defined as the changes in RTL normalized to the number of cell division ($|\frac{\Delta RTL}{\Delta PDL}|$), was significantly higher in IGF-I-treated cells than in the control cells, while there were no significant differences observed between the GH-treated and control cells (Fig 2C and 2D), indicating that IGF-I, and not GH, shortened telomeres *in vitro*.

It has been reported that telomere shortening causes cellular senescence via the p53-p21 pathway [21] and IGF-I enhances cellular senescence in confluent human fibroblasts [20]. We explored whether IGF-I itself induces cellular senescence using human fibroblasts. Our study design is illustrated in S1A Fig. Fibroblasts treated with GH or IGF-I for 10 days (PDL of 10–11) showed comparable expression of p53 and p21 to vehicle-treated cells at the mRNA and protein levels (S1B–S1F Fig). However, after stimulation of GH or IGF-I for 27 days (PDL of 21–22), IGF-I, but not GH, treatment significantly increased the expression of p53 and p21 mRNA (Fig 3A and 3B). IGF-I also increased p53, serine-phosphorylated p53, and p21 protein levels (Fig 3C–3E). Considering the number of PDL, these results suggest that after the telomere shortening caused by IGF-I treatment, induction of the p53-p21 pathway occurred. Comparably, not GH but IGF-I increased senescence-associated β-galactosidase (SA β-gal) activity at day 38 (PDL of 28–29) (Fig 4A–4C). Regarding the cell growth curve, as previously

