

GPC3 in terms of IGF2-induced cell proliferation, FGF2-induced cell proliferation was markedly suppressed by overexpressed GPC3 in HY-Toff cells. As for the inhibitory growth factors, BMP-7 suppressed the growth rate of HY-Toff cells in the absence of GPC3 expression, whereas the activity of BMP-7 was significantly blocked under conditions of an overexpression of GPC3. On the other hand, TGF- $\beta$  had no effect on the cell proliferation of HY-Toff cells in the presence or absence of GPC3 overexpression. Overexpression of GPC3 also could not block the actions of HB-EGF.

#### GPC3 binds FGF2 in vivo

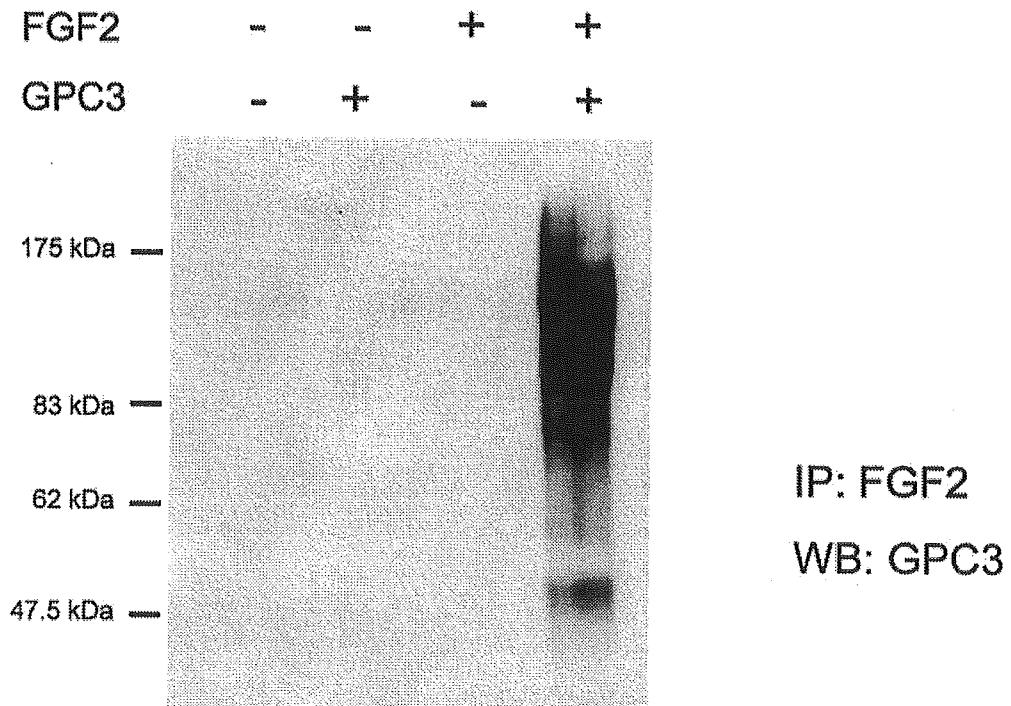
It has been previously demonstrated that GPC3 binds to FGF2 *in vitro*.<sup>12</sup> To confirm that GPC3 binds to FGF2 *in vivo*, FGF2 was cotransfected in HY-Toff-GPC3 cells and the interaction of GPC3 with FGF2 was investigated by immunoprecipitation assay. First, whole lysate was immunoprecipitated with an anti-GPC3 antibody bound to protein G beads, and the immunoprecipitates were ana-

lyzed by Western blot analysis with anti-FGF2 antibody. As shown in Figure 7a, FGF2 bound to GPC3 was detected. In the same manner, GPC3 interacting with FGF2 was confirmed by immunoprecipitation of FGF2, followed by Western blotting of GPC3 (Fig. 6b). The binding of GPC3 and FGF2 was thus demonstrated *in vivo*.

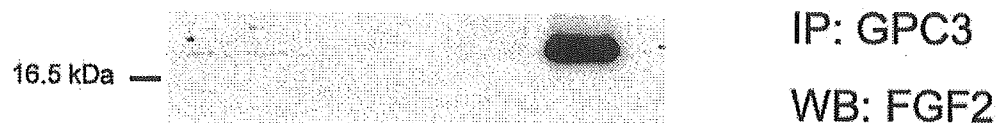
#### Enhanced expression of GPC3 inhibits the BMP-7 signaling pathway

To examine the effects of GPC3 on the BMP-7 signaling pathway, we compared the effects of the luciferase activity induced by BMP-7 in the presence or absence of enhanced expression of GPC3. In this model, ligand-induced functional activation is monitored by means of a luciferase reporter gene linked in its promoter region to a BMP-responsive element of Smad6. BMP-7-induced transcriptional activation of 3GC2-Lux was suppressed by the overexpression of GPC3. With high doses of BMP-7, the increase of luciferase activity was about 2.5-fold, in the absence of expres-

A.



B.



**FIGURE 6**—Binding of GPC3 and FGF2 protein in HY-Toff cells. FGF2 was transiently transfected in HY-Toff-GPC3 cells and the expression of GPC3 was controlled by administration of Dox. The complex of FGF2 and GPC3 was co-immunoprecipitated by the anti-FGF2 (a) or anti-GPC3 (b) antibody. The coreprotein of GPC3 is truncated in the vicinity of N-terminus and the smaller size of coreprotein instead of the expected 65 kD is detected in this experiment (Oizumi *et al.*, unpublished data). IP, immunoprecipitation; WB, Western blotting.

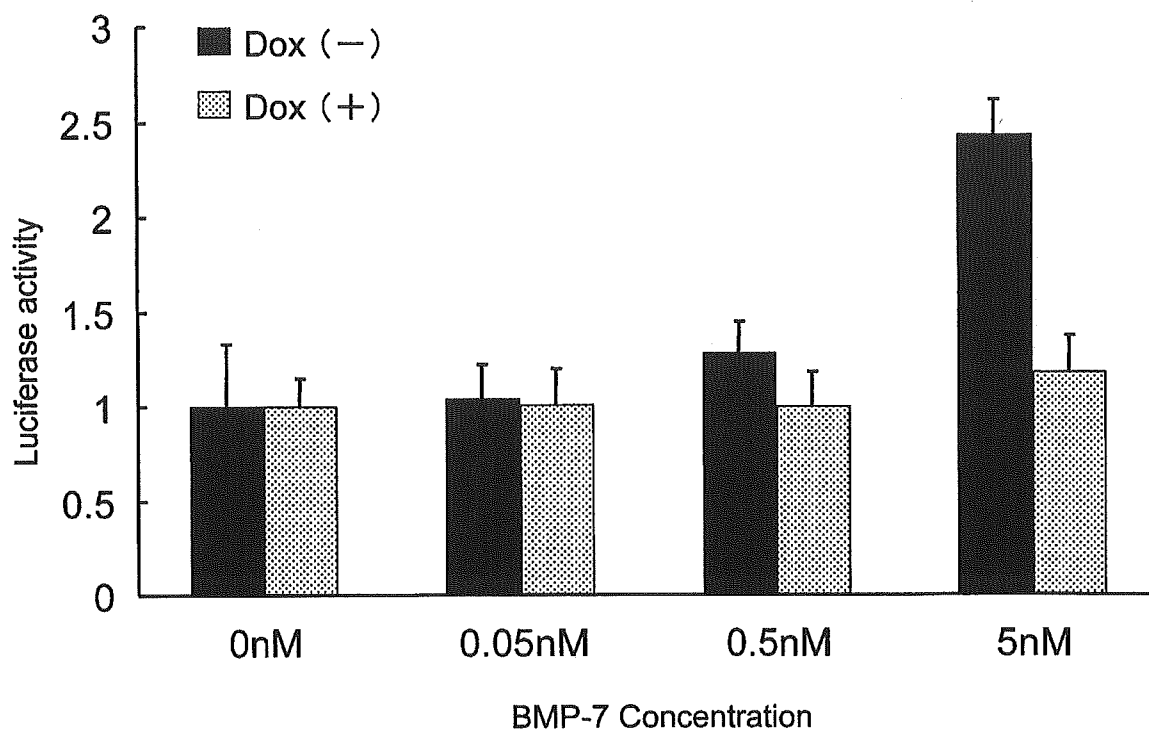


FIGURE 7 – Effects of enhanced GPC3 expression on BMP-7 signaling in HY-Toff cells. A fragment of the mouse Smad6 gene promoter, including the BMP-responsive element, was inserted into pGL2-Basic, and transcriptional activation activity was determined by luciferase assay in the presence (black bars) or absence (dotted bars) of Doxycycline using HY-Toff cells. Results are shown as fold-induction compared to the control cultures in the absence of BMP-7. Error bars represent standard deviations.

sion of GPC3. In contrast, overexpressed GPC3 strongly suppressed luciferase activity, even at the highest dose of BMP-7 (Fig. 7).

