the protein with anti-S100A4 antibody (Fig. 4D). S100A4 also increased ANP accumulation, a molecular hypertrophy marker, by more than 30 fold in the culture medium in a time dependent manner (Fig. 4E).

Next, we investigated the S100A4 effects on cardiac myocyte numbers by counting viable cells. As depicted in Fig. 5A, cardiac myocyte numbers were significantly higher in the presence than in the absence of S100A4. This could be the result of either increased survival, proliferation or a combination of both. To address this, we first determined possible anti-apoptotic activity. In two different assays including DNA fragmentation ELISA and caspase 3 Western blotting, S100A4 prominently inhibited apoptosis elicited either by serum deprivation or by doxorubicin (Fig. 5B and C). To determine whether S100A4 could also stimulate cardiac myocyte proliferation, we measured the levels of a marker of proliferation, PCNA, by densitometric

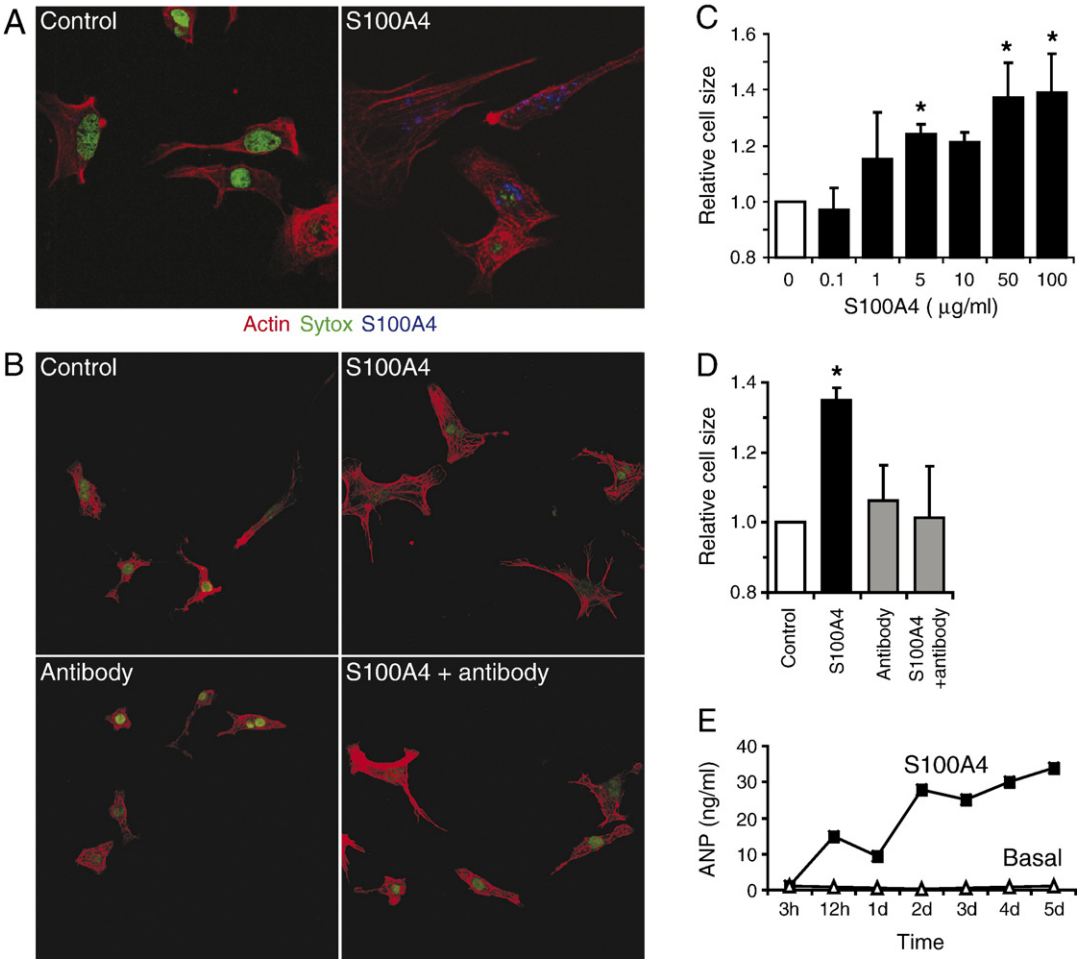

Fig. 4. Recombinant extracellular S100A4 induces hypertrophy of cardiac myocytes *in vitro*. A, Cardiac myocytes were treated 30 min with 5 µg/ml oligomeric S100A4, and stained for His-tagged S100A4 (blue), f-actin (red) and nuclei (green). B, C, and D, Cardiac myocytes were treated 5 days with S100A4 protein (50 µg/ml unless otherwise indicated), and stained for f-actin and nuclei. B, Representative images of cardiac myocytes cultured in control conditions, treated with S100A4, anti-S100A4 antibody, or S100A4 pre-incubated with anti-S100A4 antibody are shown. C, Concentration-response dependency and D, Antibody inhibition of S100A4 induced cardiac myocyte growth was quantified by morphometrical analysis. Data represent average values from at least three individual experiments and is expressed as relative cell size normalized to untreated controls. E, S100A4 stimulated ANP release from cardiac myocyte cultures. Medium was harvested at indicated time points after stimulation with S100A4 or from control cultures (Basal). Data are representative from two experiments performed in triplicates.

