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# Adiponectin Mediated APPL1-AMPK Signaling Induces Cell Migration, MMP Activation, and Collagen Remodeling in Cardiac Fibroblasts

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## ABSTRACT

Defects in adiponectin action have been implicated in the development of cardiac dysfunction in obesity and diabetes. Cardiac fibroblasts play an important role in regulating extracellular matrix remodeling yet little is known regarding the direct effects of adiponectin on cardiac fibroblasts. In this study, we first demonstrated temporal relocalization of cellular APPL1 in response to adiponectin in primary cardiac fibroblasts and that siRNA-mediated knockdown of APPL1 attenuated stimulation of AMPK by adiponectin. The cell surface content of MT1-MMP and activation of MMP2 were induced by adiponectin and these responses were dependent on AMPK signaling. Enhanced MMP activity facilitated increased fibroblast migration in response to adiponectin which was also prevented by inhibition of AMPK, with no change in cell proliferation observed. Collagen and elastin immunofluorescence demonstrated reorganization of the extracellular matrix in accordance with increased MMP activity, whereas quantitative mRNA analysis, $^{3}$H-proline incorporation and picrosirius red assays showed no change in intracellular or extracellular total collagen levels in response to adiponectin. In summary, these data are the first to report the adiponectin stimulated APPL1-AMPK signaling axis in cardiac fibroblasts and characterize MT1-MMP translocation, MMP2 activity and cell migration as functional outcomes. These effects may be of significance in heart failure associated with obesity and diabetes. J. Cell. Biochem. 115: 785–793, 2014. 

**KEY WORDS:** ADIPONECTIN; APPL1; EXTRACELLULAR MATRIX; COLLAGEN; MATRIX METALLOPROTEINASE; CARDIAC FIBROBLAST

The growing epidemic of obesity and diabetes has caused a disturbing and rapid increase in the incidence of heart disease [Abel et al., 2008; Despres, 2012]. Extensive studies have indicated that altered circulating levels of adiponectin are associated with cardiac remodeling and dysfunction in obesity and diabetes [Abel et al., 2008; Park and Sweeney, 2013]. The majority of studies indicate a cardioprotective role of adiponectin which is mediated via regulation of multiple myocardial remodeling events [Hui et al., 2012; Park and Sweeney, 2013]. The metabolic, anti-hypertrophic and anti-apoptotic effects of adiponectin are well established and adiponectin has also been shown to mediate anti-fibrotic effects [Schram and Sweeney, 2008; Park and Sweeney, 2013].

The cardiac extracellular matrix (ECM) is a highly structured interstitial network of proteins composed mainly of collagens that surrounds the contractile cardiomyocytes. Remodeling of the ECM is widely accepted to have broad implications on cardiac function [Weber et al., 2013]. Fibrosis is commonly observed in various models of heart disease in rodents and in human failing hearts [Shahbaz et al., 2010; Fowlkes et al., 2013]. Cardiac fibroblasts are the most prevalent cell type in the heart, and regulate ECM dynamics through the expression and regulation of collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs) [Miner and Miller, 2006; Spinale, 2007]. Acute and chronic stressors such as myocardial infarction and hypertension have been shown to induce MMP expression and activity to degrade structural ECM proteins. After myocardial infarction, cardiac fibroblasts migrate to the damaged area to participate in repair [Zhang et al., 2012; Spinale et al., 2013]. This can initially aid contractile function but renewal of matrix with

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poorly structured collagen increases myocardial stiffness and contributes to dysfunction. A more detailed understanding of adiponectin's effects and mechanism of action on cardiac fibroblasts is needed.

Many cardioprotective effects of adiponectin have been shown to be mediated via AMPK [Turer and Scherer, 2012] and recent work in skeletal muscle, cardiomyocytes, endothelial cells and hepatocytes have shown APPL1, an adaptor protein containing an $NH_2$-terminal BAR (Bin/Amphiphysin/Rvs) domain, a PH (pleckstrin homology) domain, a COOH-terminal PTB (phosphotyrosine-binding) domain, and a leucine zipper motif, plays an essential role in activation of AMPK by adiponectin [Mao et al., 2006; Cheng et al., 2007, 2009; Zhou et al., 2009; Fang et al., 2010; Deepa et al., 2011; Liu et al., 2012]. However, adiponectin stimulated APPL1-AMPK signaling in cardiac fibroblasts, and the functional consequences, have not yet been shown.

In the present study, we used primary rat cardiac fibroblasts to examine the direct effects of adiponectin on MMP expression, localization and activities and cell migration. Changes in collagen isoform expression and extracelluar collagen content and structure were determined. We also investigated if adiponectin's effects were mediated via AMPK and whether APPL1 was critical for AMPK activation.

