Reduced scar maturation and contractility lead to exaggerated left ventricular dilation after myocardial infarction in mice lacking AMPKα1

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Abstract

Cardiac fibroblasts (CF) are crucial in left ventricular (LV) healing and remodeling after myocardial infarction (MI). They are typically activated into myofibroblasts that express alpha-smooth muscle actin (α-SMA) microfilaments and contribute to the formation of contractile and mature collagen scars that minimize the adverse dilatation of infarcted areas. CF predominantly express the α1 catalytic subunit of AMP-activated protein kinase (AMPKα1), while AMPKα2 is the major catalytic isoform in cardiomyocytes. AMPKα2 is known to protect the heart by preserving the energy charge of cardiac myocytes during injury, but whether AMPKα1 interferes with maladaptive heart responses remains unexplored. In this study, we investigated the role of AMPKα1 in modulating LV dilatation and CF fibrosis during post-MI remodeling. AMPKα1 knockout (KO) and wild type (WT) mice were subjected to permanent ligation of the left anterior descending coronary artery. The absence of AMPKα1 was associated with increased CF proliferation in infarcted areas, while expression of the myodifferentiation marker α-SMA was decreased. Faulty maturation of myofibroblasts might derive from severe down-regulation of the non-canonical transforming growth factor-beta1/p38 mitogen-activated protein kinase (TGF-β1/p38 MAPK) pathway in KO hearts. In addition, lysyl oxidase (LOX) protein expression was dramatically reduced in the scar of KO hearts. Although infarct size was similar in AMPK-KO and WT hearts subjected to MI, these changes resulted in compromised scar contractility, defective scar collagen maturation, and exacerbated adverse remodeling, as indicated by increased LV diastolic dimension 30 days after MI. Our data genetically demonstrate the centrality of AMPKα1 in post-MI scar formation and highlight the specificity of this catalytic isoform in cardiac fibroblast/myofibroblast biology.

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1. Introduction

Cardiac fibroblasts (CF) maintain myocardial extracellular matrix (ECM) homeostasis under normal physiological conditions. These cells are also crucial in the reparative response to myocardial infarction (MI) and are involved in the pathogenesis of cardiac remodeling. They are typically activated into myofibroblasts that are normally not found in healthy adult hearts [1]. Myofibroblasts repair necrotic areas, forming scars to preserve myocardial integrity [2]. They express alpha-smooth muscle actin (α-SMA) microfilaments and rapidly mount healing responses that involve contractile granulation tissue deposition and collagen fibrillogenesis, both resulting in mature and stabilized scars [3,4]. Although these cells are essential to prevent cardiac rupture, injured tissue removal and scar establishment are associated, in the long-term, with deleterious changes in the ventricular architecture [5]. More so, CF proliferative and migratory responses are activated after MI and participate in excessive deposition of ECM proteins, in both infarcted and non-infarcted myocardia, even in later post-infarct phases [6,7]. The highly-organized architecture of the myocardial interstitium is, therefore, progressively replaced by a thickened and poorly-organized structure that reduces myocardial elasticity and contributes to altered systolic and/or diastolic function.

Excessive cardiac collagen degradation by tissue-based, Zn²⁺-dependent matrix metalloproteases (MMP) contributes to left ventricular (LV) dilatation [8]. During the first few days after MI, MMP are activated to injury sites, by reactive oxygen species and cytokines. Up-regulation of tissue inhibitors of metalloproteases (TIMP) ends the degenerative repair phase about 7 days after necrosis onset. However, MMP expression is further increased in patients with systolic heart failure, worsening LV dilatation and contributing to maladaptive remodeling [9].

Transforming growth factor-beta1 (TGF-β1), the major isoform in the heart, and the downstream Smad family are markedly up-regulated in infarcted hearts [10–12]. Angiotensin II signaling in CF and macrophages is required to stimulate TGF-β1 synthesis [13]. Thrombospondin-1 induction is involved in its proteolytic activation. TGF-β1 signaling pathways promote tissue fibrosis and ECM remodeling by inducing CF myofibrodifferentiation and proliferation, and by promoting ECM protein synthesis as well as matrix deposition. In addition, TGF-β1 suppresses MMP activity, notably through TIMP up-regulation [10,14]. Its mode of action entails phosphorylation of Smad proteins, which subsequently move to the nucleus where they activate the transcription of target genes [15]. Importantly, TGF-β1 activates Smad-independent signals, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase and p38 mitogen-activated kinase (p38 MAPK) [16–18]. The up-regulation of all these pathways has also been associated with myofibroblast activation.