#### DISCUSSION

Recently, the overexpression of the mRNA for GPC3 has been demonstrated in embryonal tumors,<sup>16</sup> colon cancer<sup>17</sup> and HCC.<sup>18,19</sup> Our data based on oligonucleotide array analysis shows that GPC3 mRNA is highly expressed in 72% of liver cancer tissues even in 50% of WD. Moreover, the positive rate of GPC3 mRNA is higher than that of AFP, a tumor marker for HCC. This fact suggests that GPC3 is overexpressed in the early stage of HCC progression and may play a pivotal role in hepatocarcinogenesis. We also investigated expression of other members of glypican families using oligonucleotide array data, because glypican 1 has been reported to be upregulated in various cancers, such as pancreatic cancer and breast cancer.<sup>26,27</sup> U95A chip of GeneChip contains Glypican 1, 3, 4 and 5, and our data shows that glypican members except GPC3 were not upregulated in HCC compared to noncancerous tissues.

It has not been demonstrated that the GPC3 protein is in fact overexpressed in these cancers and this cancer-related cell line. Accordingly, to confirm overexpression of the GPC3 protein in HCC, we generated an anti-GPC3 monoclonal antibody and demonstrated it by Western blot and immunohistochemical analysis using hepatoma cell lines and surgically resected specimens. Although there was a slight discrepancy between the positive rate in HCC on Western blot and Northern blot analysis, the intensity of the expression of the GPC3 mRNA and 65 kDa protein in the resected specimens were significantly correlated. In the hepatoma cell lines HepG2, Hep3B, HT17, HuH6, HuH7 and PLC/PRF/5,

which exhibited moderate to high levels of GPC3 mRNA, they also exhibited a high expression of GPC3 in translation. In contrast, HLE and Li7, in which the GPC3 mRNA was not demonstrated, there was no signal of the GPC3 protein via Western blot analysis. Immunohistochemical analysis revealed a clear pattern of immunoactivity at the cell surface of HCC and, therefore, led us to believe that GPC3 protein is also overexpressed in HCC.

However, an upregulation of GPC3 in cancerous tissues would be contradictory to the function of this molecule as it is generally understood, and therefore, we endeavored to elucidate how an upregulation of GPC3 could exert a positive effect on cancer cell growth. We first observed whether an overexpression of GPC3 in HY-Toff cells is able to accelerate their progression. However, the cell proliferation rate was not altered by the presence or absence of GPC3 expression. We then directed attention to studying the effects of GPC3 on the heparin-binding growth factors, IGF2 and FGF2, as well as on the TGF- $\beta$  superfamily, BMP-7 and TGF- $\beta$ 3 because the HSPGs have been shown to interact with the heparin-binding growth factors and have been implicated in the signaling mediated by the TGF- $\beta$  families.<sup>1,2,28</sup>

It is controversial whether GPC3 can exert control over the activity of IGF2. Pilia *et al.* reported that GPC3 forms a complex with IGF2 and negatively regulates IGF2 activity by competing for IGF2 binding with the signaling receptor.<sup>11</sup> At the same time, IGF2R, which is known to be a negative regulator of IGF2, in IGF2-deficient mice does not exhibit any in its levels.<sup>29</sup> In addition, no direct interaction between IGF2 and GPC3 to date has been reported.<sup>12</sup> And our data from a cell growth assay did show that an enhanced expression of GPC3 was not able to alter cell proliferation induced by IGF2.

HSPGs are known to be coreceptors for FGF2, strongly promoting FGF-FGFR binding and the subsequent activation of the receptor.<sup>30,31</sup> For example, syndecan-2 promotes FGF2-mediated proliferation,<sup>32</sup> and glypican-1 stimulates the FGF2 signaling pathway.<sup>26</sup> On the other hand, it has also been reported that endothelial proteoglycans inhibit FGF2 binding and mitogenesis.<sup>6</sup> Mali *et al.* demonstrated that an overexpression of syndecan-1 inhibits FGF2-induced growth promotion and at the same time does not control the binding of FGF2 at the cell surface.<sup>5</sup> Consistent with these data, our findings show that an enhanced expression of GPC3 in HY-Toff cells inhibits the stimulatory effect of FGF2 on cell proliferation through the interaction between GPC3 and FGF2, which is compatible with the function of GPC3.

Like FGF2, HB-EGF is known to have strong affinity for heparin,<sup>33</sup> and the growth rate of HY-Toff cells was accelerated by HB-EGF in cell growth assay. In our study, induced expression of GPC3 did not affect cell proliferation, which suggests that HB-EGF signaling is independent of GPC3.

In contrast, the overexpression of GPC3 was also shown to exert control over the inhibitory growth factor, BMP-7. BMP-7 is known to control tubule cell proliferation and apoptosis in a dose-dependent manner via Smad1-dependent and -independent pathways,<sup>10</sup> and GPC3 has been demonstrated to modulate the effects of BMP-7 in the kidney of the GPC3-/mouse.<sup>4,34</sup> Our data depict similar effects of GPC3 on BMP-7 in liver cancer, that is, BMP-7 controls the cell proliferation of hepatoma cell lines and an enhanced expression of GPC3 is able to exert control over the BMP-7-induced inhibitory effects in hepatoma cell proliferation. Moreover, by means of reporter gene assay with the Smad6 pro-

motor, which contains a BMP-responsive element, we showed that overexpression of GPC3 actually inhibits the transcriptional activation induced by BMP-7. This function of GPC3 exerting control over the effect of an inhibitory growth factor is consistent with the extent of the upregulation of this molecule in HCC in our data.

In our study, we have demonstrated an overexpression of GPC3 in HCC. This molecule has been identified as a suppression of cell proliferation, and therefore, the fact that GPC3 interacts with FGF2 by inhibiting the activity of this growth factor is not surprising. At the same time, GPC3 also acts as a negative regulator of the inhibitory growth factor, BMP-7, controlling its transcriptional activation. Thus, GPC3 is able to not only inhibit cell proliferation in certain contexts but also can promote carcinogenesis in liver and other forms of cancer in others. To elucidate precisely, such different actions in these differing contexts will be important to understand the role GPC3 plays in the promotion of or protection against carcinogenesis in different biologic milieu. In addition, the ability of HCC cells to express GPC3 at high levels hints that GPC3 is possibly secreted in the serum of HCC patients and may serve as a new tumor marker for HCC.