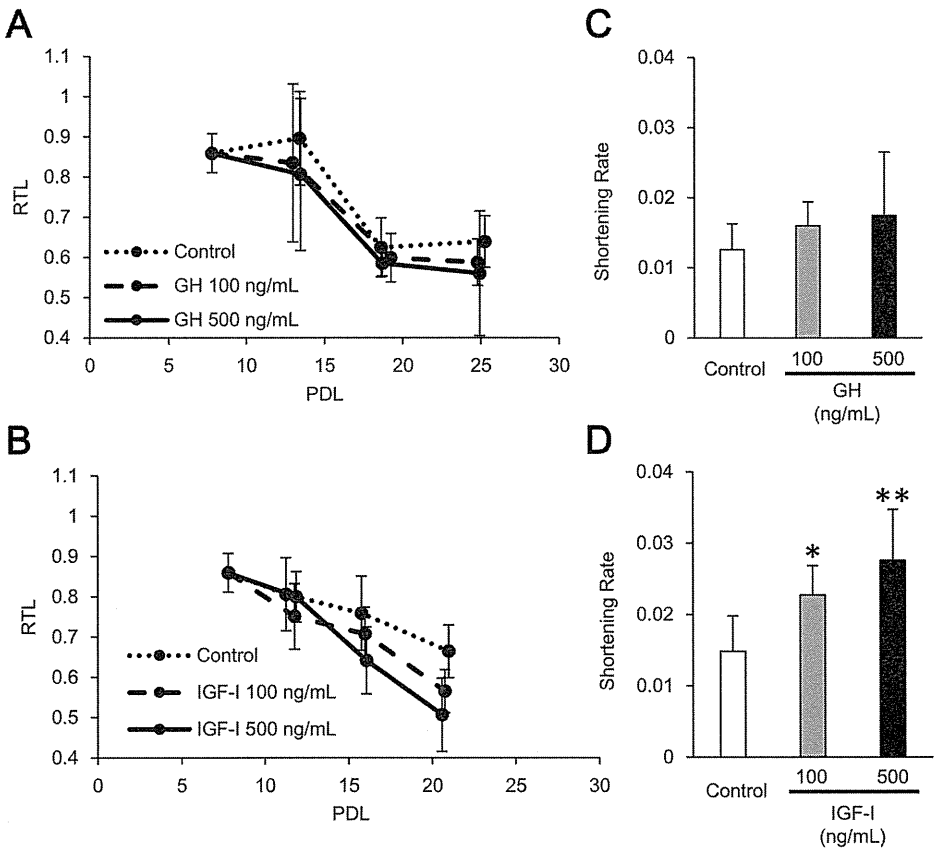

Fig 2. Telomere length in human fibroblasts with GH and IGF-I treatment. A, B, Longitudinal changes in relative telomere length in human fibroblasts treated with GH and IGF-I. **C, D,** Telomere shortening rate in human fibroblasts treated with GH and IGF-I. All data are expressed as mean ± standard deviation. Data were compared using one-way analysis of variance followed by post hoc Fisher's least significant difference test. *$P < 0.05$, **$P < 0.01$. PDL, population doubling levels; RTL, relative telomere length.

doi:10.1371/journal.pone.0140189.g002

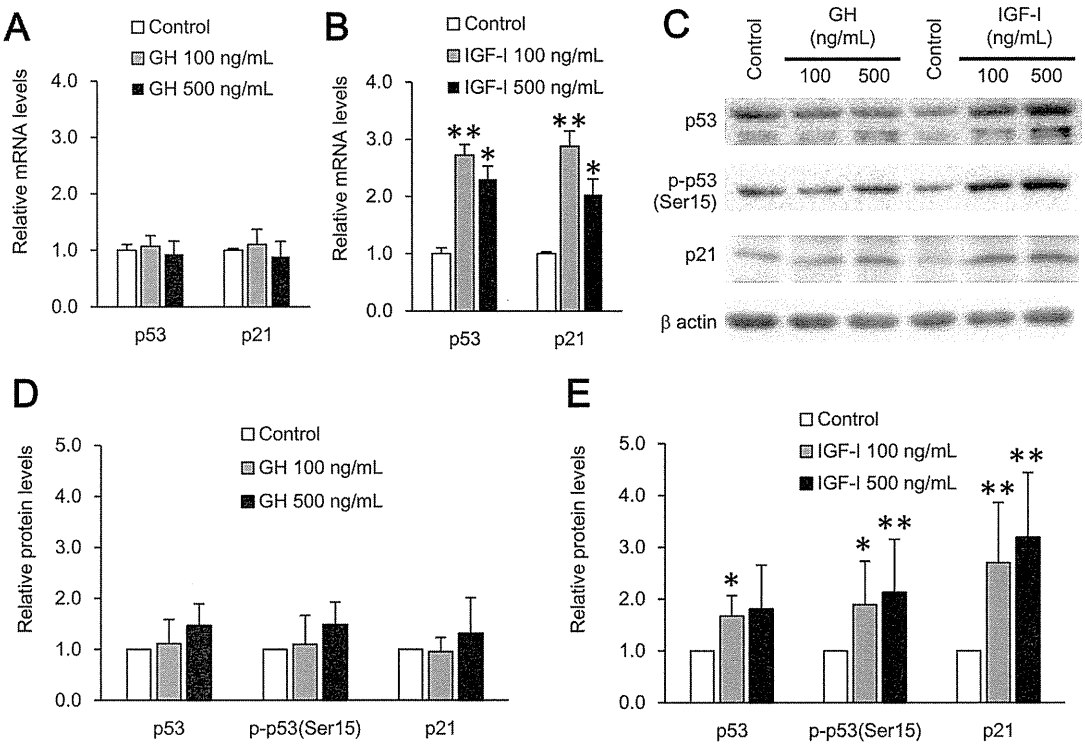

Fig 3. IGF-I enhanced p53-p21 pathway in human fibroblasts. A, B, p53 and p21 mRNA expression in human fibroblasts treated with GH or IGF-I for 27 days (PDL of 20–21). The expressions were measured using quantitative RT-PCR and normalized to β-actin. **C,** Immunoblotting analysis of p53, serine-phosphorylated-p53 (p-p53), and p21 proteins in human fibroblasts treated with GH or IGF-I for 27 days (PDL of 20–21). Anti-p-p53 protein at serine 15 antibody was used for the detection of p-p53. **D, E,** Densitometric analysis of p53, p-p53, and p21 protein levels normalized to β-actin. All data are expressed as mean ± standard deviation. Data were compared using one-way analysis of variance followed by post hoc Fisher's least significant difference test. $^{*}P < 0.05$, $^{**}P < 0.01$; PDL, population doubling levels.

