The EMBO Journal (2008) 27, 3104–3115 | © 2008 European Molecular Biology Organization | All Rights Reserved 0261-4189/08
www.embojournal.org

THE EMBO JOURNAL

$P2Y_6$ receptor-$G\alpha_{12/13}$ signalling in cardiomyocytes triggers pressure overload-induced cardiac fibrosis

Motohiro Nishida[1], Yoji Sato[2], Aya Uemura[1], Yusuke Narita[1], Hidetoshi Tozaki-Saitoh[3], Michio Nakaya[1], Tomomi Ide[4], Kazuhiro Suzuki[2], Kazuhide Inoue[3], Taku Nagao[2] and Hitoshi Kurose[1,*]

[1]Department of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan, [2]National Institute of Health Sciences, Tokyo, Japan, [3]Department of Molecular and System Pharmacology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan and [4]Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Cardiac fibrosis, characterized by excessive deposition of extracellular matrix proteins, is one of the causes of heart failure, and it contributes to the impairment of cardiac function. Fibrosis of various tissues, including the heart, is believed to be regulated by the signalling pathway of angiotensin II (Ang II) and transforming growth factor (TGF)-β. Transgenic expression of inhibitory polypeptides of the heterotrimeric G12 family G protein ($G\alpha_{12/13}$) in cardiomyocytes suppressed pressure overload-induced fibrosis without affecting hypertrophy. The expression of fibrogenic genes (TGF-β, connective tissue growth factor, and periostin) and Ang-converting enzyme (ACE) was suppressed by the functional inhibition of $G\alpha_{12/13}$. The expression of these fibrogenic genes through $G\alpha_{12/13}$ by mechanical stretch was initiated by ATP and UDP released from cardiac myocytes through pannexin hemichannels. Inhibition of G-protein-coupled P2Y6 receptors suppressed the expression of ACE, fibrogenic genes, and cardiac fibrosis. These results indicate that activation of $G\alpha_{12/13}$ in cardiomyocytes by the extracellular nucleotides-stimulated $P2Y_6$ receptor triggers fibrosis in pressure overload-induced cardiac fibrosis, which works as an upstream mediator of the signalling pathway between Ang II and TGF-β.

The EMBO Journal (2008) **27,** 3104–3115. doi:10.1038/emboj.2008.237; Published online 13 November 2008
Subject Categories: signal transduction
Keywords: fibrosis; G protein; purine receptor

Introduction

Heart failure is the final cardiac stage that is observed in nearly all forms of cardiovascular diseases. Structural remodelling of the heart, including myocardial hypertrophy and fibrosis, is a key determinant for the clinical outcome of heart failure (Cohn *et al*, 2000; Berk *et al*, 2007). A variety of evidence indicates that the initial phase in the development of myocardial hypertrophy involves neurohumoral factors, such as endothelin (ET)-1, angiotensin (Ang) II and norepinephrine, and their receptors being coupled to G proteins of the G_q, G_{12}, and G_i families (Sadoshima and Izumo, 1997; Gohla *et al*, 2000; Arai *et al*, 2003). These agonists induce hypertrophic gene expression in cardiomyocytes through the Ca^{2+}-dependent pathway (Onohara *et al*, 2006). Previous studies using transgenic or conditional knockout mice clearly revealed that the G_q family protein predominantly regulates the pathogenesis of hypertrophy (Adams *et al*, 1998; Wettschureck *et al*, 2001). Therefore, it is well recognized that the $G\alpha_q$-mediated Ca^{2+} signalling pathway has an important function in the development of cardiac hypertrophy.

Cardiac fibrosis is characterized by excessive deposition of extracellular matrix (ECM) proteins, such as collagen type I and type III (Brown *et al*, 2005). A variety of growth factors, such as Ang II, ET-1, transforming growth factor (TGF)-β, connective tissue growth factor (CTGF) and periostin, have been reported to promote fibrotic responses of the heart (Katsuragi *et al*, 2004; Zhang *et al*, 2006; Berk *et al*, 2007). Although cardiac fibrosis is accompanied by maladaptive cardiac hypertrophy that eventually results in heart failure, the mechanism of the induction of cardiac fibrosis and its pathophysiological function have yet to be understood.

The relationship between Ang II and TGF-β for induction of fibrosis is a well-established one (Rosenkranz, 2004; Berk *et al*, 2007). In many types of cells, it has been reported that Ang II stimulation regulates TGF-β expression and activation. TGF-β mediates some Ang II-induced responses, and the blockade of Ang II-mediated signalling partially suppresses TGF-β-induced fibrosis (Xu *et al*, 2008). Other signalling molecules for mediating fibrosis are Rho and Rho-associated kinase (ROCK), which are known as downstream effectors of Ang II (Nishida *et al*, 2005). It has been reported that targeted deletion of ROCK suppressed the development of cardiac fibrosis induced by pathological hypertension (Rikitake *et al*, 2005; Zhang *et al*, 2006). ROCK is a downstream mediator of Rho, a small GTP-binding protein (Amano *et al*, 2000), and Rho is reported to exert an effect as one of the downstream mediators of $G\alpha_{12/13}$ (Kozasa *et al*, 1998). We have reported previously that α subunits of the G_{12} family protein ($G\alpha_{12}$ and $G\alpha_{13}$: $G\alpha_{12/13}$) participate in Ang II-, ET-1-, and α_1-adrenergic receptor agonist-induced cardiomyocyte hypertrophy (Maruyama *et al*, 2002; Arai *et al*, 2003; Nishida *et al*, 2005). However, the pathophysiological function of $G\alpha_{12/13}$ in cardiac hypertrophy and fibrosis *in vivo* is still unknown.

$G\alpha_{12}$ and $G\alpha_{13}$ appear to be expressed ubiquitously (Simon *et al*, 1991), and a lack of $G\alpha_{13}$ in mice results in embryonic lethality because of the defective organization of the vascular system (Offermanns *et al*, 1997). Therefore, we generated mice with a cardiomyocyte-specific overexpression of $G\alpha_{12/13}$-specific inhibitory polypeptide, which mimics the tissue-

*Corresponding author. Department of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan.
Tel.: +81 92 642 6884; Fax: +81 92 642 6884;
E-mail: kurose@phar.kyushu-u.ac.jp

Received: 4 June 2008; accepted: 15 October 2008; published online: 13 November 2008

specific knockout of G$\alpha_{12/13}$. Using these mice, we tested the hypothesis that G$\alpha_{12/13}$ signalling contributes to pressure overload-induced cardiac hypertrophy and associated events *in vivo*.

Results

Cardiac-specific expression of p115-RGS attenuates pressure overload-induced cardiac fibrosis

Previous studies using transgenic or conditional knockout mice clearly revealed that Gα_q predominantly regulates the development of hypertrophy (Adams *et al*, 1998; Wettschureck *et al*, 2001). In addition, we reported previously that G$\alpha_{12/13}$ also have an important function in agonist-induced hypertrophic responses of cardiomyocytes using a G$\alpha_{12/13}$-specific inhibitor, a regulator of the G protein signalling domain of p115RhoGEF (p115-RGS) (Maruyama *et al*, 2002; Arai *et al*, 2003; Nishida *et al*, 2005). We generated mice with a cardiomyocyte-specific overexpression of p115-RGS protein to test the hypothesis that G$\alpha_{12/13}$ signalling contributes to pressure overload-induced cardiac hypertrophy *in vivo* (Supplementary Figure 1A–C). Ang II stimulation caused Rho activation in the hearts of wild-type (WT) mice, and the activation was completely suppressed in transgenic (p115-Tg) mice (Supplementary Figure 1D). This result confirmed that receptor-stimulated activation of G$\alpha_{12/13}$ signalling is inhibited in the p115-Tg heart. Pressure overload was induced by surgical transverse aortic constriction (TAC)

