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IGF-I enhances cellular senescence via the reactive oxygen species–p53 pathway

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ABSTRACT

Cellular senescence is characterized by growth arrest, enlarged and flattened cell morphology, the expression of senescence-associated β-galactosidase (SA-β-gal), and by activation of tumor suppressor networks. Insulin-like growth factor-I (IGF-I) plays a critical role in cellular growth, proliferation, tumorigenesis, and regulation of aging. In the present study, we show that IGF-I enhances cellular senescence in mouse, rat, and human primary cells in the confluent state. IGF-I induced expression of a DNA damage marker, γH2AX, the increased levels of p53 and p21 proteins, and activated SA-β-gal. In the confluent state, an altered downstream signaling of IGF-I receptor was observed. Treatment with a reactive oxygen species (ROS) scavenger, *N*-acetylcystein (NAC) significantly suppressed induction of these markers, indicating that ROS are involved in the induction of cellular senescence by IGF-I. In p53-null mouse embryonic fibroblasts, the IGF-I-induced augmentation of SA-β-gal and p21 was inhibited, demonstrating that p53 is required for cellular senescence induced by IGF-I. Thus, these data reveal a novel pathway whereby IGF-I enhances cellular senescence in the ROS and p53-dependent manner and may explain the underlying mechanisms of IGF-I involvement in tumorigenesis and in regulation of aging.

1. Introduction

Normal somatic cells cultured *in vitro* display a limited ability to divide and eventually enter a state of irreversible proliferative arrest termed replicative cellular senescence [1]. The definition of cellular senescence has expanded to include the phenotypically similar growth arrest, which is caused by various cellular stresses, including DNA damage and oxidative stress [2]. Irreversible growth arrest is also induced by the expression of activated oncogenes, such as Ras [3], Raf [4], and by activation of tumor suppressor genes [5,6]. Moreover, cellular senescence is characterized by enlarged, flattened cell morphology [7], the expression of senescence-associated β-galactosidase (SA-β-gal) [8], activation of tumor suppressor networks [9], and by the appearance of senescence-associated heterochromatic loci (SAHF) [10].

Induction of cellular senescence results in the accumulation of tumor suppressors p19Arf and p53. Cellular senescence caused by p53 is associated with the regulation of p53-dependent genes (e.g., *p21*), which participate in cell cycle arrest. Accordingly, recent studies indicate that cellular senescence functions as an important tumor-suppressive mechanism to restrict tumor development [11,12]. On the other hand, activation of cellular senescence and the aging process are closely related. The number of senescent cells increases with age, senescent cells are present at sites of age-related pathology, and increased senescent cells are causally related to tissue aging—decrements in neurogenesis, hematopoiesis, and pancreatic function [13]. A trade-off between tumor suppression and aging is seen in mice that express constitutively hyperactive forms of p53;—while these animals are remarkably tumor-free, they show multiple signs of accelerated aging [5].

Insulin-like growth factor-I (IGF-I) plays a critical role in the regulation of cellular growth and proliferation, and reduced IGF-I signaling results in growth retardation [14]. The IGF-I/insulin pathway also controls life span and aging in organisms ranging from worms to mammals. In contrast to its deleterious effects on growth and proliferation, reduced IGF-I signaling has beneficial effects on longevity [15]. The effect of IGF-I on tumor formation in humans is demonstrated by the finding that patients with acromegaly who display elevated levels of serum IGF-I, exhibit an increased risk of colon and thyroid cancer, which are thought to be driven by the proliferative effect of IGF-I [16]. Conversely, individuals with growth hormone (GH) receptor deficiency (GHRD), also known as Laron syndrome, who display GH insensitivity and congenital IGF-I deficiency, and individuals with congenital isolated GH deficiency, who also have very low IGF-I levels, appear to be protected from the development of cancer [17,18]. Recently, link between the IGF-I axis and the p53 pathway has emerged. Specifically, p53 negatively regulates IGF-I signaling and biological

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activity by a number of mechanisms [19]. On the other hand, IGF-I induces p53 expression [20,21]; however, the pathophysiological significance and underlying mechanisms remain unknown.