## MATERIALS AND METHODS

### MATERIALS

Recombinant globular adiponectin was produced in-house as previously detailed by [Palanivel et al., 2007]. Dulbecco's modified eagle's medium (DMEM), trypsin, antibiotic/antimycotic and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Life Technologies, Inc., Burlington, ON, Canada). All culture plates were BD Falcon™ brand and purchased from BD Biosciences (Mississauga, ON, Canada). The MF20 antibody was a kind gift from Dr. J.C. McDermott (York University, Toronto, ON, Canada) and von Willebrand factor antibody was purchased from Affinity Biologicals, Inc. (Ancaster, ON, Canada). Anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-APPL1, anti-β-actin primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology (New England Biolabs Ltd, Whitby, ON, Canada). The anti-collagen antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and the anti-elastin antibody was obtained from Elastin Products Company, Inc. (Owenville, MO). Western Lightening Plus ECL was obtained from PerkinElmer (Woodbridge, ON, Canada). TRIzol® Reagent and all siRNAs used in this study were purchased from Ambion Inc. (Life Technologies, Inc.), Lipofectamine® 2000 Transfection Reagent was obtained from Invitrogen (Life Technologies, Inc.), and Alexa Fluor® 488 goat anti-rabbit antibody was obtained from Molecular Probes (Life Technologies, Inc.). The FITC-labeled goat anti-mouse antibody was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The MT1-MMP (MMP-14) antibody was purchased from Acris Antibodies, Inc. (Cedarlane Labs, Burlington, ON, Canada). VECTASHIELD® mounting medium with DAPI was obtained from Vector Laboratories (Burlington, ON, Canada). RNeasy MinElute Cleanup and RT$^2$ First Strand kits were purchased from QIAGEN, Inc. (Mississauga, ON, Canada). $^3$H-proline and $^3$H-thymidine were purchased from Amersham Biosciences (GE Healthcare Lifesciences, Baie d'Urfe, QC, Canada). Amicon Ultra-15 Centrifugal Filter Units and Compound C were obtained from EMD Millipore (Billerica, MA). The Pierce BCA Protein Assay kit was purchased from Thermo Scientific (Fisher Canada, Nepean, ON, Canada). The SIGMAFAST OPD kit was purchased from Sigma-Aldrich (Oakville, ON, Canada). The custom PCR array for collagens was purchased from SABiosciences (QIAGEN, Inc.).

### ISOLATION, CULTURE, AND ADIPONECTIN TREATMENT OF NEONATAL RAT CARDIAC FIBROBLASTS

Neonatal cardiac fibroblasts (CFs) were isolated from 3- to 4-day-old Wistar rats as previously described [Palanivel et al., 2007]. Briefly, excised hearts were digested with 0.15% trypsin for 1 h at room temperature. Trypsin was then neutralized with DMEM containing 10% FBS, and digested cells were centrifuged (10 min at 2,000 rpm), resuspended in DMEM containing 10% FBS and 1% antibiotic/antimycotic and plated onto culture plates. Following incubation at 37°C for 1 h in a 5% $CO_2$ atmosphere, cardiomyocytes remaining in suspension were removed, and attached fibroblasts were replenished with complete growth medium described above. Cardiac fibroblast purity was assessed through MF20 (cardiomyocyte) and von Willebrand factor (endothelial cell) staining [Palanivel et al., 2007] which showed less than 1% staining for non-fibroblast cells. CFs were passaged twice, grown to 100% confluence (or as otherwise indicated below) and then starved with serum-free DMEM for at least 3 h prior to treatment with recombinant globular adiponectin (1.0 μg/ml).

### WESTERN BLOT ANALYSIS

Cells lysis and protein sample preparation for Western blot was conducted according to methods detailed by Vu et al. [2011]. Primary anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-APPL1, and anti-β-actin antibodies were used at 1:1,000 dilutions, and appropriate HRP-conjugated secondary antibodies were used at 1:10,000 dilutions. Proteins were detected by chemiluminescence, quantified by densitometry using Scion Image software (Scion Corp., Frederick, MD) and then normalized to either β-actin or total AMPK protein levels as appropriate.

### siRNA TRANSFECTION OF CARDIAC FIBROBLASTS

CFs were grown to ~30–50% confluence in 12-well plates, and then transfected for 4–6 h with 100 nM control or APPL1 siRNA using Lipofectamine® 2000 Transfection Reagent according to the manufacturer's instructions. The APPL1 siRNA sequence is as follows: GCUUAGUUCUUGUCAUGCAtt. Adiponectin treatment was commenced 48 h post-transfection and APPL1 knockdown efficiency was assessed by Western blot as detailed above (see Western Blot Analysis Section).

### RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from cultured CFs using TRIzol® Reagent according to the manufacturer's instructions, and purified using the RNeasy MinElute Cleanup Kit to attain an $A_{260}/A_{280}$ ratio between 1.9 and 2.0. First-strand cDNA, synthesized from 1 μg RNA using the RT$^2$

First Strand kit, was used in a custom PCR array comprising 96-well plates pre-coated with primers for collagens-I, -III, and -IV. Quantitative real-time PCR was conducted using a Chromo4™ Detection system (Bio-Rad Laboratories Canada Ltd, Mississauga, ON, Canada) according to cycling conditions outlined by the PCR array manufacturer. Data were analyzed using $RT^2$ Profiler PCR Array Data Analysis software (Version 3.5; QIAGEN, Inc.) and normalized to GAPDH mRNA expression.

### WOUND SCRATCH MIGRATION ASSAY

Fibroblast migration in response to adiponectin treatment was assessed using the wound scratch assay. Briefly, a sterile 200 μl pipette tip was used to scratch a vertical line through CFs grown to confluence in 12-well plates. Scratched wells were then thoroughly washed with PBS to remove unattached cells, starved overnight in serum-free DMEM, and then pretreated for 1 h with DMSO or 20 μM Compound C before adiponectin treatment. Following the indicated adiponectin treatment times, cells were fixed in 90% methanol for 30 min at −20°C, however prior to fixation, 1 or 2 unscratched wells were also freshly scratched with a sterile 200 μl pipette tip to serve as "fresh scratch" controls. The cells were washed once more with PBS and then mounted using VECTASHIELD® mounting medium with DAPI. Fluorescent images were obtained using an Olympus BX51 confocal microscope (Olympus, Seattle, WA) with a 20× objective, and fibroblast migration was assessed as the closure of the scratch wound in arbitrary length units using Inkscape software (www.inkscape.org).