doi:10.1371/journal.pone.0140189.g003

described, IGF-I-treated cells proliferated faster than control cells in the first 10 days of stimulation, whereas GH did not show any such growth effect (S3A and S3B Fig). However, the effect on proliferation of IGF-I did not last more than 2 weeks and eventually each cell showed similar doubling time in the logarithmic growth phase (Fig 4D and 4E). Intriguingly, fibroblasts treated with IGF-I reached the Hayflick limit approximately 5 PDL earlier than GH- or vehicle-treated cells (Fig 4D and 4E), clearly demonstrating that IGF-I induces cellular senescence. Furthermore, we examined the effect of GH or IGF-I on IL-6 mRNA expression, because it has been reported that cellular senescence is associated with an inflammatory response through production of inflammatory cytokines or chemokines (senescence-associated secretary phenotype; SASP) [22]. Indeed, IGF-I-treated cells showed increased mRNA expression of IL-6 (S4 Fig).

Discussion

In the present study, we demonstrated that patients with acromegaly exhibited shorter telomeres than the age-, sex-, smoking-, and diabetes-matched patients with NFPA in peripheral leukocytes. In addition, there was a significant negative correlation between telomere length

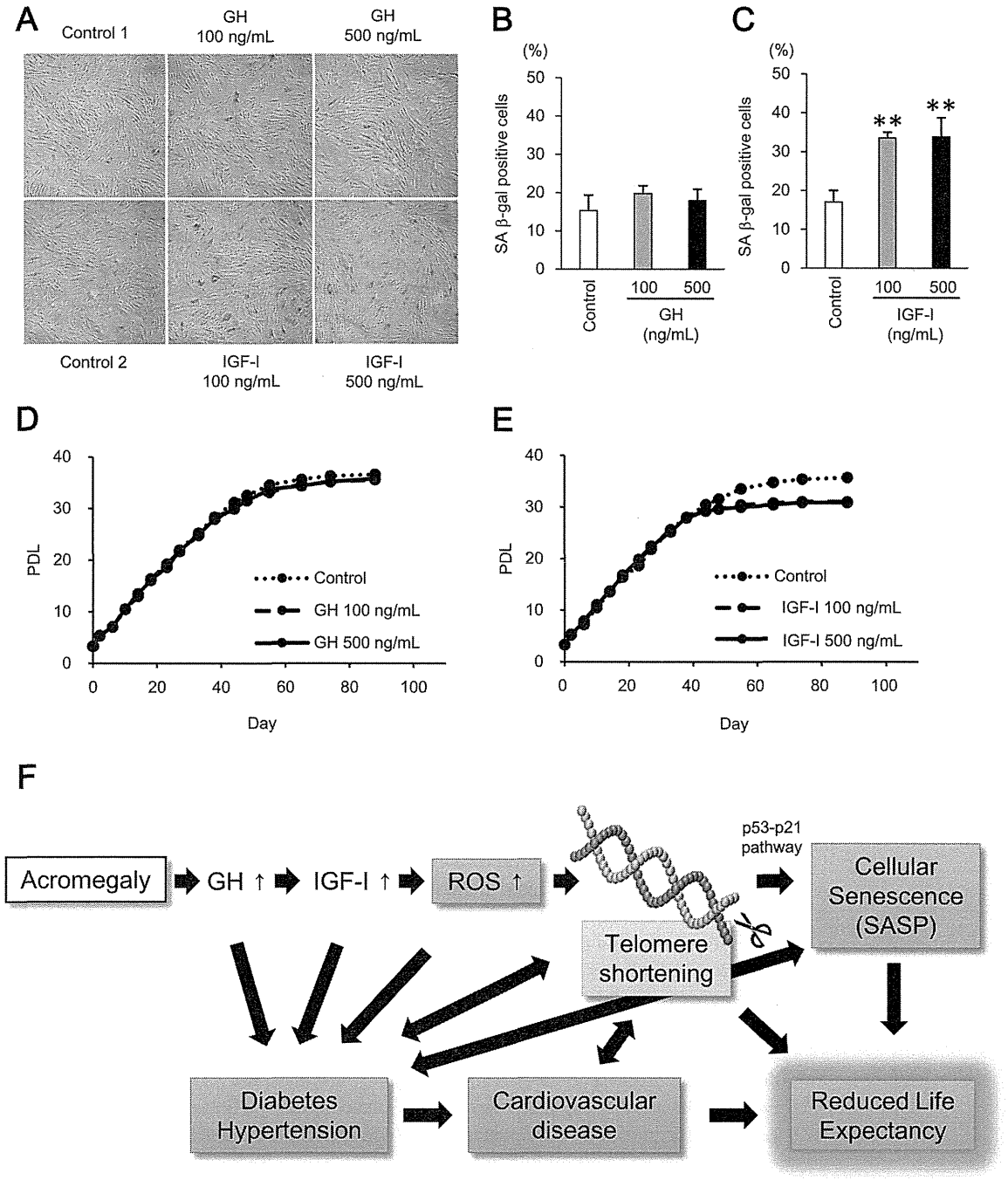

Fig 4. IGF-I induced cellular senescence in human fibroblasts. A, Senescence-associated β-galactosidase staining in human fibroblasts treated with GH or IGF-I for 38 days (PDL of 28–29). **B, C,** Rate of senescence-associated β-galactosidase activity (SA β-gal)-positive cells. **D, E,** Cell growth curves of human fibroblasts treated with GH or IGF-I. **F,** Proposed mechanistic model, in which excess secretion of GH and IGF-I causes increased comorbidities and mortality in patients with acromegaly. Data are expressed as mean ± standard deviation. Data were compared using χ^2 test followed by Tukey's honestly significant difference test. $^{**}P < 0.01$; PDL, population doubling levels.

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and disease duration in acromegalic patients. These data suggest that excessive secretion of GH or IGF-I is associated with telomere shortening.