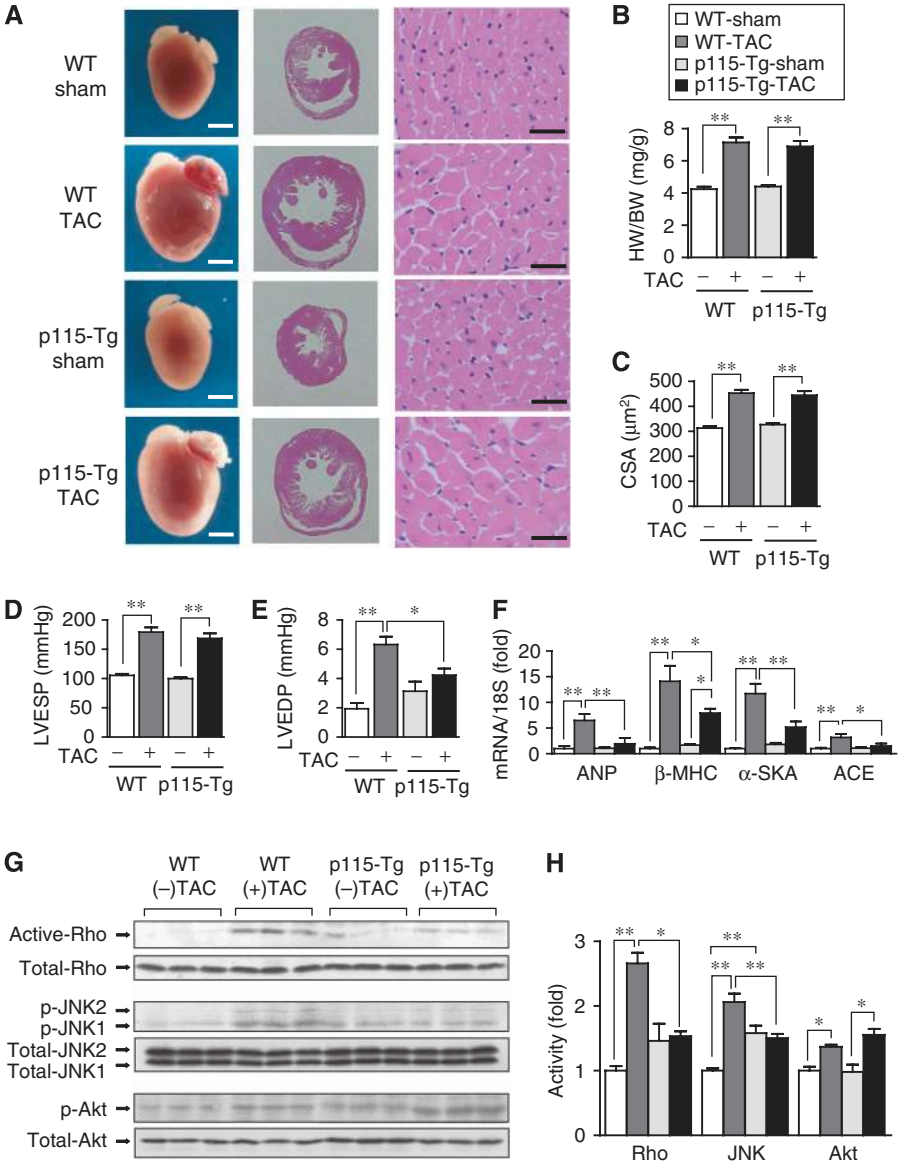

Figure 1 p115-RGS suppresses pressure overload-induced cardiac dysfunction but not hypertrophy. (**A–F**) TAC-induced increase in the size of the heart and cardiomyocytes (A–C), left ventricular (LV) pressure (D, E), and the expression of hypertrophic genes (F) in WT and p115-Tg mice. (A) Gross appearance of the hearts and H&E-stained mid-transverse sections of hearts isolated from WT and p115-Tg mice 6 weeks after sham or TAC surgery. Left (white) scale bar, 2 mm; right (black) scale bar, 30 μm. (B and C) Heart weight (HW) to body weight (BW) ratios (B) and cross-sectional areas (CSA) of cardiomyocytes (C). (D and E) LV pressure. LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure. (F) Expression of ANP, β-MHC, α-SKA, and ACE mRNAs. (**G**, **H**) Activation of Rho, JNK, and Akt induced by 1 week of TAC in WT and p115-Tg hearts. Error bars indicate s.e.m.; $n=3$–5 (C), $n=17$–23 (B, D, E), $n=3$ (F, H). Representative result of hearts from sham ($n=3$) and TAC surgery ($n=5$) was shown (A). * indicates $P<0.05$ and ** indicates $P<0.01$.

in WT and p115-Tg mice. The increase in size of the p115-Tg heart is essentially the same as that in WT mice (Figure 1A–C). TAC of p115-Tg mice increased left ventricular end-systolic pressure (LVESP) to the same extent as that in WT mice (Figure 1D), indicating that pressure overload by TAC was equally performed. TAC induced a significant elevation of left ventricular end-diastolic pressure (LVEDP) in WT mice. However, there was no alteration in p115-Tg mice (Figure 1E). Although the LV systolic function in p115-Tg mice was slightly reduced in sham operation compared with that in WT mice, there was no further impairment by TAC (Figure 1E and Supplementary Table 1). These results suggest that systolic and diastolic function of the p115-Tg heart is not impaired after TAC. TAC in WT mice strongly increased the expression of messenger ribonucleic acid (mRNA) of classical markers of pathological hypertrophy in myocardium, atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and α-skeletal muscle actin (α-SKA) (Figure 1F). However, the expression of these genes in p115-Tg hearts was less than half of that in WT hearts. We have reported that $G\alpha_{12/13}$ mediate activation of Rho and c-Jun NH_2-terminal kinase (JNK) in cultured cardiomyocytes (Maruyama *et al*, 2002; Arai *et al*, 2003; Nishida *et al*, 2005). Pressure overload increased the activities of Rho and JNK in WT hearts, but the activation of Rho and JNK was significantly suppressed in TAC of p115-Tg hearts (Figure 1G and H). However, TAC-induced Akt activation was not suppressed in p115-Tg hearts, suggesting that p115-RGS specifically inhibits $G\alpha_{12/13}$-mediated signalling in the heart. As the TAC-induced Rac activation was also suppressed in p115-Tg hearts (Supplementary Figure 1E), $G\alpha_{12/13}$ may mediate TAC-induced JNK activation through Rho- and Rac-dependent pathways in mouse hearts as well as rat cardiac myocytes.

As overproduction of ECM protein causes ventricular stiffness leading to the impairment of diastolic function (Berk *et al*, 2007), we examined the involvement of $G\alpha_{12/13}$ in pressure overload-induced cardiac fibrosis. TAC increased the expression of collagen types I and III proteins in the interstitial tissue in WT mice, as determined by picrosirius red staining (Figure 2A). In contrast, TAC-induced collagen deposition in p115-Tg mice was less than half of that in WTs (Figure 2B). The relationship between heart weight to body weight ratio (HW/BW) and collagen expression in WT hearts reveals that the degree of hypertrophy correlates positively with severity of fibrosis (Figure 2C). In contrast, the correlation between HW/BW and collagen expression in p115-Tg hearts also indicates that TAC-induced cardiac fibrosis was significantly suppressed despite the development of cardiac hypertrophy. TAC increased the expression of mRNAs for procollagen type I and type III, fibrogenic factors (CTGF (Hahn *et al*, 2000), periostin (Katsuragi *et al*, 2004), and TGF-βs (Zhang *et al*, 2000)) in WT hearts (Figure 2D). These increases were significantly attenuated in p115-Tg hearts. Furthermore, TAC increased the expression of periostin, mature TGF-βs, and angiotensin-converting enzyme (ACE) in WT hearts, but these increases were attenuated also in p115-Tg hearts (Figure 2E). It has been

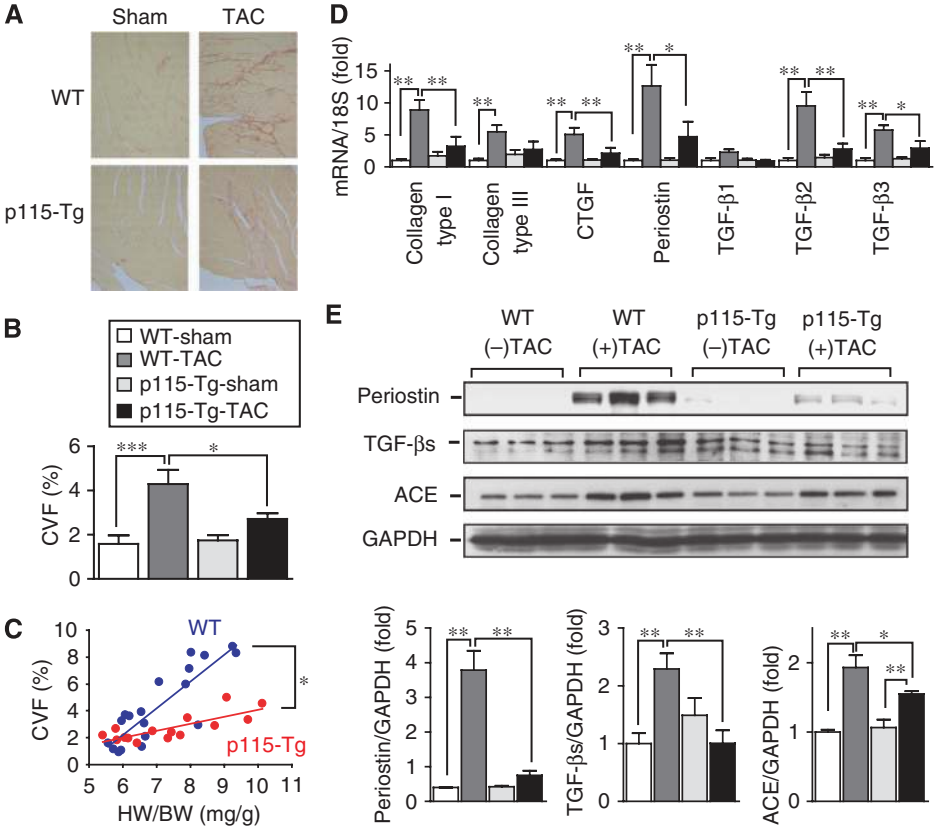

Figure 2 $G\alpha_{12/13}$ mediate pressure overload-induced cardiac fibrosis. (**A**) LV sections stained by picrosirius red. (**B**, **C**) Collagen volume fraction (CVF) (B) and correlations between HW/BW and CVF (C). (**D**) Expression of collagen type I, type III, CTGF, periostin, and TGF-β1-3 mRNAs. (**E**) Expression of periostin, mature TGF-βs, and ACE proteins. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels were used as an internal control. Error bars indicate s.e.m.; $n=15$–19 (B), $n=3$ (D, E). Representative picrosirius red staining sections of hearts from WT ($n=15$–19) and p115-Tg ($n=15$–16) mice were shown (A). * indicates $P<0.05$ and ** indicates $P<0.01$.

postulated that activation of the renin–angiotensin–aldosterone system (RAAS) and increased levels of active TGF-β1 has an important function in pressure overload-induced cardiac fibrosis (Berk *et al*, 2007). Pressure overload increased three TGF-β mRNA isoforms and proteins in WT hearts, and these increases were suppressed in p115-Tg hearts. These results suggest that $G\alpha_{12/13}$ mediate pressure overload-induced cardiac fibrosis by an increase in induction of CTGF, periostin, and TGF-βs.

