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Growth Hormone Receptor Deficiency Is Associated with a Major Reduction in Pro-Aging Signaling, Cancer, and Diabetes in Humans

Jaime Guevara-Aguirre *et al.*

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Editor's Summary

Clues to a Cancer- and Diabetes-Free Life

In the 1958 film *Live Fast and Die Young*, two reckless sisters threaten to burn out early. Similarly, one theory of aging predicts that a faster metabolism leads to a shorter life. Does this trade-off also apply to age-related disease? A new study by Guevara-Aguirre *et al*. offers clues that address this seminal question. The authors' findings stem from studies of a unique group of Ecuadorian people who have a mutation in the *growth hormone receptor* (*GHR*) gene and a resulting insulin-like growth factor –1 (IGF-1) deficiency, which stunts their growth. These descendants of Spanish conversos, Jews who converted to Christianity to avoid the Inquisition, almost never get diabetes or cancer as a result, the authors postulate, of the privileged metabolic status that arises from their altered hormonal state. Relative to controls, these subjects show lower insulin concentrations and higher insulin sensitivity, and when stressed, their cells tend to self-destruct rather than accumulate mutations and DNA damage—all features that are known to promote cell protection in model organisms.

For 22 years, this group of 99 related Ecuadorians—most of whom are homozygous for an A-to-G splice site mutation at position 180 in exon 6 of the *GHR* gene—has been monitored extensively, so that their health details are well documented. From this reservoir of data, plus information about the diseases of family members as well as causes of death of those relatives who have died, the authors deciphered that the Ecuadorian subjects who carried the *GHR* mutation had an abnormally low incidence of cancer and diabetes. The group showed only one case of nonlethal cancer and no cases of diabetes, whereas the controls ––unaffected relatives––developed cancer (17%) and diabetes (5%) at rates similar to those of the Ecuadorian population as a whole.

To illuminate the underlying reason for the subjects' freedom from these diseases, the authors focused on the components carried in their blood. In experiments on cultured human epithelial cells, Guevara-Aguirre *et al*. found that low concentrations of one of these, IGF-1, was responsible for preventing oxidative DNA damage when the cells were exposed to the oxidizing agent H_2O_2 and for promoting cell death when stress-related DNA damage did occur, a checkpoint that averts cancer-promoting behavior by abnormal cells. Analysis of the participating cell signaling pathways identified activation of the transcription factor FoxO under conditions of low IGF-1 as a likely mediator of these effects. Further, the lower blood insulin concentrations and higher insulin sensitivity in these subjects likely account for the absence of diabetes in this population.

Although it is difficult to prove that alterations in IGF-1 amounts are responsible for the cancer- and diabetes-free lives of these Ecuadorian people, genetic work from several model organisms suggests that this is so. In yeast, mutations in genes that encode components of a growth-promoting pathway protect against age-dependent genomic instability, and mutations in the insulin/IGF-1–like signaling pathway increase life span and reduce abnormal cellular proliferation in worms. Mice with defects in GH and IGF-1 live exceptionally long lives, with delayed appearance of age-dependent mutations and cancer. The Ecuadorians do not live longer-than-normal lives compared with their compatriots, but rather die in due course from causes of death other than cancer and diabetes complications. Thus, the metabolic inverse of "live fast and die young"—a slowed metabolism yields a longer life—is not supported by the current findings. But a life free from two dreaded diseases may be considered a desirable trade-off.

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AGING

Growth Hormone Receptor Deficiency Is Associated with a Major Reduction in Pro-Aging Signaling, Cancer, and Diabetes in Humans