AMP-activated protein kinase (AMPK) is a highly-conserved eukaryotic protein serine/threonine kinase that senses cellular energy status and coordinates global metabolic responses to energy deprivation [19,20]. It is a heterotrimer consisting of a catalytic α-subunit and 2 regulatory subunits, β and γ. Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, and γ3). Its activation requires phosphorylation of a threonine residue (Thr172) in the activation loop of the α-subunit. At least 2 upstream AMPK kinases have been identified, including LKB1 and calcium and calmodulin-dependent kinase kinase-beta [21–23]. AMPKα2 is the most abundant cardiac subunit in murine cardiomyocytes [24]. Its beneficial metabolic role in post-ischemic and failing hearts has been studied extensively [25–29]. AMPKα1 is also present in the heart. It is predominantly expressed in other cardiac cell types, namely, endothelial cells [30], smooth muscle cells [31], mesenchymal stem cells [32] and fibroblasts [33]. All these cell types participate in cardiac repair after MI. While it is known that AMPK exerts anti-proliferative [32,34,35], anti-fibrotic [36,37] and angiogenic [38–40] effects independently of its role in energy homeostasis, the potential protective contribution of AMPKα1 against adverse ventricular remodeling has never been investigated in vivo. Our study was therefore undertaken to elucidate its function in modulating LV dilatation and CF fibrosis during post-MI remodeling.

Our data indicate that in AMPKα1-deficient hearts (knockout, KO), AMPKα1 paucity is associated with increased number of proliferative CF, fewer myofibroblasts and altered collagen maturation in infarcted areas. All these changes lead to increased end-diastolic volume (EDV) of KO hearts after MI. Our results highlight the role of AMPKα1 in CF/myofibroblast biology, providing new perspectives and potential therapeutic approaches that could counter the adverse LV remodeling of infarcted hearts.

2. Materials and methods

2.1. Reagents and antibodies

SAMs peptide was kindly provided by Dr. V. Stroobant (Ludwig Institute, Université catholique de Louvain, Brussels, Belgium). γ4-P-ATP (Perkin Elmer), Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fischer), Lipofectamine RNAiMAX, Opti-MEM, small interfering RNA (siRNA) Negative Control, siRNA PRKAA1, Click-IT Edu Alexa Fluor 488 flow cytometry assay kits, Hoechst 33342 (Invitrogen), 35-mm μ-dish culture inserts (Ibidi), picrosirius red, TGF-β1 (Sigma Aldrich), luminescence horseradish peroxidase (HRP) substrate, Entellan mounting buffer (Millipore), collagenase (Roche) and Percoll (GE Healthcare) were procured from the suppliers named. The antibodies used were: AMPKα1 and α2 (Kinasource); Vimentin, cardiac Troponin I (Santa Cruz); Smad2, phospho-Smad2 (S465/467), p38, phospho-p38 (T180/Y182), GADPH and eukaryotic elongation factor 2 (eEF2) (Cell Signaling), α-SMA, Lysyl oxidase (LOX) (Sigma-Aldrich), Collagen type 1a (Calbiochem), CD45 (Becton Dickinson), and Ki-67 (Abcam). Iso type control antibodies from goat and rabbit were purified by affinity chromatography and kindly provided by Prof. Charles Pilette (IREC Institute, Brussels, Belgium).

2.2. Animals

Animal handling was approved by the Animal Research Committee of Université catholique de Louvain (2012/UCL/MD/003) and confirmed to American Heart Association Guidelines for the Use of Animals in Research. Experiments were performed on 12–16-week-old AMPKα1 null mice (KO) and wild type (WT) females. MI was induced by left anterior descending coronary artery (LAD) ligation. The animals were anesthetized with tribromoethanol (Avertin™)-Temgesic (310 mg/kg, 0.1 mg/kg, i.p.) and their tracheas were intubated. After left-sided thoracotomy at the fourth intercostal space, the LAD was ligated with 6-0 silk thread. Infarction was confirmed by ventricle cyanosis. The sham procedure consisted of similar surgery, except no ligation was undertaken.

2.3. Primary human cardiac fibroblasts (HCF): culture, AMPKα1 silencing and treatments

HCF were purchased from Cell Applications, Inc. (San Diego, CA, USA) and cultured according to the manufacturer’s recommendations. AMPKα1 was silenced by reverse transfection of 30 nM siRNA with Lipofectamine RNAiMAX, following the manufacturer’s instructions. Cells were seeded at 7500/cm² and cultured for 48 h to allow inhibition of AMPKα1 expression prior to treatment or experimentation. For cell proliferation analysis, cells between 2 and 5 passages were serum-starved for 24 h prior to treatment. For cell migration analysis, 12,000 cells were seeded per well with μ-dish culture inserts. The inserts were removed 2 days after transfection, and cell migration was assessed in serum-free medium. For Western blot analysis of Smad2, collagen and α-SMA expression, the cells were serum-starved for 24 h prior to treatment with 10 ng/ml of TGF-β1 for 30 min or 48 h.
2.4. Isolation of murine non-myocyte cells from cardiac muscle tissue

Adult mice ventricular non-myocytes (mostly fibroblasts) were prepared from 12 week-old mice hearts after general anesthesia induced by nembutal injection (Ceva Santé Animale). Hearts were minced in small pieces in glucose 1% solution. After sedimentation, solution was replaced by collagenase 2%. Five cycles of 15 min incubation at 37 °C in collagenase 2% solution with stirring were performed before acquiring separated cells. Non-myocytes were isolated by Percoll centrifugation at 1000 g for 30 min, at 15 °C. Cells were washed two times and lysed for Western blot analysis.