### DETECTION OF CELL SURFACE MT1-MMP

Cell surface MT1-MMP was quantified in intact CFs using an MT1-MMP antibody, which recognizes an extracellular epitope, and the SIGMAFAST OPD kit as previously described [Ceddia et al., 2005]. Briefly, adiponectin-treated cells were washed with PBS and fixed on ice for 3 min with 3% paraformaldehyde (PFA). The cells were then washed and incubated for 10 min with 1% glycine to neutralize the PFA, blocked for 30 min in 5% goat serum, incubated on ice for 1 h with MT1-MMP antibody (1:500 dilution in blocking buffer) and then incubated for 1 h at 4°C with HRP-linked anti-mouse antibody (1:1,000). Cells were then washed with PBS, incubated for 30 min at room temperature with OPD reagent (0.8 ml/well), following which 200 μl of 3 M HCl was added to stop the reaction. An absorbance reading was taken for 1 ml of each sample at 492 nm using a spectrophotometer.

### MMP2 ACTIVITY BY GELATIN ZYMOGRAPHY

Media was collected from CFs grown in six-well plates and concentrated using Amicon Ultra-15 Centrifugal Filter Units following adiponectin treatment at indicated times. The protein content of the resulting concentrated conditioned media was determined using the Pierce BCA Protein Assay kit, and then equal amounts of protein from media (25 μg) were resolved by SDS–PAGE on a 10% polyacrylamide gel containing 0.3% gelatin. The gel was washed for 1 h in a solution of 2.5% Triton X-100, briefly rinsed with deionized water, and then incubated for 18 h at 37°C in 1 M Tris–HCl (pH 7.6) containing 100 mM $CaCl_2$ to activate MMP gelatin degradation. MMP activity was stopped using a 10 min wash in 1 M Tris–HCl (pH 7.6) containing 100 mM EDTA. Gels were then fixed and stained in a solution containing 50% methanol, 10% acetic acid and 0.25% Coomassie Blue R-250. Areas of gelatin degradation, which were correlated with known molecular weights of MMP2 isoforms, indicated MMP activation. MMP2 activity was quantified by densitometric analysis of degraded areas using Scion Image software (Scion Corp.).

### IMMUNOFLUORESCENT IMAGING OF ENDOGENOUS APPL1 AND MT1-MMP, AND SECRETED COLLAGEN AND ELASTIN

CFs were seeded onto 25 mm coverslips. For APPL1 and MT1-MMP imaging, CFs were grown to ~30–50% confluence in DMEM containing 10% FBS before adiponectin treatment. For collagen imaging, CFs were initially grown as above for 1 day, and then cultured for 3 days in DMEM containing 10% goat serum prior to adiponectin treatment. For elastin imaging, CFs were cultured as described above for collagen imaging, however cells were grown in DMEM containing 10% FBS. Following adiponectin treatment at indicated times, cells cultured for APPL1, MT1-MMP and collagen I imaging were gently washed with PBS, fixed with 3% PFA for 30 min at room temperature, and then incubated with 1% glycine for 10 min at room temperature to quench PFA. Alternatively, cells cultured for elastin imaging were fixed for 30 min with 90% methanol at −20°C. After fixation, the cells were then blocked at room temperature in either 5% goat serum for 1 h for APPL1 and MT1-MMP imaging, 1% horse serum for 30 min for collagen imaging or 1% goat serum for 30 min for elastin imaging, followed by incubation at room temperature with rabbit anti-APPL1 (1:1,000 dilution in respective blocking buffer), mouse anti-MT1-MMP (1:200), rabbit anti-collagen I (1:100) or rabbit anti-elastin (1:100) antibody for 1 h. Cells were then incubated at room temperature with AlexaFluor 488 goat anti-rabbit (1:1,000) or FITC-labeled goat anti-mouse (1:1,000) secondary antibody respectively for 1 h, followed by a final wash with PBS, and mounting on glass microscope slides using VECTASHIELD® mounting medium with DAPI. Immunofluorescent images were obtained using an Olympus BX51 confocal microscope (Olympus) with 20× and 60× objectives.

### $^3$H-PROLINE AND $^3$H-THYMIDINE INCORPORATION ASSAYS

Pro-collagen synthesis and fibroblast proliferation was assessed by measurement of cellular $^3$H-proline and $^3$H-thymidine uptake respectively as previously outlined [Madani et al., 2006]. Briefly, CFs were treated with or without adiponectin in the presence of $^3$H-proline or $^3$H-thymidine (1 μCi/ml final concentration) for the indicated times. At the end of each treatment period, cells were incubated for 30 min with ice-cold 5% trichloroacetic acid (TCA) at 4°C. The resulting acid precipitate was then solubilized overnight in 0.5 ml of 0.5 N NaOH at 37°C and neutralized with 0.5 ml 0.5 N HCl per well. The radioactivity of each sample was measured in a liquid scintillation counter and corrected for total protein content using the Pierce BCA Protein Assay kit.

### PICROSIRIUS RED DETECTION OF SECRETED COLLAGEN

CF conditioned media was collected and concentrated following adiponectin treatment, and subsequent protein content was determined as described above (see *MMP2 Activity By Gelatin*

*Zymography*). Equal amounts of concentrated media (~60 μg) was then dried at 37°C in wells of a 96-well plate. To stain collagen, 0.1% picrosirius red solution was added to dried wells for 1 h at room temperature. Wells were then washed with 10 mM HCl, and the stain was eluted with 0.1 M NaOH for 5 min. Eluate absorbance was read at 540 nm using a spectrophotometer, and the collagen content of each eluate was quantified based on a collagen standard curve.