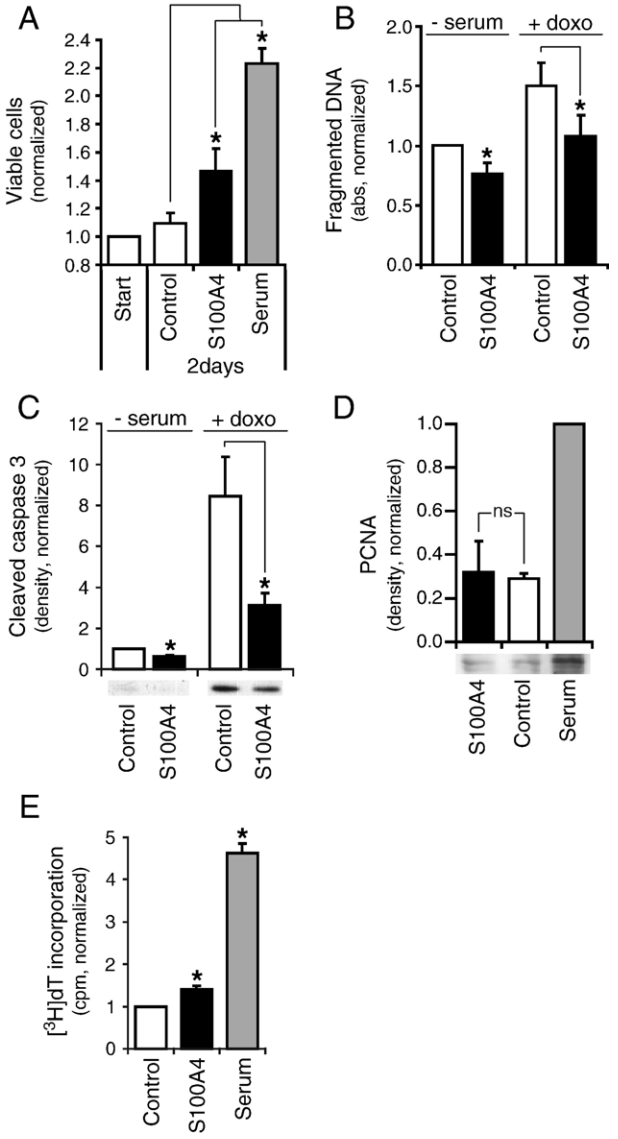

Fig. 5. Cardiac myocyte viability is increased in S100A4 treated cultures by inhibition of apoptosis. A, Viable cells were counted at the beginning of the experiment ("Start"). Cells in remaining wells were treated with S100A4 (50 μg/ml) or serum (5%), cultured another two days, and counted (2 days). Values represent averages of six individual experiments and are normalized to "Start". B and C, Apoptosis was induced by serum deprivation (-serum) and by doxorubicin treatment (1 μM; +doxo) in the presence or absence of S100A4 (50 μg/ml). After 24 h of incubation, B, cellular fragmented DNA was isolated and quantified by ELISA or, C, cell lysates were immunoblotted for caspase 3. Values are averaged from at least four independent experiments performed in duplicate or triplicate and normalized to serum deprived control cells. D, PCNA protein levels were quantified by densitometric Western blotting after 48 h of treatment. Values are averaged from four independent experiments performed in duplicates and normalized to serum treated samples; ns, nonsignificant. E, DNA synthesis was quantified by [6-^{3}H]-thymidine incorporation. Cells were treated 24 h with S100A4 (50 μg/ml) or serum (5%) in the presence of [6-^{3}H]-thymidine. Values are averaged from three independent experiments performed in triplicates and normalized to control. Inserted in C and D are images of representative immunoblots.

immunoblotting (Fig. 5D), as well as DNA synthesis using a [6-^{3}H]-thymidine incorporation assay (Fig. 5E). Though S100A4 did not increase PCNA levels, [6-^{3}H]-thymidine incorporation was increased 30%, as compared to a more than 350% increase with serum. This DNA synthesis may therefore reflect hypertrophy-related DNA synthesis rather than proliferation [32]. Collectively, these data suggest that S100A4 promotes cardiac myocyte growth and survival.

