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H. 知的財産権の出願・登録状況（予定を含む。）

１．特許取得

１）「心筋形成能のある成体骨髄由来細胞」国内（第372826号、平成11年12月28日）

「心筋細胞への分化能を有する成体骨髄由来細胞」国際（PCT/JP00/001148、平成13年2月28日）

「心筋細胞への分化能を有する骨髄由来細胞」国際（PCT/JP00/07741、平成13年11月2日）

「心筋細胞への分化能を有する細胞」国際（PCT/JP00/09323、平成13年12月27日）

出願人：協和醗酵株式会社

２）「骨の再生方法」

発明者：梅澤明弘、秦順一、立石哲也、牛田多加志、陳国平

出願日：第251365号、平成13年8月22日

出願人：梅澤明弘、牛田多加志、独立行政法人産業技術総合研究所

３）「間葉系細胞から膵β細胞を形成する方法」

発明者：梅澤明弘、伊澤良兼

出願日：平成14年4月17日

出願番号　特願2002-115201、

出願人：大塚製薬株式会社

２．実用新案登録

なし

３．その他

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
分担研究報告書

ヒト幹細胞の完全ヒト型培養システムの開発と臨床材料の提供

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研究要旨
本研究ではヒト骨髄由来間葉系幹細胞の完全ヒト型培養法の開発を目指した。すなわち、動物由来培養成分を完全に除去した培地を調整しヒト幹細胞の安全性を確保することを目的とした。本年度は、添加血清低濃度化と各種の液性因子の添加という両者の条件を検定するために、遺伝子導入によって寿命延長させたヒト幹細胞を樹立し、増殖に重要な液性因子を同定した。また、完全無血清化により細胞接着性の低下等の特徴変化が見られたが、微量の血清添加で制御可能であることを明らかにした。

A. 研究目的

間葉系幹細胞は心筋、骨、軟骨、脂肪など様々な組織に分化することが明らかになっており、再生医療への利用が注目されている。再生医療に用いるためには生体から分離した細胞を体外で拡大培養させることで大量の細胞量を確保する必要が有る。そのためには安全且つ迅速に大量の細胞を得る培養方法を確立しなければならない。細胞培養には一般に牛血清を含む培地が用いられるが、安全確保のためには感染リスクのある牛血清の使用は極力回避する必要がある。これまで、表皮角化細胞など種々の細胞について無血清培養化は成功しているが、間葉系幹細胞においては臨床応用に対応しうる無血清培地は確立していない。ただし、無血清化の検討の為には増殖性評価のスクリーニングに耐えうる安定した形質を有する細胞が必要である。これまでの研究からヒト間葉系幹細胞に TERT、E6、E7 を遺伝子導入することにより細胞寿命を延長し、形質も安定した細胞を得ることに成功している。そこで、本研究では臨床応用可能な安全性の高い培地の作製を目指し、寿命延長化した間葉系幹細胞を用い細胞増殖性を指標に血清代替組成のスクリーニングを行い、培地の無血清化を試みた。

B. 研究方法

1) ヒト間葉系細胞の寿命延長

ヒト骨髄間葉系細胞を限外希釈法でサブクローニングをして得られた細胞に、レトロウィルスを用いて TERT、E6、E7 を遺伝子導入した。これにより複数のヒト寿命延長骨髄間葉系幹細胞クローンを得た。

2) 増殖性試験

間葉系幹細胞を 24 穴プレートに播種し、

各組成を添加した後、４～５日培養後のWST-1 による細胞量並びに顕微鏡観察による細胞形態を評価した。骨髄より細胞を採取し、至適化した培地と従来培地（１０％血清含有培地）でそれぞれ継代培養を行い、それぞれの細胞増殖動態を比較検討した。

（倫理面への配慮）

本研究では、ヒト由来細胞を用いた研究が予定されている。機関の外部委員を含めた倫理審査委員会において生命倫理、安全管理を厳重に審査する。倫理委員会の承認かつ実施施設の長の許可を得て、全ての研究を遂行する。国立成育医療センター研究所においては、ヒト間葉系細胞の培養に関し、研究面において既に倫理審査を受け、承認を受けている（国立成育医療センター研究所、受付番号８８、平成１６年７月承認)。実験動物を用いる研究については、各施設の動物実験指針に準拠して研究を実施する。特に、動物愛護と動物福祉の観点から実験動物使用は、目的に合致した最小限にとどめる。またその際、麻酔等手段により苦痛を与えない等の倫理的配慮をおこなう。実験者は、管理者と相互協力のもと適切な環境のもと飼育管理を行う。

C. 研究結果

ヒト骨髄液から浮遊細胞と接着細胞を分離し、得られた接着細胞のうち増殖能の良好な骨髄間質細胞を限外希釈法でサブクローニングした。得られた細胞に、レトロウィルスを用いて TERT、E6、E7 を遺伝子導入した。こうして得た複数のクローンのうち安定した形質を有する UEET-12 と名付けた細胞を主に用いた。

まず、MEM 系、HAM 系或いはこれらの改変・混合培地など約 30 種類の基礎培地から低血清下で良好な増殖性を示す培地を選定した。ついで、入手可能な既知の増殖因子（十数種類）について増殖性を検討した。その結果、UEET-12 については PDGF が極めて良好な、更に EGF、bFGF、LIF についても良好な増殖性を示した。より充分な増殖を得る為、PDGF に他の増殖因子を組み合わせた場合も検討した。その結果、LIF 或いは VEGF の添加により相乗的効果が認められた。これら増殖因子に対する反応性は細胞のクローン間で共通する傾向はあるものの、最適な増殖因子、及びその組合せは異なった。よって、最終的な増殖因子の最適化は複数のクローンでの成績を総合的に評価した。

更に、ホルモン、脂質など約２０種類の成分を評価・至適化することで改良培地を得た。このようにして得られた改良培地では従来培地と異なり無血清化でも１０％血清存在化と同等の増殖性を示した。ただし継代培養に供した場合、無血清下では著しい接着性の低下が認められた。接着性の低下はフラスコ表面をファイブロネクチンコートすることで緩和しうるが、充分な接着性を回復するに至らなかった。ただし、１％程度の低濃度の血清を加えることで接着機能の低下を抑制することができた。そこで、最終的に１％の血清成分を含む改良培地組成を確立した。

ヒト間葉系幹細胞を従来の１０％牛血清含有培地と本改良培地で継代培養したのちFACS

により細胞表面マーカーの発現を調べたが相違は認められなかった。また、ヒト骨髄液を等量に分け、１０％牛血清含有培地と本改良培地で分離培養を行ったところ、本改良培地では培養初期において多数のクローン増殖が認められ、増殖能が高く、１４日間の培養で１０％牛血清含有培地より約１００倍量の細胞数を得ることができた。

