Mechanically activated Piezo1 channels of cardiac fibroblasts stimulate p38 mitogen-activated protein kinase activity and interleukin-6 secretion

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Running title: Cardiac fibroblast Piezo1 activation induces IL-6 secretion

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Abstract
Piezo1 is a mechanosensitive cation channel with widespread physiological importance; however, its role in the heart is poorly understood. Cardiac fibroblasts help preserve myocardial integrity and play a key role in regulating its repair and remodeling following stress or injury. Here, we investigated Piezo1 expression and function in cultured human and mouse cardiac fibroblasts. RT-PCR experiments confirmed that Piezo1 mRNA in cardiac fibroblasts is expressed at levels similar to those in endothelial cells. Results from the Fura-2 intracellular Ca\(^{2+}\) assay validated Piezo1 as a functional ion channel that is activated by its agonist, Yoda1. Yoda1-induced Ca\(^{2+}\) entry was inhibited by Piezo1 blockers (gadolinium and ruthenium red) and was reduced proportionally by siRNA-mediated Piezo1 knockdown or in murine Piezo1\(^{-/-}\) cells. Results from cell-attached patch-clamp recordings on human cardiac fibroblasts established that they contain mechanically activated ion channels and that their pressure responses are reduced by the Piezo1 knockdown. Investigation of Yoda1 effects on selected remodeling genes indicated that Piezo1 activation increases both mRNA levels and protein secretion of interleukin-6 (IL-6), a pro-hypertrophic and profibrotic cytokine, in a Piezo1-dependent manner. Moreover, Piezo1 knockdown reduced basal IL-6 expression from cells cultured on collagen-coated softer substrates. Multiplex kinase activity profiling, combined with kinase inhibitor experiments and phospho-specific immunoblotting, established that Piezo1 activation stimulates IL-6 secretion via the p38 mitogen-activated protein kinase downstream of Ca\(^{2+}\) entry. In summary, cardiac fibroblasts express mechanically activated Piezo1 channels coupled to secretion of the paracrine signaling molecule IL-6. Piezo1 may therefore be important in regulating cardiac remodeling.

Introduction
Cardiac fibroblasts play important roles in the normal physiology of the heart and in its response
We hypothesized that Piezo1 plays an important role in cardiac fibroblast function by regulating Ca\(^{2+}\) entry and downstream signaling.

Our data provide evidence that Piezo1 acts as a functional Ca\(^{2+}\)-permeable mechanosensitive ion channel in murine and human cardiac fibroblasts, and that its activation by Yoda1 is coupled to secretion of interleukin (IL)-6, a cytokine that is important in the response to cardiac injury and hypertrophic remodeling. We further revealed that Piezo1-induced Ca\(^{2+}\) entry was coupled to IL-6 expression via activation of p38 MAP kinase.

## Results

### Piezo1 expression and activity in cardiac fibroblasts

Messenger RNA encoding Piezo1 was detected in cultured cardiac fibroblasts from both mouse and human hearts (Fig. 1A,B). In a comparison with endothelial cells, which are known to express high levels of this channel (14), Piezo1 mRNA expression levels in murine cardiac fibroblasts were similar to those observed in murine pulmonary endothelial cells, and those in human cardiac fibroblasts were similar to those in human saphenous vein endothelial cells and human umbilical vein endothelial cells (HUVECs) (Fig. 1A,B). Using a magnetic antibody cell separation (MACS) technique (15), we confirmed that Piezo1 was expressed in the fibroblast-enriched (Col1a2-positive) fraction of freshly isolated cells from mouse heart at a similar level to that in the endothelial cell-enriched (Pecam-1-positive) fraction (Fig. 1C,D). Piezo1 mRNA levels were 20 times higher in isolated cardiac fibroblasts than Myh6-positive cardiomyocytes when normalized to the average of three separate housekeeping genes (Fig. 1C,D).

Having demonstrated that cardiac fibroblasts express Piezo1 mRNA, we investigated whether the Piezo1 protein was able to form a functional ion channel. Using the Fura-2 Ca\(^{2+}\) indicator assay, it was found that Yoda1, a Piezo1 agonist (11), elicited an increase in intracellular Ca\(^{2+}\) in murine and human cardiac fibroblasts (Fig. 1E,F). Consistent with the Yoda1-induced increase in intracellular Ca\(^{2+}\) being due to influx of extracellular Ca\(^{2+}\) through an ion channel, the Ca\(^{2+}\) signal was reduced by >90% when extracellular Ca\(^{2+}\) was absent in human and mouse cardiac fibroblast cultures (Fig. 1E,F).
Concentration-response data for Yoda1 inmurine cardiac fibroblasts revealed a marked effect at 0.3 μM and the maximal response was generated at 10 μM; the 50% maximum effect (EC$_{50}$) of Yoda1 was estimated to be 0.72 μM (Fig. 1G). This was almost identical to the EC$_{50}$ observed in human cardiac fibroblasts in similar experiments (Fig. 1H) and comparable with those for mouse Piezo1 heterologously expressed in HEK T-REx$^\text{TM}$-293 cells, where the EC$_{50}$ of Yoda1 was 0.33 μM (Fig. 1I).