Activation of $G\alpha_{13}$ in cardiomyocytes induces cardiac fibrosis

We also generated mice with cardiomyocyte-specific overexpression of a constitutively active (CA) mutant of $G\alpha_{13}$ protein (CA-$G\alpha_{13}$) (Supplementary Figure 2). The CA-$G\alpha_{13}$ heterozygous mice did not show an increase in heart size, compared with WT mice (Supplementary Figure 3A and B). However, collagen deposition was significantly increased (Supplementary Figure 3C). The expression of mRNA for CTGF was strongly increased in CA-$G\alpha_{13}$ mice (Supplementary Figure 3D). The expression of mRNA for ACE and protein expression were slightly increased (Supplementary Figure 3E). The expression of mRNAs for periostin and TGF-βs was not increased, but proteins of periostin and TGF-βs were increased in CA-$G\alpha_{13}$ hearts. As CA-$G\alpha_{13}$ increased proteins but not mRNAs for periostin and TGF-βs, $G\alpha_{13}$ may participate in the stabilizing of periostin and TGF-βs proteins. These results also suggest that pressure overload-induced expression of fibrogenic factors is mediated by $G\alpha_{13}$. In contrast to CTGF, the expression of hypertrophy-related genes (ANP and β-MHC) was not increased in CA-$G\alpha_{13}$ heart, consistent with the inability of CA-$G\alpha_{13}$ to induce hypertrophy. However, strong activation of $G\alpha_{13}$ signalling may induce cardiac hypertrophy as well as fibrosis, as CA-$G\alpha_{13}$ homozygous mice showed a significant increase in heart size (data not shown). The LV function of CA-$G\alpha_{13}$ mice was significantly decreased compared with that of WT mice (Supplementary Figure 3F and G). These results suggest that $G\alpha_{12/13}$ mediate cardiac fibrosis and dysfunction induced by pressure overload.

Extracellular nucleotides mediate mechanical stretch-induced $G\alpha_{12/13}$ activation through purinergic receptors

As heterotrimeric G proteins are activated primarily by receptor stimulation, it is reasonable to assume that pressure overload activates $G\alpha_{12/13}$-coupled receptors. As mechanical stretch of cardiomyocytes is frequently used as an *in vitro* model of pressure overload, we examined which G protein-coupled receptor(s) are involved in mechanical stress-induced $G\alpha_{12/13}$ activation. As activation of small GTP-binding protein Rho is a sensitive marker of $G\alpha_{12/13}$ activity (Kozasa *et al*, 1998), we measured Rho activity as an index of the magnitude of $G\alpha_{12/13}$ signalling. Mechanical stretch of cardiomyocytes increased Rho activity, and this increase was sustained for 30 min (Figure 3A). Overexpression of p115-RGS completely inhibited mechanical stretch-induced Rho activation at early time and 30 min (Figure 3B and C). As a mutation in the RGS domain of p115RhoGEF loses the interaction with $G\alpha_{12/13}$ (Bhattacharyya and Wedegaertner, 2003), we expressed the mutated p115-RGS to examine whether the effects of p115-RGS are specific for inhibition of interaction with $G\alpha_{12/13}$. Expression of the interaction-deficient mutant of p115-RGS did not affect mechanical stretch-induced Rho activation. In addition, treatment with *Pertussis* toxin, an uncoupler of receptor-G_i interaction, did not suppress mechanical stretch-induced Rho activation. These results suggest that mechanical stretch activates Rho through $G\alpha_{12/13}$. It has been reported that Ang type 1 receptor (AT1R) is activated by mechanical stretch without the involvement of Ang II, and AT1R antagonist blocks mechanical stretch-induced G_q activation and hypertrophic responses (Zou *et al*, 2004). However, mechanical stretch-induced Rho activation through $G\alpha_{12/13}$ was not attenuated by treatment with not only CV11974 (AT1R antagonist) but also PD123319 (AT2R antagonist), propranolol (β adrenergic receptor (AR) antagonist), prazosin (α_1AR antagonist), BQ123 (ET type A receptor antagonist), BQ788 (ET type B receptor antagonist) and CGP20712A (selective β_1AR antagonist) (Figure 3C and Supplementary Figure 4A and B). Mechanical stretch increases intracellular Ca^{2+} concentration through mechanosensitive cation channels (Christensen and Corey, 2007). However, treatment with an inhibitor of stretch-sensitive channels GsMTx4, intracellular Ca^{2+} chelator BAPTA-AM, and L-type Ca^{2+} channel blocker nitrendipine did not suppress mechanical stretch-induced Rho activation (Figure 3C and D). The Src family kinase substrate p130Cas has been reported to function as a mechanosensor (Sawada *et al*, 2006), but an Src inhibitor, PP2, did not affect mechanical stretch-induced Rho activation (data not shown). In contrast, treatment with apyrase, an ATP/ADP scavenging enzyme, completely blocked mechanical stretch-induced Rho activation (Figure 3D and Supplementary Figure 4C). Treatment with another ATP scavenging enzyme, hexokinase II, or purinergic receptor antagonists, suramin and PPADS, also suppressed mechanical stretch-induced Rho activation. Furthermore, extracellular treatment with ATP, ADP, UTP, and UDP, but not adenosine, increased Rho activity (Supplementary Figure 4D–F). The extracellular nucleotide-stimulated Rho activation was completely suppressed by the expression of p115-RGS (Supplementary Figure 4G). Mechanical stretch actually activated $G\alpha_{12}$ and $G\alpha_{13}$, which were completely suppressed by treatment with suramin (Figure 3E and F). These results suggest that extracellular nucleotides mediate mechanical stretch-induced $G\alpha_{12/13}$ activation through purinergic receptors in rat cardiomyocytes.

Pannexin-1 mediates mechanical stretch-induced release of nucleotides

Extracellular ATP in the cardiovascular system may originate from different cellular sources, such as perivascular sympathetic nerve endings (Burnstock, 1972), activated platelets, endothelial cells, and inflammatory cells. It has also been postulated that connexin and pannexin hemichannels are involved in ATP release caused by mechanical stimulation in cardiac myocytes (Suadicani *et al*, 2000; Shestopalov and Panchin, 2008). Mechanical stretch of cardiomyocytes increased extracellular ATP concentration (Figure 4A). Treatment with hemichannel inhibitors, carbenoxolone, and 1-heptanol, suppressed both mechanical stretch-induced Rho activation and the increase in extracellular ATP concentration (Figures 3D and 4B). As the increase in extracellular ATP was not affected by p115-RGS and P2 receptor antagonists (PPADS and suramin), $G\alpha_{12/13}$ do not participate in ATP release, but rather mediate mechanical stretch-induced Rho activation.