D. 考察

無血清化には至らなかったが、１％血清及び増殖因子（ヒト型組替え体）、ヒト由来蛋白質を含む改良培地を確立した。本培地は血清成分を自己血清に代替することで完全ヒト型合成培地とすることができる。これにより本件研究の目標である「臨床試験に適応できる安全な培地」を提供することが可能である。患者から採取可能な自己血清の量的限界を考慮すると、血清成分を１％に低下させることは、１０～２０％量の患者自己血清を含有する培地と比較して、その有用性は明らかである。また、優れた増殖性を示す点は培養期間を短縮する上で有利である。

本研究では間葉系幹細胞のクローンにより最適な増殖因子が必要なことを明らかにした。ある分化特性を有する細胞だけを選択的に増殖させるクローン選択培地が作製できる可能性がある。また、分裂回数に限界がある初代細胞においては大量の細胞を得る為には細胞寿命をのばすことが必要である。細胞寿命には活性酸素や浸透圧などの培養ストレスが関与している。より大量の細胞を得る為には今後はこれら培養ストレスを除去する検討が必要である。

E. 結論

ヒト間葉系幹細胞に対して優れた増殖性を示す低血清ヒト型合成培地を確立した。

F. 健康危険情報

なし

G. 研究発表

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該当なし。

H. 知的財産権の出願・登録状況（予定を含む。）

１． 特許取得

なし

２． 実用新案登録

なし

３． その他

なし

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研究成果の刊行に関する一覧表

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発表者氏名	論文タイトル名	発表雑誌名	巻号	ページ	出版年
Kiyokawa N, Sekino T, Matsui T, Takenouchi H, Mimori K, Tang W, Matsui J, Taguchi T, Katagiri YU, Okita H, Matsuo Y, Karasuyama H, Fujimoto J.	Diagnostic Importance of CD179a/b as Markers of Precursor B-Cell Lymphoblastic Lymphoma.	Modern Pathol	17	423-429	2004
Takenouchi H, Kiyokawa N, Taguchi T, Matsui J, Katagiri YU, Okita H, Okuda K, Fujimoto J.	Shiga toxin binding to globotriaosyl ceramide induces intracellular signals that mediate cytoskeleton remodeling in human renal carcinoma-derived cells.	J Cell Sci	117	3911-3922	2004
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Mechanical stress activates angiotensin II type 1 receptor without the involvement of angiotensin II

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The angiotensin II type 1 (AT1) receptor has a crucial role in load-induced cardiac hypertrophy. Here we show that the AT1 receptor can be activated by mechanical stress through an angiotensin-II-independent mechanism. Without the involvement of angiotensin II, mechanical stress not only activates extracellular-signal-regulated kinases and increases phosphoinositide production *in vitro*, but also induces cardiac hypertrophy *in vivo*. Mechanical stretch induces association of the AT1 receptor with Janus kinase 2, and translocation of G proteins into the cytosol. All of these events are inhibited by the AT1 receptor blocker candesartan. Thus, mechanical stress activates AT1 receptor independently of angiotensin II, and this activation can be inhibited by an inverse agonist of the AT1 receptor.

Cardiac hypertrophy is not only an adaptational state before cardiac failure, but also an independent risk factor of major cardiac events[1]. It is thus very important to understand the molecular mechanism that underlies the development of cardiac hypertrophy. Although various humoral factors such as vasoactive peptides, catecholamines, cytokines and growth factors can contribute to the development of cardiac hypertrophy during the increase in haemodynamic load, the initial stimulus mechanical stress is the most important contributory factor[2].

To dissect the mechanism of how mechanical stress induces cardiac hypertrophy, we and others have developed an *in vitro* device by which stretch stimuli can be imposed on cultured cardiomyocytes[3,4]. Mechanical stretch induces the activation of many protein kinases including extracellular-signal-regulated kinases (ERKs), reprogramming of gene expression, and cardiomyocyte hypertrophy in cultured cardiomyocytes[5]. Pretreatment of cardiomyocytes with angiotensin II (AII) type (AT1) receptor blockers significantly attenuates all of these mechanical-stretch-induced events[6,7]. Furthermore, many animal and clinical studies have shown that AT1 receptor blockers induce regression of cardiac hypertrophy and prevent progression of heart failure, resulting in a reduction in cardiac morbidity and mortality[8–12].

These basic and clinical studies have suggested that the local renin–angiotensin system (RAS) is activated by haemodynamic overload and that the AT1 receptor has a crucial role in the development of load-induced cardiac hypertrophy; however, it remains unclear how the AT1 receptor is activated by mechanical stress. It has been reported that AII is stored in cardiomyocytes and that mechanical stretch induces the secretion of stored AII into the culture medium, resulting in the induction of cardiomyocyte hypertrophy by the autocrine mechanism[6]. Here we have examined the possibility that mechanical stress can directly activate the AT1 receptor without the involvement of AII.

RESULTS

Marginal roles of endogenous AII in cardiomyoctes

Mechanically stretching cardiomyocytes by 20% for 8 min activated ERKs, and this activation was significantly inhibited by an AT1 receptor blocker, candesartan, as reported previously[7] (Fig. 1a, lanes 2 and 4). The magnitude of the stretch-induced activation of ERKs was the same as that induced by exposing the cardiomyocytes to 10^{-8} to 10^{-7} M AII (Fig. 1a, lanes 7, 8). These results indicate that the AT1 receptor is crucially involved in the stretch-induced activation of ERKs in cardiomyocytes and suggest that AII is secreted from cardiomyocytes into the culture medium by stretch.

We first used radioimmunoassay to measure AII in culture medium conditioned by stretching cardiomyocytes for 8 min, but did not detect a significant increase in AII concentration after stretch (AII without stretch, $0.7 \pm 1.6 \times 10^{-12}$ M; AII with stretch, $2.0 \pm 3.5 \times 10^{-12}$ M; not significant; Fig. 1b). We then carried out a bioassay using human embryonic kidney 293 cells expressing the