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Review Article

## Vascular cell senescence and vascular aging<sup>☆</sup>

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### Abstract

Vascular cells have a finite lifespan when cultured *in vitro* and eventually enter an irreversible growth arrest called “cellular senescence”. A number of genetic animal models carrying targeted disruption of the genes that confer the protection against senescence *in vitro* have been reported to exhibit the phenotypes of premature aging. Similar mutations have been found in the patients with premature aging syndromes. Many of the changes in senescent vascular cell behavior are consistent with the changes seen in age-related vascular diseases. We have demonstrated the presence of senescent vascular cells in human atherosclerotic lesions but not in non-atherosclerotic lesions. Moreover, these cells express increased levels of pro-inflammatory molecules and decreased levels of endothelial nitric oxide synthase, suggesting that cellular senescence *in vivo* contributes to the pathogenesis of human atherosclerosis. One widely discussed hypothesis of senescence is the telomere hypothesis. An increasing body of evidence has established the critical role of the telomere in vascular cell senescence. Another line of evidence suggests that telomere-independent mechanisms are also involved in vascular cell senescence. Activation of Ras, an important signaling molecule involved in atherogenic stimuli, induces vascular cell senescence and thereby promotes vascular inflammation *in vitro* and *in vivo*. It is possible that mitogenic-signaling pathways induce telomere-dependent and telomere-independent senescence, which results in vascular dysfunction. Further understanding of the mechanism underlying cellular senescence will provide insights into the potential of antisenescence therapy for vascular aging.

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**Keywords:** Senescence; Aging; Atherosclerosis; Telomere; Ras; Akt; Inflammation; Cyclin-dependent kinase inhibitor

### 1. Introduction

Cellular senescence is the limited ability of primary human cells to divide when cultured *in vitro* and is accompanied by a specific set of phenotypic changes in morphology, gene expression and function. These changes in phenotype have been implicated in human aging [1]. This hypothesis, the hypothesis of cellular aging, was first described by Hayflick [2] and supported by evidence that cellular senescence and the division potential of human primary cultures are dependent on donor age [3] and that the growth potential of cultures correlates well with mean maximum lifespan of the species from which the cultures are derived [4]. Human primary cultures derived from the patients with premature aging syndromes, such as Werner syndrome and Bloom syn-

drome, are known to have shorter lifespan than the cultures from age-matched healthy populations [5], thus supporting the hypothesis of aging. However, until recently, little attention has been paid on the potential impact of vascular cell senescence *in vivo* on age-related vascular disorders.

In the past decades, significant progress has been made in our understanding of the mechanisms underlying cellular senescence. One widely discussed hypothesis is the telomere hypothesis of senescence [6]. A growing body of evidence has demonstrated a critical role of telomere and telomerase in regulating not only cellular lifespan but also organismal aging. However, recent findings suggest that cellular senescence can also be induced by DNA damage, cellular stress or oncogenic activation, which is independent of replicative age [7]. For example, the constitutive activation of mitogenic stimuli by expression of oncogenic Ras induces senescent phenotypes [8–10]. Thus, it is possible that atherogenic stimuli increase cell turnover at the sites of atherosclerosis, thereby promoting telomere shortening, whereas it also re-

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sults in activation of proliferative signals that potentially induce senescence independent of telomere shortening.

In this review, we will describe recently accumulating evidence that supports the hypothesis of cellular aging in the vasculature and discuss the potential of antisenesescence therapy for age-related human vascular disorders.

## 2. Occurrence of vascular cell senescence in vivo

Vascular cells have a finite lifespan in vitro and eventually enter an irreversible growth arrest called cellular senescence. Flattened and enlarged cell morphology is known to be one of the characteristics of cellular senescence [11]. Expression of negative regulators for the cell cycle machinery, such as p53 and p16, is increased with cell division and thereby promotes growth arrest [12]. Primary cultured cells undergoing cellular senescence in vitro express the increased activity of  $\beta$ -galactosidase ( $\beta$ -gal) when assayed at pH 6, which is distinguishable from endogenous lysosomal  $\beta$ -gal activity that can be detected at pH 4. This activity, senescence-associated  $\beta$ -gal (SA  $\beta$ -gal) activity, has been shown to correlate with cellular aging and thus is regarded as a biomarker for cellular senescence [13]. The in vitro growth properties of vascular cells derived from human atherosclerotic plaque are impaired, and they develop senescence earlier than those from normal lesions [14,15]. The histology of the lesions of human atherosclerosis has been extensively studied, and these studies have demonstrated that there are endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) that exhibit the morphological features of cellular senescence [16,17]. These suggest the occurrence of cellular senescence in vivo. Recently, this notion has been confirmed by cytochemical analysis in vivo using SA  $\beta$ -gal activity. Fenton et al. [18] have successfully detected SA  $\beta$ -gal-positive vascular cells in rabbit carotid arteries subjected to vascular injury. With repeated denudation, the accumulation of SA  $\beta$ -gal-positive cells was markedly enhanced. The authors have recently demonstrated SA  $\beta$ -gal-positive vascular cells in human atherosclerotic plaque of the coronary arteries obtained from the patients who had ischemic heart disease [19]. SA  $\beta$ -gal-positive cells were predominately localized on the luminal surface of atherosclerotic plaque and identified as ECs, but no positive cells were observed in the internal mammary arteries from the same patients where atherosclerotic changes were minimally observed. In advanced plaque, however, SA  $\beta$ -gal-positive VSMCs were detected in the intima but not in the media [20], which may represent extensive replication in the lesions, as observed in the arteries subjected to double-denudation (Fig. 1). SA  $\beta$ -gal-positive cells exhibit increased expression of p53 and p16, alternative markers for cellular senescence, in human atheroma, suggesting the further evidence of in vivo senescence. These cells also show impaired function, such as the decreased expression of endothelial nitric oxide synthase (eNOS) and the increased expression of pro-inflammatory molecules

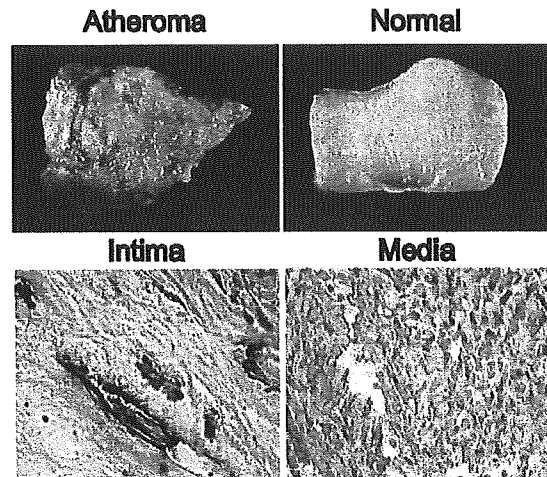


Fig. 1. Senescent vascular cells in human atheroma. Photographs of the luminal surface of human atheroma (atheroma, left) and non-atherosclerotic normal artery (normal, right) stained with SA  $\beta$ -gal staining. SA  $\beta$ -gal activity was observed in human atheroma but not in normal arteries (upper panel). A double staining for SA  $\beta$ -gal and  $\alpha$ -smooth muscle actin of the sections of atheroma identified SA  $\beta$ -gal-positive cells as VSMCs in the intima but not in the media (lower panel).

[20]. Thus, cellular senescence in vivo may contribute to the pathogenesis of vascular aging.

## 3. Role of cellular senescence in vascular pathophysiology

Age-associated changes in the blood vessels include a decrease in compliance and an increase in inflammatory response that promote atherogenesis [21]. It has also been reported that angiogenesis is impaired with advanced age [22,23], whereas aging decreases the antithrombotic property of the endothelium [24]. A number of studies have reported that many of the changes in senescent vascular cell behavior are consistent with known changes seen in age-related vascular diseases, suggesting a critical role of cellular senescence in vascular pathophysiology. The production of nitric oxide (NO) and the eNOS activity are reduced in senescent human ECs [25]. Induction of NO production by shear stress is also decreased in senescent ECs [26]. A decline in the eNOS activity of senescent ECs is attributable to a decrease in expression of eNOS protein as well as in eNOS phosphorylation mediated by Akt [27]. The levels of prostacyclin production are significantly decreased with in vitro aging of ECs [28], whereas senescent ECs upregulate plasminogen activator inhibitor-1 [29]. All these alterations are likely involved in the impairment of endothelium-dependent vasodilation but also increased sensitivity of thrombogenesis in human atherosclerosis. The interaction between monocytes and ECs is enhanced by EC senescence [30], thereby promoting atherogenesis. This appears to be mediated by upregulation of adhesion molecules and pro-inflammatory

cytokines as well as a decrease in the production of NO in senescent ECs [19,26]. It is reported that the ability to form capillary structures in vitro is reduced in senescent ECs [31]. Bone marrow-derived circulating endothelial progenitor cells (EPCs) are known to participate in postnatal neovascularization and vascular repair [32,33]. The in vitro growth property and function of bone marrow-derived EPCs are impaired in the patients with coronary artery disease and negatively correlate with risk factors including age [34,35]. Thus, aging may promote senescence of EPCs as well as ECs, resulting in decreases in angiogenesis and vascular healing.