3.5. S100A4 effects on cardiac myocytes are ERK1/2 dependent

The signaling processes triggering cellular effects of S100A4 are not well understood, but at least in neurons include the ERK1/2 MAP kinase pathway. We therefore investigated if S100A4 protein affected ERK1/2 phosphorylation state by Western blotting. This approach revealed increased ERK1/2 phosphorylation within 3 min of S100A4 treatment and a maximal response at 30 min (Fig. 6A). In contrast, S100A4 failed to activate two other canonical growth and survival pathways including inositol phosphate accumulation and Akt kinase phosphorylation (data not shown). To ascertain the importance of the ERK cascade, we blocked ERK1/2 activity using the MEK1/2 inhibitor U0126. Interestingly, this abolished both cardiac myocyte hypertrophy and pro-survival effects, suggesting ERK1/2 activation is required for S1004A stimulation of these effects (Fig. 6B and C). In contrast, U0126 did not significantly alter cardiac myocyte size or survival induced by 5% serum.

4. Discussion

S100A4 was first characterized as a factor promoting metastasis (reviewed in [6]). However, today S100A4 has several other functions ascribed, including cell motility [8,10,12,33], neuronal differentiation [13,14,34], angiogenesis [8,11], and cardiomyogenesis [4]. Here, we identify S100A4 as a member of the cardiac hypertrophy gene program with growth and survival promoting activity on cardiac myocytes suggesting this protein exerts a paracrine cardioprotective effect in response to injury.

Several pieces of evidence presented in this report and data from others support a possible paracrine function for S100A4 in hearts. Firstly, we show that S100A4 expressing cells, including fibroblast-like, endothelial, and inflammatory cells, increase in numbers upon cardiac injury. These cell types have been reported to release S100A4 as a paracrine factor or passively due to cell death [9,12,24,31]. Secondly, we present evidence that extracellular derived S100A4 protein is uptaken by cardiac myocytes and stimulates cell growth as well as survival in myocyte cultures. Similarly, we have previously reported that S100A4 is secreted from parietal endoderm cells and in turn enhances cardiomyogenesis in embryonic stem cells [4], whereas others have shown that release of S100A4 is promoted in pulmonary vascular disease and in turn leads to proliferation and migration of pulmonary artery smooth muscle cells [12,35]. Thirdly, S100A4 is known to exert effects on cell types that participate in cardiac remodeling, including endothelial