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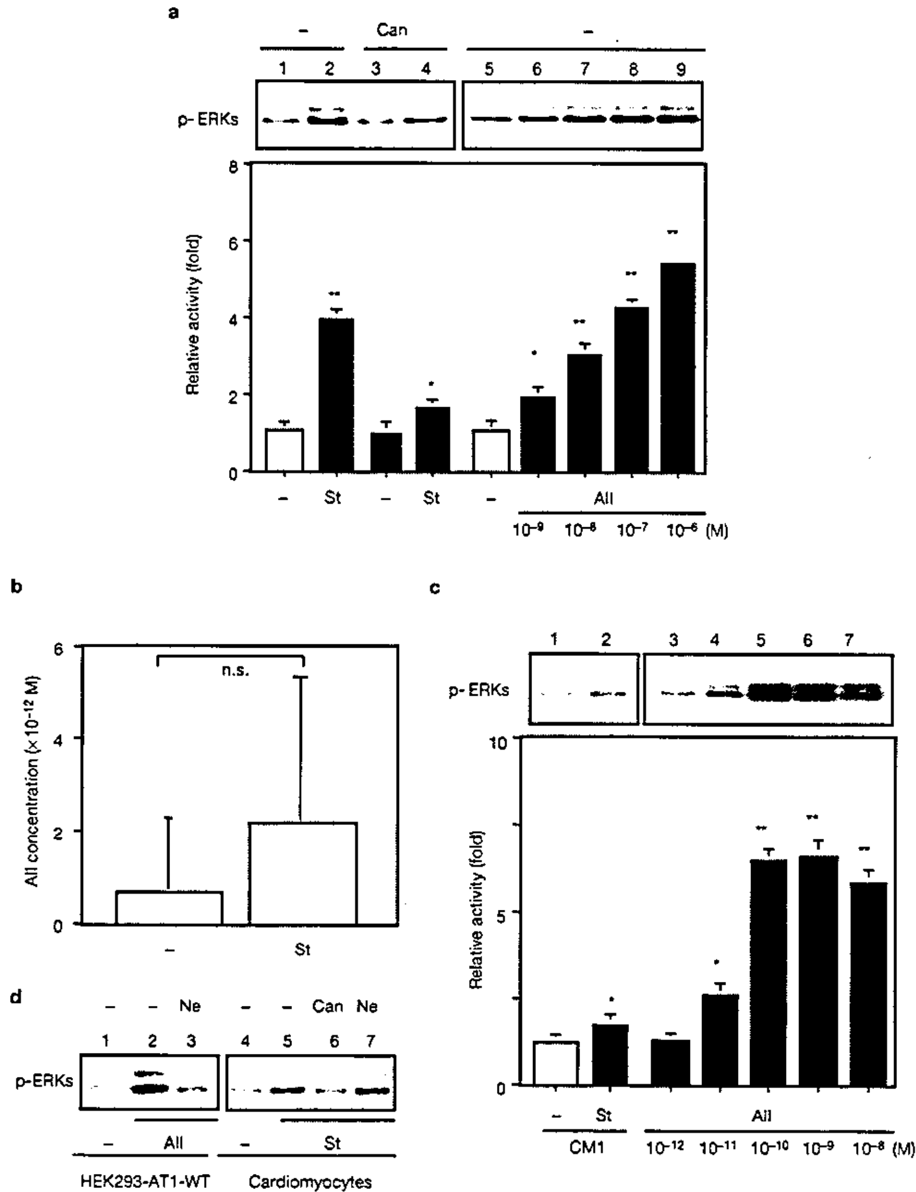

Figure 1 Activation of ERKs by mechanical stretch, AII and conditioned medium. **(a)** Cultured cardiomyocytes of neonatal rats were pretreated with 10^{-7} M candesartan (Can) or vehicle (–), and either stretched by 20% (St) or incubated with the indicated concentrations of AII for 8 min. Activation of ERKs was determined by using antibodies against phosphorylated ERKs (p-ERKs). The relative kinase activities of an ERK of relative molecular mass 42,000 (M_r 42K) were determined by scanning each band with a densitometer. Results are shown as the fold increase in activity over unstimulated cells and are the mean ± s.e.m. of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus vehicle. **(b)** The culture medium from cardiomyocytes conditioned by stretch (St) or no stretch (–) was measured for AII by radioimmunoassay. Results are the mean ± s.e.m. of six samples. n.s., not significant. **(c)** HEK293-AT1-WT cells were exposed to conditioned medium collected before (–) or after stretching (St) cardiomyocytes for 8 min (CM1) or to the indicated concentrations of AII. Relative kinase activities of the 42K ERK were determined as in **a**. *$P < 0.05$, **$P < 0.01$, as compared with vehicle. **(d)** HEK293-AT1-WT cells or cardiomyocytes were subjected to AII or stretch for 8 min without (–) or with (Ne) the presence of a neutralizing antibody to AII (10 μg ml^{-1}; Cortex Biochem).

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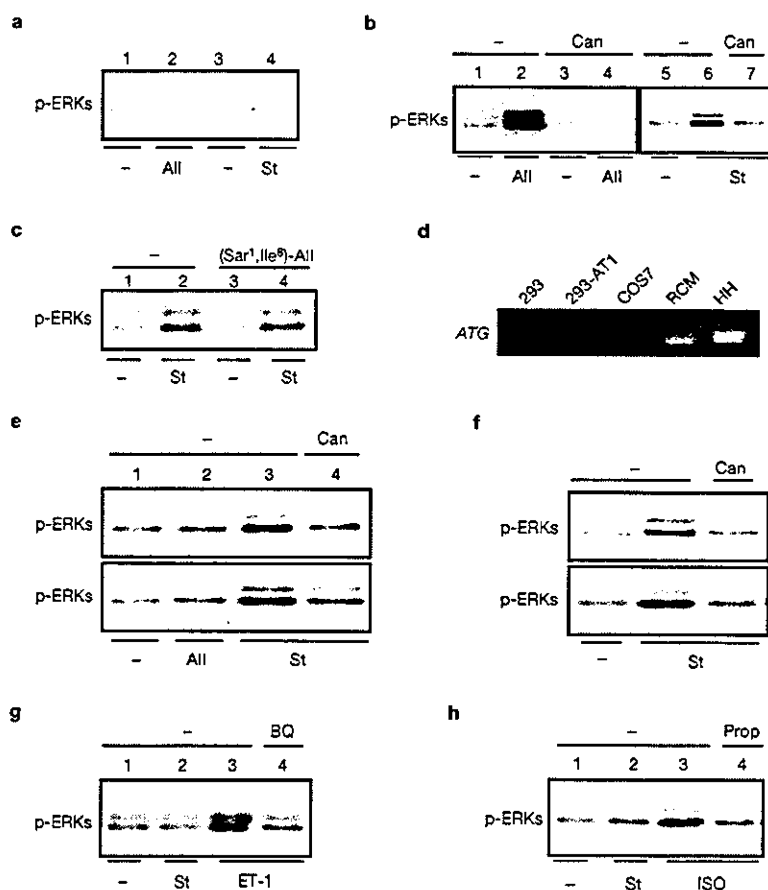