2.5. AMPK assay

AMPK activity was measured, as described previously [31], after the immunoprecipitation of 50 μg of tissue extracts with 10 μg of AMPKα1 or AMPKα2 antibodies.

2.6. Western blotting

Cells and tissues were lysed in cold buffer containing 50 mM Heps, 1 mM dithiothreitol, 50 mM KCl, 0.1% Triton X-100 and supplemented with a cocktail of inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail). Protein content was measured by the Bradford method with bovine serum albumin (BSA) as reference. 10 μg (in vitro studies) or 30 μg (in vivo studies) of proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes and blocked with milk or BSA. The membranes were probed with antibodies targeting AMPKα1 or α2 (1/1000), vimentin (1/2000), cardiac troponin I (1/5000), Snail2 (1/1000), phospho-Snail2 S465/467 (1/1000), p38 (1/1000), phospho-p38 T180/Y182 (1/1000), lysozyme oxidase (LOX) (1/5000), GAPDH (1/5000), EEF2 (1/2000), α-SMA (1/100,000), and collagen type Iα (1/500), overnight at 4 °C. After incubation with appropriate secondary antibody for 1 h, bound antibodies were visualized with luminescent HRP substrate and quantified by the ImageJ program (version 1.440, National Institutes of Health, Bethesda, MD, USA). Band intensities were normalized relative to those of loading controls on the same gel. The loading controls were anti-GAPDH or anti-EF2 antibodies. Immunoblotting with respective anti-total antibodies was performed on a different gel.

2.7. RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from cells and infarcted/sham tissues with RNeasy mini-kit (Qiagen) before storage at −80 °C. mRNAs were quantified with NanoDrop (Thermo Scientific). Reverse transcription was performed with iScript™ CDNA synthesis kit (Bio-Rad Laboratories). RT-qPCR was undertaken in IQ5 apparatus (Bio-Rad Laboratories) with qPCR core kit for SYBR Green (Eurogentec). Primers were designed with Primer Express software (Applied Biosystems). GAPDH served as housekeeping gene. Primer sequences are presented in Supplementary Table S1. Primers for interleukin-6 (IL-6) and IL-1β were kindly provided by Prof. Thomas Michiels (De Duve Institute, Brussels, Belgium) [41].

2.8. Tissue processing before histological analysis

Mice were injected with Nembutal (Ceva Santé Animale) and heparin (LEO Pharma) at 3 and 30 days after LAD ligation. Their hearts were excised, rinsed with phosphate-buffered saline (PBS) and stopped with 250 mM KCl.

2.9. Immunohistochemistry

The hearts were fixed for 24 h in 4% paraformaldehyde and embedded in paraffin. CD45 immunostaining was performed as described elsewhere [30]. Deparaffinized and rehydrated 5-μm sections were processed for Ki-67 or picrosirius red staining.

Antigens were retrieved with Tris/EDTA (pH 9.0) from a pressure cooker before permeabilization in Tris-buffered saline containing 0.025% Triton X-100. The slices were blocked in 10% fetal bovine serum (FBS) and 1% BSA solution. Anti-Ki-67 antibody (1/500) was incubated overnight before washing and inactivation of endogenous per-oxidases with 0.3% H2O2. Secondary antibody coupled with HRP was applied for 1 h and the slices were washed. Ki-67 was revealed with diaminobenzidin and slices counterstained with hematoxylin. Finally, the sections were dehydrated and mounted in Entellan buffer. Stained slides were digitized by automated whole-slide image capture, with a Mirax Midi scanner (Carl Zeiss). Randomly-selected images were extracted with Mirax Viewer software (Carl Zeiss). Eight and 16 pictures from sham and infarcted mice respectively were analyzed with ImageJ software (Version 1.440, National Institutes of Health, Bethesda, MD, USA).

2.10. Immunohistofluorescence

Hearts were mounted in O.C.T.-embedding compound and frozen at −80 °C. Heart sections (10-μm thick) were permeabilized and saturat- ed in 0.1% Triton X-100, 10% FBS in PBS solution at room temperature. CF proliferation was assessed by double staining with Ki-67 (1/1000) and vimentin (1/100) antibodies. Primary antibodies were incubated overnight at 4 °C. Slices were washed and incubated with Alexa488 and Alexa594 antibodies (1/1000) for 30 min at room temperature. Finally, nuclei were stained with Hoechst 33342 (1/20,000) and slices were mounted in medium for fluorescence analysis (Vector Laboratories). Pictures were shot by Zeiss Axiosimager.z1 and ApoTome module (Carl Zeiss). Eight randomly-selected images from each mouse were quanti- fied by Imagej software (Version 1.440, National Institutes of Health, Bethesda, MD, USA).