### STATISTICAL ANALYSIS

Data are expressed as mean values ± SEM (n), where n represents the number of experiments conducted. Student's *t* tests were used to determine significant differences ($P < 0.05$) between groups. All statistical analyses were conducted using SigmaStat 3.5 Software (Systat Software, Inc., San Jose, CA).

## RESULTS

### ACTIVATION OF AMPK BY ADIPONECTIN WAS APPL1 DEPENDENT

Immunofluorescent imaging of APPL1 localization in CFs revealed a diffuse cytosolic localization under unstimulated conditions (Fig. 1A). Adiponectin treatment induced an initial (10 and 15 min) redistribution of APPL1 to the cell membrane where it is likely to directly bind with adiponectin receptors. Changes in APPL1 were clearly time-dependent with nuclear and perinuclear localization of APPL1 evident after 30 min (Fig. 1A). We next used siRNA to knockdown APPL1 expression (Fig. 1B, top) and showed that adiponectin stimulated AMPK phosphorylation was significantly inhibited after APPL1 knockdown (Fig. 1B,C). siRNA transfection achieved ~65% APPL1 knockdown as determined by Western blot (data not shown). Of note, we observed a slight increase in basal AMPK phosphorylation after APPL1 knockdown, with no changes in total AMPK expression (Fig. 1B).

### ADIPONECTIN STIMULATED MT1-MMP TRANSLOCATION TO THE CELL SURFACE AND MMP2 ACTIVATION, AND THESE EFFECTS WERE MEDIATED VIA AMPK

To characterize the effects of adiponectin on MMPs in CFs, we first examined cell surface localization of MT1-MMP. This was achieved by antibody-based detection of an exofacial epitope in intact cells and quantitative analysis of cell surface MT1-MMP showed over twofold increased levels following adiponectin treatment (Fig. 2A,B). This observation was supported by qualitative immunofluorescent data (Fig. 2C). To examine mechanisms of this response, we first studied the consequences of reducing APPL1 expression using siRNA and demonstrated that the adiponectin response was significantly blunted (Fig. 2A). In addition, after pretreatment of cells with Compound C to inhibit AMPK the adiponectin-stimulated translocation of MT1-MMP to the cell surface was also significantly attenuated (Fig. 2B). Zymographic analysis of CF conditioned media demonstrated an increase in MMP2 activation by adiponectin treatment, which was significantly attenuated by pretreatment with Compound C (Fig. 2C).

### ADIPONECTIN INDUCED CARDIAC FIBROBLAST MIGRATION VIA AMPK SIGNALING

We performed the wound-scratch assay to measure the effects of adiponectin on CF migration. Adiponectin significantly increased CF

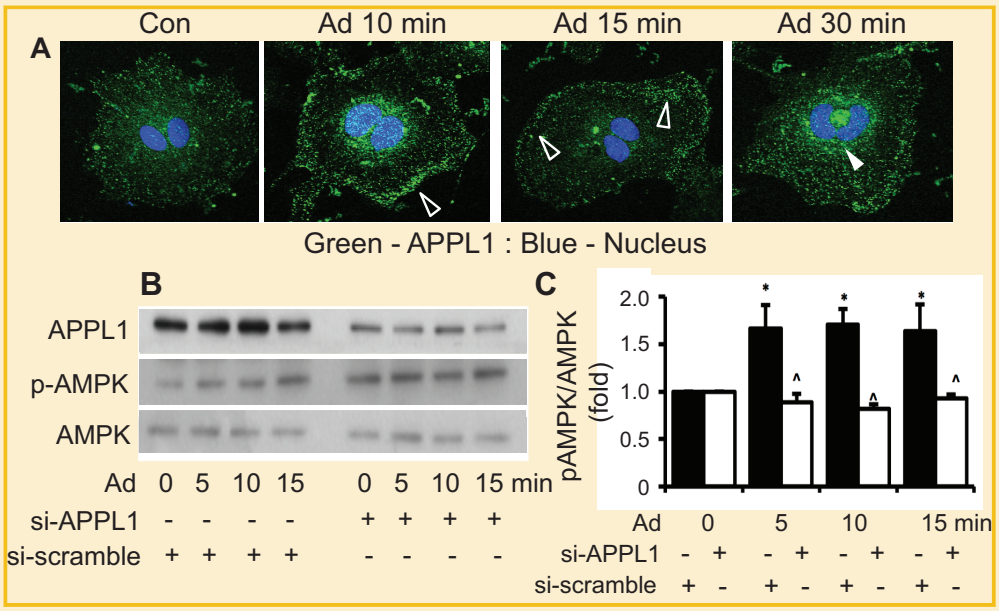


**Fig. 1.** Activation of AMPK by adiponectin was APPL1 dependent. A: Immunofluorescent images of APPL1 (green) in cardiac fibroblasts stimulated with adiponectin (1 μg/ml) for 10, 15, and 30 min. Cell nuclei were also stained with DAPI (blue). Representative images of n = 3 experiments are shown. Open arrows indicate membrane localization. Closed arrow indicates peri-nuclear localization. B: APPL1 or non-specific control siRNA transfected cardiac fibroblasts were treated with adiponectin for 5, 10, and 15 min, and immunoblotted for phospho-AMPK, total AMPK, and APPL1 to verify knockdown efficiency. Representative Western blots are shown from n = 3 experiments. C: Phospho-AMPK was quantified and normalized to total AMPK. Data represent mean values ± SEM from n = 3 experiments. *Significant difference ($P < 0.05$) from control. ^Significant difference ($P < 0.05$) from adiponectin treatment group at corresponding time point.