#### 4. Telomere-dependent vascular cell senescence

Telomeres are non-nucleosomal DNA–protein complexes at the end of chromosomes that serve as protective caps and are substrates for specialized replication mechanisms. As a consequence of semi-conservative DNA replication, the extreme termini of chromosomes are not duplicated completely, resulting in successive shortening of telomeres with each cell division. Critically short telomere was thought to trigger the onset of cellular senescence, but recent studies suggest that the single-strand telomeric overhang and associated proteins are key components for signals of senescence [36,37]. Telomerase is a ribonucleoprotein that adds telomeres onto chromosome ends with its RNA moiety as a template. Forced expression of the catalytic component of telomerase TERT in telomerase-negative human fibroblasts results in the stabilization of telomere length and extension of cell lifespan [38]. This observation has established the importance of telomere shortening in human cellular senescence. It has been also reported that there is another mechanism of telomere maintenance without telomerase activity, called telomerase-independent alternative lengthening of telomeres (ALT) [39]. In the field of vascular biology, it has been shown that telomere shortening with cell divisions occurs in human-cultured ECs and VSMCs, and that introduction of TERT extends cell lifespan of human ECs and VSMCs, suggesting a critical role of telomere shortening in vascular cell senescence as well [31,40,41]. It is likely that shortened telomeres are in some way sensed in a cell, and that a pathway is activated that results in exit from cell cycle (Fig. 2). The p53, p21 and p16 proteins and their downstream effectors are important for cellular senescence, and thus are likely to be a part of the telomere-response pathway. However, less is known about what links telomeres and these factors. Until present, several telomeric-binding proteins have been identified that contribute to the integrity of telomere functions and are potentially involved in the telomere-response pathway [42]. These include protection of telomeres-1 (Pot-1) [43], Ku [44], telomeric repeat-binding factor 1 and 2 (TRF1 and TRF2, respectively) [45]. Pot-1 is identified as a telomeric protein that binds to a tip of telomere and is thought to constitute the telomere shortening signal [46]. The protein

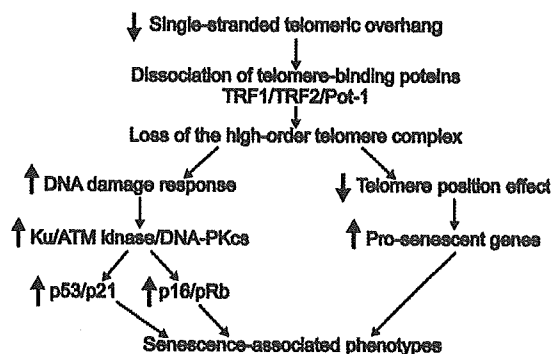


Fig. 2. Putative telomere-responsive pathways. As cells age, single-stranded telomeric overhangs are eroded, resulting in the dissociation of telomeric proteins. Loss of the high-order telomere complex is recognized as DNA breaks, thereby activating the molecules involving DNA repair that also play a critical role for telomere maintenance. These signals of DNA damage response are conducted to the cell cycle machinery. Telomere dysfunction also results in a release of telomere position effects that have the impact on expression of the genes at subtelomeric regions.

Ku, originally defined by its role in the repair of chromosomal DNA breaks, is found at telomeres and is necessary for normal telomere maintenance and functions. Genetic ablation studies demonstrated an essential role of Ku in mediating the telomere-response pathway [47]. Likewise, the catalytic subunit of the DNA-dependent protein kinase complex [48] and ATM kinase [49], both of which function in the double-strand break repair, have been implicated in the signal pathway of telomere shortening. Recently, it has been proposed that telomeres form large duplex loops, and telomeric proteins, TRF1 and TRF2, are essential for their formation [50,51]. Particularly, inhibition of TRF2 function is reported to cause cellular senescence in a p53/p16-dependent manner [19,52]. Thus, it is supposed that, as a result of telomere shortening, the efficacy of forming a high-order telomeric complex is impaired, leading to a release of various telomeric proteins from telomeres that elicit signals for cellular senescence (Fig. 2). Cytologically, telomeric regions are heterochromatic, implying that local DNA folding is increased. Positioning a gene in a telomeric heterochromatic region can impose telomere position effect on that gene. Telomere position effect, which has been characterized in the yeast and recently in human cells, is known to induce the reversible silencing of the gene [53]. Consequently, the modification of gene expression by telomere position effect may also contribute to the signaling pathway for cellular senescence.

#### 5. Telomere shortening and vascular dysfunction

There is evidence indicating that telomere shortening occurs in human vasculature, which may be related to age-associated vascular diseases [54]. In most of the previous reports mentioned, changes in cell phenotypes associated with senescence were studied in vascular cell populations

undergoing replicative senescence, thus suggesting telomere-dependent vascular dysfunction. However, it remains unclear whether phenotypic changes in senescent vascular cells result from telomere dysfunction. Inhibition of TRF2 has been shown to induce either cellular senescence or apoptosis in various cells by destroying telomere loop structure [51,55]. The authors have demonstrated that the introduction of a dominant-negative form of TRF2 into human ECs induces a growth arrest with phenotypic characteristics of cellular senescence [19]. Telomere dysfunction significantly increases expression of pro-inflammatory molecules and reduces the activity of eNOS, suggesting a causal link between telomere and vascular dysfunction associated with senescence.

Telomerase-deficient mice have been developed and found to reveal a normal phenotype at the first generation presumably because of mice having much longer telomeres [56,57]. Telomeres are shortened with successive generations, and they become infertile at the sixth generation due to the impairment of reproductive system. Some aspects of the late generation mice mimic age-associated phenotypes. They exhibit shortened lifespan and a reduced capacity to respond to stress, such as wound healing and hematopoietic ablation [58]. The ability of neovascularization is reduced in the late generation of telomerase-deficient mice [59]. Decreased vessel formation may be attributable to the impaired function and replicative capacity of ECs induced by telomere shortening. Recently, the heart in late generation of telomerase-deficient mice has been shown to mimic the end-stage-dilated cardiac myopathy in humans [60].

## 6. Telomerase

Since early studies reported that telomerase activity was detected in cancer cells but not normal somatic cells, the idea emerged that telomerase activity might be essential for tumor growth [61]. Multiple tumor-suppressor pathways are likely to repress telomerase expression in normal somatic cells [62]. Increasing evidence has suggested that telomerase activity regulates cell proliferation in normal somatic cells by telomere lengthening or upregulating growth-controlling genes in a telomere length-independent manner [57,63]. Human ECs and VSMCs express telomerase activity, which is drastically activated by mitogenic stimuli via a protein kinase C-dependent pathway [64] but the activity declined with *in vitro* aging due to a decrease in expression of TERT, leading to telomere shortening and cellular senescence [41,65]. Introduction of TERT prevents endothelial dysfunction associated with senescence, such as a decrease in eNOS activity and an increase in monocyte binding to ECs [19,26]. Immortalized human ECs (TERT-ECs) have been established by introduction of TERT [31]. TERT-ECs appear to retain EC characteristics including various cell surface markers. When examined in Matrigel, they form capillary-like structures in response to extracellular matrix signals as efficiently as early

passage of ECs, whereas senescent or transformed ECs do not. In addition, TERT-ECs are more resistant to apoptotic induction than pre-senescent ECs. They maintain a normal growth control and exhibit no transformed phenotype. These telomerized human ECs are functional *in vivo* as demonstrated by the Matrigel implantation mouse model [66]. In this model, whereas primary human EC-derived vessel density decreased with time after implantation, telomerized ECs maintained durable vessels, indicating that telomerase activity is important for the maintenance of a microvascular phenotype.