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Mutations in growth signaling pathways extend life span, as well as protect against age-dependent DNA damage in yeast and decrease insulin resistance and cancer in mice. To test their effect in humans, we monitored for 22 years Ecuadorian individuals who carry mutations in the growth hormone receptor (*GHR*) gene that lead to severe GHR and IGF-1 (insulin-like growth factor–1) deficiencies. We combined this information with surveys to identify the cause and age of death for individuals in this community who died before this period. The individuals with GHR deficiency exhibited only one nonlethal malignancy and no cases of diabetes, in contrast to a prevalence of 17% for cancer and 5% for diabetes in control subjects. A possible explanation for the very low incidence of cancer was suggested by in vitro studies: Serum from subjects with GHR deficiency reduced DNA breaks but increased apoptosis in human mammary epithelial cells treated with hydrogen peroxide. Serum from GHR-deficient subjects also caused reduced expression of *RAS*, *PKA* (protein kinase A), and *TOR* (target of rapamycin) and up-regulation of *SOD2* (superoxide dismutase 2) in treated cells, changes that promote cellular protection and life-span extension in model organisms. We also observed reduced insulin concentrations (1.4 μU/ml versus 4.4 μU/ml in unaffected relatives) and a very low HOMA-IR (homeostatic model assessment–insulin resistance) index (0.34 versus 0.96 in unaffected relatives) in individuals with GHR deficiency, indicating higher insulin sensitivity, which could explain the absence of diabetes in these subjects. These results provide evidence for a role of evolutionarily conserved pathways in the control of aging and disease burden in humans.

INTRODUCTION

Reduced activity of growth hormone (GH) and insulin-like growth factor–1 (IGF-1) signaling proteins or of their orthologs in nonhuman organisms and the activation of stress resistance transcription factors and antioxidant enzymes contribute to extended life span and protection against age-dependent damage or diseases (*1*–*16*). Pathways that regulate growth and metabolism also promote aging and genomic instability, a correspondence that is conserved in simple eukaryotes and mammals (*7*). In yeast, life span–extending mutations in genes such as *SCH9*, the homolog of mammalian *S6K* (S6 kinase), protect against age-dependent genomic instability (*17*–*19*). Similarly, mutations in the insulin/IGF-1–like signaling pathway increase life span and reduce abnormal cellular proliferation in worms, and mice deficient in GH and IGF-1 are not only long-lived but also show delayed occurrence of age-dependent mutations and neoplastic disease (*20*–*23*). Among the frequently detected mutations in human cancers are those that activate two major signaling proteins downstream of the IGF-1 receptor (IGF-1R)—Ras and Akt—and those in the IGF-1R itself (*24*, *25*). This is in agreement with a potential role for the IGF-1 signaling pathway in promoting age-dependent mutations that lead to the activation of proto-oncogenes and for oncogenes in exacerbating the generation of additional mutations and changes required for cancer progression (*26*). It has been proposed that the growth-promoting and antiapoptotic functions of the IGF-1 pathway underlie its putative role in cancer development and progression (*27*). This link is supported by some population studies but not others, which instead indicate a modest association between high IGF-1 concentrations and increased risk of certain cancers (*27*, *28*).

GH may also promote insulin resistance. For example, age-dependent insulin resistance is reduced in GH- and GH receptor (GHR)–deficient mice (*29*–*32*), and GH replacement therapy can exacerbate insulin resistance in GH-deficient individuals, apparently because it causes a switch from glucose metabolism to lipolysis (*33*).

Here, we have monitored an Ecuadorian cohort with GHR deficiency (GHRD), which results in IGF-1 deficiency, for 22 years and investigated the effect of these deficiencies on cellular responses to stress and on markers of cancer and diabetes. We show that the fundamental link between pro-growth pathways, oxidative stress, age-dependent genomic instability, and cellular damage observed in yeast (*2*, *15*, *17*–*19*), worms, and mice (*5*, *6*, *20*–*23*, *34*) is conserved in humans.