Figure 2 AII-independent activation of ERKs by mechanical stretch in cells overexpressing AT1 receptors. (a) HEK293 cells were stretched by 20% (St) or exposed to 10^{-7} M AII for 8 min. (b) HEK293-AT1-WT cells were stimulated by mechanical stretch or AII in the absence (–) or presence of 10^{-7} M candesartan (Can). (c) HEK293-AT1-WT cells were stretched in the absence (–) or presence of 10^{-7} M (Sar^1,Ile^8)-AII. (d) Expression of the *ATG* gene analysed by RT–PCR. 293, HEK293 cells; 293-AT1, HEK293-AT1-WT cells; RCM, cardiomyocytes of neonatal rats; HH, human heart. (e) HEK293 (top) and COS7 cells (bottom) were transiently transfected with AT1-mut1, which does not bind to AII. Cells were stimulated with mechanical stretch or AII without (–) or with candesartan (Can). (f) Cardiomyocytes prepared from neonatal (top) and adult (bottom) *ATG*$^{-/-}$ mice were pretreated with candesartan, and then stimulated with mechanical stretch. (g, h) COS7 cells transiently transfected with ET1A (g) or β2-AR (h) receptors were pretreated with an ET1A antagonist BQ123 (BQ), a β-AR blocker propranolol (Prop) or vehicle (–), and then stimulated with mechanical stretch, ET-1 or ISO.

wild-type mouse AT1a receptor (hereafter denoted HEK293-AT1-WT cells). The medium conditioned by stretching cardiomyocytes for 8 min only slightly activated ERKs in HEK293-AT1-WT cells (Fig. 1c, lane 2). Its activity was equivalent to that induced by incubating HEK293-AT1-WT cells with 10^{-12} to 10^{-11} M AII (Fig. 1c, lanes 3 and 4), a value that was similar to the concentration of AII measured in the medium by radioimmunoassay. These results suggest that AII, even if secreted, would not be enough to induce the full activation of the AT1 receptor observed after stretch.

To confirm whether secreted AII has a marginal role in the stretch-induced activation of ERKs, we blocked AII activity with a neutralizing antibody to AII. Although the antibody abolished the activation of ERKs induced by 10^{-7} M AII (Fig. 1d, lane 3), it did not significantly suppress the stretch-induced activation of ERKs (Fig. 1d, lane 7).

Stretch activates ERKs through the AT1 receptor without AII

Neither mechanical stretch nor AII (10^{-7} M) activated ERKs in HEK293 cells (Fig. 2a). In HEK293-AT1-WT cells, however, both mechanical stretch and AII activated ERKs (Fig. 2b, lanes 2 and 6). Pretreatment with the AT1 receptor blocker candesartan inhibited the activation of ERKs induced not only by AII (Fig. 2b, lane 4) but also by mechanical stretch in HEK293-AT1-WT cells (Fig. 2b, lane 7). Basal ERK activity was also decreased by candesartan (Fig. 2b, lane 3) in HEK293-AT1-WT cells, and similar results were obtained in COS7 cells transiently expressing AT1-WT.

Mechanical stretch activated ERKs in COS7 cells expressing AT1-WT (see Supplementary Information Fig. S1) but not in COS7 cells (data not shown). Candesartan also reduced the basal and the stretch-enhanced ERK activities in these cells (see Supplementary Information Fig. S1). However, a competitive inhibitor for AII, (Sar^1,Ile^8)-AII, did

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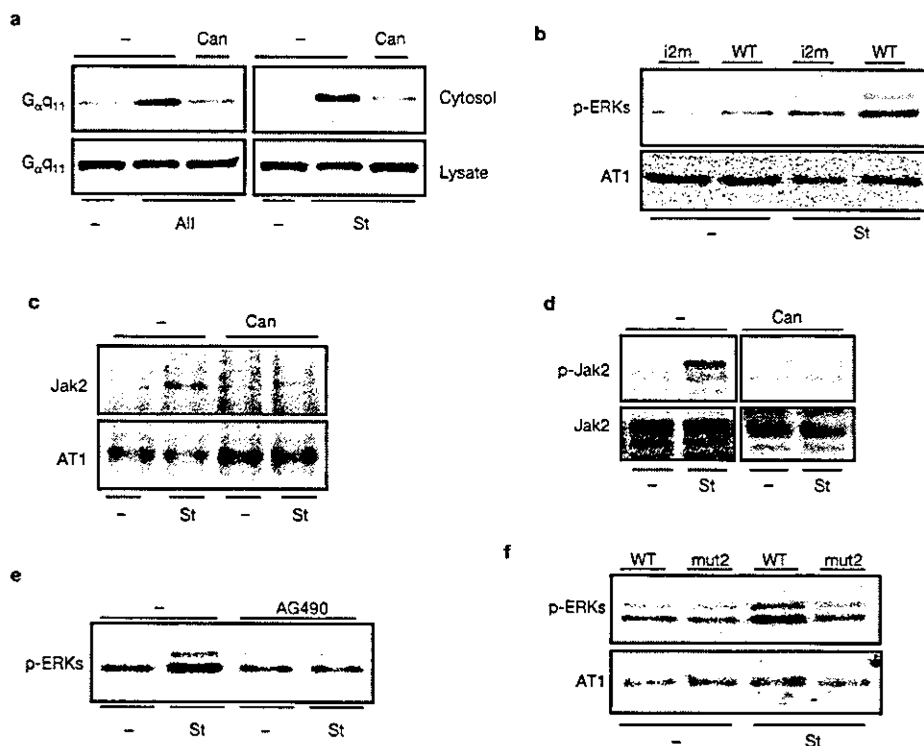

Figure 3 Activation of G proteins and Jak2 by mechanical stretch, and their effects on ERK activation. (a) HEK293T-AT1-WT cells, pretreated with candesartan (Can) or vehicle (–), were stimulated with AII or mechanical stretch (St) for 5 min. Aliquots of the cytosol (top) and whole lysates (bottom) were resolved by SDS–PAGE. Blotted membranes were incubated with an antibodies against $G_\alpha q_{11}$. (b) COS7 cells were transfected with AT1-i2m (i2m), which lacks a binding domain for G proteins, or the wild-type AT1 receptor (WT), and stretched for 8 min (top). Receptor transfection was verified by an antibody against the AT1 receptor (bottom). (c) HEK293T-AT1-WT cells, pretreated with candesartan (Can) or vehicle (–), were subjected to mechanical stretch for 5 min. Total cell lysates were immunoprecipitated by an antibody against the AT1 receptor and the immunocomplexes were subjected to immunoblotting with antibodies against Jak2 (top) or the AT1 receptor (bottom). (d) HEK293T-AT1-WT cells, pretreated with candesartan (Can) or vehicle (–), were subjected to mechanical stretch. Blotted membranes were incubated with antibodies against phosphorylated Jak2 (top) or total Jak2 (bottom). (e) HEK293T-AT1-WT cells, pretreated with tyrphostin AG490 or vehicle (–), were stretched by 20% for 8 min. (f) COS7 cells were transfected with AT1-mut2 (mut2), which lacks a binding domain for Jak2, or the wild-type AT1 receptor (WT), and stimulated by mechanical stretch for 8 min (top). Receptor transfection was verified by an antibody against the AT1 receptor (bottom).

not attenuate stretch-induced ERK activation in HEK293-AT1-WT cells (Fig. 2c, lane 4), although this inhibitor abolished AII-induced ERK activation in the same cells (data not shown).