2.11. Picrosirius red staining and collagen matrix quality

At 30 days after LAD ligation, collagen was stained on sections deparaffinized, rehydrated and treated with 0.2% phosphomolybdic acid. Slices were incubated in 1.3% picrosirius red solution for 2 h at room temperature before washing in hydrochloric acid and mounting. Staining was digitized by automated whole-slide image capture, with a Leica SCN400 (Leica Microsystems). Myocardial fibrosis was analyzed on 3 to 5 heart sections per animal with Digital Image Hub software (Leica Microsystems). Areas to be analyzed were selected by ImageJ, while positive areas were assessed by FRIDA (version 1.0, The Johns Hopkins University).

Collagen fiber quality was further investigated by Zeiss Axioskop 40 microscopy (Carl Zeiss) with a polarizer filter, to quantify thick, tightly-packed collagen fibers as orange-red, and thin, loosely-assembled, immature fibers as yellow-green [42]. Analysis was conducted with FRIDA (version 1.0, The Johns Hopkins University).

2.12. HCF proliferation assay

HCF proliferation was measured by FACSCalibur flow cytometry (Becton Dickinson), with Click-iT Edu flow cytometry assay kit, accord- ing to the manufacturer’s recommendations. The data were analyzed by FLOWJO software (version 8.8.7, Tree Star & Leland Stanford Junior University).
2.13. HCF migration assay

Migration was evaluated by refilling of free areas between cell populations initially separated by culture inserts. HCF were seeded at 1.2 $\times$ 10^4 cells/well in 35-mm dishes. After 48 h, the culture inserts were removed, and cellular reservoirs, initially separated by a 500-μm thick wall, were allowed to migrate. Three pictures were taken by IX71 Olympus phase-contrast microscopy (Olympus Corporation), at the times of analysis indicated in the figure legends for each condition. Images were assessed and free area refilling was estimated by the TScratch program (version 1.0, CSElab).

2.14. Echocardiography

Echocardiography was performed with a Vevo 2100 Imaging System (Visualsonics, Toronto, ON, Canada). The mice were anesthetized by isoflurane inhalation at concentrations of 5% (induction) and 0.5% (maintenance), in 100% oxygen.

Infarct size was ascertained by analysis of regional LV wall motion, based on the standardized myocardial segmentation model employed by physicians to image human hearts [43]. A 16-segment murine model quantified regional wall motion in each segment as normal or akinetic. Echoes were acquired at baseline before and at 1 and 30 days after LAD ligation. LV dimensions were measured by 2-dimensional (B-mode) parasternal long-axis view, at EDV and end-systole volume (ESV), with deduction of ejection fraction percentage (EF%).

2.15. Statistical analysis

Data were expressed as means ± SD. Echocardiographic measurements and histological and molecular analyses in sham-operated and infarcted mice were undertaken in independent groups. The significance of the differences between mean values of the different groups was ascertained by unpaired Student’s t-test and 2-way ANOVA for simple and multiple comparisons, respectively. The Bonferroni correction was applied to p-values in multiple comparisons. P < 0.05 was considered as significant.

3. Results

3.1. AMPKα1 is the predominant catalytic isofrom seen during healing after MI

Early mortality (<24 h), mainly related to the surgical LAD ligation procedure, was similar in KO and WT mice (mortality rate: 27.2% in KO versus 22.6% in WT controls). No deaths were observed up to 4 weeks after surgery in both groups. Post-MI remodeling was associated with altered AMPKα1 activity in infarcted regions. Seven days after MI, AMPKα1 activity more than doubled in WT hearts (Fig. 1A). This increase in AMPKα1 activity was coupled with a 2-fold increment in AMPKα1 protein content (Figs. 1B, C). In contrast, AMPKα2 protein content decreased in both WT and KO infarcts (Figs. 1B, D). This probably resulted from cardiomyocytes death, as reflected by the dramatic decrease in cardiac troponin I in the necrotic area (Figs. 1B, F). As expected, AMPKα1 activity was undetectable in KO hearts, without any compensatory increase in AMPKα2 protein content (Fig. 1D). During the healing process, necrotic areas were colonized by CF, as attested by vimentin detection in infarcted areas after 7 days (Figs. 1B, E). Only AMPKα1 was expressed in isolated murine non-myocytes (mostly CF), as in HCF (Fig. 1G). Therefore, increased AMPKα1 expression and concomitant AMPKα2 decrease in infarcted myocardia, might result, at least in part, from necrotic tissue invasion by CF.