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RESULTS

Ecuadorian cohort

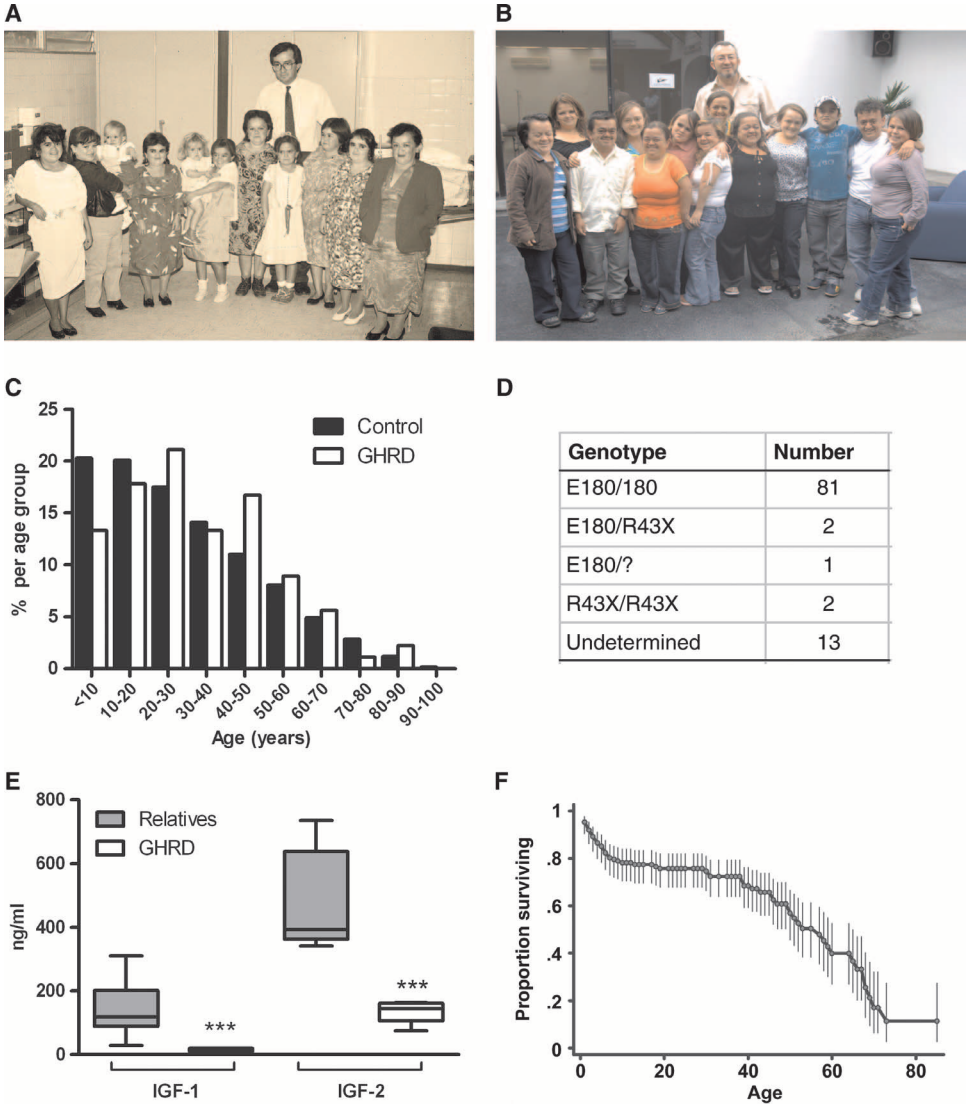
The study subjects were 99 individuals with GHRD who have been followed by one of the authors (J.G.-A.) at the Institute of Endocrinology, Metabolism and Reproduction (IEMYR) in Ecuador since 1988 (Fig. 1, A and B). Of these, nine subjects died during the course of monitoring. The age distribution of the 90 living GHRD subjects and the control Ecuador population is shown in Fig. 1C (*35*). Using a questionnaire (table S1), we collected mortality data for 53 additional GHRD subjects who died before 1988 and obtained information on illnesses and cause of death for 1606 unaffected first- to fourth-degree relatives of the GHRD subjects. The GHRD cohort was identified on the basis of the severe short stature of the subjects (Fig. 1, A and B) (*36*–*38*) and confirmed by genotyping (Fig. 1D). Most GHRD subjects in this cohort were homozygous for an A-to-G splice site mutation at position 180 in exon 6 of the *GHR* gene (Fig. 1D). This mutation, termed E180, results in a protein that lacks eight amino acids in its extracellular domain and is possibly misfolded and degraded (*39*). Two GHRD subjects were homozygous for the R43X mutation, which results in a truncated GHR protein as a result of a premature stop codon (Fig. 1D) (*40*), and two GHRD subjects were E180/R43X heterozygotes (Fig. 1D).

To confirm IGF deficiency in this cohort, we measured IGF-1 and IGF-2 concentrations (Fig. 1E) in 13 relatives and 16 GHRD subjects ranging in age from 20 to 50 years, including those whose serum was later used for in vitro studies. Serum IGF-1 ranged from 29 to 310 ng/ml (mean, 144) among relatives, but was ≤20 ng/ml in all GHRD subjects (Fig. 1E). Serum IGF-2 ranged from 341 to 735 ng/ml (mean, 473) among relatives, but was below 164 ng/ml in all GHRD subjects (Fig. 1E). There was no overlap in the range of IGF-1 and IGF-2 serum values between GHRD subjects and relatives ($P < 0.0001$) (Fig. 1E).