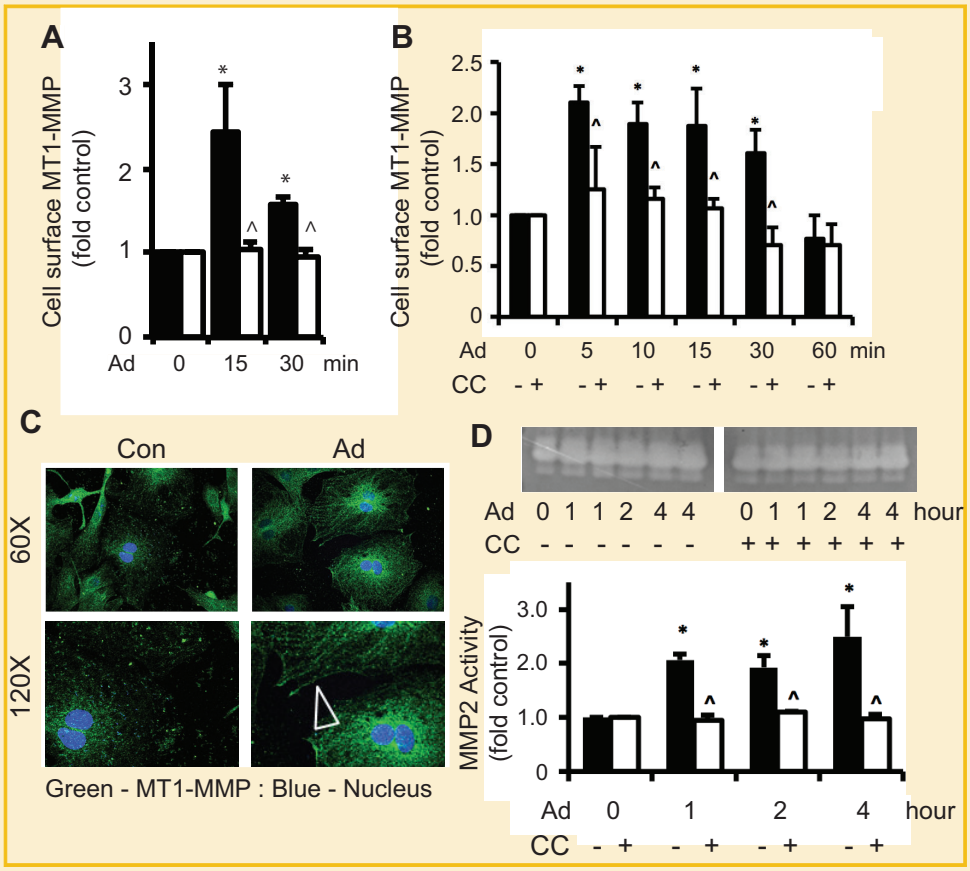


**Fig. 2.** Adiponectin stimulated MT1-MMP translocation to the cell surface and MMP2 activation, and these effects were mediated via AMPK. Cell surface MT1-MMP was examined by OPD assay in cardiac fibroblasts that were treated with adiponectin (1 $\mu$g/ml) at indicated time points and following (A) knockdown of APPL1 using siRNA versus scrambled control or (B) 1 h pre-treatment with either vehicle (DMSO) or 20 $\mu$M Compound C. Data represent mean values ± SEM from n = 3. C: Representative immunofluorescent images from n = 3 experiments are shown of MT1-MMP (green) localization in adiponectin treated cardiac fibroblasts. Cell nuclei were also stained with DAPI (blue). Open arrow indicates MT1-MMP membrane localization. Top images were taken using a 60× objective, and bottom images were digitally enhanced 2× to achieve 120× magnification. D: MMP2 activation was analyzed by gelatin zymography in conditioned media collected from 1, 2, and 4 h adiponectin treated fibroblasts that were pre-treated for 1 h with either DMSO or 20 $\mu$M Compound C. A representative gel, indicating inactive (~68 kDa) and active (~62 kDa) MMP2 isoforms, is also shown in (C). MMP2 activity represents the MMP2 active/inactive ratio. Data represent mean values ± SEM from n = 3 experiments. The black bars are scrambled siRNA and the white bars are siAPPL1 siRNA. *Significant difference ($P < 0.05$) from control. ^Significant difference ($P < 0.05$) from adiponectin treatment group at corresponding time point.

migration after 40, 60, and 120 min of treatment (Fig. 3A,B). Adiponectin had no significant effect on cell proliferation measured via $^{3}$H-thymidine incorporation (Fig. 3C). Compound C significantly attenuated adiponectin-stimulated CF migration (Fig. 3A,B). There was no significant difference in cell migration between DMSO only, Compound C only, or "fresh scratch" wells (data not shown).

### ADIPONECTIN DOES NOT ALTER COLLAGEN SYNTHESIS AND SECRETION BUT INDUCES COLLAGEN AND ELASTIN REMODELING

Under basal conditions, CFs secrete and assemble a collagen I matrix with a wispy and homogenous appearance (Fig. 4A). In keeping with the enhanced MMP activity, degradation of this collagen matrix was observed after 1 and 3 h of adiponectin treatment (Fig. 4A). Interestingly, a restored collagen matrix was observed 24 h after adiponectin treatment. This newly formed matrix appeared more heterogeneous with larger collagen fibril aggregates, giving a thickened and patchy appearance (Fig. 4A). Examination of intracellular pro-collagen synthesis by $^{3}$H-proline incorporation (Fig. 4B) and total secreted collagen in CF conditioned media measured by picrosirius red staining (Fig. 4C) showed no change in response to adiponectin treatment. Using quantitative PCR analysis we also found that expression of collagen-I, -III, and -IV isoforms were not altered by adiponectin (Fig. 4D). The CF elastin matrix also appeared to be similarly degraded upon adiponectin treatment (Fig. 4E).