3.2. Absence of AMPKα1 is associated with increased expression of fibrotic genes in infarcted areas

The transcript levels of well-known fibrotic factors involved in collagen synthesis and degradation were assessed by RT-qPCR in infarcted and remote areas of WT and KO hearts. At day 7 after MI, abundant collagen type I and type III expression was detected in infarcted regions of both WT and KO hearts (Figs. 2A, B). However, a further increase in collagen type I and type III mRNA contents was observed in KO mice (Figs. 2A, B). Collagen expression remained low in remote myocardium areas, and was comparable in WT-MI and KO-MI mice hearts (Supplementary Figs. S1A, B). MMP and TIMP play an important role in ECM remodeling after MI, and it is known that their expression increases rapidly in the heart post-MI [9]. MMP (13, 14, 2 and 9) and TIMP (1 and 2) were up-regulated in both WT-MI and KO-MI infarcted zones, compared to their respective sham controls (Figs. 2C–H). TIMP3 and TIMP4 were not regulated in WT-MI and KO-MI hearts (Fig. 2I). KO-MI mice showed a further increase in the collagenases MMP13, MMP14, TIMP1 and TIMP2 mRNA content (Figs. 2C–D, G–H). In contrast, mRNA expression of the gelatinases MMP2 and MMP9 was not significantly different between WT-MI and KO-MI hearts (Figs. 2E–F). Finally, most MMP and TIMP did not present significant increases in remote LV areas at 7 days after MI (Supplementary Fig. S1), except for MMP14 and TIMP1 (Supplementary Figs. S1D, G).

3.3. Myocardial inflammatory responses are not affected in the absence of AMPKα1 after MI

As post-infarct inflammatory responses increase MMP and fibrosis, we carried out immunohistochemical staining of CD45-positive cells (leukocytes) in cardiac tissue sections to study inflammatory cell infiltration post-MI. Infiltration of CD45-positive cells in infarcted zones increased drastically at 3 days after MI although no significant difference was observed between WT-MI and KO-MI hearts (Supplementary Figs. S2A, B). In addition, mRNA expression of pro-inflammatory cytokines (IL-6 and IL-1β) at 3 days post-MI was assessed by RT-qPCR. Increased inflammatory cell infiltration was associated with heightened mRNA expression of IL-6 and IL-1β in infarcted myocardia (Supplementary Figs. S2C, D). Once again, their expression was not significantly different between WT-MI and KO-MI hearts, indicating that the inflammatory process was not affected by the absence of AMPKα1.

3.4. AMPKα1 deficiency increases CF proliferative and migratory capacities

The augmented expression of fibrotic genes in KO-MI hearts could have been due to proliferative CF accumulation in myocardia. We identified proliferating CF by dual immunohistofluorescent staining with the proliferation marker Ki-67 along with vimentin, a fibroblast marker. Fig. 3A indicates that Ki-67 is enriched in MI compared to sham hearts, at 3 days post-MI. Quantification of Ki-67/vimentin-positive cells demonstrated a clear increase of proliferating fibroblasts in infarcted areas from KO compared to WT mice (Fig. 3B). Isotype control antibodies confirmed the specificity of vimentin and Ki-67 primary antibodies (Supplementary Fig. S3). Immunohistochemical staining of Ki-67-positive cells showed that proliferative cells remained significantly more numerous in infarcted areas from KO mice at 30 days post-MI, compared to WT mice (Fig. 3C). These results were reinforced by in vitro experiments on HCF. Their transfection with specific siRNA targeting AMPKα1 led to a 95% decrease in AMPKα1 protein levels (Supplementary Figs. S4A, B) and increased their proliferation rate, either in the presence or in the absence of serum (Fig. 3D). The data demonstrate that AMPKα1 plays a key role in CF proliferation at baseline and upon stimulation by growth factors. We could not exclude that increased CF migration into infarcted zone also contributed to the growing number of proliferative cells in infarcted areas of KO hearts.
Indeed, in addition to increasing proliferation, the absence of AMPKα1 stimulated the HCF migration rate (Fig. 3E).