## 7. Telomere-independent vascular cell senescence

Signals other than extended proliferation have been shown to result in cells developing a phenotype indistinguishable from that of senescent cells at the end of their replicative lifespan. For example, the constitutive activation of mitogenic stimuli by expression of oncogenic Ras or E2F induce a senescent phenotype [8,67]. Cellular senescence triggered by mitogenic stimuli is independent of replicative age, and these signals act before the replicative limits of cells. Hence, it is apparently telomere independent and thus termed as premature senescence. The constitutive activation of Ras provokes premature senescence in vascular cells, which is associated with accumulation of the proteins p53 as well as p16 [20]. Activation of extracellular signal-regulated kinase (ERK) appears to be critical for Ras-induced senescence since inhibition of ERK restores cell growth arrest elicited by Ras activation [20], whereas introduction of an active form of ERK results in premature senescence involving p53 and p16 [9]. p38 mitogen-activated protein kinase (MAPK) is also implicated in Ras-induced senescence. p38 MAPK is activated in a ERK-dependent fashion, thus indicating that Ras promotes premature senescence by sequentially activating the ERK and p38 MAPK pathway [68].

## 8. Cell cycle regulators

It is clear that cellular senescence entails the activation of several tumor-suppressor proteins and inactivation of several oncoproteins, each of which ultimately engages either the p53 or pRb pathway and interacts with each other at multiple levels (Fig. 3). p53 activity and in some cases protein levels are increased in senescent cells. The mechanisms responsible for p53 activation in senescent cells remain elusive, however some molecular details are emerging. One cause of p53 activation may be an increase in expression of p14, a tumor-suppressor protein encoded by the INK4a locus. p14 activates p53 through a mechanism involving sequestration of MDM2, a protein that promotes degradation of p53 [69]. p14 is induced by oncogenic Ras and E2F [70], whereas TBX2, a member of T-box family of transcription factors, suppresses p14 expression [71]. Another cause of p53 activation is the



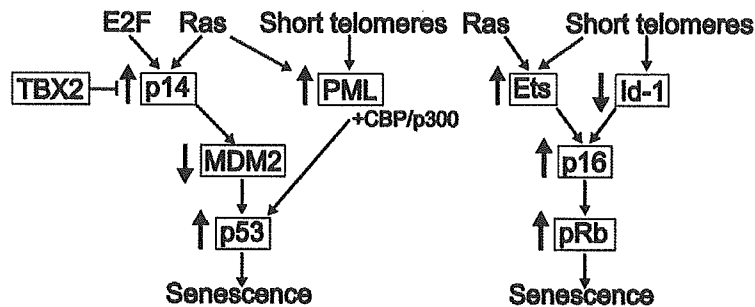


Fig. 3. Control of cellular senescence by the p53/p21 and p16/pRb pathways. Shown are the consequences of senescence-inducing signals on cell cycle regulators in the p53/p21 and p16/pRb pathways. Senescence-inducing signals, such as oncogenic Ras and E2F, increase expression of p14, whereas TBX2 represses p14 promoter activity, thus counteracting with each other. p14 sequesters MDM2, leading to an increase in p53 activity. Signals, such as oncogenic Ras, telomere shortening and possibly other signals increase expression of PML, which interacts with CBP/p300 and stimulates p53 activity. Oncogenic Ras stimulates the activity of Ets that induces p16 transcription by promoting its phosphorylation. Short telomeres lead to an increase in Ets and a decrease in Id, a protein that inhibits Ets activity, resulting in the accumulation of p16. p16 inhibits the cyclin-dependent kinases that phosphorylate pRb, leading to an increase in its active form.

promyelocytic leukemia (PML) tumor suppressor. PML is induced by replicative senescence and Ras activation by unknown mechanisms [72,73]. PML interacts with CBP/p300 acetyltransferase, which acetylates p53, thus stimulating p53 activity. Recently, p53 is found at telomeres and ablation of p53 function restores adverse effects of telomere loss [74], suggesting active roles of p53 in telomere maintenance as well as the telomere-response pathway. pRb exists in hypophosphorylated form that binds to E2F and inhibits cell cycle progression in senescent cells because of high levels of the cyclin-dependent kinase inhibitors, p21 and p16. p21 is transcriptionally induced at least partly by p53, although p53-independent, post-transcriptional mechanisms also contribute to an increase in p21 expression in senescent cells [75]. p16, another tumor-suppressor protein encoded by INK4a locus, increases in part because Ets, a transcription factor that stimulates p16 expression, is induced by senescent signals including telomere shortening and Ras activation, whereas Id1, a protein that inhibits Ets activity, is decreased in senescent cells [76]. It is demonstrated that ectopic expression of the cyclin-dependent kinase inhibitors, such as p21, p16 and p14, causes premature senescence [77], suggesting pivotal roles in the signaling pathways of senescence.

p53 immunoreactivity is present in vascular cells in areas with chronic inflammation of human atheroma, while a few cells positive for p53 immunoreactivity are found in control arteries [78]. p21 immunoreactivity is also detected in human atheroma but not in normal lesions and is colocalized with p53. Forced expression of cyclin-dependent kinase inhibitors induces premature senescence that is associated with cell dysfunction in cultured vascular cells (Minamino et al. unpublished data). These observations suggest a pathological role of p53 and p21 in atherogenesis. However, their precise roles remain unclear. It is demonstrated that atherosclerosis is aggravated in p53/apolipoprotein E (ApoE) double-knockout mice through an increase in p53-controlled proliferation [79]. In contrast, the study using perivascular collar model in ApoE-knockout mice shows that p53 overexpres-

sion results in a marked decrease in the cellular and extracellular contents in the cap lesions, leading to spontaneous plaque rupture [80]. Thus, in the clinical settings, elevated expression of p53 and cyclin-dependent kinase inhibitors may be deleterious in human atherosclerosis.

## 9. Ras-induced senescence and vascular inflammation

It has been demonstrated that various molecules including growth factors, vasoactive peptides and oxidative stresses, such as ROS and oxidized low-density lipoproteins, are induced during the lesion formation and regulate numerous critical cell functions, thereby contributing to atherogenesis [81]. These stimuli function as mitogens for vascular cells through the signaling cascades that activate Ras [81]. Inhibition of Ras has been reported to prevent intimal formation after vascular injury, suggesting a critical role of Ras activation in VSMC proliferation [82]. In addition to its role in cell proliferation, we have found that constitutive activation of Ras induces vascular cell senescence that is associated with vascular inflammation [20]. Activation of Ras drastically increased expression of pro-inflammatory cytokines partially through ERK activation in cultured vascular cells. Introduction of Ras into balloon-injured arteries enhanced vascular inflammation as well as senescence compared with control-injured arteries (Fig. 4). Moreover, senescent cells express inflammatory molecules in human atherosclerotic plaque, and ERK is activated in these cells, suggesting that telomere-independent mechanisms may also contribute to vascular cell senescence in human atherosclerosis. Consistent with our findings, functional inhibition of Ras has been demonstrated to suppress pro-inflammatory molecules, thereby reducing lesion formation in ApoE-deficient mice [83]. Moreover, angiotensin II, an important atherogenic molecule that activates the Ras-signaling pathway, has been demonstrated to promote vascular cell senescence as well as vascular inflammation [84]. Thus, it is assumed that atherogenic stimuli may