The GHRD cohort shows high mortality from common diseases of childhood (Fig. 1F) (*41*). Because of this, we considered only individuals who survived to at least age 10 for further analysis of diseases in this cohort. Of the 30 deaths among GHRD subjects (data from both monitoring and surveys) older than 10, 9 were due to age-related diseases (8 from cardiac disease, 1 stroke) and 21 were due to non–age-related causes. Compared to their relatives, GHRD subjects died much more frequently from accidents, alcohol-related causes, and convulsive disorders (Fig. 2A).

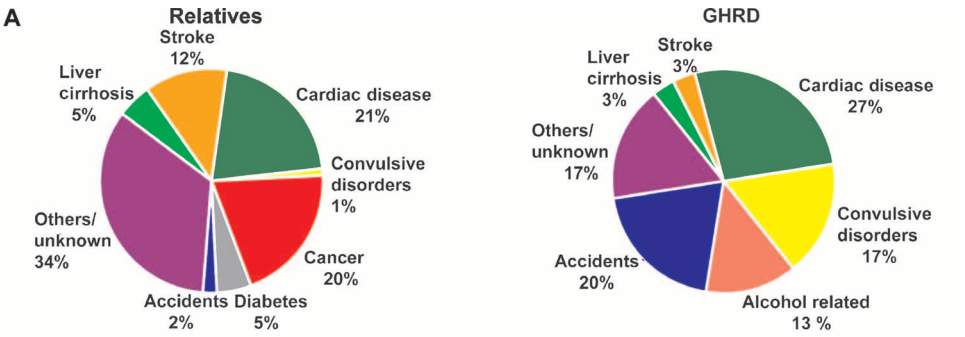
Cancer was not a cause of death in GHRD subjects of any age group; however, it accounted for 20% of deaths in the relatives (Fig. 2, A and B). Stomach cancer was the predominant cause of cancer-related mortality in the relatives (Fig. 2C), which is consistent with the high incidence of this cancer in Ecuador (*42*). Among deaths in each age group, the proportion from cancer was lower in the GHRD subjects than in relatives (based on the exact hypergeometric distribution as implemented in StatXact 7, $P = 0.003$). Cancer accounted for 17% of all diseases in the relatives (Fig. 2D). Of all the GHRD subjects monitored since 1988, only one was diagnosed with cancer, a papillary serous epithelial tumor of the ovary in 2008. After treatment, she has remained cancer-free.

We did not observe any mortality or morbidity due to type 2 diabetes in the GHRD cohort, although diabetes was



Genotype	Number
E180/180	81
E180/R43X	2
E180/?	1
R43X/R43X	2
Undetermined	13

Fig. 1. Ecuadorian cohort. (**A** and **B**) Several members of the GHRD cohort with J.G.-A. in (A) 1988 and (B) 2009. (**C**) Age distribution for 90 living GHRD subjects and the Ecuadorian (Control) population. (**D**) Genotypes of the GHRD cohort. All GHRD subjects were identified on the basis of their short stature and very low serum IGF-1 levels. Undetermined, subjects whose genotypes have not been confirmed. (**E**) Serum IGF-1 and IGF-2 levels in 13 unaffected relatives and 16 GHRD subjects. ***$P < 0.0001$. (**F**) Survival of the GHRD cohort.



B

	Relatives		GHRD	
Age	Total deaths	Cancer deaths (%)	Total deaths	Cancer deaths
10-30 yr	60	3 (5)	5	0
30-50 yr	208	46 (22.1)	12	0
50-70 yr	370	84 (22.7)	12	0
70-90 yr	446	89 (20)	1	0