These results indicate that expression of the AT1 receptor provides HEK293 and COS7 cells with the ability to respond to stretch, for which there might be two possible mechanisms. First, AII is stored in these cells and secreted by stretch, but the cells do not respond to secreted AII because they lack the AT1 receptor. Second, the AT1 receptor is activated by stretch without the involvement of AII.

The medium conditioned by stretching HEK293-AT1-WT cells did not activate ERKs in HEK293-AT1-WT cells (see Supplementary Information Fig. S2). Analysis by polymerase chain reaction with reverse transcription (RT–PCR) did not detect the transcript of the angiotensinogen gene (*ATG*) in HEK293, COS7 or HEK293-AT1-WT cells even after 50 cycles, although it was detected in cardiomyocytes of neonatal rats and human hearts (Fig. 2d). Taken together, these results suggest that mechanical stretch activates the AT1 receptor in both HEK293-AT1-WT cells and COS7 cells expressing the AT1 receptor without the secretion of AII.

To confirm whether or not AT1 receptor is activated by mechanical stretch without involving its ligand, we stretched HEK293 and COS7 cells expressing an AT1 receptor whose binding site for AII was mutated by the replacement of Lys 199 with glutamine (AT1-mut1)[13]. AII did not activate ERKs in cells expressing AT1-mut1 (Fig. 2e, lane 2), indicating a lack of coupling between the mutated receptor and AII. In the same AT1-mut1 cells, however, ERKs were strongly activated by mechanical stretch, and this activation was inhibited by candesartan (Fig. 2e, lanes 3 and 4).

To confirm further activation of the AT1 receptor by mechanical stretch without the involvement of AII, we stretched cardiomyocytes prepared from *ATG*-deficient ($ATG^{-/-}$) mice, in which AII is not detected[14]. Mechanical stress activated ERKs in the cardiomyocytes prepared from both neonatal (Fig. 2f, top) and adult $ATG^{-/-}$ mice (Fig. 2f, bottom). Pretreatment of these cells with candesartan markedly suppressed the activation of ERKs (Fig. 2f). These results indicate that mechanical stress may activate ERKs in cardiomyocytes through the AT1 receptor even in the absence of AII.

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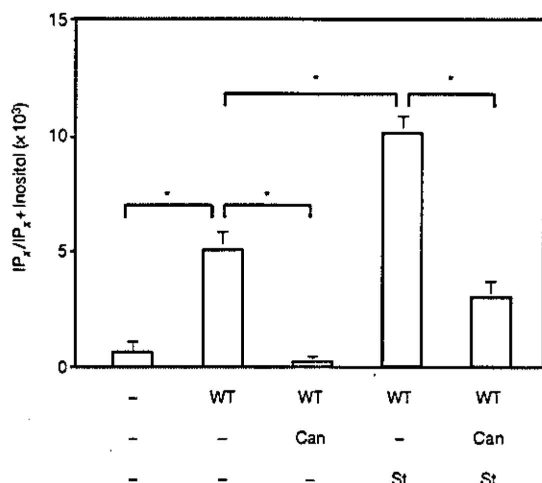

Figure 4 Mechanical-stretch-stimulated production of inositol phosphates through the AT1 receptor. COS7 cells (–) or COS7 cells transfected with AT1-WT (WT) were labelled with myo-[^{3}H]inositol 24 h after transfection. After 24 h of labelling, cells were incubated with vehicle (–) or candesartan (Can) for 5 h at 37 °C. The accumulation of inositol phosphates was measured as described in Methods. Some COS7 cells transiently transfected with AT1-WT (WT) were subjected to stretching (St) for 45 min or no stimulus (–) in the presence of 5 mM LiCl. *$P < 0.05$.

The AT1 receptor is a guanine-nucleotide-binding protein (G-protein)-coupled receptor (GPCR), a member of a large family of cell-surface receptors that contain common structural features characterized by seven transmembrane helices essential for signal transduction[15–17]. Activation of other GPCRs, such as the receptors of endothelin 1 (ET-1) and catecholamines, also induces cardiomyocyte hypertrophy[18,19]. We therefore tested whether mechanical stretch can activate these receptors in a ligand-independent manner. We stretched COS7 cells overexpressing either the wild-type ET-1 type A (ET1A) receptor (Fig. 2g) or the wild-type β2-adrenoceptor (β2-AR; Fig. 2h). Whereas ET-1 and isoproterenol (ISO) activated ERKs, mechanical stretch did not evoke significant activation of ERKs in these transfected cells. These results suggest that the activation of GPCRs by mechanical stretch without the involvement of their ligands is not a general phenomenon but specific to some GPCRs including the AT1 receptor.

G proteins and Jak2 are activated by stretch

As a member of the GPCR family, the AT1 receptor evokes intracellular signals through G proteins[20,21]. We therefore examined whether mechanical stress could activate G proteins through the AT1 receptor. Stimulation with either AII or mechanical stretch induced the redistribution of $G_{\alpha}q_{11}$ subunits into the cytosol of HEK293-AT1-WT cells and this redistribution was inhibited by pretreatment with candesartan (Fig. 3a), suggesting that $G_{\alpha}q_{11}$ is activated by mechanical stretch as well as by AII.

To determine whether an interaction between the AT1 receptor and G proteins has a role in the activation of ERKs, we transfected an AT1 receptor mutant that does not couple to G proteins (AT1-i2m)[22] into COS7 cells. Activation of ERKs by mechanical stretch was weaker in the AT1-i2m-transfected cells than in those overexpressing AT1-WT (Fig. 3b), suggesting that coupling of G proteins to the AT1 receptor is partly involved in the stretch-induced activation of ERKs.

Non-receptor-type tyrosine kinases such as the Janus kinase (Jak) family and the Src family may be important in AT1 receptor signalling[22,23]. The AT1 receptor activates the Src–Ras–ERK pathway independently of G-protein coupling through the association and activation of Jak2 (refs 22,24,25). Mechanical stretch induced association of Jak2 with the AT1 receptor (Fig. 3c) and phosphorylation of Jak2 (Fig. 3d) in HEK293-AT1-WT cells. Pretreatment of the cells with candesartan significantly suppressed association with the AT1 receptor and phosphorylation of Jak2 (Fig. 3c, d). Mechanical stretch did not activate ERKs in HEK293-AT1-WT cells that had been pretreated with AG490, a specific inhibitor of Jak2 (Fig. 3e), or in COS7 cells expressing an AT1-mutant (AT1-mut2)[25] that lacks a binding domain for Jak2 (Fig. 3f). These results suggest that activation of Jak2 is crucially involved in the stretch-induced activation of ERKs.