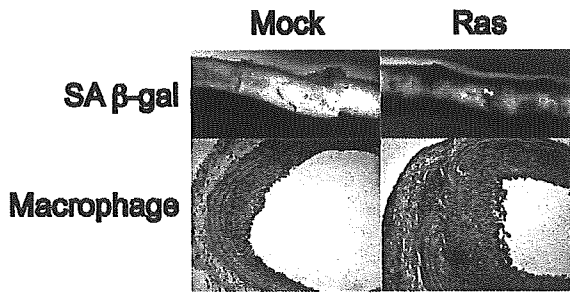


Fig. 4. Activation of Ras induces senescence and inflammation in vivo. The adenoviral vector encoding *H-ras*V12 (Ras) or the empty vector (Mock) was transduced into rat carotid arteries injured by a balloon catheter. It is known that accumulation of macrophages is minimally involved in the lesion formation in this model. Whereas only a little SA  $\beta$ -gal activity was found in mock-infected injured arteries, transduction of adeno-Ras into injured arteries increased SA  $\beta$ -gal activity (upper panel). The area of accumulated macrophages (brown) in the intima was markedly increased in Ras-infected injured arteries compared with mock-infected injured arteries (lower panel), indicating a causal relationship between Ras activation and vascular inflammation. Adapted from Ref. [20] with permission.

initially promote cell proliferation, and when overstimulated to proliferate, mitogenic-signaling pathways may induce telomere-dependent and telomere-independent senescence, which results in vascular dysfunction. Vascular inflammation is known to induce degradation of extracellular matrix by various proteinases, such as collagenases and gelatinases, and by inhibition of matrix production [85]. Therefore, decreased cellularity and enhanced inflammation associated with vascular cell senescence may contribute to plaque vulnerability.

## 10. Genetic models for aging

Many molecular mechanisms have been suggested to contribute to human aging and age-associated disease. Recent genetic analyses have demonstrated that reduction-of-function mutations of the signaling pathway of insulin/insulin-like growth factor-1 (IGF-1)/phosphatidylinositol-3 kinase (PI3K)/Akt (also known as protein kinase B) extends the longevity of the nematode *Caenorhabditis elegans* [86–92]. The forkhead transcription factor DAF-16, which is phosphorylated and thereby inactivated by Akt, plays an essential role in this longevity pathway [93,94]. More recently, it has been reported that the genes regulating longevity are conserved in organisms ranging from yeast to mice. The mutation of *Sch9*, which is homologous to *Akt*, extends the lifespan of yeast [95] and mutations that decrease the activity of insulin/IGF-1-like pathway increase the longevity of fruit flies [96] and mice [97,98]. These mutations that extend the lifespan are associated with increased resistance to oxidative stress, which is mediated in part by an increase in expression of antioxidant genes [99–101]. In mammalian cells, activation of Akt has been reported to induce cell proliferation and survival toward tumorigenesis [102–104].

The insulin pathway has also been shown to be essential for the maintenance of normal metabolic homeostasis [105]. Restriction of caloric intake extends the longevity of yeast, worms, fruit flies, mice and probably humans and postpones or prevents age-dependent deterioration and chronic diseases [91]. Since calorie restriction associates with the persistent decrease in the insulin signals, one might think that the insulin pathway could be involved in human aging and age-associated diseases, especially in the patients with diabetes.

Mice models that exhibit an early onset of phenotypes associated with aging have been reported. These include mouse mutants carrying targeted disruption of the genes involving DNA damage repair, such as *ku86* [106], *XPD* [107] and *BRCA1* [108]. Activation of p53 [109] as well as telomerase deficiency [58] also cause premature aging, which is characterized by reduced longevity, osteoporosis, organ atrophy and a diminished stress tolerance. All these molecules have been implicated in cellular senescence. More importantly, cellular senescence in vivo has been detected in premature aging mice [108]. Thus, these results provide in vivo evidence that links cellular senescence to organismal aging.

## 11. Conclusion

Accumulating evidence indicates a critical role of cellular senescence in organismal aging and age-related disease including atherosclerosis. Young adult bone marrow-derived EPCs have been shown to restore aging-impaired angiogenic function [110]. Chronic treatment with EPCs from young non-atherosclerotic ApoE-deficient mice prevents atherosclerosis progression in ApoE-deficient recipients despite persistent hypercholesterolemia [111]. Moreover, introduction of telomerase into EPCs has been reported to extend cell lifespan and to increase the efficacy of vasculogenesis in vivo [112]. These reports indicate that progressive progenitor cell deficits contribute to age-associated vascular dysfunction and suggest the potential utility of cell-based antisense therapy as a novel therapeutic strategy for vascular aging. Further understanding of mechanisms underlying cellular senescence will provide new insights into the pathogenesis of age-associated vascular disorders.

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Perspectivce

# Akt-Induced Cellular Senescence

## Implication for Human Disease

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### KEY WORDS

Akt, insulin, diabetes mellitus, atherosclerosis, senescence

### ABSTRACT

Reduction-of-function mutations in components of the insulin/insulin-like growth factor-1/Akt pathway have been shown to extend the lifespan in organisms ranging from yeast to mice. It has also been reported that activation of Akt induces proliferation and survival of mammalian cells, thereby promoting tumorigenesis. We have recently shown that Akt activity increases with cellular senescence and that inhibition of Akt extends the lifespan of primary cultured human endothelial cells. Constitutive activation of Akt promotes senescence-like arrest of cell growth via a p53/p21-dependent pathway, leading to endothelial dysfunction. This novel role of Akt in regulating the cellular lifespan may contribute to various human diseases including atherosclerosis and diabetes mellitus.

Normal human somatic cells have a finite lifespan in vitro and eventually show irreversible growth arrest called cellular senescence. As cells age in vitro, significant phenotypic changes occur. The cells become flattened and enlarged, as well as expressing different genes such as the tumor suppressor gene p53 and cyclin-dependent kinase inhibitors including p21<sup>Waf1/Cip1</sup> and p16<sup>Ink4a</sup>.<sup>1</sup> Signals other than extended proliferation have also been shown to produce a phenotype which is indistinguishable from that of senescent cells at the end of their replicative lifespan.<sup>2</sup> For example, constitutive activation of mitogenic stimuli, DNA damage, or oxidative stress can all prematurely induce cellular senescence.<sup>3-5</sup> In senescent vascular cells, expression of pro-inflammatory molecules is increased and production of vasodilators is decreased,<sup>6-9</sup> both of which are well-known changes in the vascular tissues of elderly persons that contribute to the pathogenesis of vascular aging.<sup>10</sup> There is also in vivo evidence cellular senescence.<sup>11,12</sup> Vascular cells with a similar phenotype to that of senescent vascular cells in vitro have been detected in human atheroma tissues, but not in normal arteries.<sup>6,7</sup> Such reports suggest that cellular senescence may play an important role in human aging and age-associated diseases. Alternatively, the mechanisms underlying cellular senescence may also regulate the lifespan of the entire organism and vice versa. This concept is supported by recent studies on mice that exhibit early onset of phenotypes associated with aging. These include mouse mutants with targeted disruption of the genes that are involved in the repair of DNA damage, such as *ku86*,<sup>13</sup> *XPD*<sup>14</sup> and *BRCAl*.<sup>15</sup> Activation of p53 as well as telomerase-deficiency also cause premature aging,<sup>16,17</sup> which is characterized by reduced longevity, osteoporosis, organ atrophy and a diminished stress tolerance. All of these molecules have been implicated in cellular senescence. More importantly, cellular senescence has been detected in the tissues of mice with premature aging.<sup>15</sup> These results provide further in vivo evidence that links cellular senescence to aging of the organism.