3.5. AMPKα1 deficiency impairs myodifferentiation in infarcted myocardia

We questioned whether the changes in fibrotic gene expression seen in the infarcts of KO mice were linked with increased CF myodifferentiation. At 7 days post-MI, α-SMA expression increased dramatically in infarcted zones from both WT and KO mice relative to their respective sham-operated controls (Figs. 4A, B). Strikingly, it was significantly lower in KO-MI compared to WT-MI hearts, indicating that the myodifferentiation process was impaired in KO hearts. It did not result from altered canonical signaling of the profibrotic cytokine TGF-β. Indeed, Smad2 level and phosphorylation were significantly higher in necrotic areas of both WT and KO hearts after MI, compared to their respective sham-operated controls (Fig. 4C), as already described.
by Hao et al. [44]. However, the ratio of phosphorylated Smad2 to total Smad2 was not significantly different between WT-MI and KO-MI hearts (quantification not included). These results were supported by in vitro experiments on HCF. Treatment with 10 ng/ml TGF-β1 led to a significant increase in the phosphorylation state of Smad2 and collagen type I expression, which was not affected by decreased AMPKα1 (Supplementary Fig. S5A). Since AMPK has been shown to regulate myodifferentiation via up-regulation of the p38 MAPK pathway downstream of TGF-β [45], the ratio of phosphorylated p38 to total p38 MAPK was evaluated in infarcted hearts (Figs. 4C, D). At day 3, this ratio was decreased by 60% and 84% in WT and KO infarcts, respectively, indicating that a more pronounced downregulation of p38 activity might impair myodifferentiation in KO infarcts. Once again, the requirement of AMPKα1 to regulate TGF-β-induced p38 phosphorylation in fibroblasts was supported by in vitro experiments. Since the transfection of HCF with scramble or specific siRNA targeting AMPKα1 induced by itself p38 phosphorylation (data not included), we resorted to the use of murine embryonic fibroblasts lacking AMPKα1. Fig. S5B shows that...
Fig. 3. AMPKα1 deficiency increases CF proliferative and migrant capacities. (A) Double immunofluorescent staining of proliferative CF (Ki-67-positive, red fluorescence; vimentin-positive, green fluorescence) in infarcted myocardia at 3 days post-MI. Scale bar = 20 μM. Quantitative analysis appears in (B). Data quantification of immunoochemical staining of infarcted areas with Ki-67 at 30 days post-MI is shown in (C). For immunofluorescent staining, data are means ± SD of 3 and 9 mice for sham and MI-operated animals, respectively. For immunohistochemical staining, data are means ± SD of 3 mice per group. **P < 0.001 compared to corresponding sham values; #P < 0.05 and ##P < 0.01 represent statistical differences between WT and KO. (D) Fold increase in proliferation rate of AMPKα1-deleted HCF relative to scramble-transfected HCF. Proliferation was assessed by Edu incorporation in the absence or presence of FBS 1%. Data are means ± SD of 3 independent experiments. *P < 0.05 and **P < 0.01 compared to respective scramble values. (E) Fold increase in migration rate of AMPKα1-deleted HCF relative to scramble-transfected HCF. HCF migration was measured at 2 h, 4 h and 6 h after insert removal. Data are means ± SD of 3 independent experiments. *P < 0.05 compared to respective scramble values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
AMPK

We therefore measured collagen volume fraction by analysis. An earlier study suggests that ECM secretion reaches maximum by (Fig. 5A). Collagen deposition in infarcted areas and scar size did not revealed thick collagenous scars in both WT and KO infarcted hearts staining myocardial sections with sirius red at this time point. It re-

mice). Interstitial post-MI (n = 41, 8.3 ± 0.5 in KO mice; n = 33, 8.1 ± 0.4 in WT hearts). These changes resulted in compromised scar contractility, defective scar collagen maturation, and exacerbated LV dilatation 30 days after MI.

3.6. AMPKα1 deficiency does not alter collagen content but affects scar maturation in infarcted myocardia

Changes in fibroblastic properties during healing impact scar formation. An earlier study suggests that ECM secretion reaches maximum by 4 weeks [46]. We therefore measured collagen volume fraction by staining myocardial sections with sirius red at this time point. It revealed thick collagenous scars in both WT and KO infarcted hearts (Fig. 5A). Collagen deposition in infarcted areas and scar size did not differ significantly between WT and KO mice (Figs. 5B, C). This result confirmed our echocardiography data showing similar infarct sizes assessed semi-quantitatively by number of akinetic segments at day 1 post-MI (n = 41, 8.3 ± 0.5 in KO mice; n = 33, 8.1 ± 0.4 in WT mice). Interstitial fibrosis was not different between WT and KO hearts (Figs. 5D, E). While we did not detect any quantitative differences in collagen deposition between WT and KO 30 days after MI, we demonstrated qualitative differences in collagen fibers. Sirius red polarization microscopy revealed a 3-fold increase in the ratio of yellow-green to orange-red fibers in KO compared to WT infarcts (Figs. 6A, B). These differences between polarization colors strongly suggest that collagen fibers are packed less tightly in KO compared to WT infarcts or that the fibers themselves are composed of excess, immature, thin collagen, such as collagen type III. Since lysyl oxidase (LOX) plays a crucial role in the cross-linking of collagen fibrils, LOX protein expression was examined in WT and KO infarcts. Its expression was induced in WT infarcts, compared to sham-operated controls but was completely blunted in KO infarcts (Fig. 6C). Interestingly, this downregulation was not due to an effect on gene expression (Supplementary Fig. S6). In agreement with the in vivo data, AMPKα1 deficiency drastically reduced LOX protein in HCF (Fig. 6D).

p38 phosphorylation was induced in WT fibroblasts but not in AMPKα1 KO fibroblasts, upon TGF-β1 stimulation.