Senescence is associated with both activation of the expression of a number of inflammatory genes and with the senescence-associated secretory phenotype (SASP). Intriguingly, senescent cells secrete inflammatory cytokines, such as interleukin-6 (IL6) and IL8, which reinforce the senescent phenotype in an autoparacrine manner [22,23]. Moreover, anti-proliferative cytokines such as interferon-β [24] and transforming growth factor-β [25] induce reactive oxigen species (ROS)-dependent cellular senescence. Taken together, these data highlight the importance of regulation of cellular senescence by auto-, para-, and endocrine factors. Thus, we aimed to investigate the role of IGF-I in the regulation of cellular senescence.

2. Materials and methods

2.1. Cell culture

VSMCs were isolated from thoracic aorta of 3-day-old Sprague–Dawley rats by enzymatic digestion [26]. MEF were derived from $p53^{+/+}$ and $p53^{-/-}$ mice as previously described [27], and p53 knockout mice were obtained from Riken (Tsukuba, Japan). Cells passaged 3–7 times were used for experiments. All mouse experiments were performed according to the guidelines of the Animal Ethics Committee of the Kobe University Graduate School of Medicine. Human fibroblasts were derived from a 17-year-old man and a 27-year-old woman as previously described [28]. Experiments were performed using cells passaged 3–7 times and cells were maintained in DMEM containing 10% FBS. IGF-I stimulation was performed in serum-free DMEM as previously described [29]. The human study was approved by the Kobe University Graduate School of Medicine Ethics Committee.

2.2. Materials

Recombinant human IGF-I was obtained from the Fujisawa Pharmaceutical Company Ltd., (Osaka, Japan). *N*-Acetyl-cysteine (NAC) and Tiron (4,5-dihydroxy-1,3-benzene -disulfonic acid, disodium salt monohydrate) were purchased from Sigma (St Louis, MO). The following antibodies were used in immunoblotting: H2AX (Upstate Biotechnology), p53, phosphor-Rb, p16 (Cell Signaling), p21 (Santa Cruz Biotechnology), pERK, pAkt (Cell Signaling), and β-actin (Sigma).

2.3. Immunoblotting

Cells were lysed in lysis buffer containing 50 mM Tris HCl pH 8, 150 mM NaCl, 1 mM EDTA, 1% NP-40, protease (Nacalai Tesque, Kyoto, Japan) and phosphatase (Pierce Thermo Fisher Scientific, IL) inhibitor cocktail (25). Proteins were loaded in Laemmli buffer, subjected to SDS–PAGE, and transferred to a PVDF membrane. The membrane was incubated with primary antibody overnight at 4 °C. After incubation with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, bands were visualized with Chemi-Lumi One L solution (Nacalai Tesque, Kyoto, Japan) using the LAS-3000 CCD Imaging System (Fujifilm Corporation, Tokyo, Japan).

2.4. Quantitative real time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). Subsequently, 500 ng of RNA was subjected to cDNA synthesis using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative real time PCR was performed with SBYR mix Ex Taq™II (Takara, Japan) and an ABI PRISM 7500 Real-Time PCR System (Applied Biosystem, Tokyo, Japan). β-Actin was employed as internal control. The sequence of primers used are as follows: p21 forward, ACGGTGGAACTTTGACTTCG and reverse, GACCCAGGGCTCAGG AGAT; SESN1 forward, ACTGGAAAGCGTTAGGCAGA and reverse, TGGACA GCATAAGCAGATGG; GADD45 forward, CCCTCATTCGTGCTTTCTGT and reverse, GGCTCTTGTCGTTCTCCAGT; β-actin forward, TGA CCCTGAAGTACCC CATT and reverse, GGGGTGTTGAAGGTCTCAAA.

2.5. Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase (SA-β-gal) staining was performed as previously described [30]. Briefly, cells were fixed with PBS containing 3.7% formaldehyde for 5 min at room temperature, washed, and incubated with SA-β-gal staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl P3-d-galactoside (X-Gal) (Zymoresearch, CA), 40 mM citric acid/sodium phosphate buffer (pH 6.0), 5 mM potassium ferricyanide (Nacalai Tesque, Kyoto, Japan), 5 mM potassium ferrocyanide (Nacalai Tesque, Kyoto, Japan), 150 mM NaCl, and 2 mM MgCl2. After 20 h, X-Gal-positive blue staining cells were counted in 5 random fields visualized by bright field microscopy (Olympus CKX31, 20× magnification).