Gadolinium (Gd$^{3+}$) and ruthenium red, both non-specific inhibitors of mechanosensitive ion channels including Piezo1 (10), were used to investigate the pharmacology of the channel. Murine cardiac fibroblasts pre-incubated with these inhibitors exhibited significantly reduced Yoda1-evoked Ca$_{2+}^+$ entry (>70% reduction in both cases) (Fig. 2A). Additionally, the Yoda1-evoked Ca$_{2+}^+$ entry was significantly reduced by >50% by Dooku1 (Fig. 2A), an analogue of Yoda1 that has antagonist properties against Yoda1 (7). Piezo1-specific siRNA, which decreased Piezo1 mRNA expression in cardiac fibroblasts derived from Piezo1$^{+/\text{c}}$ hearts compared with WT (Fig. 2B), was transfected into Piezo1-overexpressing HEK-293 cells and HUVECs (16). The data were similar in human cardiac fibroblasts, where again all three inhibitors significantly reduced the Ca$_{2+}^+$ influx in response to Yoda1 (Fig. S1). The Ca$_{2+}^+$ entry elicited by Yoda1 application was also investigated in cardiac fibroblasts isolated from a global heterozygous (Het) Piezo1$^{+/\text{c}}$ mouse line. RT-PCR analysis confirmed the predicted 50% reduction in Piezo1 mRNA expression in cardiac fibroblasts derived from Piezo1$^{+/\text{c}}$ hearts compared with WT (Fig. 2B). The Ca$_{2+}^+$ response to Yoda1 was reduced by 40% (Fig. 2C), whereas the response to ATP was similar in cells from WT and Piezo1$^{+/\text{c}}$ mice (Fig. 2D). Piezo1-specific siRNA, which decreased Piezo1 mRNA expression by 80% in murine cardiac fibroblasts (Fig. 2E), reduced Yoda1-evoked Ca$_{2+}^+$ entry by a similar level (Fig. 2F), whereas control siRNA was without effect. Similar results were obtained with human cardiac fibroblasts (Fig. 2G,H). Thus, the Yoda1 response was dependent upon Piezo1 and proportional to its expression level.

Together, these data established that Yoda1-induced Ca$_{2+}^+$ entry in cardiac fibroblasts is dependent on Piezo1 expression and that the channel has the expected pharmacological properties.

**Cardiac fibroblasts contain mechanically-activated currents**

To investigate if cardiac fibroblasts contain mechanically-activated ion channels we made cell-attached patch recordings from human cardiac fibroblasts. Mechanical force was applied to the patches using a fast pressure-clamp system that generated calibrated suction pulses (pressure pulses) in the patch pipette and therefore increased membrane tension (Fig. 3A). Increased pressure caused increased inward currents up to a limit (Fig. 3A, B). The currents were noisy macroscopic currents, suggesting the presence of multiple individual channels that summed to generate the overall current. The amplitude of the current was highly variable between patches, as seen by the large standard error in Fig. 3B. Despite this variability we estimated that the pressure required for 50% activation was -61.3 mmHg (Fig. 3B).

**The mechanically-activated channel activity is Piezo1-dependent**

To test if the currents were Piezo1-dependent we systematically compared two matched groups of human cardiac fibroblasts: one transfected with control siRNA and the other transfected with Piezo1 siRNA. Because the transfection efficiency was estimated to be 90% at best, we expected that at least 1 in every 10 recordings would be from a non-transfected cell. To compensate for this limitation we made a large number of individual recordings before analyzing the data (45 in total). Our overall impression was that most Piezo1 siRNA-transfected cells showed no meaningful current in response to pressure steps (Fig. 3C). Data for all 45 recordings are shown in Fig. 3D,E. The high variability was again observed but it was also visually apparent that pressure-activated currents were more common in the control siRNA group than the Piezo1 siRNA group (compare Fig. 3D and Fig. 3E). In only 2 patches of the Piezo1 siRNA group were clear pressure-activated currents observed (Fig. 3E). We suspected that these latter 2 recordings were from non-transfected cells (assuming 90% transfection efficiency). Formal analysis using two-way ANOVA on all data in the two groups for all pressure steps (no data were excluded) indicated that currents in the Piezo1 siRNA group were statistically different (smaller) than those in the control siRNA group. These data
suggest that human cardiac fibroblasts express mechanically activated Piezo1 channels.

**Yoda1-induced Piezo1 activation is coupled to increased Il6 gene expression**
To gain insight into the functional role of Piezo1 activation in cardiac fibroblasts, the effect of 24 h treatment with 0.5-10 μM Yoda1 on the expression of selected remodeling genes in murine fibroblasts was investigated by RT-PCR (Fig. 4A-G). Prolonged Yoda1 treatment did not modulate Piezo1 gene expression (Fig. 4A); nor did it affect expression of genes involved in ECM turnover, including collagens I and III (Col1a1, Col3a1) or matrix metalloproteinases (MMPs) 3 and 9 (Mmp3, Mmp9) (Fig. 4B-E). However, a concentration-dependent increase in mRNA expression of the inflammatory/hypertrophic cytokine IL-6 was observed; with significant 2- to 4-fold increases observed in response to 2-10 μM Yoda1 (Fig. 4F). This was not a generic inflammatory response since Il1b mRNA levels remained unaffected by Yoda1 treatment (Fig. 4G). Prolonged exposure to Yoda1 did not affect cell viability (Fig. 4H,I), although it did appear to induce a morphological change in the cells towards a more spindle-like, less rhomboid shape (Fig. 4J).

**Piezo1 activation stimulates IL-6 expression and secretion via p38 MAP kinase**
Given our recent findings on the potential importance of cardiac fibroblast-derived IL-6 in modulating cardiac hypertrophy (15), and the causative link between mechanical stimulation and cardiac hypertrophy (17), we proceeded to interrogate the mechanism by which Piezo1 activation was coupled to IL-6 expression. Firstly, we explored whether mechanical activation of cardiac fibroblasts was coupled to IL-6 expression and whether this occurred via a Piezo1-dependent mechanism. Cyclic stretching (1 Hz, 10% stretch) of human cardiac fibroblasts for 24 h had no effect on Il6 mRNA levels or IL-6 protein secretion (Fig. 5A). However, during these experiments it became apparent that PIEZO1 gene silencing significantly reduced basal IL-6 expression at both mRNA and protein levels (independently of stretch) when cells were grown on BioFlex plates (Fig. 5A), but not when they were maintained on regular tissue culture plastic (Fig. 5E). The BioFlex plates have a stiffness (Young’s modulus) ~1000 times less than standard tissue culture plates and were coated with type I collagen. Thus, activation of Piezo1 by chemical activation (Yoda1), or in response to altered substrate composition/stiffness, was coupled to increased IL-6 expression in cardiac fibroblasts.