4. Discussion

In this work, we provide evidence that the role of AMPK during MI extends beyond the metabolic control of cardiomyocytes. Our data demonstrate that AMPKα1 regulates the fibrotic properties of CF during post-MI remodeling. CF are pivotal in ECM maintenance and remodeling [4,6,7]. AMPKα1 deficiency was associated with increased number of proliferative CF in infarcted areas but these fibroblasts exhibited a myodifferentiation defect associated with decreased p38 MAPK activity. In addition, LOX expression was dramatically reduced in KO infarcts. These changes resulted in compromised scar contractility, defective scar collagen maturation, and exacerbated LV dilatation 30 days after MI.

Regulation of energy metabolism is a key function of AMPK in the heart [47,48]. During myocardial ischemia, reduced oxygen flux instantly induces an increase in AMP/ATP ratio with subsequent AMPK activation [49]. Several studies including ours show that, in isolated-perfused heart experiments, the catalytic α2 subunit is vital in metabolic myocardial protection during ischemia [25–27]. More recently, the protective effect of the α2 isoform has been demonstrated in an in vivo coronary occlusion model [29]. AMPK was activated by intraperitoneal injection of A-769662, a non-nucleoside thienopyridone that strongly promotes AMPK activation. A-769662 administration reduced infarct size and apoptosis after MI, via a mechanism implicating AMPKα2 activation in cardiomyocytes. Therefore, one may postulate that AMPKα1 in cardiomyocytes does not play a major role in limiting cell death during ischemia. Our own work supports this concept as we showed that, in an

![Image](Fig. 4. AMPKα1 deficiency impairs myodifferentiation in infarcted myocardia. (A, B) Western blot analysis and quantification of α-SMA expression in infarcted areas of WT and KO hearts at 7 days after MI. (C) Western blot analysis of Smad2 and p38 MAPK expression and phosphorylation in infarcted areas of WT and KO hearts at 3 days after MI. (D) Data quantification of p38 MAPK. Data are means ± SD. n = 3 and 4 for sham and MI-operated animals, respectively. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to corresponding sham values; and *P < 0.05 represents statistical difference between WT and KO.)
in vivo coronary occlusion model, AMPKα1 deficiency did not affect infarct size (Table 1 and Fig. 5) or cardiomyocyte apoptosis (data not reported). However, besides the well-known metabolic protective role of AMPKα2, AMPKα1 could regulate some other cellular aspects of heart responses after MI, like the control of cardiomyocyte contractility [50] or the release of paracrine factors in the remote myocardium. Paracrine factors include growth factors, hormones, cytokines, microRNAs or metabolites such as ATP or adenosine [51–53]. The release of ATP by cardiomyocytes has been recently reported to allow early CF activation and lead to fibrosis [53]. Adenosine provided by ATP breakdown could have similar downstream effects [54].

Cardiac remodeling, occurring after any form of cardiac injury, contributes to contractile dysfunction. In this respect, non-muscle cells residing in the interstitium, namely, fibroblasts, endothelial cells, smooth muscle cells and cardiac stem cells, are important [45,55–57]. In contrast to cardiomyocytes, they mainly express the AMPKα1 catalytic isoform [30–33]. To evaluate the functions of these cells, and specifically of this catalytic isoform, in post-MI cardiac remodeling, we submitted AMPKα1 KO mice to LAD ligation. Interestingly, we found that AMPKα1 activity was increased in infarcted areas of WT hearts. It was associated with the up-regulation of α1 protein, the latter being probably the primary mechanism responsible for elevation of AMPKα1 activity. In contrast, α2 protein was downregulated in infarcts, probably because of cardiomyocytes losses in this region, while cells expressing the α1 isoform had to sustain processes, such as angiogenesis, inflammation and fibrosis. Indeed, in myocardial vascular endothelial cells, AMPK is known to regulate vascular endothelial growth factor expression and angiogenesis [38–40,58]. In addition, we have recently shown that AMPKα1 counteracted cardiac vascular hyperpermeability and exerted a protective action against myocardial edema induced by endotoxemia [30]. Its anti-inflammatory effect on the endothelium has also been demonstrated in vitro [59]. Our data showed that
AMPKα1 deficiency did not affect myocardial capillary density (Supplementary Fig. S7) or inflammatory cell infiltration level in infarcted myocardia (Supplementary Fig. S2). In contrast, changes in the fibrotic properties of CF were detected in KO infarcts. Specifically, their proliferation was increased. The link between AMPK and cell proliferation has already been investigated in vitro, in several cell types [32,35,60]. AMPK has been postulated to be a cell cycle metabolic checkpoint and its activation reportedly leads to the phosphorylation of several proteins involved in cell cycle regulation [61,62]. In neonatal rat CF, inhibitory crosstalk between ERK and AMPK has been proposed as a new mechanism that growth factors utilize to promote CF proliferation [34]. Although cell proliferation was enhanced, fibroblast transformation to myofibroblasts was impaired in KO infarcts. Indeed, α-SMA expression was significantly reduced. This myodifferentiation defect observed in