2.6. Statistics

Results were presented as the mean ± SEM and analyzed by ANOVA for multiple groups. A value of $p < 0.05$ was considered significant. The data shown in the figures are representative of 3 or 4 independent experiments.

3. Results

3.1. IGF-I induces cellular senescence in rat, mouse, and human primary cells

To clarify the role of IGF-I in the regulation of cellular senescence, we first investigated the effect of IGF-I on the proliferation status of rat vascular smooth muscle cells (VSMCs). We could not detect any significant changes in the SA-β-gal activity as a marker of cellular senescence (Fig. 1A and B); however, we observed a strong induction of SA-β-gal activity following IGF-I stimulation in the confluent cells (Fig. 1A–C). One of reactive oxygen species (ROS), H_2O_2, which is known to stimulate cellular senescence, also induced SA-β-gal activity on the confluent state (Fig. 1A–C). Similar results were also observed in mouse and human fibroblasts (data not shown). The effect of IGF-I stimulation was concentration-dependent (Fig. 1D). We subsequently examined the levels of p53 and p21 proteins, which are the other markers for cellular senescense. As expected, the expression of both p53 and p21 was increased by IGF-I stimulation in a concentration-dependent manner (Fig. 2A and B). In contrast, no significant changes were observed in the levels of phosphorylated Rb or p16 protein (data not shown). These result demonstrated that IGF-I enhances cellular senescence on the confluent state and suggested it might be via p53-dependent pathway. A time course experiment revealed that at 24 h after IGF-I stimulation, levels of γH2AX protein—a marker for DNA damage—were significantly increased (Fig. 2C), followed by augmentation of p53 protein levels (Fig. 2D). Subsequently, expression of the p21 protein significantly increased after 48 h (Fig. 2E). These results suggested that IGF-I induced cellular senescence via DNA damage-p53-p21 pathway. To verify the effect of induction of p53 by IGF-I, we analyzed the mRNA expression of p53 target genes. The expression of *p21*, the antioxidative stress protein Sestrin1 (*SESN1*), and the growth arrest and DNA