We then probed the mechanism further in murine cardiac fibroblasts using Yoda1 as a stimulus. Il6 mRNA expression increased in a time-dependent manner over a 2-24 h period of Yoda1 treatment (Fig. 5B). This increase correlated with an increase in IL-6 protein secretion (Fig. 5C). Yoda-1 induced IL-6 secretion was reduced by approximately 50% in cardiac fibroblasts isolated from hearts of Piezo1−/− mice compared with those from WT hearts (Fig. 5D), in keeping with the reduction in Piezo1 expression and Ca2+ response in these cells (Fig. 2B,C). IL-6 secretion was unchanged following treatment of cardiac fibroblasts with compound ‘2e’ (Fig. 5D), an inactive analogue of Yoda1 (16) that did not induce Ca2+ entry in murine or human cardiac fibroblasts or in HEK T-REx-293 cells heterologously expressing mouse Piezo1 (Fig. S2A-C). The Yoda1-induced increase in IL-6 secretion was dependent on Piezo1, as evidenced by attenuation of the secretion in murine cardiac fibroblasts transfected with Piezo1-specific siRNA (Fig. 5E). The same was true in human cardiac fibroblasts, where the Yoda1-evoked increase in Il6 mRNA expression was attenuated by Piezo1-specific siRNA, and again the inactive Yoda1 analogue 2e had no effect (Fig. S2D,E). These data establish that Yoda1 induces IL-6 expression in cardiac fibroblasts via a Piezo1-dependent mechanism.

PamChip multiplex serine/threonine kinase activity profiling was used to assess differences in kinase activity following treatment of murine cardiac fibroblasts with Yoda1 for 10 min. Combinatorial analysis of phosphorylation of 140 peptide substrates revealed a hierarchical list of predicted serine/threonine kinases that were activated downstream of Piezo1 (Fig. 6A, Table S1). Within the top 20 hits, two major kinase families were identified; MAP kinases (extracellular signal-regulated kinases ERK1/2/5, c-Jun N-terminal kinases JNK1/2/3, p38 mitogen-activated protein kinases p38α/β/γ/δ) and cyclin-dependent kinases (CDKs1-7,9,11) (Fig. 6A). The primary role of CDK-family kinases is to phosphorylate cell cycle proteins and thereby
Functional Piezo1 channels coupled to a rapid rise in intracellular Ca\(^{2+}\); (ii) these channels are mechanosensitive; and (iii) Piezo1 activation is coupled to secretion of IL-6 via a p38 MAPK-dependent pathway. These data establish a link between cardiac fibroblast Piezo1 and secretion of paracrine signaling molecules that can modulate cardiac remodeling.

We established that murine and human cardiac fibroblasts express Piezo1 mRNA at levels similar to those found in endothelial cells from various sources, which are known to express high levels of functional Piezo1 (14). Piezo1 mRNA expression was 20 times higher in cardiac fibroblasts than cardiomyocytes. The EC\(_{50}\) values of Yoda1 acting on endogenous Piezo1 in murine and human cardiac fibroblasts (0.72 \(\mu\)M and 0.71 \(\mu\)M respectively) were comparable with our previous report of 0.23 \(\mu\)M in HUVECs (16). When artificially over-expressed in cell lines, our data on mouse Piezo1 in HEK T-REx-293 cells (EC\(_{50}\) = 0.33 \(\mu\)M) were broadly comparable with human Piezo1 expressed in HEK T-REx-293 cells (EC\(_{50}\) = 2.51 \(\mu\)M) [15], and somewhat lower than those originally reported in HEK293T cells transiently transfected with mouse (EC\(_{50}\) = 17.1 \(\mu\)M) or human (EC\(_{50}\) = 26.6 \(\mu\)M) Piezo1 (11). It is worth noting that we could only use Yoda1 at concentrations up to 10 \(\mu\)M due to solubility problems, so these EC\(_{50}\) values are estimates.

Further studies investigating p38 activation revealed that Yoda1-induced p38 phosphorylation occurred in a concentration-dependent manner and that p38 was not activated in response to the inactive Yoda1 analogue 2e (Fig. 7B). Piezo1-specific siRNA reduced Yoda1-induced activation of p38\(\alpha\) in both murine (Fig. 7C) and human (Fig. 7D) cardiac fibroblasts, confirming the role of Piezo1 in Yoda1-induced p38 activation. The p38 inhibitor SB203580 significantly reduced the Yoda1-induced increase in p38 phosphorylation (Fig. 7E). Furthermore, p38 activation following Yoda1 treatment was shown to be dependent on the presence of extracellular Ca\(^{2+}\) (Fig. 7F), indicating that it is Ca\(^{2+}\) entry through the Piezo1 channel that is important for downstream signaling. Together, these data demonstrate that Ca\(^{2+}\)-induced p38 MAPK activation is essential for inducing the expression and secretion of IL-6 in response to Piezo1 activation.

Discussion
The three main findings of this study are that (i) human and mouse cardiac fibroblasts express functional Piezo1 channels coupled to a rapid rise in intracellular Ca\(^{2+}\); (ii) these channels are mechanosensitive; and (iii) Piezo1 activation is coupled to secretion of IL-6 via a p38 MAPK-dependent pathway. These data establish a link between cardiac fibroblast Piezo1 and secretion of paracrine signaling molecules that can modulate cardiac remodeling.
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Prolonged exposure to Yoda1 increased IL-6 mRNA and protein expression in a Piezo1-dependent manner in both human and mouse cardiac fibroblasts. IL-6 is a pleiotropic pro-inflammatory cytokine that promotes cardiac fibroblast proliferation and fibrosis (22), as well as cardiac hypertrophy through its actions on cardiomyocytes (23), and is readily secreted by cardiac fibroblasts in culture (24). Mechanical stretching of cardiac fibroblasts induces expression of several pro-fibrotic cytokines (1,25), although IL-6 expression does not appear to be mechanically regulated in human cardiac fibroblasts (26), as we found in the present study. This is in contrast to fibroblasts from various other non-cardiac sources that have been shown to secrete IL-6 in response to mechanical stretch (27-29).