## DISCUSSION

In this study, we present evidence for a role of adiponectin-stimulated APPL1-AMPK signaling in MMP activation and cell migration in cardiac fibroblasts. The precise role of adiponectin in heart failure is not fully established, however the majority of studies suggest that adiponectin mediates cardioprotective effects and that lack of

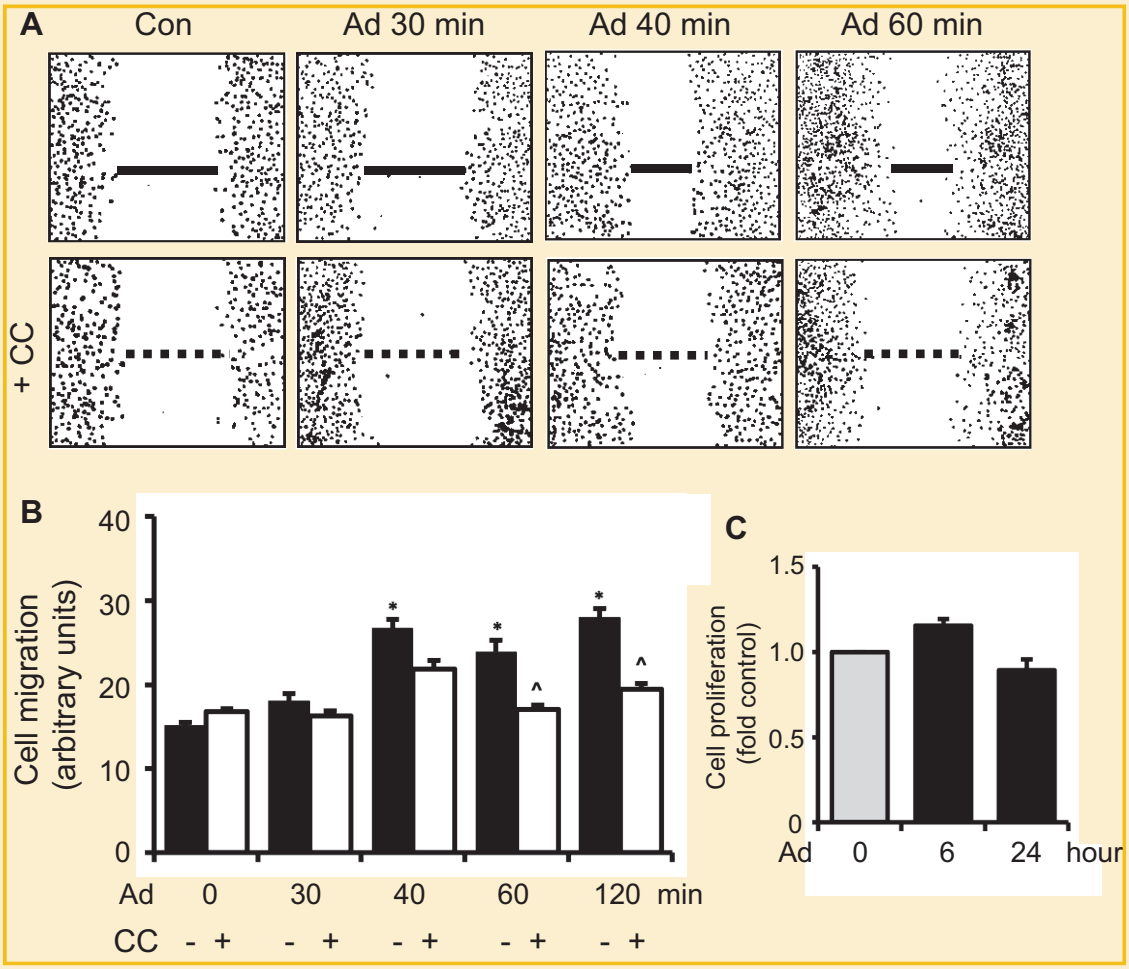


**Fig. 3.** Adiponectin induced cardiac fibroblast migration via AMPK signaling. A: Cell migration was examined using the wound scratch assay in adiponectin treated (1 $\mu$g/ml) cardiac fibroblasts following 1 h pre-treatment with either DMSO or 20 $\mu$M Compound C. Cell nuclei were stained with DAPI and imaged using fluorescent microscopy under a 20× objective. Colors were digitally inversed so that cell nuclei are shown as black against a white background. Cell migration was quantified in (B) as the reduction of the wound width, as denoted by the solid lines in (A). Data represent mean values ± SEM from n = 3 experiments, using 7–10 images per group for quantification. C: Fibroblast proliferation was assessed by $^{3}$H-thymidine incorporation following 6 or 24 h adiponectin treatment. Data represent mean values ± SEM from n = 3 experiments. *Significant difference ($P < 0.05$) from untreated control. ^Significant difference ($P < 0.05$) from adiponectin treatment group at corresponding time point.

adiponectin in obesity and diabetes leads to heart failure [Hui et al., 2012; Park and Sweeney, 2013]. This is strongly supported by studies in adiponectin knockout mice. For example, pressure overload or ischemia/reperfusion induced fibrosis was exaggerated in these mice lacking adiponectin [Shibata et al., 2004; Shibata et al., 2007]. Furthermore, angiotensin-II induced fibrosis and MMP activity was exaggerated in Ad-KO mice and the enhanced fibrosis in adiponectin knockout mouse studies could be corrected by replenishment of adiponectin [Shibata et al., 2004; Shibata et al., 2007; Fujita et al., 2008; Essick et al., 2011]. However, little is known regarding direct effects of adiponectin on cardiac fibroblasts. The main function of cardiac fibroblasts is the maintenance of ECM homeostasis to maintain structural integrity and therefore retain optimal heart function [Fan et al., 2012; Zhang et al., 2012; Spinale et al., 2013; Weber et al., 2013]. This involves the synthesis and secretion of structural proteins, such as collagen I which accounts for 80–85% of myocardial collagen expression, and MMPs which degrade structural proteins.