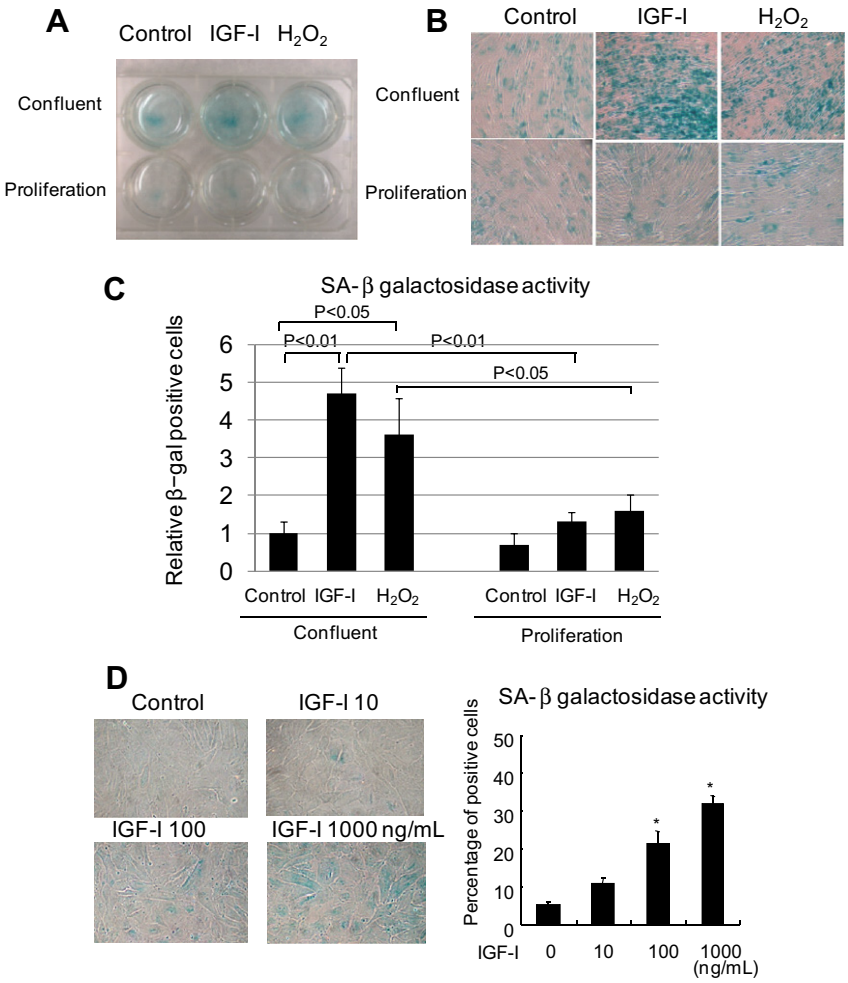

Fig. 1. IGF-I enhances cellular senescence in the confluent state in VSMCs. (A) IGF-I and H_2O_2 induced SA-β-gal activation in the confluent state but not in the log phase, proliferation status. VSMCs were incubated with IGF-I at the indicated concentrations for 5 days. (B) SA-β-gal stating. X100 (C) The quantitative analysis of SA-β-gal positive cells. Data were expressed as the mean ± SEM of 5 random fields ($n = 5$). (D) IGF-I induced SA-β-gal activation in a concentration-dependent manner. Quantitative analysis demonstrated that IGF-I significantly increased SA-β-gal-positive cells in VSMCs. Data were expressed as the mean ± SEM of 5 random fields ($n = 5$).

damage-induced gene45 (*GADD45*) were significantly increased by IGF-I stimulation, supporting the hypothesis that IGF-I stimulated the p53 transcriptional activity (Fig. 2F).To further clarify the physiological relevance, we examined whether the induction of cellular senescence by IGF-I was observed in other types of cells. As observed in rat VSMCs, IGF-I significantly increased SA-β-gal activity (Fig. 2G), as well as levels of p53 (Fig. 2H) and p21 (Fig. 2I) in human fibroblasts. Other human fibroblasts displayed similar results (data not shown). Furthermore, IGF-I also induced cellular senescence in mouse embryonic fibroblasts (MEFs) (Fig. 4E).

3.2. Cell confluence-dependent altered IGF-I receptor signaling

To further dissect the mechanisms in the intracellular signaling, we compared the downstream signaling of IGF-IR in VSMCs between in the proliferation and confluent status. We first analyzed Akt activation by IGF-I. There was no difference in the degree of phosphorylation in Akt by IGF-I treatment between in the proliferation and confluent status (Fig 3A and B). Intriguingly, although IGF-I induced strong phosphorylation of ERK in the proliferation status, the induction of phosphorylation was significantly decreased in the confluent status (Fig 3A and C).

3.3. IGF-I induces cellular senescence via the ROS–p53 pathway

To explore the underlying molecular mechanisms, we examined the involvement of ROS in the induction of cellular senescence by IGF-I. As shown in Fig. 4A, treatment with an ROS scavenger, *N*-acetylcysteine (NAC), significantly suppressed the IGF-I-induced increase in SA-β-gal activity. Furthermore, NAC treatment also suppressed the induction of γH2AX, p53, and p21 proteins (Fig. 4B–D), indicating that ROS are involved in IGF-I-induced cellular senescence. Treatment with an alternative ROS scavenger, Trion, also produced similar results (data not shown). In addition, we used *p53−/−* MEFs to clarify the role of p53 in IGF-I-induced cellular senescence; in the absence of p53, IGF-I-induced SA-β-gal activation (Fig. 4E) and p21 augmentation (Fig. 4F) were inhibited.