Although cyclic stretch did not modulate IL-6 expression in human cardiac fibroblasts (and no role for Piezo1 was found), it was apparent from the stretch experiments that Piezo1 siRNA could reduce basal IL-6 expression. This was evident only when cells were grown on the collagen-coated softer substrate of the BioFlex plates, but not on the rigid plastic of regular tissue culture plates. Thus, Piezo1 may play a role in detecting altered substrate stiffness and/or composition and modulate IL-6 expression accordingly. It has been shown previously that stretch-induced expression of several cardiac fibroblast genes is dependent on matrix stiffness (3,25) and hence there is a complex interplay of mechanosignaling pathways that convert these different mechanical stimuli into alterations in cellular phenotype. Further studies are required to more precisely determine the role that substrate stiffness plays in regulating IL-6 secretion in cardiac fibroblasts.

Experimental Procedures

Reagents
Yoda1 (Tocris), staurosporine (Sigma), PD98059 (Merck), SP600125 (Cambridge Bioscience) and SB203580 (Merck) were all solubilized in dimethylsulfoxide (DMSO). ATP, gadolinium and ruthenium red were all obtained from Sigma-Aldrich and dissolved in H2O. Dooku1 and compound 2e [15] were synthesized at the University of Leeds and solubilized in DMSO.

**Mouse cardiac fibroblast culture**
Adult C57BL/6J mice were euthanized according to guidelines of The UK Animals (Scientific Procedures) Act 1986. Cardiac fibroblast cultures were established from collagenase-digested hearts and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, as previously described (15). All cells were kept at 37 °C and 5% CO2 throughout the study. Cells were studied at passage 1-2. Cells were kept in serum-free DMEM for 16 h prior to treatment in order to collect RNA, lysates and conditioned media.

**Human cell culture**
Biopsies of human atrial appendage and long saphenous vein were obtained from patients undergoing elective coronary artery bypass grafting at the Leeds General Infirmary following local ethical committee approval and informed patient consent. The study complied with the principles of the Declaration of Helsinki. Cells were harvested and cultured as described previously for cardiac fibroblasts (35) and saphenous vein endothelial cells (36). Experiments were performed on human cardiac fibroblasts from passages 2-5. Serum-free DMEM was used for 1 h prior to treatment in order to collect RNA, lysates and conditioned media. HUVECs were purchased from Lonza and cultured in Endothelial Cell Growth Medium (EGM-2) (Lonza), supplemented with 2% fetal calf serum and EGM-2 SingleQuots Kit (Lonza). HUVECs were used at passage 2.

**Mouse pulmonary endothelial cell culture**
CD146+ pulmonary endothelial cells (PECs) were isolated with immunomagnetic microbeads (Miltenyi) and cultured in MV2 medium (Promocell), supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were studied at passage 1.

**HEK T-Rex-293 cell culture**
pcDNA3_mouse Piezo1_IRES_GFP, a kind gift from Artem Patapoutian (10), was used as a template to clone the mouse Piezo1 coding sequence into pcDNA4/TO. Overlapping mouse Piezo1 (forward primer 5’ GTAACAATCGCCCACTTG 3’ and reverse primer 5’ GCTTCTACTCCCTCTACGGTGC 3’) and pcDNA4/TO (forward primer 5’ GACACGAGAGGAGGTAGAAGCCCTGA TCAGCCTCGACTG 3’ and reverse primer 5’ CAATGGGGCGGAGTTGTTAC 3’) PCR products were assembled using Gibson Assembly (New England Biolabs) (37). This construct does not contain tetracycline operator sequences. HEK T-Rex-293 cells (Invitrogen) were transfected with pcDNA4/TO-mPiezo1 using Lipofectamine 2000 (Invitrogen) and treated with 200 μg/ml zeocin (InvivoGen) to select for stably transfected cells. Individual clones were isolated and analyzed for expression using Yoda1 and intracellular Ca2+ measurements. HEK T-Rex-293 cells were maintained in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Sigma-Aldrich). Non-transfected HEK T-Rex-293 cells were used as control cells.

**Cardiac cell fractionation**
Non-myocyte cardiac cell fractions were prepared as described previously (15). Briefly, collagenase-digested heart tissue was filtered through a 30 μm MACS smart strainer (Miltenyi) to remove cardiomyocytes. Non-myocytes were then separated into two fractions using a cardiac fibroblast magnetic antibody cell separation kit (MACS; Miltenyi). ‘Non-fibroblasts’ (Pecam1-positive endothelial cells, and leukocytes) were collected in fraction 1, and ‘fibroblasts’ (Col1a1/Col1a2/Ddr2/Pdgfra-positive) were collected in fraction 2, as previously characterized (15). Separately, adult mouse cardiomyocytes were isolated from ventricles of 8 week old WT mice. Hearts were cannulated through the aorta and perfused with perfusion buffer (124.5 mM NaCl, 10 mM HEPES, 11.1 mM glucose, 1.2 mM NaH2PO4, 1.2 mM MgSO4, 4mM KCl, 25 mM Taurine; pH 7.34) containing 10 mM butanedione monoxime for 5 min followed by perfusion buffer containing 1 mg/ml type 2 collagenase, 0.05 mg/ml protease and 12.5 μM CaCl2 for 7-15 min until suitable digestion was observed. Ventricles were gently cut in
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Perfusion solution containing 10% FBS and 12.5 μM CaCl₂ and filtered. This step was repeated in perfusion solution containing 5% FBS and 12.5 μM CaCl₂ and finally perfusion solution containing only 12.5 μM CaCl₂. The filtrate was pelleted by gravity for 5 min before resuspension in perfusion buffer containing 0.5 mM CaCl₂. This step was repeated with increasing concentrations of CaCl₂ (1.5 mM, 3.5 mM, 8 mM and 18 mM). Finally, the cell pellet was resuspended in 1 ml Trizol. RNA was extracted from cardiac cell fractions and qRT-PCR used to quantify gene expression.