Accumulating evidence demonstrates a clear link between telomere length and mortality in various conditions; for example, a pioneering study by Cawthon et al. reported that subjects aged 60 years or older with shorter telomeres revealed a significant increase in the all-cause mortality rate compared to those with normal telomere lengths [6]. Furthermore, telomere shortening is reportedly associated with increased age-related diseases such as cardiovascular disease, cerebrovascular disease, hypertension, and diabetes [11, 23, 24]. It is well known that patients with acromegaly exhibit increased morbidity of age-related disease such as cardiovascular disease, cerebrovascular disease, hypertension, and diabetes, and premature mortality [25, 26]. Taken together with the present data, it is speculated that the shortened telomeres in acromegalic patients are closely associated with these pathological conditions, although it remains unknown whether the shortened telomeres play a causal role.

When telomeres become critically short, they become dysfunctional and activate the DDR pathway that generally is caused by double-strand breaks [27]. The resulting signaling cascade induces apoptosis and/or a permanent cell cycle arrest, and cellular senescence via the p53-p21 pathway. In the present report, we have demonstrated that not GH but IGF-I induced telomere shortening and cellular senescence in human fibroblasts. Recently, it has emerged that cellular senescence plays important roles not only in cancer protection but also in the development of age-related diseases such as diabetes and atherosclerosis, which are often associated with acromegaly, and aging itself [28, 29]. The onset of these age-related diseases is reportedly associated with SASP [22]. In line with this, IGF-I-treated cells showed an increased IL-6 mRNA expression, which is known as SASP-related cytokine. In conjunction with the effect of telomere shortening, the resulting cellular senescence may be associated with the increased morbidity and mortality in acromegalic patients.