4. Discussion

Although cellular senescence was originally described as an *in vitro* cellular characteristic [1], it has recently emerged that senescence is involved not only in protection against tumor development, but also in the process of aging and age-related diseases *in vivo* [13]. In the present study, we demonstrated that IGF-I enhances cellular senescence in 3 types of cells (primary rat VSMCs,

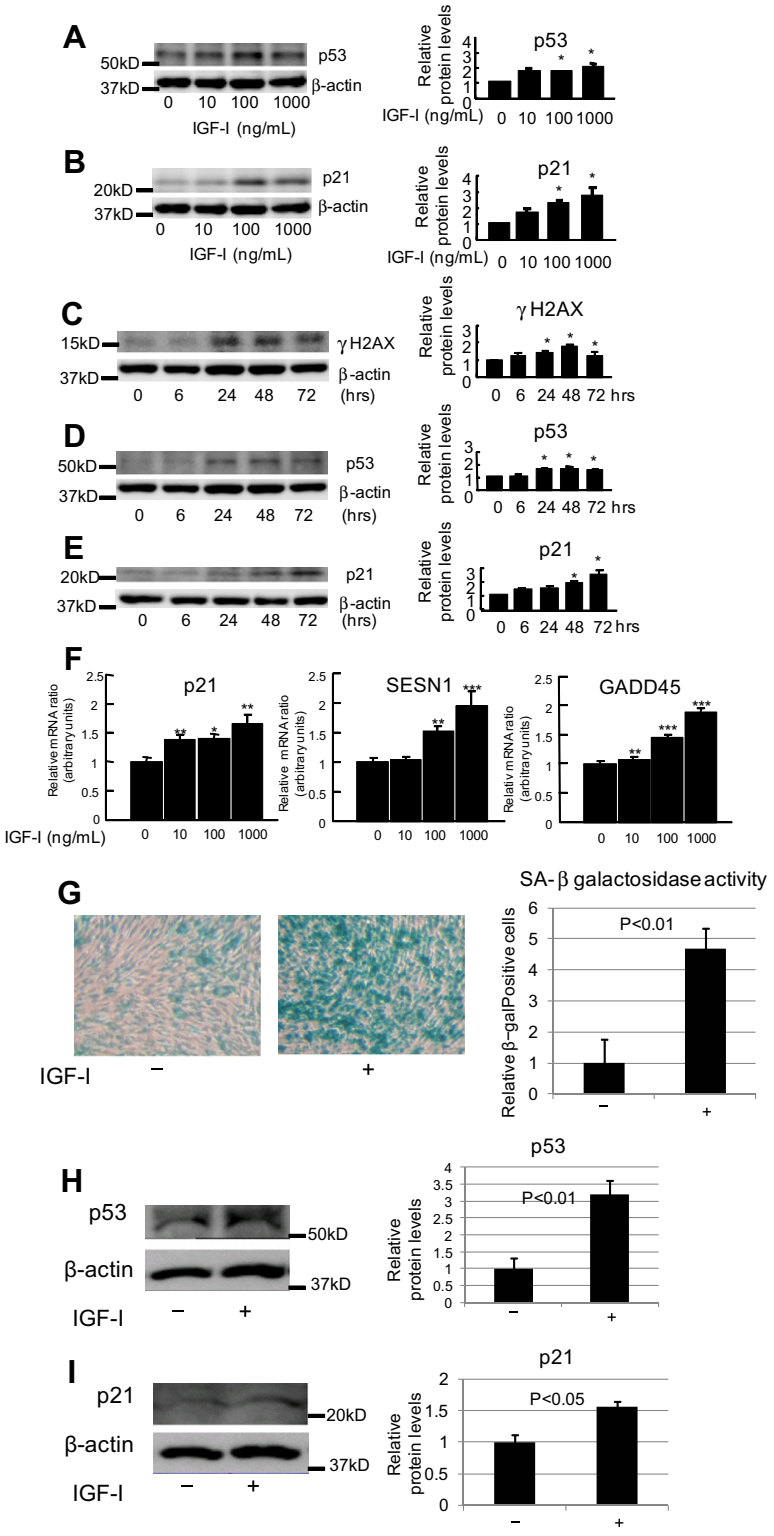

Fig. 2. IGF-I enhances cellular senescence markers in VSMCs and human fibroblasts. (A and B) IGF-I increased expression of p53 and p21 protein in a concentration-dependent manner in VSMCs. Cells were incubated with IGF-I for 72 h. Quantitative analysis demonstrated that IGF-I significantly increased p53 and p21 protein expression. IGF-I induced expression of the DNA damage marker H2AX (C), the p53 protein (D) and the p21 protein (E) in a time-dependent manner. VSMCs were incubated with 100 ng/mL IGF-I for the indicated times. Densitometric analyses were performed using data from 3 independent experiments. Each value was normalized to β-actin. (F) IGF-I induced expression of p53-target genes. After a 72 h incubation with 100 ng/mL IGF-I, the mRNA expression of *p21*, *Sestrin1*, and *GADD45* was analyzed by