Quantitative RT-PCR
For gene expression studies, cardiac fibroblasts were treated with Yoda1 or vehicle (DMSO) for the indicated time. RNA was extracted from cultured/fractionated cells with the Aurum RNA Extraction Kit (Bio-Rad). cDNA was synthesized using a reverse-transcription system (Promega). Real-time RT-PCR was performed with the ABI-7500 System, gene expression master mix and specific Taqman primer/probe sets (Thermo Fisher Scientific): mouse Coll1a1 (Mm01302043_g1), mouse Col3a1 (Mm01254476_m1), mouse Piezo1 (Mm01241545_g1), human PIEZO1 (Hs00207230_m1), mouse Il1b (Mm00434228_m1), mouse Il6 (Mm00464190_m1), human IL6 (Hs00174131_m1), mouse Mmp3 (Mm00440295_m1) and mouse Mmp9 (Mm00442991_m1). Data are routinely expressed as percentage of mouse Gapdh (Mm99999915_g1) or human GAPDH (Hs99999905_m1) housekeeping gene mRNA expression using the formula 2^{ΔC_T} x 100, in which C_T is the cycle threshold number. For analysis of cardiac cell fractions, three housekeeping genes were used to mitigate the effects of any variation in expression of housekeeping genes between cell fractions: Gapdh (Mm01302043_g1), Hprt (Mm03024075_m1) and Actb (Mm00607939_s1). Data were expressed relative to the geometric mean of the pooled housekeeping gene expression using the formula 2^{ΔC_T}.

Piezo1-modified mice
Animal use was authorized by both the University of Leeds Animal Ethics Committee and by The UK Home Office under project license P144DD006. Animals were maintained in Optimice individually ventilated cages (Animal Care Systems) at 21°C, 50-70% humidity, light/dark cycle 12 h/12 h on RM1 diet (Special Diet Services) ad libitum and bedding of Pure'ø Cell (Datesand). C57BL/6 mice carrying global disruption of the Piezo1 gene with a lacZ insertion flanked by FRT sites have been described previously (14). Identification of wild-type (WT) and Piezo1+/− mice was performed by genotyping using primers for LacZ: forward: AATGGTCTGCTGCTGCTGAAC and reverse: GGCTTCATCCACCACATACAG. Mice of varying ages and sexes were used for experiments.

Western blotting
Cells were treated with vehicle (DMSO), Yoda1 or compound 2e at the appropriate times prior to lysing. Cells were harvested in lysis buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, MiniComplete protease inhibitors (Roche), and PhosSTOP phosphatase inhibitors (Roche). A protein quantification assay was then performed using the DC Protein Assay (BioRad). 25 μg protein was loaded on a 10% polyacrylamide gel. After resolution by electrophoresis, samples were transferred to PVDF membranes and western blotting performed as described previously (38) using a primary antibody for phospho-p38 MAPK (#9215; Cell Signaling Technology). Membranes were re-probed with p38α antibody (#9228; Cell Signaling Technology) to confirm equal protein loading. Species-appropriate secondary antibodies (GE Healthcare) and ECL Prime Western Blotting Detection reagent (GE Healthcare) were used for visualization. Syngene G:BOX Chemi XT4 was used for imaging, alongside GeneSys image acquisition software for densitometric analysis.

Intracellular Ca^{2+} measurements
Changes in intracellular Ca^{2+} (Ca^{2+}) concentration were measured using the ratiometric Ca^{2+} indicator dye, Fura-2-AM and a 96-well fluorescence plate reader (FlexStationII384, Molecular Devices), controlled by SOFTmax PRO software v5.4.5. Cardiac fibroblasts and HUVECs were plated in clear 96-well plates (Corning) and HEK T-REx-293 cells in black, clear-bottomed 96-well plates (Grenier) at a confluence of 90%, 24 h before experimentation. Cells were incubated for 1 h at 37°C in standard bath solution (SBS: 130 mM NaCl, 5 mM KCl, 8 mM D-glucose, 10 mM HEPES, 1.2
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mM MgCl₂, 1.5 mM CaCl₂, pH 7.4) containing 2 
µM Fura-2, in the presence of 0.01% pluronic acid
(Thermo Fisher Scientific) to aid dispersion. During all Fura-2 assays involving cardiac fibroblasts, 2.5 mM probenecid (Sigma-Aldrich) was present to prevent extrusion of the Fura-2 indicator (39). Cells were washed with SBS and then incubated at room temperature for 30 min, during which time inhibitors were added if applicable. Stimuli were injected at 60 sec of recording. The change in intracellular Ca²⁺ concentration (ΔCa²⁺) was measured as the ratio of Fura-2 emission (510 nm) intensities at 340 nm and 380 nm.

**Gene silencing**

Murine cardiac fibroblasts were grown to 80% confluence and transfected with 10 nM Piezo1-specific Silencer Select Pre-Designed siRNA (4390771, siRNA ID: s107968, Life Technologies) or Silencer Select Negative Control No. 1 siRNA (4390843, Life Technologies), using Lipofectamine RNAiMAX Reagent (Life Technologies) in OptiMEM (Gibco), as per the manufacturer’s instructions. Medium was replaced with full-growth media 24 h later. For human cardiac fibroblasts, cells were grown to 90% confluence and transfected with 20 nM Piezo1-specific Silencer Select Pre-designed siRNA (#4392420, siRNA ID: s18891, Thermo Fisher Scientific) or ON-TARGETplus Non-targeting Pool siRNA (#D-01810-10-20, Dharmacon) using Lipofectamine 2000 in OptiMEM, as per the manufacturer’s instructions. Medium was replaced with full-growth medium after 4.5 h. Cells were used for experimentation 48 h after transfection.

**Patch-clamp electrophysiology**

Ionic currents were recorded through cell-attached patches using standard patch-clamp technique in voltage-clamp mode. Patch pipettes had resistance of 4–6 MΩ when filled with pipette solution. Ionic solution of composition (mM) CsCl 145, MgCl₂ 2, HEPES 10, ATP 5, GTP 0.1, EGTA 1 (titrated to pH 7.2 using CsOH) was used in the pipette. The bath solution was SBS. Recordings were made with an Axopatch-200B amplifier (Axon Instruments, Inc., USA) equipped with Digidata 1550B and pClamp 10.6 software (Molecular Devices, USA) at room temperature. Currents were filtered at 2 kHz and digitally sampled at 20 kHz. Data were analyzed using pClamp 10.6, MicroCal Origin 2018 (OriginLab Corporation, USA) software package. All recordings were made blind i.e. without knowledge of which cells had been transfected with control or Piezo1 siRNA.