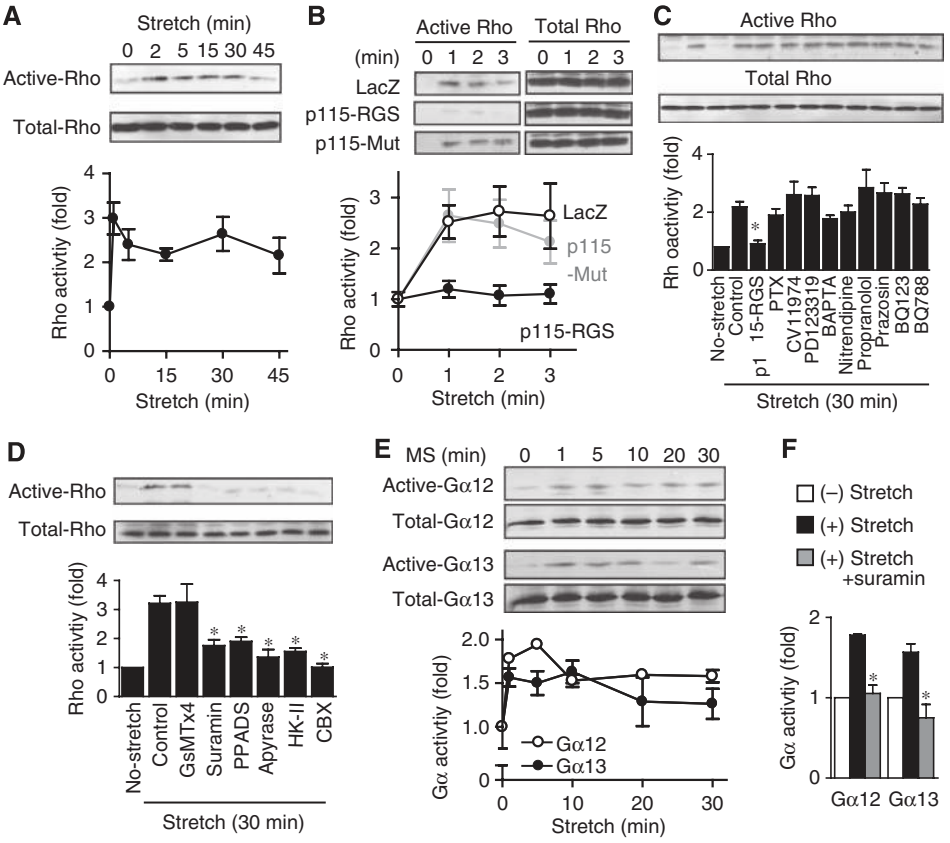

Figure 3 Mechanical stretch activates $G\alpha_{12/13}$ and Rho through purinergic receptor. (**A**) Time course of Rho activation by mechanical stretch. (**B**) $G\alpha_{12/13}$-mediated Rho activation by mechanical stretch. Cells were transfected with GFP, p115-RGS, and inactive mutant of p115-RGS (p115-Mut) by electroporation. (**C**, **D**) Effects of various inhibitors on Rho activation by mechanical stretch. Cells were treated with CV11974 (2.5 μM), PD123319 (2.5 μM), BAPTA-AM (3 μM), nitrendipine (1 μM), propranolol (1 μM), prazosin (10 μM), BQ123 (3 μM), BQ788 (3 μM), GsMTx4 (1 μM), apyrase (1 U/ml), hexokinase (HK)-II (100 μg/ml), suramin (100 μM), PPADS (100 μM), carbenoxolone (CBX; 20 μM), and *Pertussis* toxin (PTX; 100 ng/ml for 12 h) 5 min before mechanical stretch. (**E**) Time courses of $G\alpha_{12}$ and $G\alpha_{13}$ activation by mechanical stretch (MS). (**F**) Effects of suramin on $G\alpha_{12}$ and $G\alpha_{13}$ activation. Cells were pretreated with suramin (100 μM) 5 min before mechanical stretch. Error bars indicate s.e.m.; $n=4$ (A, D) and $n=3$ (B, C, E, F). * indicates $P<0.05$ versus control.

The function of all connexins as gap junction channels or hemichannels is strongly dependent on Ca^{2+}, but the function of pannexin-1 is independent of Ca^{2+} (Shestopalov and Panchin, 2008). As mechanical stretch-induced Rho activation was independent of Ca^{2+} (Figure 3C) and a low concentration of carbenoxolone (but not 1-heptanol) inhibited mechanical stretch-induced ATP release (Figure 4B), pannexin-1 appears to be a prime candidate for an ATP release channel. Pannexin-1 and pannexin-2 mRNAs, but not pannexin-3 mRNA, were expressed in mouse hearts and rat cardiomyocytes (Supplementary Figure 5). The expression of pannexin-1 mRNA was increased by pressure overload (Supplementary Figure 5A). Treatment with siRNAs for pannexin-1 induced a 50% decrease in pannexin-1 mRNA levels (Supplementary Figure 5B). The mechanical stretch-induced ATP release was decreased by about 50% in pannexin-1 siRNA-treated cardiomyocytes (Figure 4C). These results suggest that pannexin-1 mediates ATP release by mechanical stretch in rat cardiomyocytes.

Involvement of $P2Y_6$ receptor in mechanical stretch-induced fibrotic responses

We also examined which receptor subtype(s) is involved in mechanical stretch-induced $G\alpha_{12/13}$ activation. RT–PCR analysis showed that mouse hearts express mRNAs coding $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ receptors (Supplementary Figure 6). Among them, mRNA levels of $P2Y_2$ and $P2Y_6$ receptors were upregulated in TAC hearts and CA-$G\alpha_{13}$ hearts. We also found that neonatal cardiomyocytes express mRNAs coding $P2Y_1$, $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ receptors (data not shown). Treatment with MRS2578, a selective $P2Y_6$ receptor antagonist, suppressed mechanical stretch-induced Rho activation in a concentration-dependent manner, with an IC_{50} value of about 0.1 μM (Figure 5A and B). In contrast, treatment with MRS2179 (a $P2Y_1$ receptor antagonist), AR-C67719MX (a $P2Y_{12}$ receptor antagonist), and 8-SPT (an adenosine receptor antagonist) did not suppress mechanical stretch-induced Rho activation. As Rho is reported to regulate the expression levels of CTGF (Hahn *et al*, 2000) and periostin (Butcher *et al*, 2007), we examined the effects of P2Y receptor antagonists on the expression of these fibrogenic factors. Mechanical stretch increased expression of CTGF mRNA, which had been completely suppressed by the expression of p115-RGS, and by treatment with suramin, PPADS, and MRS2578 (Figure 5C and D). Mechanical stretch increased the expression of TGF-β2 mRNA but did not affect the expression of TGF-β1 and -β3 mRNAs, and the induction of TGF-β2 mRNA was also suppressed by suramin, PPADS, and MRS2578. In addition, mechanical stretch increased periostin proteins two-fold, which had been completely suppressed

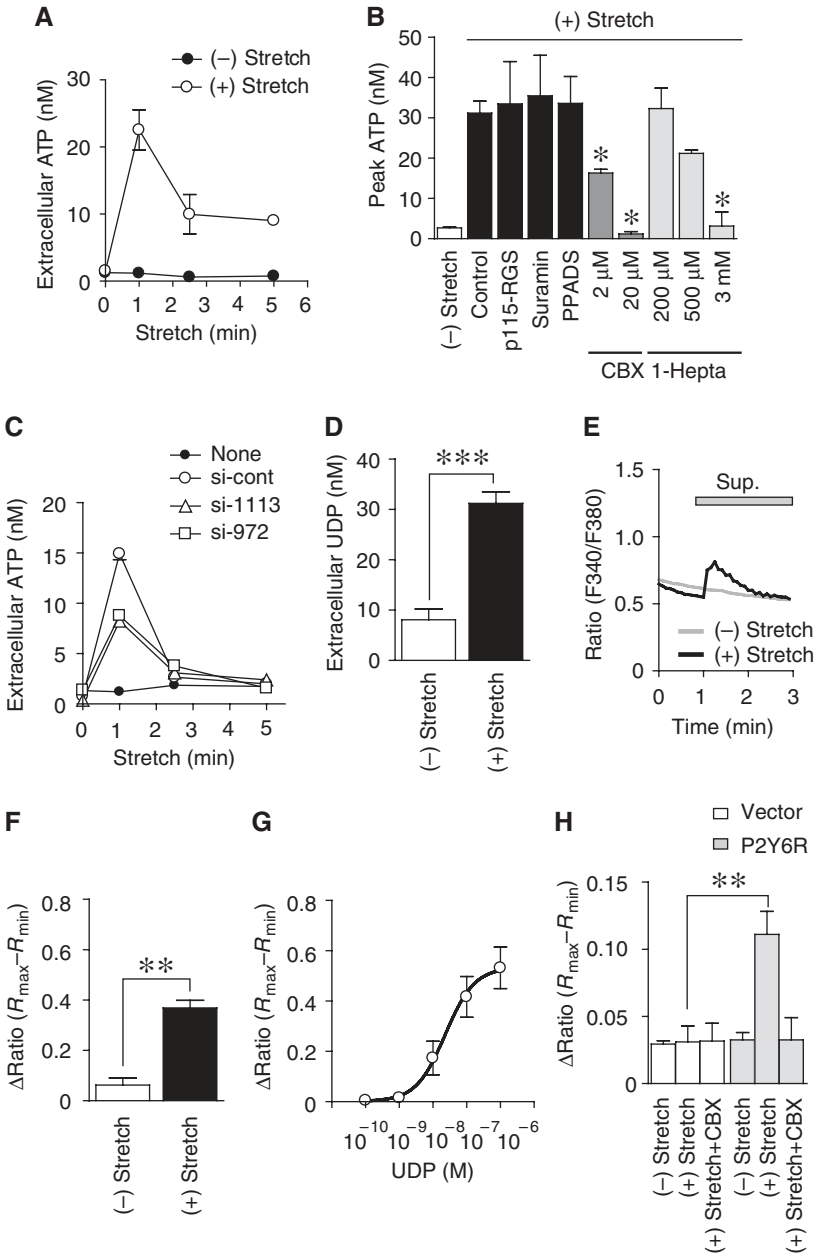

Figure 4 Pannexin 1 mediates mechanical stretch-induced release of nucleotides. (**A**) Time course of ATP release by mechanical stretch. Cells were treated with ARL67156 (50 μM) 20 min before mechanical stretch. (**B**) Effects of inhibitors on the increase in extracellular ATP concentration induced by mechanical stretch. Cells were treated with suramin (100 μM), PPADS (100 μM), carbenoxolone (CBX), and 1-heptanol (1-Hepta) 20 min before mechanical stretch. (**C**) Effects of pannexin-1 siRNAs on mechanical stretch-induced ATP release. (**D**) UDP concentration in the culture medium from control cardiomyocytes ((−) stretch) or mechanically stretched cells for 10 min ((+) stretch). (**E**, **F**) Time courses of changes (E) and peak increases (F) in $[Ca^{2+}]_i$ of $P2Y_6$ receptor-expressing HEK293 cells. The changes in $[Ca^{2+}]_i$ were determined after the substitution of external solution with the culture medium (Sup.) of mechanically stretched cells. (**G**) Concentration-dependent maximal increases in $[Ca^{2+}]_i$ induced by UDP in $P2Y_6$ receptor-expressing HEK293 cells. (**H**) Peak increases in $[Ca^{2+}]_i$ of control (vector) and $P2Y_6$ receptor-expressing H9c2 myoblasts. The changes in $[Ca^{2+}]_i$ were determined after the addition of the culture medium from control cardiomyocytes ((−) stretch) or mechanically stretched cells ((+) stretch) for 10 min with or without 2 μM of carbenoxolone (+CBX). Error bars indicate s.e.m.; $n=4$ (A) and $n=3$ (B–H). * indicates $P<0.05$, ** indicates $P<0.01$ and *** indicates $P<0.001$.