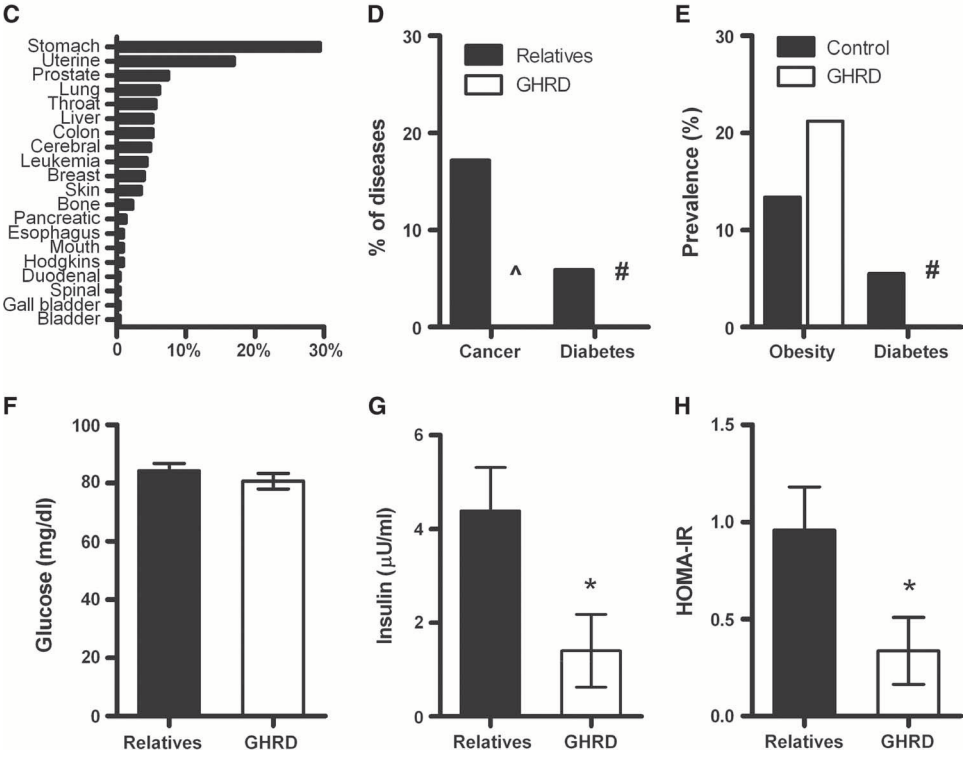


Fig. 2. Diseases and mortality in the Ecuadorian cohort. (**A**) Causes of death in unaffected relatives and GHRD subjects. (**B**) Percentage of cancers per age group in unaffected relatives and GHRD subjects. (**C**) Percent distribution of cancer deaths in the unaffected relatives. (**D**) Percentage of cancer and type 2 diabetes in unaffected relatives and GHRD subjects. Data are shown as a percentage of all diagnosed/reported diseases. ^, one case of cancer; #, no case of diabetes has been recorded. (**E**) Percent prevalence of obesity and type 2 diabetes in Ecuador (Control) and GHRD subjects. #, no case of diabetes has been recorded in the GHRDs. Obesity prevalence in Ecuador is based on WHO reports for 2010 (*42*), whereas that for GHRD subjects was defined as body mass index (BMI) > 30 kg/m^2. Prevalence of type 2 diabetes in Ecuador was obtained from (*43*). (**F** to **H**) Fasting glucose (F), fasting insulin (G), and insulin resistance (H) as indicated by HOMA-IR in relatives and GHRD subjects. Data represent means ± SEM for 13 control and 16 GHRD samples. *P < 0.05.