Restriction of calorie intake is known to extend longevity in organisms ranging from yeast to mice and to prevent age-dependent deterioration such as cancer, impaired immune function, and increased inflammation.<sup>18,19</sup> Calorie restriction decreases the plasma levels of glucose, insulin, and insulin-like growth factor-1 (IGF-1). Recent genetic analyses have demonstrated that reduction-of-function mutations in the glucose or IGF-1-like signaling pathways also extend the lifespan of many organisms, suggesting that these pathways may underlie the mechanism of longevity related to calorie restriction.<sup>18,20</sup> Among the molecules that exist in these pathways, the Ser/Thr kinase Akt is remarkably well conserved across a broad range of species and is involved in a diverse array of cellular processes.<sup>21</sup> Although mutations that reduce Akt activity prolong the longevity of yeast and nematodes,<sup>22,23</sup> activation of Akt has been reported to induce proliferation and survival of mammalian cells, thereby promoting tumorigenesis.<sup>21,24,25</sup> In a study that was recently published in *EMBO J*,<sup>26</sup> we showed that Akt activity increases with cellular senescence and that inhibition of Akt extends the lifespan of primary cultured human endothelial cells.

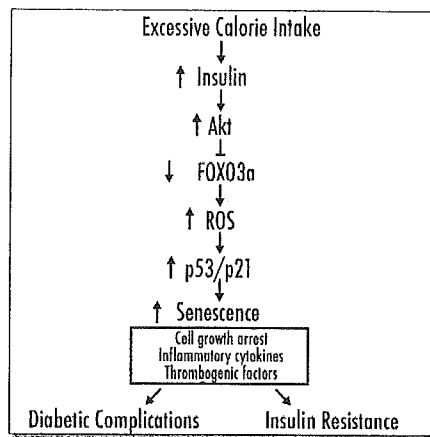


Figure 1. Signaling pathway of insulin/Akt-induced senescence. Excessive calorie intake increases the levels of insulin and results in activation of Akt in the target tissues. Constitutive activation of Akt induces cellular senescence via a p53/p21-dependent pathway, which is mediated by the forkhead transcription factor (FOXO3a), which regulates cellular levels of reactive oxygen species. Accumulation of senescent cells in the target organs causes diabetic complications and promotes insulin resistance because of their senescent phenotypes such as increased production of inflammatory cytokines and thrombogenic factors.

Constitutive activation of Akt promotes senescence-like arrest of cell growth via a p53/p21-dependent pathway. This action is at least partly mediated by the forkhead transcription factor, which regulates cellular levels of reactive oxygen species. Our findings reveal a novel role of Akt in regulating the cellular lifespan and suggest that the mechanism of longevity is conserved in primary cultured human cells.

What is the implication of these findings for human disease? Since various growth factors that contribute to atherosclerosis have been shown to increase Akt activity,<sup>27</sup> atherogenic stimuli may activate Akt in the vasculature and thus promote atherogenesis. Consistent with this notion, activation of Akt is observed in human atheroma tissues, but not in normal arteries.<sup>26</sup> Constitutive activation of Akt in human endothelial cells not only leads to cellular senescence but also to vascular dysfunction such as impaired angiogenesis and increased inflammation.<sup>26</sup> In a similar way, Akt-induced senescence may contribute to other age-associated diseases. Cell division is essential for the survival of multicellular organisms that contain various renewable tissues, but it also puts the organism at risk of developing cancer. Thus, complex organisms have evolved at least two cellular mechanisms to prevent oncogenesis, which are apoptosis and cellular senescence.<sup>2</sup> In this respect, Akt-induced senescence can be seen as an anti-tumorigenesis mechanism since gain-of-function mutations in the Akt signaling pathway are common in human cancers. Finally, Akt is known to be involved in signaling pathways that mediate the metabolic effects of insulin in several physiologically important target tissues.<sup>28</sup> Insulin regulates energy metabolism after food intake by promoting the uptake and storage of glucose, amino acid and fat, while simultaneously antagonizing the catabolism of fuel reserve. The basic role of insulin as a signal that informs the organism of nutritional abundance appears to be well conserved among species.<sup>28</sup> We have shown that insulin also increases p53 activity and expression of p21 and that it promotes cellular senescence in an Akt-dependent manner.<sup>26</sup> Since hyperinsulinemia is a basic feature of type 2 diabetes

mellitus, insulin-induced senescence may have the direct impact on diabetic complications such as vasculopathy and nephropathy. An inevitable feature of type 2 diabetes mellitus is insulin resistance.<sup>28</sup> Some previous studies have suggested that a decline of Akt activity in response to insulin might lead to insulin resistance.<sup>29</sup> In contrast, some recent studies have shown that basal Akt activity in the tissues of diabetic patients tends to be higher than in normal subjects and that there is no significant difference in the response of Akt to insulin stimulation.<sup>30</sup> Increased plasma and tissue levels of pro-inflammatory cytokines and pro-thrombogenic factors have been demonstrated to exaggerate insulin resistance and to contribute to diabetic complications,<sup>31,32</sup> and both of these changes are well-known features of senescent cells in vitro.<sup>12</sup> Accordingly, we propose that type 2 diabetes mellitus can be regarded as a premature aging syndrome in which the dysregulation of insulin/Akt signaling promotes cellular senescence, leading to various complications. This suggests that anti-senescence therapy might be effective for the treatment of diabetic complications and insulin resistance.

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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<sup>1</sup>. 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<sup>2</sup>.

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<sup>3,4</sup>. 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<sup>5</sup>. Pretreatment of cardiomyocytes with angiotensin II (AII) type (AT1) receptor blockers significantly attenuates all of these mechanical-stretch-induced events<sup>6,7</sup>. 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<sup>8-12</sup>.

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<sup>6</sup>. 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 cardiomyocytes

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<sup>7</sup> (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.

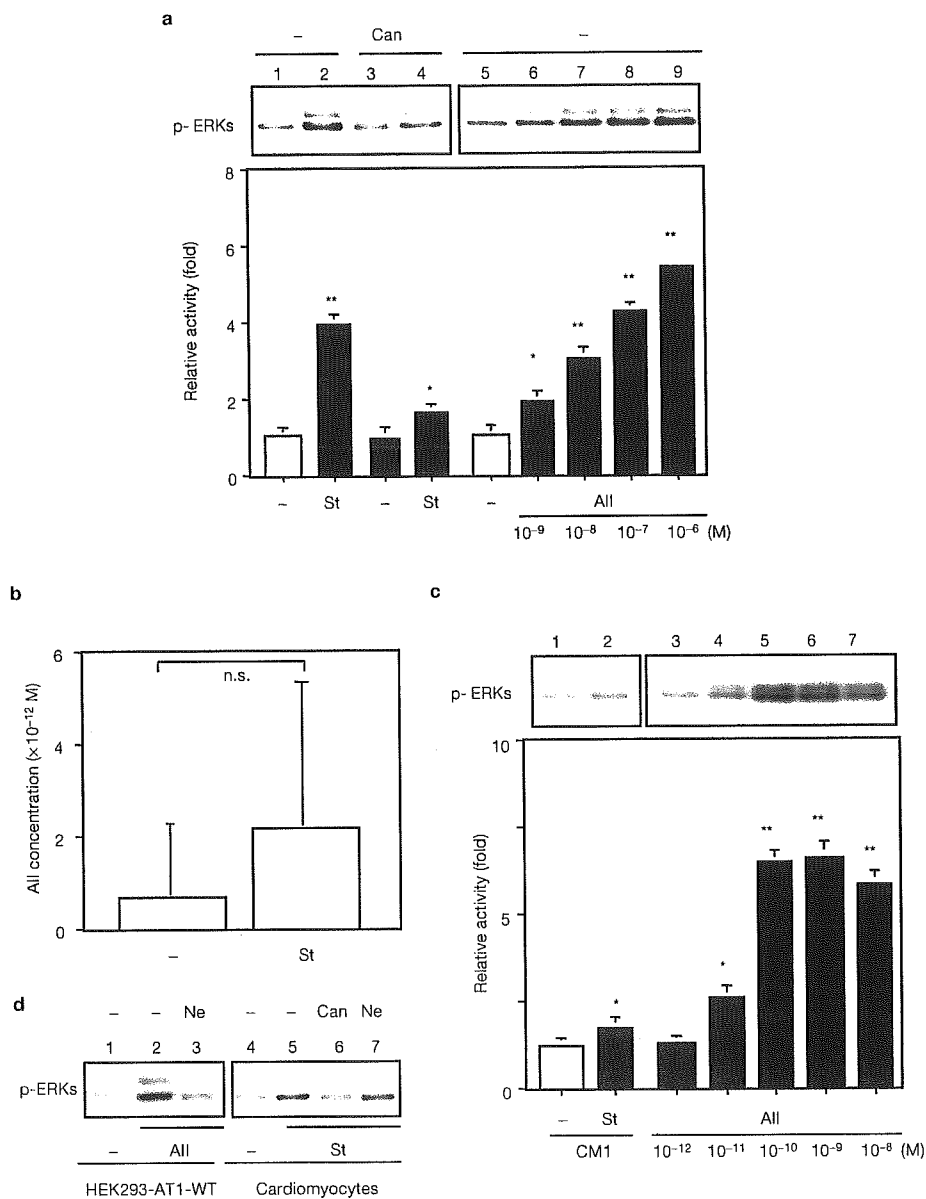
We first used radioimmunoassay to measure AII in culture medium conditioned by stretching cardiomyocytes for 8 min, but did not detect a significant increase in AII concentration after stretch (AII without stretch,  $0.7 \pm 1.6 \times 10^{-12}$  M; AII with stretch,  $2.0 \pm 3.5 \times 10^{-12}$  M;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