by the expression of p115-RGS, and by treatment with suramin, PPADS, and MRS2578 (Figure 5E). These increases were not affected by the expression of G protein-coupled receptor kinase 2–RGS, a $G\alpha_q$-specific RGS domain (Nishida *et al*, 2005; Onohara *et al*, 2006), nor by the treatment with $P2Y_1$ receptor antagonist (MRS2179), $P2Y_{12}$ receptor antagonist (AR-C67719MX), and 8-SPT (an adenosine receptor antagonist). As $P2Y_2$ receptor-selective antagonist is not commercially available, we examined the involvement of the $P2Y_2$ receptor in mechanical stretch-induced Rho activation with siRNAs. The treatment with $P2Y_2$-specific siRNAs decreased the mRNA by about 50% but did not suppress Rho activity (Figure 5F). In contrast, the treatment with $P2Y_6$-specific siRNAs decreased the mRNA by about 70% and significantly suppressed mechanical stretch-induced Rho activation in cardiomyocytes. These results suggest

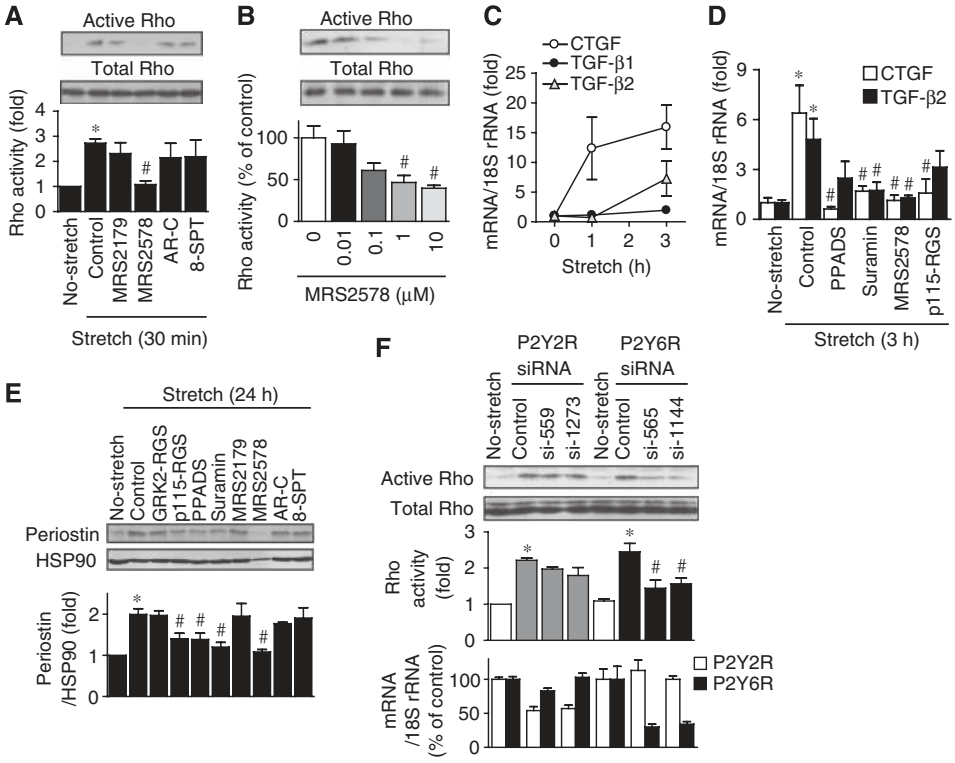

Figure 5 Involvement of $P2Y_6$ receptor in mechanical stretch-induced Rho activation and expression of fibrogenic factors. (**A**) Effects of MRS2179 ($P2Y_1$ receptor antagonist, 10 μM), MRS2578 ($P2Y_6$ receptor antagonist, 10 μM), AR-C69931MX ($P2Y_{12}$ receptor antagonist, AR-C; 1 μM), and 8-(p-sulphophenyl) theophylline (8-SPT; adenosine receptor antagonist, 10 μM) on Rho activation. (**B**) Concentration-dependent inhibition of Rho activation by MRS2578. (**C**) Time courses of changes in CTGF and TGF-β1 and -β2 mRNA expressions induced by mechanical stretch. (**D**) Effects of inhibitors on the expression of CTGF and TGF-β2 mRNAs induced by mechanical stretch for 3 h. (**E**) Effects of various inhibitors on the expression of periostin induced by mechanical stretch for 24 h. The expression was plotted by the ratio to HSP90. (**F**) Effects of siRNAs of $P2Y_2$ and $P2Y_6$ receptors on mechanical stretch-induced Rho activation. Lower graph shows the expression levels of $P2Y_2$ and $P2Y_6$ receptor mRNAs. Error bars indicate s.e.m.; $n=3$ (A, B, D, E), $n=5$ (C) and $n=6$ (F). * indicates $P<0.05$ versus no-stretch and # indicates $P<0.05$ versus control.

that the $P2Y_6$ receptor predominantly regulates the mechanical stretch-induced activation of fibrotic signalling in cardiomyocytes.

As the $P2Y_6$ receptor is mainly activated by UDP (Vassort, 2001), and uridine nucleotides are known to be released by mechanical stretch (Lazarowski and Boucher, 2001), we also examined whether UDP is released by mechanical stretch. Mechanical stretch of cardiomyocytes increased extracellular UDP concentration three-fold (Figure 4D). In addition, treatment of $P2Y_6$ receptor-overexpressing HEK293 cells with supernatant from mechanically stretched rat cardiomyocytes significantly increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (Figure 4E). The magnitude of maximal increase in $[Ca^{2+}]_i$ induced by the supernatant was equivalent to the peak $[Ca^{2+}]_i$ increase induced by 30 nM of extracellular UDP (Figure 4F and G). As H9c2 myofibroblasts do not express $P2Y_1$ and $P2Y_2$ receptors, we further examined the effects of nucleotides on $[Ca^{2+}]_i$ increase using H9c2 cells. Treatment of vector-expressing H9c2 cells with UDP, ATP, or the supernatant of stretch-activated cardiomyocytes did not show any significant increases in $[Ca^{2+}]_i$, but the treatment with the supernatant significantly increased $[Ca^{2+}]_i$ in $P2Y_6$ receptor-overexpressing H9c2 cells (Figure 4H). This $[Ca^{2+}]_i$ increase was completely suppressed by the treatment of cardiomyocytes with carbenoxolone, suggesting that pannexin-1 mediates mechanical stretch-induced UDP release. Furthermore, treatment of cardiomyocytes with 3-phenacyl-UDP, a highly selective $P2Y_6$ receptor agonist, increased Rho activity in a concentration-dependent manner (Supplementary Figure 4H). These results suggest that extracellular UDP predominantly mediates mechanical stretch-induced $P2Y_6$ receptor activation in cardiomyocytes.

Inhibition of $P2Y_6$ receptors attenuates pressure overload-induced cardiac fibrosis in vivo

We next examined whether purinergic receptors actually participate in pressure overload-induced cardiac fibrosis *in vivo*. Treatment with MRS2578 after TAC significantly suppressed pressure overload-induced collagen deposition without affecting cardiomyocyte hypertrophy (Figure 6A–C). Treatment with MRS2578 significantly suppressed LV dysfunction induced by pressure overload (Figure 6D and E and Supplementary Table 3). Furthermore, the treatment with MRS2578 suppressed the increases in mRNA expressions of ANP, β-MHC, procollagen type I, periostin, and TGF-β2 by pressure overload (Figure 6F). We also found that MRS2578 inhibited pressure overload-induced Rho activation and TAC-induced increases in expression of periostin, mature TGF-βs, and ACE proteins (Figure 6G and H). Furthermore, we found that treatment with suramin also suppressed pressure overload-induced collagen deposition and LV dysfunction (Supplementary Figure 7 and Supplementary Table 4). These results suggest that inhibition of $P2Y_6$ receptors

actually attenuates pressure overload-induced cardiac fibrosis and LV dysfunction.

Discussion

Remodelling of the heart, including accumulation of ECM and an associated change in ventricular geometry, is a common feature of heart failure. In this study, we found that $G\alpha_{12/13}$ mediate cardiac fibrosis without the development of hypertrophy induced by pressure overload. We reported previously that $G\alpha_{12/13}$ mediate agonist-induced hypertrophic responses of cardiomyocytes. However, we also found that mechanical stretch-induced increases in NFAT- and BNP-dependent transcriptional activities are not suppressed in p115-RGS-expressing myocytes (Supplementary Figure 8). These results suggest that activation of $G\alpha_{12/13}$ is not involved in mechanical stress-induced NFAT and BNP expression. $G\alpha_{12/13}$ are activated by extracellular ATP and UDP that are released by mechanical stretch. The nucleotides released through pannexin-1 hemichannels activate $G\alpha_{12/13}$-mediated Rho