In this study we first examined the effect of adiponectin on principal MMP isoforms. MT1-MMP is a membrane type MMP and is translocated to the cell surface by various stimuli [Schram et al., 2008, 2011]. In this location, MT1-MMP can mediate proteolysis of matrix components but, perhaps most importantly, it is an important activator of other MMP isoforms which are secreted from cells as inactive zymogens [Koziol et al., 2012]. Indeed, cleavage of MMP2 to its active form can be mediated via MT1-MMP and the coordination of MT1-MMP and MMP2 activities in various cell types plays an important role in cell migration [Sato and Takino, 2010; Gu et al., 2012]. In keeping with these dogma we show here that in cardiac fibroblasts adiponectin increased cell surface MT1-MMP levels and also increased MMP2 activity in extracellular media. Furthermore, we observed that adiponectin could enhance cell migration via a

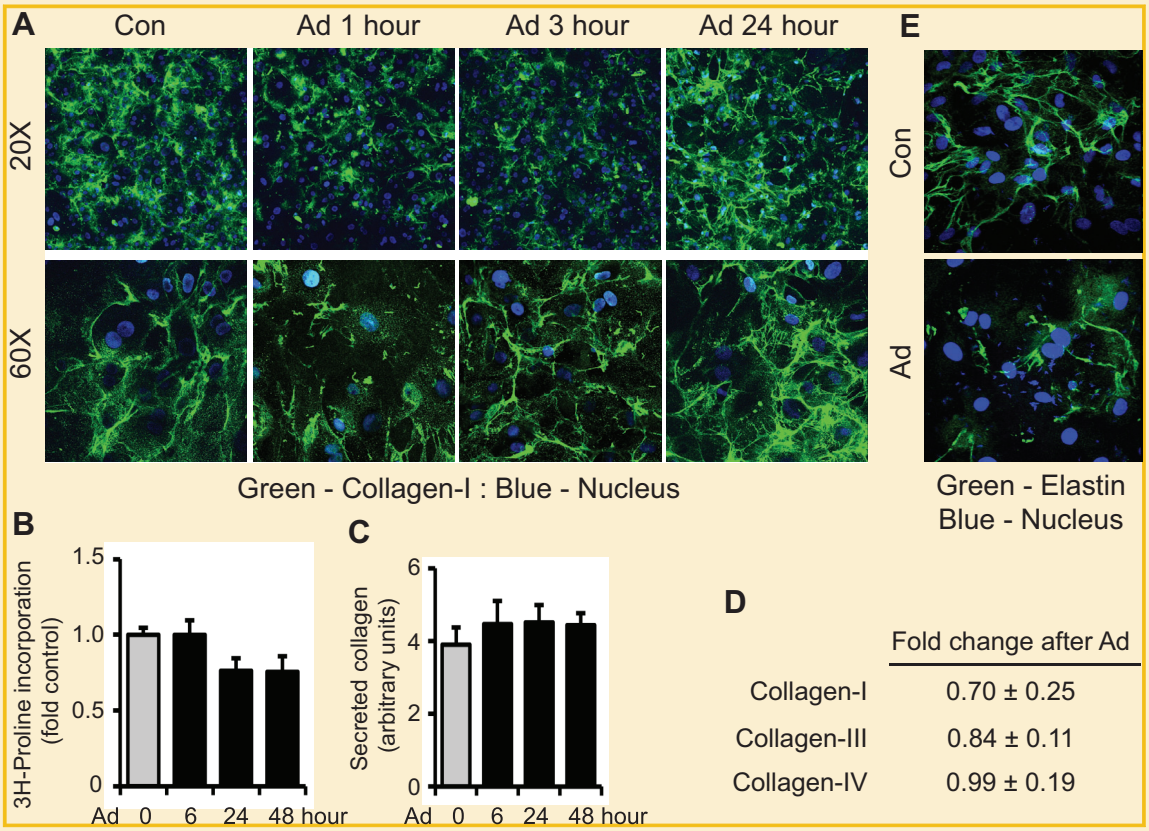


**Fig. 4.** Adiponectin does not alter collagen synthesis and secretion but induces collagen and elastin remodeling. A: Immunofluorescent images of extracellular collagen I (green) secreted from cardiac fibroblasts at 20× and 60× magnification. Cells were treated with adiponectin (1 $\mu$g/ml) for 1, 3, and 24 h. Cell nuclei were also stained with DAPI (blue). Representative images from n = 3 experiments are shown. B: Intracellular pro-collagen synthesis was assessed by $^{3}$H-proline incorporation following adiponectin treatment for 6, 24, or 48 h. Data represent mean values ± SEM from n = 3 experiments using three wells per group for quantification. C: Total secreted collagen was measured in fibroblast conditioned media following adiponectin treatment for 6, 24, or 48 h by picrosirius red staining. Data represented as mean arbitrary units ± SEM from n = 7 experiments. D: Collagen-I, -III and -IV mRNA expression was examined in cardiac fibroblasts following 24 h adiponectin treatment by quantitative real-time PCR. Transcript expression was normalized with GAPDH mRNA abundance. Data represent mean values ± SEM from n = 3. E: Immunofluorescent images of extracellular elastin (green) secreted from cardiac fibroblasts at 60× magnification. Cells were treated with adiponectin for 3 days. Cell nuclei were also stained with DAPI (blue). Representative images from n = 3 experiments are shown.