quantitative real-time PCR. The value of each target gene was normalized to β-actin (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control. (G) IGF-I induced SA-β-gal activation in human fibroblasts. After 5 days of incubation with 100 ng/mL IGF-I, SA-β-gal staining was performed to detect senescent cells. Data were expressed as the mean ± SEM of 5 random fields ($n = 5$). IGF-I increased p53 (H) and p21 (I) protein levels in human fibroblasts. After a 72 h of incubation with 100 ng/mL of IGF-I, immunoblotting was performed.

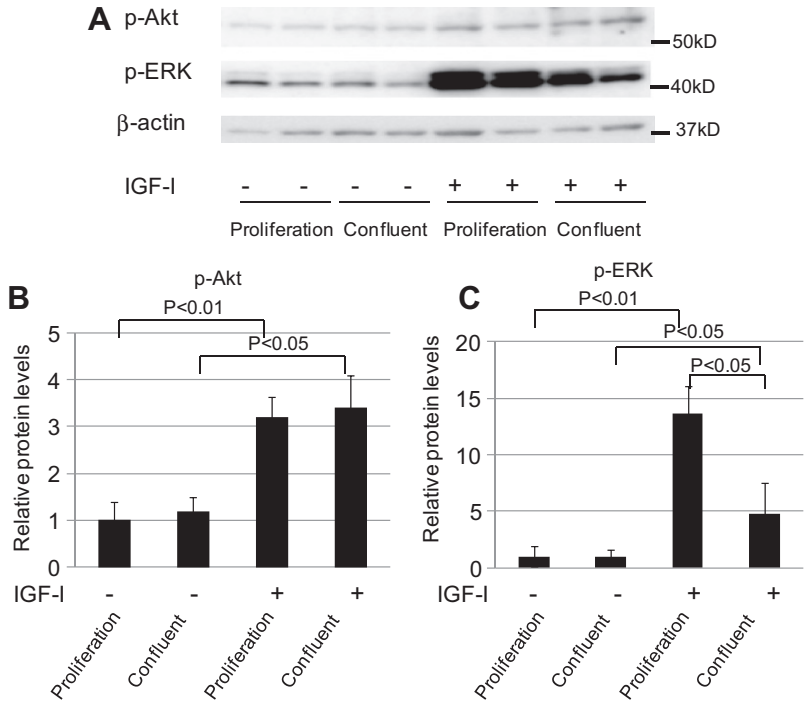

Fig. 3. Altered IGF-I receptor signaling between in the proliferation and confluent status. (A) IGF-I induced Akt and ERK phosphorylation in VSMCs. Although there was no change in the phosphorylation of Akt induced by IGF-I stimulation between the proliferation and confluent status (B), ERK phosphorylation was significantly decreased in the confluent status than in the proliferation status (C). After a 15 min incubation with 100 ng/mL of IGF-I, immunoblotting was performed.

human fibroblasts, and MEFs), suggesting a common effect of IGF-I on various cell types and species. Moreover, these data imply an important role for IGF-I function in tumorigenesis and in the regulation of life span.