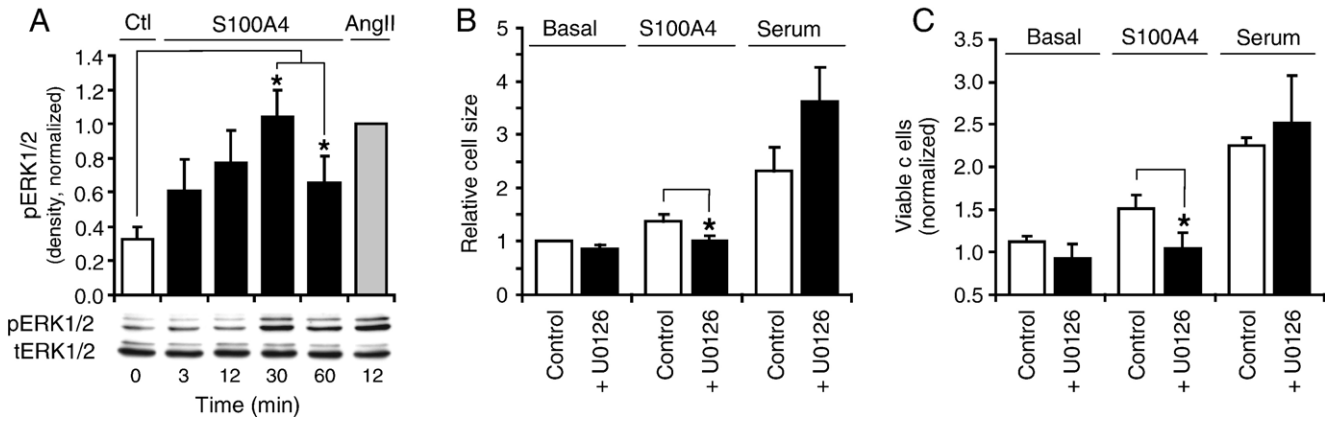

Fig. 6. Growth and survival effects of recombinant S100A4 protein on cardiac myocytes are ERK1/2 dependent. A, Cardiac myocytes were treated with S100A4 (50 μg/ml) or AngII (100 nM) for the indicated times. Hereafter, lysates were immunoblotted for total (tERK1/2) and phosphorylated ERK1/2 (pERK1/2). Values represent average of pERK1/2:tERK1/2 density ratios normalized to the pERK1/2:tERK1/2 ratio of AngII treated cells from at least three independent experiments. Inserts: images of representative immunoblots. B and C, Cardiac myocytes were cultured in absence (Control) or presence (+U0126) of 10μM U0126 and treated without (Basal) or with S100A4 (50 μg/ml) or serum (5%). In B, cell growth was quantified by morphometrical analysis after 5 days of treatment and relative cell sizes of U0126 treated cultures were compared with that for equivalent control cultures. In C, viable cells were counted after two days of culture. Values represent averages of at least three individual experiments and are expressed relative to untreated controls (Basal, Control).

cells (motility, metalloproteinase secretion and angiogenesis) [8–11] and vascular smooth muscle cells as described above [12]. Fourthly, S100A4 appears to be involved in the regenerative processes of peripheral [36] and central nervous systems [37], as it is higher expressed in astrocytes bordering sites of neural injuries, and elicits neuronal differentiation [13,14] and survival [34] in primary cultures of rat neurons.

The S100A4 protein in the infarct border zone myocytes is not produced by the cardiac myocytes themselves, but must instead be released, actively or passively, by interstitial cells and in turn be uptaken by the myocytes. One can speculate on the mechanisms specifically supporting uptake in infarct border zone myocytes rendering all other myocytes free from this extracellularly derived S100A4 protein. The receptor mediating the binding and/or uptake of S100A4 may be induced by the hostile milieu of the ischemic area, promoting the ability of cardiac myocytes to respond to increased extracellular levels of S100A4 by hypertrophic growth and survival. Moreover, given S100A4's ability to increase cell motility and metalloproteinase secretion, the myocytes may be partaking in tissue remodelling. Taken together, these observations prompt speculation towards a cardioprotective activity of S100A4, thus suggesting a parallel supportive role of paracrine S100A4, recruited after injury, in cardiac and neural tissue.

Our observation of increased S100A4 expression in hypertrophic hearts is supported by the observation that cardiac myocytes of isoproterenol treated rats are immunopositive for S100A4 protein [27]. However, the generalized myocyte localization of S100A4 noted in that publication is in sharp contrast with our observations in both our rat models and human patients. Infarct border zones, in which we do observe S100A4 positive myocytes is ischemic and isoproterenol treatment probably also induces myocardial oxygen deprivation as an effect of the strong β-adrenergic stimulation, partially explaining this apparent discrepancy.

It has been shown that paracrine S100A4 can function via more than one cell surface receptor, possibly explaining its many and diverse effects; but the nature of a cardiac S100A4 receptor is unknown. In all, or at least most, cases, it seems that only oligomeric forms of S100A4 have activity on surface receptors [4,8,13,14]. The purified recombinant S100A4 protein used in the present study was not size fractionated, but includes both dimeric and oligomeric S100A4 [14]. In our initial studies of the signaling pathways involved in S100A4 promoted growth and survival of cardiac myocytes, we found that ERK1/2 was phosphorylated and that the MEK1/2–ERK1/2 pathway likely was required for the cellular effects. These data are consistent with observations in cultured neurons, in which neuritogenic effects of S100A4 were also dependent on ERK1/2 activation [14]. Notably, the S100A4 induced phosphorylation of ERK1/2 was atypical with a delayed maximum activation in both our experiments in cardiac myocytes and in the reported experiments in neurons [14].

It is well-described that kidney fibrosis, pulmonary fibrosis, and corneal wound healing also coincides with high S100A4 levels, evidently owing to an increased number of activated, S100A4 expressing fibroblasts [38–40]. S100A4 may therefore have general roles in diseases involving inflammation, fibrosis, remodeling, and/or metastasis. Functions specific to cardiac disease could prove very complex, but holds clear future clinical implication and warrants further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.03.027.

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