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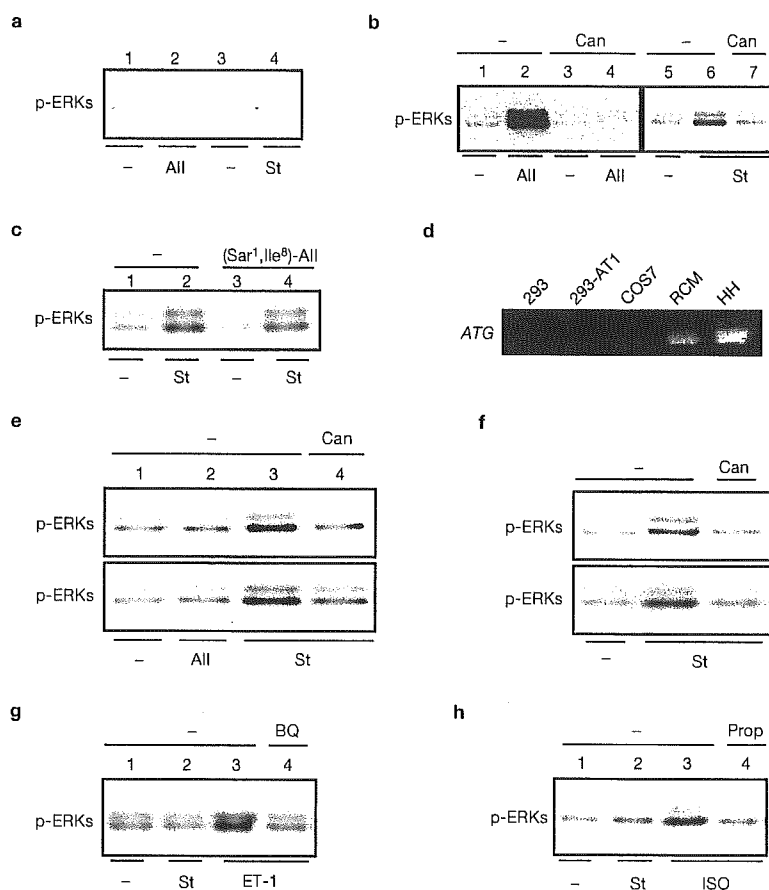
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**Figure 1** Activation of ERKs by mechanical stretch, AII and conditioned medium. (a) Cultured cardiomyocytes of neonatal rats were pretreated with  $10^{-7}$  M candesartan (Can) or vehicle (-), and either stretched by 20% (St) or incubated with 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  $\pm$  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  $\pm$  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 ( $10 \mu\text{g ml}^{-1}$ ; Cortex Biochem).



**Figure 2** All-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<sup>1</sup>,Ile<sup>8</sup>)-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*<sup>-/-</sup> mice were pretreated with candesartan, and then stimulated with mechanical stretch. (g, h) COS7 cells transiently transfected with ET1A (g) or  $\beta$ 2-AR (h) receptors were pretreated with an ET1A antagonist BQ123 (BQ), a  $\beta$ -AR blocker propranolol (Prop) or vehicle (-), and then stimulated with mechanical stretch, ET-1 or ISO.

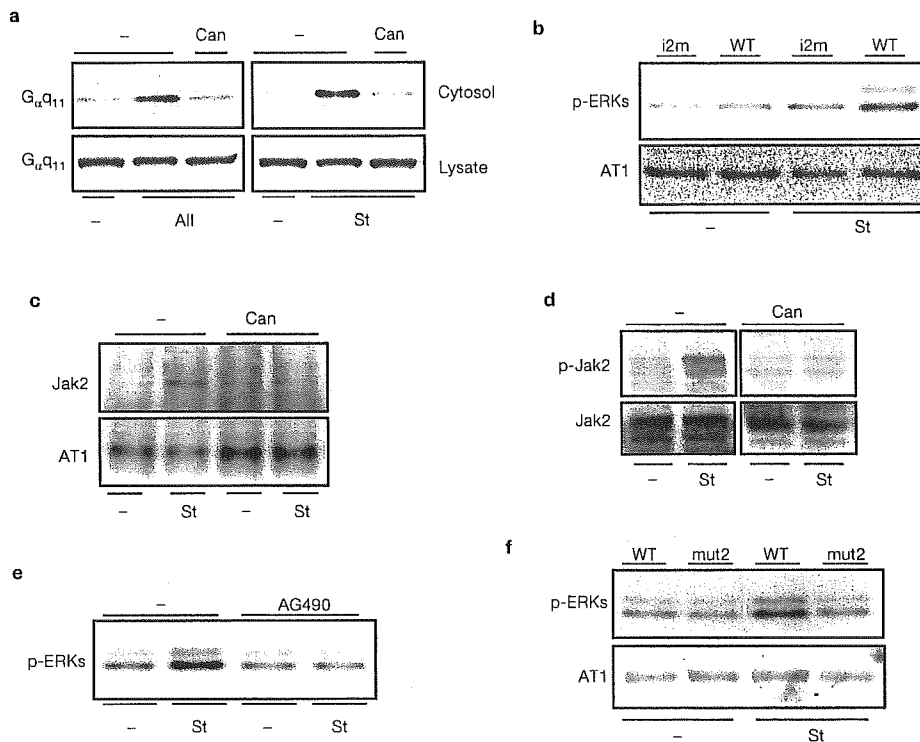
wild-type mouse AT1a receptor (hereafter denoted HEK293-AT1-WT cells). The medium conditioned by stretching cardiomyocytes for 8 min only slightly activated ERKs in HEK293-AT1-WT cells (Fig. 1c, lane 2). Its activity was equivalent to that induced by incubating HEK293-AT1-WT cells with  $10^{-12}$  to  $10^{-11}$  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<sup>1</sup>,Ile<sup>8</sup>)-AII, did



**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 antibodies against  $G_{\alpha q11}$ . (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)<sup>13</sup>. 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*<sup>-/-</sup>) mice, in which AII is not detected<sup>14</sup>. Mechanical stress activated ERKs in the cardiomyocytes prepared from both neonatal (Fig. 2f, top) and adult *ATG*<sup>-/-</sup> 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.