**Cell viability assay**

Cardiac fibroblasts were plated at 80% confluence and incubated overnight (37°C, 5% CO₂). Vehicle (DMSO), Yoda1 (10 µM) or staurosporine (1 µM) were applied the following day and cells were incubated for 24 h prior to commencing the LIVE/DEAD cell viability assay (ThermoFisher Scientific), according to the manufacturer’s instructions. Cells were imaged using the IncuCyte ZOOM Live Imaging System (Essen Bioscience). The total number of fluorescent cells in each well was calculated using inbuilt algorithms, using an average from 9 images per well of a 12 well plate. The mean data are shown as the number of live cells relative to the total number of cells.

**Stretch experiments**

Human cardiac fibroblasts were seeded at 1x10⁵ cells/well on collagen-coated membranes (BioFlex 6-well culture plates). 72 h after transfection, cells were stretched whilst in serum-free medium using an FX-4000 or FX-5000 Flexercell Tension System (Flexcell International) to equibiaxially elongate the cell-seeded elastic membrane against a loading post. Elongation at 10% strain and 1 Hz was applied to the cells for 24 h. A 6-well stretching plate was housed inside the incubator (37°C, 5% CO₂) alongside unstimulated cells adhered to BioFlex plates which served as static controls. RNA was extracted and conditioned media collected. qRT-PCR and ELISA were used to quantify gene and protein expression respectively.

**ELISA**

Conditioned media were collected, centrifuged to remove cellular debris and stored at -20 °C for subsequent analysis. The concentration of IL-6 in media was measured by ELISA (M6000, R & D Systems), according to the manufacturer's
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instructions. Samples were diluted 1:5-1:30 prior to analysis.

**Multiplex kinase activity profiling**

PamGene Serine-Threonine Kinase (STK) multiplex activity assays were used to investigate STK activity. Murine cardiac fibroblasts were treated with vehicle or Yoda1 for 10 min, collected and lyzed using the M-PER lysis buffer (Thermo Fisher Scientific) containing Halt phosphatase and Halt protease inhibitor cocktails (Pierce) for 30 min on ice. A protein quantification assay was then performed as stated above and samples were snap-frozen in liquid nitrogen. 1.2 μg protein was loaded onto each STK PamChip array. The phosphorylation of PamChip peptides were monitored by the PamStation 12 (‘s-Hertogenbosch, Netherlands), following the manufacturer’s protocols described previously (40). Signal intensities were analyzed using PamGene’s BioNavigator software as Yoda1-treated versus DMSO treatment after 10 min. Permutation analysis resulted in a specificity score (mapping of peptides to kinases) and a significance score (difference between treatment groups) for each kinase. The combined score was used to rank and predict top kinase hits.

**Statistical analysis**

OriginPro 2015 (OriginLab) and GraphPad Prism version 7.05 (GraphPad Software) were used for data analysis, and OriginPro 2015 was used to prepare the charts and graphs. Averaged data are presented as mean ± SEM, where n represents the number of independent experiments and N indicates the total number of replicates within the independent experiments. Data were log-transformed prior to statistical analysis. For comparisons between two sets of data, paired or unpaired Student’s t-test were used as appropriate.

For multiple comparisons with a single factor, a one-way ANOVA was used (P values in figure legends) with Tukey post-hoc test (P values on figures). For analysis of two factors, a regular or repeated measures two-way ANOVA was used (P values in figure legends), with Sidak post-hoc test (P values on figures). P<0.05 was considered statistically significant. For IC₅₀ determination, data were normalized to vehicle and curves were fitted using the Hill1 equation.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