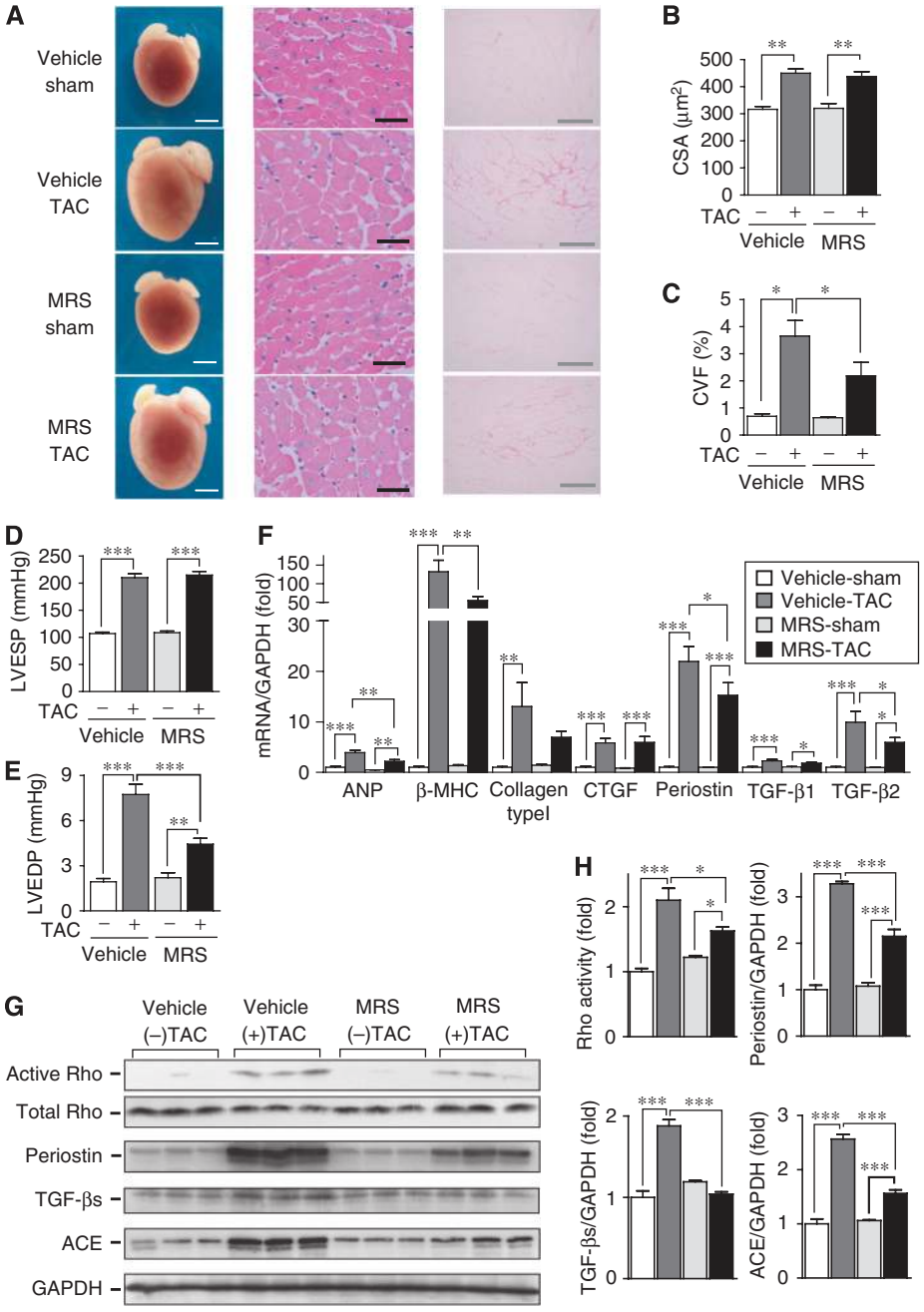

Figure 6 Involvement of $P2Y_6$ receptors in pressure overload-induced cardiac fibrosis. Effects of MRS2578 (3 mg/kg/day, started from 3 days after TAC surgery) on TAC-induced fibrosis and hypertrophy (**A**–**C**), and LV functions (**D**, **E**). (A) Hearts (left; scale bar, 2 mm), H&E staining (center; scale bar, 30 μm), and picrosirius red staining (right; scale bar, 200 μm). (B) CSA of cardiomyocytes. (C) Results of cardiac fibrosis. (**F**) Expression of hypertrophic and fibrogenic genes. (**G**) Effects of MRS2678 on TAC-induced Rho activation and the expression of periostin, mature TGF-βs, ACE, and GAPDH. GAPDH was used as an internal control. Error bars indicate s.e.m.; $n=3$–8 (B, C), $n=8$–12 (D, E), $n=5$–7 (F), and $n=3$ (G, **H**). Representative result of hearts from vehicle-sham ($n=3$), vehicle-TAC ($n=7$), and MRS-sham ($n=3$), and MRS-TAC mice ($n=8$) was shown (A). * indicates $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$.

activation leading to the induction of fibrogenic factors, such as CTGF and periostin. Furthermore, inhibition of purinergic receptors attenuates the TAC-induced cardiac fibrosis and LV dysfunction. These results indicate that activation of $G_{12/13}$-coupled purinergic receptors in cardiomyocytes by extracellular nucleotides stimulate the secretion of fibrogenic factors and trigger pressure overload-induced cardiac fibrosis (Figure 7). Purinergic receptors are classified into two families: P2X and P2Y. P2X receptors are transmitter-gated channels and consist of 7 subtypes. P2Y receptors are G protein-coupled receptors and are divided into eight subtypes. We found that the $P2Y_6$ receptor predominantly regulates mechanical stretch-induced Rho activation and the expression of fibrogenic factors in rat cardiac myocytes (Figure 5). We also found that inhibition of $P2Y_6$ receptors suppressed cardiac fibrosis and diastolic dysfunction induced by pressure overload (Figure 6). These results suggest that $P2Y_6$ receptors in cardiomyocytes have an important function in pressure overload-induced cardiac fibrosis.

It has been reported that CTGF has an important function in cardiac fibrosis. In contrast to CTGF, the function of periostin remains to be determined. Extracellular application of periostin induced re-entry of cardiomyocytes into the cell cycle, and reduced fibrosis whereas improving cardiac functions (Kühn *et al*, 2007). However, analysis of knockout and transgenic mice reveals that periostin is involved in myocardial infarction-induced fibrosis and impairment of ventricular functions (Oka *et al*, 2007; Shimazaki *et al*, 2008). They also demonstrated that pressure overload-induced hypertrophic responses and fibrosis are regulated by periostin. The present results are consistent with the findings that periostin is involved in pressure-overload-induced cardiac fibrosis.

It is interesting to note that the $G_{12/13}$-mediated pathway regulates fibrosis, and the $G_{q/11}$-mediated pathway regulates hypertrophy. Two different G proteins regulate two distinct responses: fibrosis and hypertrophy. Many groups using transgenic and knockout mice have reported that suppression of hypertrophy leads to the inhibition of fibrosis. However, we demonstrated that fibrosis and hypertrophy are independent processes, as revealed by expressing p115-RGS to block $G\alpha_{12/13}$ functions. Therefore, $G\alpha_{12/13}$-mediated signalling leading to cardiac fibrosis may turn on after hypertrophy is already developed. This speculation is supported by the finding that pannexin-1 mRNA in the heart is upregulated by pressure overload (Supplementary Figure 5A). Thus, the process of the hypertrophied heart depositing ECM proteins *in vivo* may be triggered by the release of ATP and UDP from myocytes during transition from hypertrophy to heart failure.

There are three structurally distinct TGF-βs (Bujak and Frangogiannis, 2007). TGF-β1 is a prevalent isoform, and TGF-β2 and -β3 are expressed in limited tissues. As these three isoforms do not compensate for functions of other isoforms, each TGF-β has specific and independent roles *in vivo*. Among these three isoforms, it has been reported that TGF-β1 mediates Ang II-induced hypertrophic responses *in vivo* (Schultz Jel *et al*, 2002). Myocardial infarction increases the expression of these three TGF-β isoforms, which participate in inflammation at an early phase and cardiac remodelling at a later phase. We found that TGF-β2 mRNA was most responsive to TAC, which induces cardiac fibrosis (Figure 1). We also demonstrated that p115-RGS and the $P2Y_6$ receptor antagonist inhibit the expression of TGF-β2 mRNA by pressure overload (Figure 2 and Figure 6). TGF-β2 may be the predominant form of TGF-β for the promotion of fibrosis in the heart.

Our results also indicate that $G\alpha_{13}$ mediates pressure overload-induced expression of ACE proteins (Figure 1 and Supplementary Figure 3). Although inhibition of ACE expression has been reported to inhibit pressure overload-induced cardiac hypertrophy in rats (Baker *et al*, 1990; Zierhut *et al*, 1991), inhibition of $G\alpha_{12/13}$, upstream of ACE, did not suppress cardiac hypertrophy in mice (Figure 1). We do not have any data to explain this discrepancy. However, our data are consistent with the results of Xiao *et al*. (2008), which show that an increase in ACE expression does not augment pressure overload-induced cardiac hypertrophy in mice. In addition, pressure overload induces cardiac hypertrophy in angiotensinogen-knockout mice (Zou *et al*, 2004). Crowley *et al* (2006) have reported that Ang II induces cardiac hypertrophy in mice through stimulation of AT1 receptors in the kidney. It has been reported that the expression of a gain-of-function mutant of Ang II type 1A receptor in the