Mechanical stretch upregulates inositol phosphates

To identify other stretch-induced events, we examined the accumulation of inositol phosphates in COS-7 cells expressing AT1-WT. Overexpression of AT1-WT resulted in a roughly fivefold increase in basal inositol phosphates, as compared with untransfected cells (Fig. 4). Mechanical stretch of these AT1-WT-expressing cells further upregulated inositol phosphate production by about twofold (Fig. 4). Stretching the parental COS7 cells did not increase inositol phosphate production (data not shown). Candesartan inhibited the accumulation of inositol phosphates in COS7 cells expressing AT1-WT, as well as the stretch-induced increase in inositol phosphate production (Fig. 4).

Load-induced cardiac hypertrophy through AT1 receptor

We examined whether mechanical stress could induce cardiac hypertrophy *in vivo* through the AT1 receptor in the absence of AII. We imposed a pressure overload on the heart by constricting the transverse aorta (TAC) of adult male $ATG^{-/-}$ mice. Pressure overload for 2 weeks induced significant hypertrophy in the heart of the $ATG^{-/-}$ mice (Fig. 5a, b). Heart weight was increased from 110 ± 12 mg to 189 ± 14 mg after 2 weeks of pressure overload (Fig. 5b).

Although treatment with candesartan did not reduce blood pressure in the right carotid artery (sham operated, 78 ± 10 mmHg; TAC plus saline, 166 ± 15 mmHg; TAC plus candesartan, 160 ± 17 mmHg), the development of cardiac hypertrophy was significantly attenuated by candesartan (heart weight, 145 ± 21 mg; Fig. 5). These results suggest that mechanical stress can induce cardiac hypertrophy *in vivo* by activating the AT1 receptor without the involvement of AII.

DISCUSSION

Many basic and clinical studies have shown that RAS is crucially involved in the development of various cardiovascular diseases[9–12]. Much evidence has indicated that RAS exists in various organs, as well as in the circulation, and that local RAS has an important role in organ damage including cardiac hypertrophy[26,27]. All components of RAS, such as angiotensinogen, renin, angiotensin-converting enzyme and receptors, are present in the heart[26,27], and AII induces hypertrophy of cultured cardiomyocytes[27]. It has been reported that AII is stored in cardiomyocytes and that mechanical stretch induces the secretion of stored AII into the culture medium, resulting in the induction of cardiomyocyte hypertrophy by the autocrine mechanism[6]. Taking these observations together, haemodynamic overload has been thought to promote cardiac hypertrophy by inducing the secretion of AII in the heart. In this study, however, we have shown that mechanical stress can induce cardiomyocyte hypertrophy both *in vitro* and *in vivo* through the AT1 receptor without the involvement of AII.

Although mechanical stretch has been reported to induce the release of endogenous AII from cardiomyocytes[6], by radioimmunoassay we did not detect a significant increase in AII in the cul-

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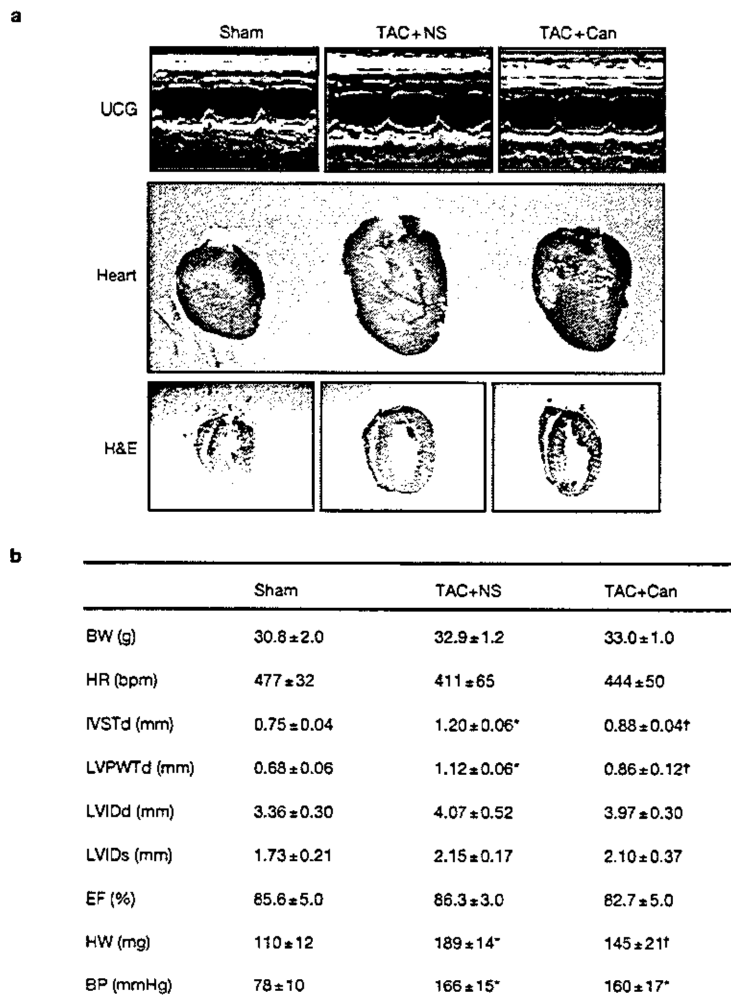

	Sham	TAC+NS	TAC+Can
BW (g)	30.8±2.0	32.9±1.2	33.0±1.0
HR (bpm)	477±32	411±65	444±50
IVSTd (mm)	0.75±0.04	1.20±0.06*	0.88±0.04†
LVPWTd (mm)	0.68±0.06	1.12±0.06*	0.86±0.12†
LVIDd (mm)	3.36±0.30	4.07±0.52	3.97±0.30
LVIDs (mm)	1.73±0.21	2.15±0.17	2.10±0.37
EF (%)	85.6±5.0	86.3±3.0	82.7±5.0
HW (mg)	110±12	189±14*	145±21†
BP (mmHg)	78±10	166±15*	160±17*