We demonstrated that cellular senescence was induced by IGF-I via the ROS–DNA damage–p53 pathway. Although it has been reported that IGF-I induces p53 expression [20,21], the physiological significance and the mechanisms underlying IGF-I function remained unclarified. The present data indicated that IGF-I induces DNA damage and increases expression of the p53 protein. Moreover, although we cannot rule out the possibility that IGF-I signaling might be affected by the absence of p53 protein, the results from *p53−/−* MEFs demonstrated that cellular senescence induced by IGF-I was p53-dependent. IGF-I is a growth factor involved in cell proliferation and promotes the development of several cancers, including colon and thyroid cancer. It is possible that IGF-I induces cellular senescence to restrain cancer development simultaneously with the stimulation of proliferation. When p53 function is impaired by mutations, IGF-I promotes more aggressive tumor growth. Indeed, mutations in the *p53* gene play a pivotal role in the development of colon [31] and thyroid cancer [32], which are reportedly associated with acromegaly.

IGF-I/insulin signaling negatively regulates aging. Although the involvement of several downstream molecules, such as FOXO and mTOR, have been proposed, the underlying molecular mechanisms have not been fully clarified [33]. The physiological relevance of cellular senescence in generating age-related phenotypes *in vivo* has been demonstrated by the finding that removal of senescent cells can prevent or delay tissue dysfunction and extend health span [34]. Furthermore, mice that express constitutively hyperactive forms of p53 are remarkably tumor-free, but these animals show multiple signs of accelerated aging [5], suggesting a pro-aging role for p53. Taken together, it is likely that IGF-I regulates aging at least in part via a mechanism involving the p53 protein and IGF-I-induced cellular senescence.

The senescent phenotype is induced in the response to multiple stressors, including dysfunctional telomeres, non-telomere DNA damage caused by UV and ROS, and excessive mitogenic signals produced by oncogenes [13]. IGF-I has been shown to stimulate ROS production, via NOX4 [29,35]. Moreover, these ROS are involved in the migration of VSMCs [35], insulin resistance in adipocytes [36], and myocyte hypertrophy [29] in the action of IGF-I in these cells. Thus, these data suggest that ROS play an important role in the biological activity of IGF-I. In addition to these effects, our data clearly demonstrate that IGF-I enhances cellular senescence via ROS production.

Oncogene-induced senescence has been observed in oncogenic forms of RAS, RAF, MEK, MOS, and BRAF [13]. Many oncogenes induce a robust DNA damage response caused by DNA hyper-replication, which lead to senescence [37]. We showed that IGF-I causes DNA damage, as demonstrated by an increase in γH2X protein, implying a common mechanism with oncogene-induced senescence. Intriguingly, mouse cells cultured in serum-free medium resist RAS-induced senescence [38,39]. Taken together with our data, it is hypothesized that IGF-I present in serum may play an important role in oncogene-induced senescence by complementary mechanisms. We also showed that IGF-I effectively enhanced senescence in confluent, but not in proliferating cells. As a mechanistic insight, we demonstrated that in the confluent state, in contrast to the pAkt, the level of pERK induced by IGF-I was down-regulated. Intriguingly, it has been reported that angiotensin-II-induced cellular senescence requires activation of Akt and down-regulation of ERK via ROS-dependent mechanisms [40]. These changes in the downstream signaling may modulate the IGF-I-induced cellular senescence.

In conclusion, we demonstrated a novel cascade for IGF-I action via ROS to induce cellular senescence in a p53-dependent manner. Although further investigation is necessary to clarify the detailed molecular mechanisms and physiological significance, the present