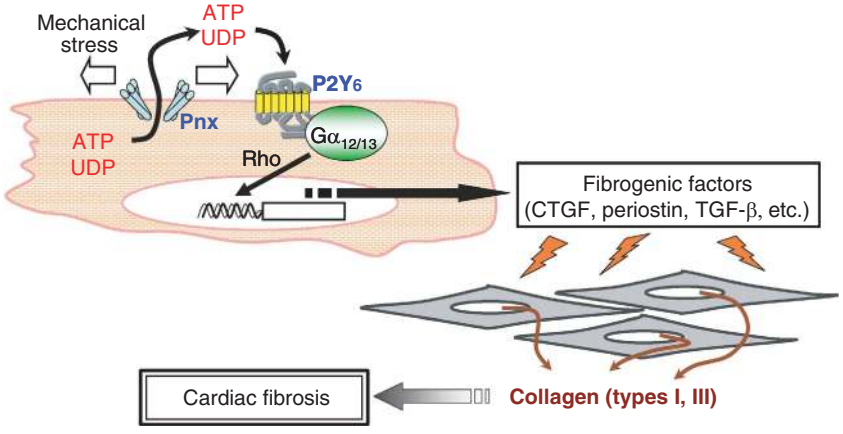

Figure 7 Schema for the mechanism of cardiac fibrosis induced by pressure overload. Mechanical stretch induces release of nucleotides through pannexin-1 hemichannels (Pnx) from cardiac myocytes, which leads to stimulation of purinergic $P2Y_6$ receptors. Nucleotide-bound $P2Y_6$ receptor activates $G_{12/13}$ proteins. Activation of $G\alpha_{12/13}$ in cardiomyocytes induces the expression of fibrogenic factors, which activate cardiac fibroblasts in a paracrine manner. The activated fibroblasts produce excessive amount of collagen types I and III, leading to induction of cardiac fibrosis.

heart causes cardiac fibrosis but not hypertrophy (Billet *et al*, 2007). Thus, increase in cardiac ACE activity induced by pressure overload may not contribute to the development of cardiac hypertrophy in mice. Using rat cardiomyocytes, we found that mechanical stretch-induced activation of JNK and p38 MAPK, but not ERK, was suppressed by p115-RGS (Supplementary Figure 8). As ERK, but not JNK and p38 MAPK, participates in cardiac hypertrophy (Liang and Molkentin, 2003), $G\alpha_{12/13}$-mediated ACE expression may participate in mechanical stress (pressure overload)-induced hypertrophy in rats but not mice.

Cardiac fibrosis is considered one of the inflammatory responses of the heart (Brown *et al*, 2005). A variety of evidence supports the idea that extracellular nucleotides function as a mediator of inflammatory responses, such as chemotaxis and phagocytosis (Chen *et al*, 2006; Idzko *et al*, 2007; Koizumi *et al*, 2007). Our data suggest that extracellular nucleotides function as a priming factor in the development of cardiac fibrosis induced by pressure overload. It is generally thought that activation of the RAAS system and increased levels of active TGF-β stimulate cardiac fibroblasts and induce ECM deposition, leading to perivascular fibrosis. CTGF, periostin, and TGF-β2 mRNAs were upregulated by pressure overload, and the increased expression of three genes were suppressed in p115-Tg mice. Furthermore, a $P2Y_6$ receptor antagonist MRS2578 suppressed the stress-induced expression of periostin and TGF-β mRNAs *in vitro* and *in vivo*. In addition to TGF-β, we demonstrate that CTGF and periostin are also involved in pressure overload-induced cardiac fibrosis. As $G\alpha_{12/13}$ mediate cardiac fibrosis, which is associated with pressure overload-induced hypertrophy, the development of drugs to block $P2Y_6$ receptors-$G\alpha_{12/13}$ signalling may be a novel strategy for heart failure.

The interrelationship between Ang II and TGF-β is well established. The blockade of TGF-β by an antibody and a mutated TGF-receptor suppressed some of Ang II-induced responses (Bujak and Frangogiannis, 2007). Therefore, it is reasonable to assume that Ang II stimulates TGF-β expression, which leads to ECM deposition. As the blockade of the $P2Y_6$ receptor with MRS2578 suppressed the expression of ACE mRNA, and the blockade of $G\alpha_{12/13}$ suppressed the expression of TGF-β mRNAs, ATP and UDP work as an upstream regulator of the Ang II-TGF-β system. This also suggests that extracellular nucleotide-stimulated $G\alpha_{12/13}$ activity regulates the Ang II-TGF-β pathway through upregulation of ACE.

It has been reported that CTGF mediates some TGF-β-induced fibrogenic responses. Inhibition of CTGF synthesis or activity suppressed TGF-β-induced collagen synthesis (Perbal, 2004). It is also reported that CTGF synergizes fibrogenic responses with TGF-β by the mechanisms on the basis of the binding of CTGF to TGF-β or transcriptional suppression of Smad7 (Ruiz-Ortega *et al*, 2007). As CTGF expression was increased in a CA-$G\alpha_{13}$-Tg heart without affecting TGF-β expression, extracellular ATP and UDP may directly increase the expression of CTGF through $P2Y_6$ and $G\alpha_{12/13}$, with CTGF then promoting the production of TGF-β. Thus, extracellular nucleotides have an important function in fibrogenic responses of the heart.

Diastolic dysfunction associated with preserved systolic function is increasingly recognized as a critical cause of heart failure. As the cardiac ECM is the major determinant of myocardial stiffness during diastole, cardiac fibrosis contributes to diastolic dysfunction. We found that 6 weeks of TAC induces impairment of LV diastolic functions, which were attenuated by the inhibition of $G\alpha_{12/13}$ signalling or purinergic $P2Y_6$ receptors. As cardiac fibrosis associated with maladaptive hypertrophy is thought as a cause of impairment of cardiac function, purinergic receptors may be promising targets for the treatment of heart failure.

Materials and methods

Animals and TAC surgery

All protocols using mice and rats conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the guidelines of Kyushu University. Transgenic C57BL/6J mice expressing p115-RGS were tried to generate three times. We obtained only one line that was used in this study. Two lines of transgenic mice expressing CA-$G\alpha_{13}$ were generated (lines 1 and 5). Heterozygote of line 5 was used in this study. Age-matched male WT C57BL/6J mice were used as control. TAC surgery was performed on 8- to 10-week-old male p115-Tg and WT C57BL/6J mice. A mini-osmotic pump (Alzet) filled with saline, MRS2578, or suramin was implanted intraperitoneally 3 days after TAC into 6-week-old male C57BL/6J mice. Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Haemodynamic measurements and histological analyses

Transthoratic echocardiography was performed using ALOKA ultrasonic image analysing system (SSD-5500) equipped with 7.5 MHz imaging transducer. Blood pressure was monitored using tail-cuff system (BP-98A, Softron). LV pressure and heart rate were measured with a micronanometer catheter (Millar 1.4F, SPR 671, Millar Instruments). Histological analyses can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Isolation of cardiomyocytes and transfection

Cultures of neonatal rat cardiac myocytes and adenoviral infection were performed as described previously (Nishida *et al*, 2000). Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Pulldown assay and western blot analysis

Methods for pulldown assay and western blot analysis can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Measurement of extracellular nucleotides concentration

The determination of extracellular ATP concentration (2×10^5 cells per well) was performed using ATP Bioluminescence Assay Kit CLSII (Roche). The concentration of extracellular UDP in the supernatant of culture medium was analysed with an HPLC system (Jasco) as described previously (Koizumi *et al*, 2007). Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Measurement of mRNA expressions

Real-time RT–PCR was performed as described (Nagamatsu *et al*, 2006; Nishida *et al*, 2007). Sequences for PCR primers and Taqman probes were described in Supplementary information (Supplementary Table 5). The PCR primers used for expression analysis of P2Y receptors are described in Supplementary Table 6. Details can be found in Supplementary methods at *The EMBO Journal* Online (http://embojournal.org).

Statistical analysis

Data were shown as means ± s.e.m. Statistical comparisons were made with two-tailed Student's *t*-test or analysis of variance followed by Student–Newman–Keuls procedure, with significance imparted at *P*-values <0.05.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Naoki Nishihara for TAC surgery and measurement of cardiac functions during the early stage of this study. We also thank Drs Makoto Tsuda (Kyushu University) and Isao Matsuoka (Takasaki University of Health and Welfare) for helpful discussion and Dr Jeffery Robbins (Cincinnati Children's Hospital Medical Center) for α-MHC promoter. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to MN and HK); from the Ministry of Health, Labour, and Welfare of Japan and the Japan Health Sciences Foundation (to YS); and from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (to MN).

References

Adams JW, Sakata Y, Davis MG, Sah VP, Wang Y, Liggett SB, Chien KR, Brown JH, Dorn II GW (1998) Enhanced Gαq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc Natl Acad Sci USA* **95:** 10140–10145

Amano M, Fukata Y, Kaibuchi K (2000) Regulation and functions of Rho-associated kinase. *Exp Cell Res* **261:** 44–51

Arai K, Maruyama Y, Nishida M, Tanabe S, Kozasa T, Mori Y, Nagao T, Kurose H (2003) Differential requirement of $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, and $G\beta\gamma$ for endothelin-1-induced c-Jun NH_2-terminal kinase and extracellular signal-regulated kinase activation. *Mol Pharmacol* **63:** 478–488

Baker KM, Chernin MI, Wixson SK, Aceto JF (1990) Renin–angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am J Physiol* **259:** H324–H332

Berk BC, Fujiwara K, Lehoux S (2007) ECM remodeling in hypertensive heart disease. *J Clin Invest* **117:** 568–575

Bhattacharyya R, Wedegaertner PB (2003) Mutation of an N-terminal acidic-rich region of p115-RhoGEF dissociates α_{13} binding and α_{13}-promoted plasma membrane recruitment. *FEBS Lett* **540:** 211–216

Billet S, Bardin S, Verp S, Baudrie V, Michaud A, Conchon S, Muffat-Joly M, Escoubet B, Souil E, Hamard G, Bernstein KE, Gasc JM, Elghozi J-L, Corvol P, Clauser E (2007) Gain-of-function mutant of angiotensin II receptor, type 1A, causes hypertension and cardiovascular fibrosis in mice. *J Clin Invest* **117:** 1914–1925