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Abbreviations

1 CDK, cyclin-dependent kinase; DMSO, dimethylsulfoxide; ECM, extracellular matrix; ERK, extracellular signal-regulated kinases; Gapdh/GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; Het, heterozygous; HUVECs, human umbilical vein endothelial cells; IL, interleukin; JNK, c-Jun N-terminal kinase; MACS, magnetic antibody cell separation; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; SBS, standard bath solution; STK, Serine-Threonine Kinase; WT, wild-type.
Figure 1. Piezo1 is expressed by cardiac fibroblasts and forms a functional ion channel. (A,B) RT-PCR analysis of Piezo1 mRNA expression in (A) murine cardiac fibroblasts (CF; n=3) compared to murine pulmonary endothelial cells (PEC; n=5), and (B) human cardiac fibroblasts (CF; n=6) compared to human saphenous vein endothelial cells (SVEC, n=3) and human umbilical vein endothelial cells (HUVEC; n=3). Expression measured as % of housekeeping control (Gapdh/GAPDH). (C) RT-PCR analysis of Piezo1 mRNA expression in the fibroblast-enriched fraction 2 (CF) and the endothelial cell-enriched fraction 1 (EC) isolated from murine heart using magnetic antibody cell separation (MACS) technique (n=4). Cardiomyocytes (CM) were isolated from separate hearts (n=2). Expression measured relative to 3 housekeeping genes (Gapdh, Actb and Hprt) and normalized to CF sample. (D) RT-PCR analysis of cell type-specific marker genes for CF (Col1a2), EC (Pecam1) and CM (Myh6) in MACS fractions, as for panel C. (E,F) Representative Ca²⁺ traces and mean ± SEM data are shown. Ca²⁺ entry evoked by 10 μM Yoda1 in murine (E) and human (F) cardiac fibroblasts in the presence or absence of extracellular Ca²⁺. ***P<0.001 (paired t test; n/N=3/9). (G-I) Ca²⁺ entry evoked by varying concentrations of Yoda1 application at 60 sec, ranging from 0.1-10 μM in murine (G) and human (H) cardiac fibroblasts and HEK T-Rex-293 cells heterologously expressing mouse Piezo1 (I). Vehicle control is illustrated by the black trace. Mean ± SEM data are displayed as concentration-response curves and fitted curves are plotted using a Hill Equation indicating the 50% maximum effect (EC₅₀) of Yoda1 (n/N=3/9).
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Figure 2. Yoda1-evoked Ca\(^{2+}\) entry is dependent on Piezo1 expression. (A) Representative intracellular Ca\(^{2+}\) traces and mean data after murine cardiac fibroblasts were exposed to 10 µM gadolinium (Gd\(^{3+}\)), 30 µM ruthenium red (RuR), 10 µM Dooku1 or vehicle for 30 min before activation of Piezo1 by application of 2 µM Yoda1. Data was normalized to vehicle-treated cells. Repeated measures 1-way ANOVA: P<0.0001, F=114.1 (n/N=3/9). Post hoc test: ***P<0.001 versus vehicle-treated cells. (B) RT-PCR analysis of Piezo1 mRNA expression in cultured murine cardiac fibroblasts isolated from wild-type (WT) and Piezo1\(^{-/-}\) (Het) mice. Expression is measured as % of housekeeping control, Gapdh. ***P<0.001 (unpaired t test, n=8). (C) Representative Ca\(^{2+}\) trace and mean data illustrating Ca\(^{2+}\) entry elicited by 10 µM Yoda in cardiac fibroblasts from WT (n/N=8/24) and Piezo1\(^{-/-}\) Het (n/N=5/15) mice. ***P<0.001 (unpaired t test). (D) Representative Ca\(^{2+}\) trace and mean data illustrating the Ca\(^{2+}\) entry evoked by 5 µM ATP in cardiac fibroblasts from wild-type (WT; n/N=4/12) and Piezo1\(^{-/-}\) (Het; n/N=3/9) mice. Unpaired t test: Not significant (NS). (E) RT-PCR analysis of Piezo1 mRNA expression following transfection of murine cardiac fibroblasts with Piezo1 siRNA, mock-transfected cells and cells transfected with control siRNA. Expression is measured as % of housekeeping control, Gapdh. Repeated measures 1-way ANOVA: P=0.0001, F=61.1 (n=3). Post hoc test: ***P<0.001 versus mock-transfected cells. (F) Representative Ca\(^{2+}\) trace and mean data showing response to 2 µM Yoda1 in murine cardiac fibroblasts transfected with Piezo1-specific siRNA, compared to mock-transfected cells and cells transfected with control siRNA. Repeated measures 1-way ANOVA: P<0.0001, F=72.6 (n/N=3/9). Post hoc test: ***P<0.001 versus mock-transfected cells. (G) As for E but in human cardiac fibroblasts. Repeated measures 1-way ANOVA: P=0.0002, F=50.8 (n/N=3/9). Post hoc test: ***P<0.001 versus mock-transfected cells. (H) As for F but in human cardiac fibroblasts. Repeated measures 1-way ANOVA: P=0.0108, F=10.6 (n/N=3/9). Post hoc test: *P<0.05 versus mock-transfected cells.
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Figure 3. Cardiac fibroblasts express mechanically-activated ionic currents that depend on Piezo1. Recordings were made from cell-attached patches on human cardiac fibroblasts. A constant holding voltage of +80 mV was applied to the patch pipette to ensure a negative membrane potential across the patch (inside relative to outside). A rapid pressure clamp system applied 200-ms negative pressure (suction) steps to the patch pipette of increasing magnitude. (A) Example data from an untransfected cell. The colour code of the pressure steps (lower panel) matches the colour code of the current traces in the upper panel. (B) Mean ± SEM data for experiments of the type shown in A in which the total current during 180-ms of each pressure pulse was summed for n=7-8 patches per data point. The fitted curve is the Boltzmann function, which gave a mid-point for 50% activation of -61.3 mmHg. (C) As for A but from a cell that had been transfected with Piezo1 siRNA. (D, E) Each colour shows the individual data for all recordings from cells transfected with control siRNA (D, n=24 recordings) or Piezo1 siRNA (E, n=21 recordings). As for B, the total current during 180-ms of each pressure pulse was summed. 2-way ANOVA: P=0.0475, F=3.95 (siRNA); P<0.0001, F=4.15 (pressure); P<0.0001, F=4.16 (interaction). In D and E, data values exceeding ~600 pA are clipped in order to maximise visibility of the majority of the data, but all data values were included in the statistical analysis. The two large current values in the Piezo1 siRNA group may have been from non-transfected cells because the transfection efficiency was estimated to be 90%.
Figure 4. Effect of Yoda1 on gene expression and cell survival in cardiac fibroblasts. (A-G) RT-PCR analysis of murine cardiac fibroblasts treated with concentrations of Yoda1 ranging from 0.5-10 μM, or DMSO vehicle, for 24 h (n=3). All mRNA expression levels were normalized to those of the housekeeping gene, Gapdh. Repeated measures 1-way ANOVA: P=0.0010, F=8.9. Post hoc test: *P<0.05, **P<0.01, ***P<0.001 versus vehicle-treated cells. (H) Live/dead cell assay performed on cultured murine cardiac fibroblasts treated with either vehicle, 10 μM Yoda1 or 1 μM staurosporine (SSP) for 24 h. Bar chart shows mean data for viable cells as a percentage of total cells. Repeated measures 1-way ANOVA: P=0.0028, F=10.6 (n=3). Post hoc test: **P<0.01, NS = not significant versus vehicle-treated cells. (I) Representative images from live/dead cell assay. Green indicates live cells; red indicates dead cells. Scale bar = 300 μm. Bottom panels are zoomed images of indicated region of top panels.
**Figure 5. Activation of Piezo1 is coupled to IL-6 expression.** (A) RT-PCR analysis of *IL6* mRNA expression (left panel) and ELISA analysis of IL-6 protein secretion (right panel) after exposure of human cardiac fibroblasts to 24 h cyclical stretch (1 Hz, 10% stretch) on collagen-coated BioFlex plates, compared to fibroblasts maintained in parallel under static conditions. Cardiac fibroblasts were previously transfected with either control or Piezo1-specific siRNA. RT-PCR data are expressed as % of housekeeping control, *GAPDH*. Repeated measures 2-way ANOVA for RT-PCR (n=3): P=0.0704, F=12.7 (siRNA); P=0.403, F=1.1 (stretch); P=0.754, F=0.13 (interaction). Post hoc test: *P<0.05. Repeated measures 2-way ANOVA for ELISA (n=3): P=0.0268, F=35.8 (siRNA); P=0.1545, F=5.0 (stretch); P=0.838, F=0.05 (interaction). Post hoc test: not statistically significant. (B,C) Murine cardiac fibroblasts were exposed to vehicle or 10 μM Yoda1 for 2-24 h before (B) measuring *Il6* mRNA levels by RT-PCR where expression is measured as % of housekeeping control, *Gapdh* or (C) analyzing conditioned medium for IL-6 levels by ELISA (both n=3). Comparison of area under curve by paired t-test: *P<0.05 for effect of Yoda1 on IL-6 expression at both mRNA and protein levels. (D) Cardiac fibroblasts from wild-type (WT; n=4) and Piezo1 +/- (Het; n=3) mice were treated with vehicle, 10 μM Yoda1 or compound 2e for 24 h before measuring IL-6 levels in conditioned medium using ELISA. 2-way ANOVA: P=0.0001, F=24.9 (compound); P=0.1041, F=3.1 (genotype); P=0.615, F=0.51 (interaction). Post hoc test: ***P<0.001, **P<0.01 for effect of Yoda1 versus vehicle. All other changes not statistically significant. (E) Mouse cardiac fibroblasts were transfected with control or Piezo1-specific siRNA and treated with either vehicle or 10 μM Yoda1 for 24 h before collecting...
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conditioned media and measuring IL-6 levels by ELISA (n=3). Repeated measures 2-way ANOVA: P=0.2349, F=2.82 (siRNA); P=0.2187, F=3.13 (Yoda1); P=0.064, F=14.2 (interaction). Post hoc test: NS = not significant versus-vehicle-treated cells.