In this study, we clearly demonstrated that not GH but IGF-I induced telomere shortening and cellular senescence in human skin fibroblasts. Generally, GH induces IGF-I production and the IGF-I exerts various actions in many cell lines, but the effect of GH-induced IGF-I is mainly depending on the ability to produce the autocrine IGF-I. Interestingly, it has been reported that human skin fibroblast has a relatively low ability to produce IGF-I by GH stimulation. For example, the concentration of IGF-I in the supernatant of the GH (20 ng/mL, 72hrs)-stimulated- fibroblasts was as low as 0.48 ± 0.09 ng/mL of IGF-I [30], which is much lower concentration of IGF-I than those we used in this study. This could explain the discrepancy in the effect on inducing telomere shortening and cellular senescence between GH and IGF-I stimulation and a presence of the IGF-I specific effect. It is also speculated that *in vivo* condition, GH induces enough amount of IGF-I mainly in the liver and the endocrine IGF-I may cause telomere shortening.

The underlying mechanisms how IGF-I induces telomere shortening and subsequent cellular senescence remains unclarified. It has been reported that various factors including ROS, defects in the telomere repair system, inflammatory reactions, and increased cellular turnover, cause telomere shortening [21]. Intriguingly, oxidative stress was enhanced both in GH-transgenic rats and patients with acromegaly [31]. In addition, it has been reported that IGF-I enhances ROS-p53 pathway and subsequent cellular senescence in cultured cells with a confluent status [20]. Bayram et al. also reported that patients with acromegaly exhibited increased oxidative stress and DNA damage [32]. Furthermore, the causal role of increased oxidative stress in telomere shortening has been reported. Human fibroblasts cultured under 40% oxygen, in which oxidative stress is increased, exhibit an accelerated rate of telomere shortening [33], and inhibition of the glutathione-dependent antioxidant system results in telomere shortening and senescence in human endothelial cells [34]. On the other hand, it is reported that

IGF-I stimulates telomerase activity in cord blood mononuclear cells treated with phytohaemaglutinin (T-cell stimulating agent). However, without phytohaemaglutinin, IGF-I did not enhance telomerase activity [35]. Also, IGF-I activates telomerase in several cancer cell lines [36]; however, this might be the cell specific effect especially in cancer cells and the effect of IGF-I on telomerase has not been reported in normal somatic cells, including lymphocytes or skin fibroblasts which have little telomerase activity. Taken together, although further investigation is needed, we speculate that the increased oxidative stress may lead to the telomere shortening in acromegalic patients. Accordingly, we propose a schematic model that explains the pathological condition in acromegaly (Fig 4F). Increased IGF-I secretion in acromegaly induces ROS, telomere shortening, and cellular senescence. These factors can be causally associated with comorbidities such as diabetes, hypertension, and cardiovascular disease, and increased mortality.

It is well known that the GH-IGF-I axis plays an essential role in the regulation of aging and longevity [37]. Genetically modified mice with reduced activity in the GH-IGF-I axis show increased life span and are protected from cancer and diabetes, two major aging-related morbidities [38, 39]. A cohort study of the Laron syndrome, in which GH receptor (GHR) is deficient and serum IGF-I levels are extremely low, demonstrated that the prevalence of cancer and diabetes was drastically decreased. Intriguingly, serum from subjects with GHR deficiency reduced DNA breaks but increased apoptosis in human mammary epithelial cells treated with hydrogen peroxide. These cells showed a decreased IGF-I signaling and the effect of the serum was completely reversed by IGF-I treatment, indicating that IGF-I signaling is responsible for these effects [40]. Taken together with the present data, although several pathways including mTOR-S6K, Foxo, oxidative stress resistance, and chronic inflammation are potentially underlying mechanisms, in which GH-IGF-I regulates aging and longevity [41], it is suggested that the telomere shortening and subsequent cellular senescence by IGF-I might be involved.