Figure 5 Cardiac hypertrophy in *ATG*$^{-/-}$ mice induced by pressure overload. Ten-week-old male *ATG*$^{-/-}$ mice, treated with saline (NS) or candesartan (Can), were subjected to a sham or TAC operation. Echocardiography and catheterization were done 2 weeks later. (a) Top, M-mode echocardiograms; middle, gross appearance of the heart; bottom, sections stained by H&E. (b) Echocardiographic results and haemodynamic parameters, shown as the mean ± s.e.m. ($n = 3$). *$P < 0.05$ versus sham operated; †$P < 0.05$ versus saline. BW, body weight; HR, heart rate; IVSTd, thickness of interventricular septum at diastole; LVPWTd, posterior wall thickness of LV at diastole; LVIDd and LVIDs, LV internal dimension at end diastole and systole, respectively; EF, ejection fraction; HW, heart weight; BP, systolic blood pressure.

ture medium after stretch. AII concentration varied considerably in the conditioned media, whereas the degree of ERK activation induced by stretch was constant, which also suggests that secreted AII is not involved in the stretch-induced activation of ERKs. A very sensitive bioassay, as well as radioimmunoassay, showed that the concentration of AII in medium conditioned by stretch was less than 10^{-11} M, which is far too low to evoke a hypertrophic response in cardiomyocytes. The reason for the difference in the role of secreted AII by stretch between the previous report[6] and this study is not clear at present. Many reports have shown that cardiomyocytes contain about 10^{-13} mol of AII per gram of cells[28–30]. Thus, even if all of the AII stored in cardiomyocytes were secreted by stretch, at most about 10^{-11} M AII would be detected in the culture medium. We detected roughly 2×10^{-12} M AII in culture medium conditioned by stretch for 8 min, consistent with the theoretical concentrations.

Although it is evident that mechanical stress is the primary trigger of cardiac hypertrophy, it is not clear how mechanical stress is received and converted into the active intracellular signalling responsible for the development of cardiac hypertrophy. Muscle LIM protein, integrins and their associated signalling machinery have been reported to be sensors for mechanical stress[31,32]. We propose that the AT1 receptor is also a receptor for mechanical stress. Mechanical stretch did not activate ERKs in HEK293 cells or COS7 cells, but expression of the AT1 receptor gave these cells the ability to respond to stretch. Bioassays using conditioned medium and RT–PCR analysis showed that there is little or no AII in HEK293 cells or COS7 cells. These results suggest that the AT1 receptor is a 'mechanical sensor' and converts mechanical stress into biochemical signals inside the cells.

This hypothesis was confirmed by results from cells expressing the AT1-mut1, which cannot bind AII, and from cardiomyocytes pre-

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pared from $ATG^{-/-}$ mice. Although AII did not activate ERKs in cells expressing AT1-mut1, mechanical stretch activated ERKs in these cells and this activation was inhibited by an AT1 receptor blocker, candesartan. Mechanical stretch activated ERKs in cardiomyocytes prepared from $ATG^{-/-}$ mice, which do not express AII, and candesartan inhibited this activation.

Because mechanical stretch did not activate ERKs in cells expressing the ET1A or β2-AR receptors, not all GPCRs are necessarily a mechanical sensor. Although we do not know at present why the AT1 receptor, but not the ET1A or β2-AR receptor, is significantly activated by mechanical stretch, there are a few possibilities. First, specificity may be due to molecules that associate with the AT1 receptor. We found that in the response to mechanical stretch, some unknown molecules bind to the AT1 receptor (our own unpublished results). Second, diversity in the structures and expression of the receptors may also determine their responsibility to mechanical stress[16,27,33].

After binding to AII, the AT1 receptor changes its conformation into an active form and stimulates G proteins through its intracellular domains[15,20,33,34]. The intracellular loops and the region between residues 312 and 318 in the carboxy-terminal tail of the AT1 receptor have been reported to be essential for coupling and activating G proteins[35]. After activation, G proteins dissociate into α- and βγ-subunits, and the α-subunit is translocated into the cytosol[34]. In addition, ligand binding to the AT1 receptor induces association of the C terminus of the AT1 receptor with Jak2, thereby resulting in activation of the Jak2–STAT3 pathway[22–25]. Although there is no direct evidence, our results suggest that, similar to AII binding to the AT1 receptor, mechanical stress induces a conformational change in the AT1 receptor by a mechanism independent of AII binding, resulting in the association and activation of G proteins and Jak2.

There are a few mechanisms by which mechanical stress might activate the AT1 receptor without the involvement of AII. First, stretching the cell membrane may directly change the conformation of the AT1 receptor. Many receptors can change their conformation between the active and inactive state under basal conditions without ligands[36,37]. Candesartan reduced the basal activity of ERKs, suggesting that part of the wild-type AT1 receptor is in an active state, and mechanical stress may increase the number of AT1 receptors in the active state. Second, mechanical stretch might activate specific mechanical sensors, which then activate the AT1 receptor from inside the cells. Potential stretch sensors, such as muscle LIM protein, integrins and stretch-sensitive ion channels, might activate the AT1 receptor, though the underlying mechanism remains to be determined.

Strong pressure overload induced cardiac hypertrophy in $ATG^{-/-}$ mice, indicating that mechanical stress can induce cardiac hypertrophy without AII. Treatment with candesartan significantly attenuated the development of cardiac hypertrophy without reducing blood pressure, suggesting that mechanical stress activates the AT1 receptor and induces cardiac hypertrophy without the involvement of AII *in vivo*. We previously reported that pressure overload induces cardiac hypertrophy in AT1a receptor knockout mice[38]. The activity of tyrosine kinases is upregulated before stretch and more strongly enhanced by mechanical stretch in AT1a-receptor-deficient cardiomyocytes as compared with wild-type cells through epidermal growth factor (EGF) receptor tyrosine kinases[39], suggesting that the AT1 receptor is not indispensable for stretch-induced cardiac hypertrophy and that some compensatory mechanisms operate and induce cardiac hypertrophy even in the absence of the AT1 receptor[38,39].

Candesartan reduced the basal activity of ERKs and inositol phosphates in cells overexpressing AT1-WT and inhibited the stretch-induced activation of ERKs and increase in inositol phosphates independently of AII, suggesting that candesartan works as an inverse agonist of the AT1 receptor. An inverse agonist of the AT1 receptor is defined as an agent that stabilizes the AT1 receptor in an inactive conformation, thereby inhibiting signals evoked by the wild-type or active AT1 receptor. As an inverse agonist, candesartan may inhibit changes in conformation of AT1 receptor and thus may efficiently suppress its activation induced by both mechanical stress and AII. Much evidence suggests that local RAS has a crucial role in injury to various organs[26,27,40]. It remains to be determined whether activation of the AT1 receptor without AII occurs in other organs, and whether inverse agonists prevent organ damage more effectively than do competitive antagonists. □

METHODS

cDNA constructs. The AT1a receptor mutants lacking binding activity with AII (AT1-mut1) or the Jak2 coupling domain (AT1-mut2) were generated by PCR from the wild-type mouse AT1 receptor (GenBank accession number S37484)[41] by replacement of Lys 199 with glutamine[13] or truncation of the C terminus (residues 312–359)[22], respectively. AT1-i2m[22], β2-AR[42] and ET1A[43] were gifts from J. Sadoshima, R J. Lefkowitz and S. Kimura, respectively. The complementary DNAs used in this study are summarized in Supplementary Information, Table 1.