**Fig. 6.** AMPKα1 deficiency affects scar maturation in infarcted myocardia. (A, B) Sirius red staining and polarization microscopy in WT and KO infarcted hearts. Scale bar = 100 μM. Representative images (A) and quantitative analyses (B) revealed more immature collagen fibers (yellow-green) in KO infarcts than in WT infarcts. Data are means ± SD of 3 animals per group. *P < 0.05 represents statistical difference between WT and KO. (C,D) Western Blot analysis of lysyl oxidase (LOX) expression in (C) infarcted areas of WT and KO hearts at 7 days after MI, and in (D) HCF deficient or not in AMPKα1. (D) Blots are presented in the lower panel and data quantification appears in the upper panel. Data are means ± SD of 3 independent experiments (***P < 0.001).
the scar tissue, but not in the remote myocardium, explains the increased LV dilatation of KO infarcts [63].

The faulty maturation of myofibroblasts did not result from impaired, canonical, Smad-dependent TGF-β signaling but was associated with significantly decreased p38 MAPK activity in KO infarcts. In 2006, Tenhunen et al. demonstrated that a marked inactivation of p38 as observed in the rat infarcted heart, due to a downregulation of the upstream MAP kinase kinase 3b (MKK3b) [64]. Our data showing that the ratio of phosphorylated p38 to total p38 was decreased in the infarcted hearts compared to sham-operated controls, are in agreement with this study. Besides MKK3b, TGF-β-activated kinase-1 (Tak-1) has been described as another upstream kinase of p38, at least in myofibroblasts [45]. Since AMPK is recognized as a direct Tak-1 substrate [65], it might contribute to p38 regulation and in turn, control the noncanonical p38-dependent regulation of α-SMA expression in the infarcted area. Our hypothesis is therefore that the absence of AMPKα1 prevents Tak-1 to activate p38 and induce α-SMA expression in KO infarcts. Accordingly, both p38 phosphorylation and α-SMA expression are downregulated in KO infarcts, compared to WT infarcts.

In the absence of AMPKα1, the CF proliferative response was increased in infarcted myocardia. It resulted in elevated levels of collagen types I and III and of the collagenases (MMP13, MMP14) responsible for decreased collagen processing and maturation enzymes than that of fibroblasts [67], we can assume that the default in collagen processing and maturation could result, not only from the decreased LOX expression in KO infarcts, but also from the lack of myofibroblastic molecular platforms.

5. Conclusion

In summary, our work presents genetic evidence that AMPKα1 deficiency in infarcted hearts aggravates adverse post-ischemic remodeling. We attribute this deleterious effect to a decreased level of LOX enzyme and less functional myofibroblasts in infarcted areas, thereby resulting in the formation of immature collagen scars and adverse cardiac remodeling.

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Disclosures

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmcc.2014.04.018.

References


Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Sham (n = 19)</th>
<th>KO Sham (n = 10)</th>
<th>WT MI (n = 19)</th>
<th>KO MI (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akinetic segments</td>
<td>0 ± 3</td>
<td>10 ± 2</td>
<td>0 ± 2</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>480 ± 52</td>
<td>461 ± 34</td>
<td>406 ± 89</td>
<td>417 ± 51</td>
</tr>
<tr>
<td>LVEDV (μl)</td>
<td>59.62 ± 9.53</td>
<td>104.57 ± 41.10</td>
<td>59.81 ± 11.39</td>
<td>143.4 ± 67.93</td>
</tr>
<tr>
<td>LVESV (μl)</td>
<td>30.28 ± 6.02</td>
<td>78.68 ± 39.33</td>
<td>28.05 ± 8.99</td>
<td>113.3 ± 78.75</td>
</tr>
<tr>
<td>EF%</td>
<td>45.66 ± 4.62</td>
<td>26.81 ± 8.58</td>
<td>53.85 ± 7.33</td>
<td>24.32 ± 10.19</td>
</tr>
</tbody>
</table>

Heart rate (HR) expressed as beats per minute (bpm); LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; and EF, ejection fraction expressed as [(LVEDV − LVESV) / LVEDV] × 100.

* P < 0.05 compared to corresponding sham values.

# P < 0.05 represents statistical difference between WT-MI and KO-MI.