Figure 6. Piezo1 activation induces MAP kinase signaling with p38 MAPK being coupled to IL-6 expression. (A) Mouse cardiac fibroblasts were stimulated with 10 μM Yoda1 for 10 min before analysing Ser/Thr protein kinase activity with PamChip multiplex kinase activity profiling (n=6). Kinases identified based on peptide substrate phosphorylation and ranked by specificity and statistical significance (only the top 40 are shown). See Table S1 for full data set. The top kinase families predicted to be activated by Yoda1 were the MAP kinases (ERK1/2/5, JNK1/2/3, p38α/β/γ/δ; blue) and the cyclin-dependent kinases (CDKs1-7,9,11; orange). (B-D) RT-PCR analysis of Il6 mRNA expression after exposure of murine cardiac fibroblasts to (B) 30 μM PD98059 (ERK pathway inhibitor), (C) 10 μM SP600125 (JNK inhibitor) or (D) 10 μM SB203580 (p38 MAPK inhibitor) for 1 h followed by treatment with vehicle or 10 μM Yoda1 for a further 24 h. Expression is measured as % of housekeeping control, Gapdh (n=7 for SB203580, n=5 for PD98059 and SP600125). Repeated measures 2-way ANOVA for B: P=0.7738, F=0.133 (PD98059); P=0.0529, F=7.41 (Yoda1); P=0.069, F=6.08 (interaction). For C: P=0.6133, F=0.299 (SP600125); P=0.0020, F=51.9 (Yoda1); P=0.475, F=0.62 (interaction). For D: P=0.0004, F=52.1 (SB203580); P=0.2942, F=1.32 (Yoda1); P=0.0028, F=23.8 (interaction). Post hoc test: ***P<0.001, NS = not significant for effect of inhibitor. (E) Murine cardiac fibroblasts were treated with vehicle or 10 μM SB203580 for 1 h and then treated with either vehicle or 10 μM Yoda1 for 24 h before collecting conditioned media and measuring IL-6 levels by ELISA (n=6). Repeated measures 2-way ANOVA: P=0.0217, F=10.8 (SB203580); P=0.0283, F=9.3 (Yoda1); P=0.0002, F=98.1 (interaction). Post hoc test: ***P<0.001 for effect of SB203580.
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Figure 7. Yoda1-induced p38 MAPK phosphorylation is dependent on Piezo1. Cellular protein samples were immunoblotted for p-p38α and reprobed with antibody for total p38α, to confirm equal protein loading. Bar charts show mean densitometric data of p-p38α normalized to p38α expression. (A) Murine cardiac fibroblasts (n=6) treated with DMSO vehicle (V) or 10 µM Yoda1 (Y) for 5-30 min. *P<0.05 versus vehicle-treated cells (repeated measures 1-way ANOVA, P=0.0085). (B) Murine cardiac fibroblasts (n=3) treated for 10 min with varying concentrations of Yoda1 (2-10 µM) or 10 µM compound 2e. *P<0.05, NS = not significant versus vehicle-treated cells (repeated measures 1-way ANOVA, P=0.0122). (C,D) Murine (C) or human (D) cardiac fibroblasts (n=3) transfected with either scrambled or Piezo1-specific siRNA before treatment with vehicle or 10 µM Yoda1 for 10 min. Repeated measures 2-way ANOVA for C: P=0.0202, F=48.0 (Yoda1); P=0.3045, F=1.87 (siRNA); P=0.0903, F=9.6 (interaction). Repeated measures 2-way ANOVA for D: P=0.0282, F=34.0 (Yoda1); P=0.2669, F=2.32 (siRNA); P=0.478, F=0.75 (interaction). Post hoc test: NS = not significant. (E) Murine cardiac fibroblasts (n=3) exposed to 10 µM SB203580 for 1 h before treatment with vehicle or 10 µM Yoda1 for 10 min. Repeated measures 2-way ANOVA for Yoda1: P=0.0312, F=30.6 (Yoda1); P=0.5898, F=0.40 (SB203580); P=0.0148, F=65.9 (interaction). Post hoc test: **P<0.01, *P<0.05, NS = not significant. (F) Murine cardiac fibroblasts (n=3) treated for 10 min with vehicle or 10 µM Yoda1 in either standard DMEM or in DMEM containing 1.75 mM EGTA to chelate...
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free Ca\(^{2+}\). Repeated measures 2-way ANOVA: P=0.0123, F=79.5 (Yoda1); P=0.5739, F=0.44 (Ca\(^{2+}\)); P=0.128, F=6.34 (interaction). Post hoc test: NS = not significant.
Mechanically activated Piezo1 channels of cardiac fibroblasts stimulate p38 mitogen-activated protein kinase activity and interleukin-6 secretion
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