Cell culture and transfection. We prepared primary cultures of cardiomyocytes from the ventricles of 1-day-old Wistar rats or $ATG^{-/-}$ mice as described[19]. Adult and neonatal cardiomyocytes of $ATG^{-/-}$ mice were prepared as described[44]. HEK293 and COS7 cells were cultured in Dulbecco's modified Eagle's medium with 10% serum. cDNAs were transfected by the calcium phosphate method as described[19]. Stable transformants were selected by the addition of hygromycin B to the cells 3 d after transfection and for all subsequent passages of the cells[41]. All cultures were transferred to serum-free conditions 48 h before stimulation.

Western blotting. Total proteins (50 μg) were size-fractionated by SDS–PAGE and transferred to Immobilon-P membranes (Millipore). The blotted membranes were incubated with antibodies against phosphorylated ERKs, phosphorylated Jak2, Jak2, AT1 or $G_{\alpha}q_{11}$ (Santa Cruz).

***ATG* gene expression.** Expression of the *ATG* gene was examined by RT–PCR using specific primers (sense, 5′-TTCAGGCCAAGACCTCCC-3′; antisense, 5′-CCAGCCGGGAGGTGCAGT-3′)[45]. We separated the PCR products on 1.2% agarose gels and visualized them by using ethidium bromide.

Detection of inositol phosphates. Accumulation of inositol phosphates was assayed in COS7 cells as described[46,47]. In brief, 24 h after transfection by the DEAE–adenovirus method[48], cells were replated in 24-well plates at 1.5×10^5 cells per well and labelled for 24 h with myo-[^{3}H]inositol (2 μCi ml^{-1}; Amersham). The cells were washed in medium containing 5 mM LiCl for 10 min, incubated with vehicle or candesartan for 5 h, and then subjected to mechanical stretch for 45 min in the presence of 5 mM LiCl. Inositol phosphates and total inositol fractions were resolved on a Dowex AG 1-X8 formate column (Bio-Rad), and inositol phosphate accumulation was estimated by determining the ratio of inositol phosphate radioactivity to the sum of inositol phosphate plus inositol radioactivity.

AII in the medium of cultured cardiomyocytes. Culture medium (2 ml per dish) was collected from dishes before and after stretching the cells by 20% for 8 min. We measured AII concentration by radioimmunoassay using two antibodies specific for AII (SRL Co.).

TAC operation. TAC operation was done on 10-week-old male $ATG^{-/-}$ mice and wild-type C52/BL6 mice[14]. A mini-osmotic pump (Alzet) filled with saline or candesartan was implanted subcutaneously in mice 3 d before TAC. All mouse protocols were approved by the guidelines of Chiba University.

Haemodynamic parameters. Transthoratic echocardiography (UCG) was done as reported[38] using a Agilent sonos 4500 (Agilent Technologies Co.) equipped

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with a 11-MHz imaging transducer. Haemodynamic measurements were taken by inserting a micronanometer catheter (Millar 1.4F, SPR 671, Millar Instruments) from the right common carotid artery into the aorta and then the left ventricle (LV). The transducer was connected to the MacLab system (AD Instruments) to record the pressure. For heart morphometry, hearts were perfused with 10% buffered formalin and subsequently embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E).

Statistics. Data are shown as mean ± s.e.m. Multiple group comparison was done by a one-way analysis of variance (ANOVA), followed by the Bonferroni procedure for comparison of means. A two-tailed Student's *t*-test was used to compare drug-treated and vehicle-treated specimens under identical conditions. Values of $P < 0.05$ were considered statistically significant.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.

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Role of Na^+–Ca^{2+} exchanger in myocardial ischemia/reperfusion injury: evaluation using a heterozygous Na^+–Ca^{2+} exchanger knockout mouse model

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Abstract

We used Na^+–Ca^{2+} exchanger (NCX) knockout mice to evaluate the effects of NCX in cardiac function and the infarct size after ischemia/reperfusion injury. The contractile function in NCX KO mice hearts was significantly better than that in wild type (WT) mice hearts after ischemia/reperfusion and the infarct size was significantly small in NCX KO mice hearts compared with that in WT mice hearts. NCX is critically involved in the development of ischemia/reperfusion-induced myocardial injury and therefore the inhibition of NCX function may contribute to cardioprotection against ischemia/reperfusion injury.
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Keywords: Sodium–calcium exchanger; Knockout mouse; Heart; Ischemia/reperfusion injury

The Na^+–Ca^{2+} exchanger (NCX) is an important electrogenic transporter in maintaining calcium homeostasis in a variety of mammalian organs [1]. NCX catalyzes electrogenic exchange of Na^+ and Ca^{2+} across the plasma membrane in either the Ca^{2+}-efflux (the forward mode) or Ca^{2+}-influx (the reverse mode), depending on the electrochemical gradients of the substrate ions. In the heart, NCX plays an important role in excitation–contraction coupling as the dominant myocardial Ca^{2+}-efflux system [2]. On the other hand, the reverse mode of NCX is associated with in cytoplasmic Ca^{2+} levels in cardiomyocytes during digitalis treatment or ischemia/reperfusion [3]. It has been reported that NCX inhibitors and NCX antisense oligonucleotides protect the heart from ischemia/reperfusion injury [4,5]. However, two putative NCX inhibitors, KB-R7943 and SEA0400, have been reported to be not specific for NCX [6]. Therefore, it remains unclear whether NCX indeed plays a crucial role in mediating Ca^{2+} influx that leads to Ca^{2+} overload and cellular injury after myocardial ischemia, reperfusion injury. Using heterozygous NCX KO mice, we examined the role of NCX in myocardial ischemia/reperfusion injury.

Materials and methods

NCX KO mice. NCX knockout (KO) mice were generated as described previously [7]. Male heterozygous KO mice and wild type (WT) littermates 12 weeks old were used. All animal experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

Electrophysiology. Ventricular cells were prepared from adult mice hearts by standard enzymatic digestion [8]. Whole-cell membrane currents were recorded by the patch-clamp method and the current–voltage relationship was obtained by voltage clamp ramp pulses as described previously [9]. Under these conditions, the Ni^{2+}-sensitive current represents NCX current [10]. All data were acquired and analyzed by the pCLAMP (version 5.5; Axon Instrument) software.

Western blot analysis. Expression levels of dihydropyridine (DHP) receptor (L-type Ca^{2+} channel) and SR Ca^{2+}-ATPase 2 (SERCA2)

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