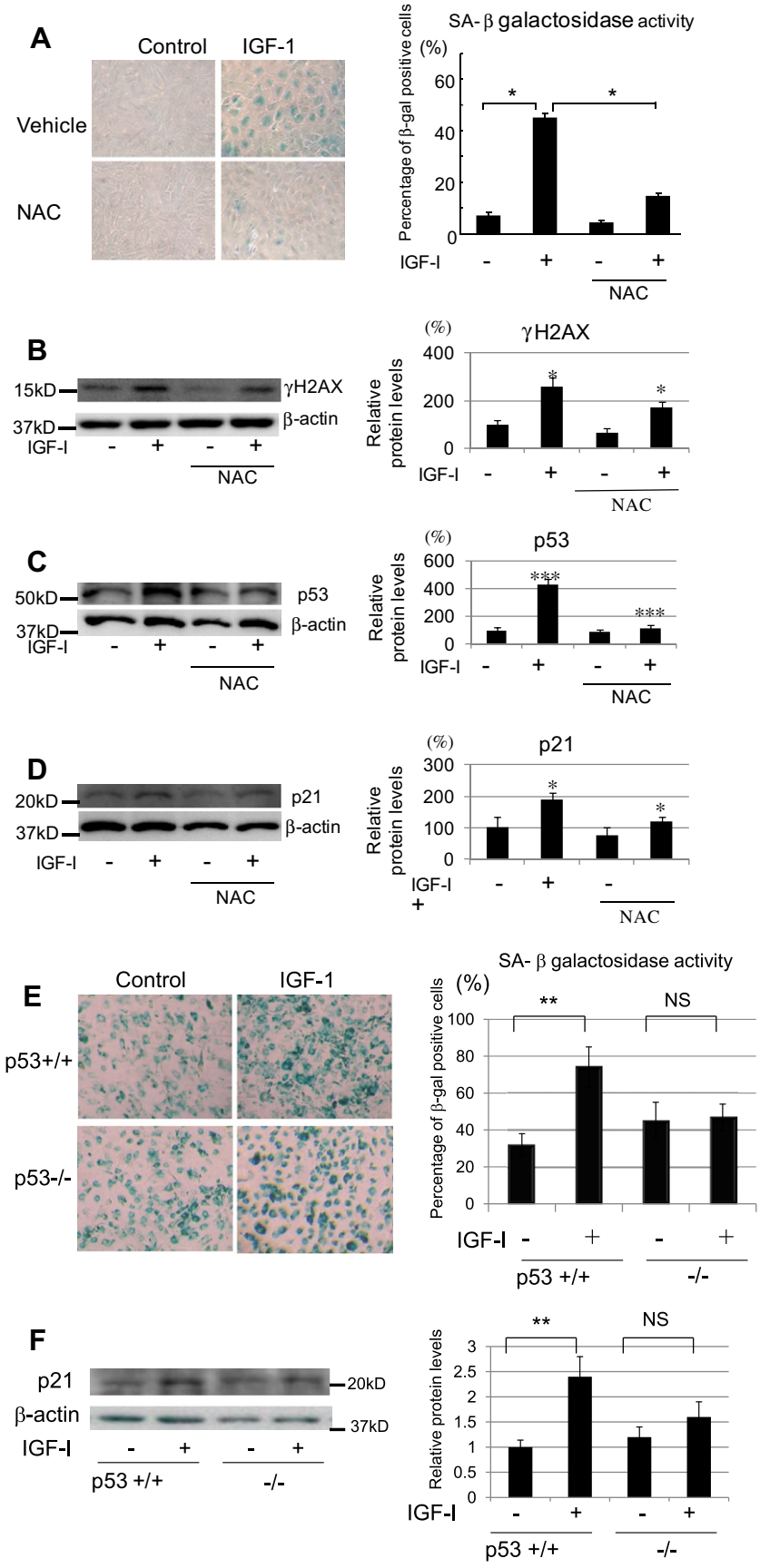

Fig. 4. ROS and p53 are involved in the IGF-I-induced cellular senescence in VSMCs. (A) Treatment with an antioxidant, *N*-acetyl cysteine (NAC) suppressed IGF-I-induced SA-β-gal activation. NAC treatment suppressed IGF-I-induced expression of γH2AX (B), p53 (C), and p21 (D). VSMCs were treated with NAC 30 min before IGF-I stimulation. Cells were incubated with or without IGF-I or NAC for 48 h for immunoblotting or for 5 days for SA-β- gal staining. (E) In contrast to *p53*+/+ MEFs, IGF-I did not increase the number of SA-β-gal-positive cells or levels of p21 protein (F) in *p53*-null MEFs. $^{*}P < 0.05$ $^{**}P < 0.01$. $^{***}P < 0.001$.

data may explain the effect of IGF-I on tumorigenesis and regulation of aging.

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