This study has several limitations. One was the inherent difficulty in the selection of control subjects. Because many factors affect telomere length, it is important to exclude confounding factors as possible; we chose age-, sex-, smoking-, and diabetes-matched patients with NFPA. Although it was not significant, there was a tendency to increase the prevalence of diabetes in patients with acromegaly, which might affect telomere lengths. Also, this study was based on a relatively small number of patients and there is a difference in the number of patients between acromegaly and NFPA group, which could cause a selection bias. Nonetheless, we demonstrated the significant difference in telomere lengths between patients with acromegaly and NFPA. Furthermore, the significant association between the disease duration and telomere length strongly supports the hypothesis that a long exposure to IGF-I could lead to DNA damage and telomere shortening. Although we have demonstrated telomere shortening in peripheral leukocytes in patients with acromegaly, it is unclear whether the same condition occurs in the other tissues. However, it has been reported that telomere shortening has been observed not only in peripheral leukocytes but also in cardiomyocytes in patients with heart failure [42] and many studies using peripheral leukocytes clearly showed the significance of telomere shortening in various diseases [43], suggesting the validity of this study.

In conclusion, we have demonstrated that patients with acromegaly exhibit shortened telomeres. The *in vitro* analysis shows that IGF-I reduces telomere length and induces cellular senescence. Although further investigation is necessary, the present data can explain, in part, the underlying mechanisms by which acromegaly exhibits the increased morbidity and mortality in association with the excess secretion of IGF-I.

Supporting Information

S1 Fig. Expression of p53 and p21 in human fibroblasts treated with GH or IGF-I. A, Study design; Human fibroblasts were treated with 100 and 500 ng/mL of GH, IGF-I, or vehicle. At day 10 and 27, quantitative RT PCR (qRT-PCR) and immunoblotting (IB) were performed. At day 38, senescence-associated β-galactosidase staining (SA β-gal) was performed. **B, C,** p53 and p21 mRNA expression in human fibroblasts treated with GH or IGF-I for 10 days (PDL of 10–11). The expression levels were measured using qRT-PCR and normalized to β-actin. **D,** Immunoblotting analysis of p53, serine-phosphorylated-p53, and p21 proteins in human fibroblasts treated with GH or IGF-I for 10 days (PDL of 10–11). Anti-phosphorylated p53 protein at serine 15 antibody was used for the detection of p-p53. **E, F,** Densitometric analysis of p53, p-p53, and p21 protein levels normalized to β-actin. Data were compared using one-way analysis of variance followed by post-hoc Fisher's least significant difference test or Kruskal—Wallis test followed by post-hoc Scheffe test. $^{*}P < 0.05$, $^{**}P < 0.01$; PDL, population doubling levels.
(TIF)

S2 Fig. Comparison of relative telomere length for the clinical indices with adjusting for the age in acromegaly. Relative telomere length was compared in patients with acromegaly after adjusting the effect of age on telomere length using analysis of covariance (ANCOVA).
(TIF)

S3 Fig. Cell growth curves of human skin fibroblasts treated with GH or IGF-I (early phase of the culture). A, GH-treated cells. **B,** IGF-I-treated cells.
(TIF)

S4 Fig. IGF-I increased senescence-associated secretary phenotype cytokine expression. A, B, IL-6 mRNA expression in human fibroblasts treated with GH or IGF-I for 27 days (PDL of 20–21). The expression levels were measured using qRT-PCR and normalized to β-actin.
(TIF)

S1 Table. Primer sequences and concentrations used for telomere measurement.
(DOC)

S2 Table. Primer sequences used in quantitative reverse transcription PCR.
(DOC)

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Author Contributions

Conceived and designed the experiments: RM YT. Performed the experiments: RM. Analyzed the data: RM HF GI YO KY HB KS HN MT WO. Contributed reagents/materials/analysis tools: SY. Wrote the paper: RM.

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