mechanism that is likely to be at least partly dependent on MT1-MMP and MMP2 mediated effects (Fig. 5). Taken together, the ability of adiponectin to coordinate MT1-MMP and MMP2 activities is likely to contribute to the cardioprotective role of this hormone via promoting fibroblast migration to areas of damaged tissue and promoting degradation of existing matrix, established as an initially favorable response to cardiac injury [Fomovsky et al., 2010; Spinale et al., 2013].

Primary cardiac fibroblasts in culture form an extracellular matrix which can be visualized with appropriate imaging approaches. We used immunofluorescent detection to show collagen-I degradation occurred after treatment of cells with adiponectin. We also noted reassembly of the collagen matrix upon prolonged culture, yet the organization of this reformed matrix appeared more disorganized and fibrous in nature, which may be analogous to adverse cardiac remodeling [Abrahams et al., 1987; Rossi, 2001]. Since there was no change in total collagen synthesis or secreted collagen levels with or without adiponectin treatment, we conclude that degraded collagen is replaced by continuous basal secretion of collagen by cardiac fibroblasts as total MMP activity subsides. Deficiencies in cardiac elastin have been reported to be involved in various forms of heart failure [Fomovsky et al., 2010; Perrotta et al., 2011] and over-expression of elastin by gene therapy prevented cardiac dilation [Li et al., 2012]. In cardiac fibroblast cultures, we found that enhanced MMP activity in response to adiponectin correlated with elastin degradation although the functional significance of this response would require further investigation.

We studied the mechanisms of adiponectin action in cardiac fibroblasts and have provided evidence for activation of APPL1-AMPK signaling which leads to the ECM related changes described above (Fig. 5). The siRNA-mediated reduction in APPL1 we achieved in our experiments was not complete, yet it was functionally significant. This may reflect the importance of the balance between APPL1 and APPL2 levels since these isoforms exhibit a yin-yang relationship [Wang et al., 2009] and the expression of APPL2 is likely to significantly override the remaining APPL1 expression. Many of adiponectins effects in various cell types have now been shown to be mediated via APPL1 with AMPK as a downstream target [Cheng et al.,

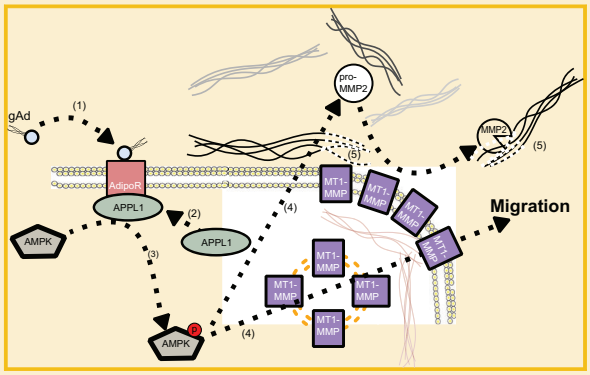


**Fig. 5.** Schematic diagram representing the main mechanisms of adiponectin-induced APPL1-AMPK signaling, MT1MMP translocation, MMP2 activation and cell migration. The schematic figure depicts an integrative summary of the data presented in this manuscript. (1) Adiponectin binding to its receptor, (2) binding of APPL1 to AdipoR and (3) subsequent activation of AMPK (4) MMP isoforms are translocated to the cell membrane (MT1-MMP) or secreted (MMP2) and (5) the inactive MMP zymogen which is secreted can be activated by MT1-MMP on the cell surface and facilitate cell migration. Abbreviations shown are for globular adiponectin (gAd) and adiponectin receptor (AdipoR).

2007; Zhou et al., 2009; Fang et al., 2010; Deepa et al., 2011; Turer and Scherer, 2012], yet this is the first demonstration of such a mechanism in cardiac fibroblasts. One previous study in fibroblasts showed that adiponectin induced the expression of interleukin-6 (IL-6) which involved ERK1/2, AMPK and p38 MAPK signaling but that this was not mediated via APPL1 [Fan et al., 2011]. Other adiponectin signaling targets such as p38 MAPK and ERK1/2 in regulation of MMPs and matrix components in cardiac fibroblasts have yet to be studied.

The effects of adiponectin described here may be mediated via endocrine responses to adipose-derived adiponectin [Dadson et al., 2011]. The presence of a local adiponectin system within the heart has been proposed [Skurk et al., 2008] and suggests that fibroblast or cardiomyocyte derived adiponectin may participate in the maintenance of the cardiac ECM and normal heart function through autocrine and paracrine actions [Dadson et al., 2011]. The concept of the heart producing a range of cardiokines has recently become established [Shimano et al., 2012] and the contribution of effects mediated by locally produced versus circulating adiponectin on cardiac remodeling is worthy of further investigation.

In summary, the data we have presented here establishes that adiponectin signals via APPL1-mediated activation of AMPK to mediate important changes in MMP isoform localization and activity. Subsequently, cell migration is enhanced and structural ECM components are degraded. Study of these events in cardiac fibroblasts further develops our understanding of the diverse functions of adiponectin in the heart and may enhance our appreciation of adiponectins role in heart failure.

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