Brown RD, Ambler SK, Mitchell MD, Long CS (2005) The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. *Annu Rev Pharmacol Toxicol* **45:** 657–687

Bujak M, Frangogiannis NG (2007) The role of TGF-β signaling in myocardial infarction and cardiac remodeling. *Cardiovasc Res* **74:** 184–195

Butcher JT, Norris RA, Hoffman S, Mjaatvedt CH, Markwald RR (2007) Periostin promotes atrioventricular mesenchyme matrix invasion and remodeling mediated by integrin signaling through Rho/PI 3-kinase. *Dev Biol* **302:** 256–266

Burnstock G (1972) Purinergic nerves. *Pharmacol Rev* **24:** 509–581

Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG (2006) ATP release guides neutrophil chemotaxis via $P2Y_2$ and A3 receptors. *Science* **314:** 1792–1795

Christensen AP, Corey DP (2007) TRP channels in mechanosensation: direct or indirect activation? *Nat Rev Neurosci* **8:** 510–521

Cohn JN, Ferrari R, Sharpe N (2000) Cardiac remodeling-concepts and clinical implications: a consensus paper from an international forum on cardiac remodeling. Behalf of an International Forum on Cardiac Remodeling. *J Am Coll Cardiol* **35:** 569–582

Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM (2006) Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc Natl Acad Sci USA* **103:** 17985–17990

Gohla A, Schultz G, Offermanns S (2000) Role for G_{12}/G_{13} in agonist-induced vascular smooth muscle cell contraction. *Circ Res* **87:** 221–227

Hahn A, Heusinger-Ribeiro J, Lanz T, Zenkel S, Goppelt-Struebe M (2000) Induction of connective tissue growth factor by activation of heptathelical receptors. Modulation by rho proteins and the actin cytoskeleton. *J Biol Chem* **275:** 37249–37435

Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MA, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow Jr JC, Lambrecht BN (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* **13:** 913–919

Katsuragi N, Morishita R, Nakamura N, Ochiai T, Taniyama Y, Hasegawa Y, Kawashima K, Kaneda Y, Ogihara T, Sugimura K (2004) Periostin as a novel factor responsible for ventricular dilation. *Circulation* **110:** 1806–1813

Koizumi S, Shigemoto-Mogami Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, Joshi BV, Jacobson KA, Kohsaka S, Inoue K (2007) UDP acting at $P2Y_6$ receptors is a mediator of microglial phagocytosis. *Nature* **446:** 1091–1095

Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, Sternweis PC (1998) p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science* **280:** 2109–2111

Kühn B, del Monte F, Hajjar RJ, Chang YS, Lebeche D, Arab S, Keating MT (2007) Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. *Nat Med* **13:** 962–969

Lazarowski ER, Boucher RC (2001) UTP as an extracellular signaling molecule. *News Physiol Sci* **16:** 1–5

Liang Q, Molkentin JD (2003) Redefining the roles of p38 and JNK signaling in cardiac hypertrophy: dichotomy between cultured myocytes and animal models. *J Mol Cell Cardiol* **35:** 1385–1394

Maruyama Y, Nishida M, Sugimoto Y, Tanabe S, Turner JH, Kozasa T, Wada T, Nagao T, Kurose H (2002) $G\alpha_{12/13}$ mediates α_1-adrenergic receptor-induced cardiac hypertrophy. *Circ Res* **91:** 961–969

Nagamatsu Y, Nishida M, Onohara N, Fukutomi M, Maruyama Y, Kobayashi H, Sato Y, Kurose H (2006) Heterotrimeric G protein $G\alpha_{13}$-induced induction of cytokine mRNAs through two distinct pathways in cardiac fibroblasts. *J Pharmacol Sci* **101:** 144–150

Nishida M, Maruyama Y, Tanaka R, Kontani K, Nagao T, Kurose H (2000) $G\alpha_i$ and $G\alpha_o$ are target proteins of reactive oxygen species. *Nature* **408:** 492–495

Nishida M, Tanabe S, Maruyama Y, Mangmool S, Urayama K, Nagamatsu Y, Takagahara S, Turner JH, Kozasa T, Kobayashi H, Sato Y, Kawanishi T, Inoue R, Nagao T, Kurose H (2005) $G\alpha_{12/13}$- and reactive oxygen species-dependent activation of c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase by angiotensin receptor stimulation in rat neonatal cardiomyocytes. *J Biol Chem* **280:** 18434–18441

Nishida M, Onohara N, Sato Y, Suda R, Ogushi M, Tanabe S, Inoue R, Mori Y, Kurose H (2007) $G\alpha_{12/13}$-mediated up-regulation of TRPC6 negatively regulates endothelin-1-induced cardiac myofibroblast formation and collagen synthesis through nuclear factor of activated T cells activation. *J Biol Chem* **282:** 23117–23128

Offermanns S, Mancino V, Revel JP, Simon MI (1997) Vascular system defects and impaired cell chemokinesis as a result of $G\alpha_{13}$ deficiency. *Science* **275:** 533–536

Oka T, Xu J, Kaiser RA, Melendez J, Hambleton M, Sargent MA, Lorts A, Brunskill EW, Dorn II GW, Conway SJ, Aronow BJ, Robbins J, Molkentin JD (2007) Genetic manipulation of periostin expression reveals a role in cardiac hypertrophy and ventricular remodeling. *Circ Res* **101:** 313–321

Onohara N, Nishida M, Inoue R, Kobayashi H, Sumimoto H, Sato Y, Mori Y, Nagao T, Kurose H (2006) TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *EMBO J* **25:** 5305–5316

Perbal B (2004) CCN proteins: multifunctional signalling regulators. *Lancet* **36:** 62–64

Rikitake Y, Oyama N, Wang CY, Noma K, Satoh M, Kim HH, Liao JK (2005) Decreased perivascular fibrosis but not cardiac hypertrophy in $ROCK1^{+/-}$ haploinsufficient mice. *Circulation* **112:** 2959–2965

Rosenkranz S (2004) TGF-β1 and angiotensin networking in cardiac remodeling. *Cardiovas Res* **63:** 423–432

Ruiz-Ortega M, Rodríguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J (2007) TGF-β signaling in vascular fibrosis. *Cardiovasc Res* **74:** 196–206

Sadoshima J, Izumo S (1997) The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu Rev Physiol* **59:** 551–571

Sawada Y, Tamada M, Dubin-Thaler BJ, Cherniavskaya O, Sakai R, Tanaka S, Sheetz MP (2006) Force sensing by mechanical extension of Src family kinase substrate p130Cas. *Cell* **127:** 1015–1026

Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR, Doetschman T (2002) TGF-β1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. *J Clin Invest* **109:** 787–796

Shestopalov VI, Panchin Y (2008) Pannexins and gap junction protein diversity. *Cell Mol Life Sci* **65:** 376–394

Shimazaki M, Nakamura K, Kii I, Kashima T, Amizuka N, Li M, Saito M, Fukuda K, Nishiyama T, Kitajima S, Saga Y, Fukayama M, Sata M, Kudo A (2008) Periostin is essential for cardiac healing after acute myocardial infarction. *J Exp Med* **205:** 295–303

Simon MI, Strathmann MP, Gautam N (1991) Diversity of G proteins in signal transduction. *Science* **252:** 802–808

Suadicani SO, Vink MJ, Spray DC (2000) Slow intercellular Ca^{2+} signaling in wild-type and Cx43-null neonatal mouse cardiac myocytes. *Am J Physiol* **279:** H3076–H3088

Vassort G (2001) Adenosine 5′-triphosphate: a P2-purinergic agonist in the myocardium. *Physiol Rev* **81:** 767–806

Wettschureck N, Rütten H, Zywietz A, Gehring D, Wilkie TM, Chen J, Chien KR, Offermanns S (2001) Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of $G\alpha_q/G\alpha_{11}$ in cardiomyocytes. *Nat Med* **7:** 1236–1240

Xiao HD, Fuchs S, Bernstein EA, Li P, Campbell DJ, Bernstein KE (2008) Mice expressing ACE only in the heart show that increased cardiac angiotensin II is not associated with cardiac hypertrophy. *Am J Physiol* **294:** H659–H667

Xu Z, Okamoto H, Akino M, Onozuka H, Matsui Y, Tsutsui H (2008) Pravastatin attenuates left ventricular remodeling and diastolic dysfunction in angiotensin II-induced hypertensive mice. *J Cardiovas Pharmacol* **51:** 61–70

Zhang D, Gaussin V, Taffet GE, Belaguli NS, Yamada M, Schwartz RJ, Michael LH, Overbeek PA, Schneider MD (2000) TAK1 is activated in the myocardium after pressure overload and is sufficient to provoke heart failure in transgenic mice. *Nat Med* **6:** 556–563

Zhang YM, Bo J, Taffet GE, Chang J, Shi J, Reddy AK, Michael LH, Schneider MD, Entman ML, Schwartz RJ, Wei L (2006) Targeted deletion of ROCK1 protects the heart against pressure overload by inhibiting reactive fibrosis. *FASEB J* **20:** 916–925

Zierhut W, Zimmer H-G, Gerdes AH (1991) Effect of angiotensin converting enzyme inhibition on pressure overload-induced left ventricular hypertrophy in rats. *Circ Res* **69:** 609–617

Zou Y, Akazawa H, Qin Y, Sano M, Takano H, Minamino T, Makita N, Iwanaga K, Zhu W, Kudoh S, Toko H, Tamura K, Kihara M, Nagai T, Fukamizu A, Umemura S, Iiri T, Fujita T, Komuro I (2004) Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II. *Nat Cell Biol* **6:** 499–506