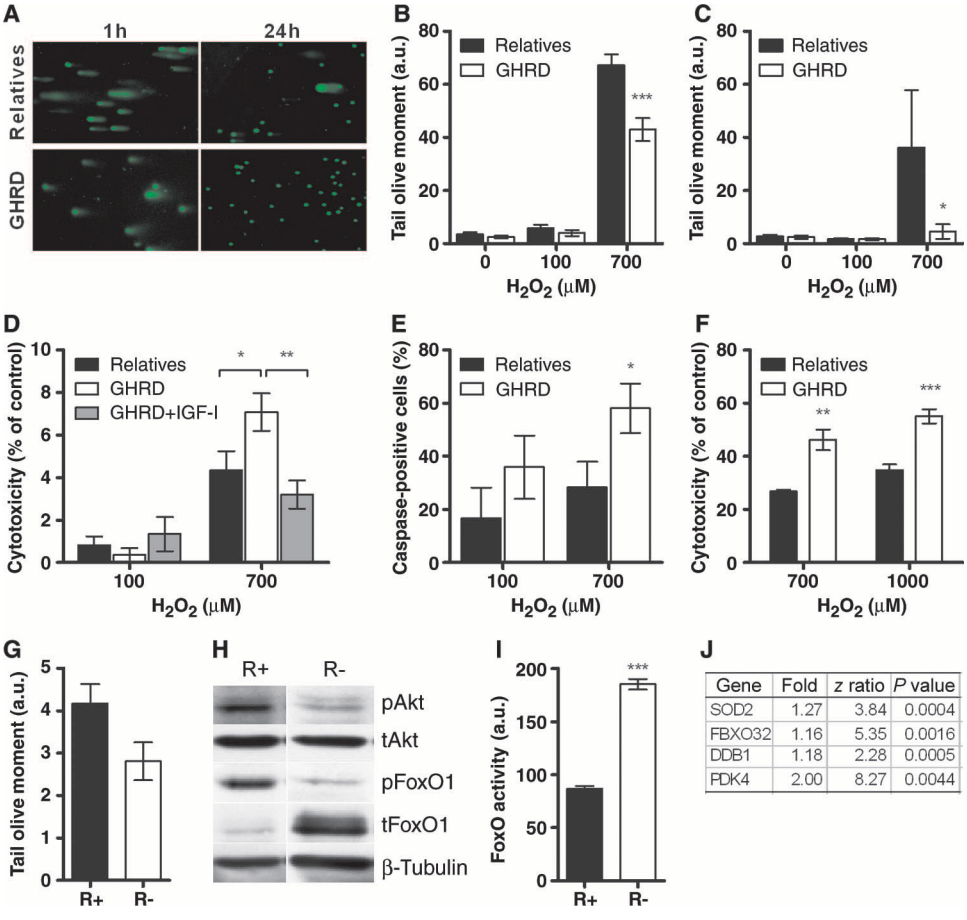
responsible for 5% of the deaths and 6% of all diseases in the relatives (Fig. 2, A and D), in agreement with the 5% prevalence of diabetes in Ecuador (Fig. 2E) (*43*). We estimated the prevalence of diabetes in the GHRD cohort as 0/90 = 0%, with 95% exact Clopper-Pearson confidence interval: 0 to 4%. To test whether the diabetes prevalence in the GHRD cohort was different from the general population prevalence of 5%, we performed an exact test of the null hypothesis that P = 0.05, based on the binomial distribution, with the type I error rate, α = 0.05. The P value was 0.02, indicating that the prevalence in the GHRD cohort is less than 5%. This is a particularly striking result considering the elevated prevalence of obesity among these GHRD individuals (21% in GHRD subjects versus 13.4% in Ecuador) (Fig. 2E) (*42*). To investigate the mechanisms that could be responsible for the observed lack of diabetes in the GHRD cohort, we measured fasting glucose and insulin concentrations in 13 relatives and 16 GHRD subjects consisting of both male and female subjects between the ages of 20 and 50. We observed no significant difference in fasting glucose concentrations between them (Fig. 2F). However, the average insulin concentration in the GHRD group was about a third of that in the relatives (Fig. 2G, P < 0.05), and the HOMA-IR (homeostatic model assessment–insulin resistance) index (*44*) indicated that GHRD subjects (HOMA-IR = 0.34) were much more insulin-sensitive than their relatives (HOMA-IR = 0.96) (Fig. 2H, P < 0.05). These results are consistent with the finding that GHRD mice and other GH-deficient mouse models have low serum insulin concentrations and are insulin-sensitive (*29*–*32*).

Although GHRD subjects may have elevated cardiac disease mortality (Fig. 2A), the mortality from vascular diseases (combining cardiac disease and stroke) appears to be similar to that of their relatives (33% of deaths in relatives versus 30% of deaths in GHRD subjects) (Fig. 2A). In agreement with studies of a human population with isolated GH deficiency (*45*), our data suggest that GHRD does not increase overall vascular disease mortality (Fig. 2A).

Effect of reduced IGF-1 signaling on DNA damage and apoptosis of damaged cells

Our studies in *Saccharomyces cerevisiae* indicate that homologs of mammalian growth

signaling pathway genes, including *TOR* (target of rapamycin), *S6K*, *RAS*, *AC* (adenylate cyclase), and *PKA* (protein kinase A), promote an age-dependent increase in DNA mutations by elevating superoxide production and increasing DNA damage independent of cell growth (*18*, *19*, *46*). Notably, the mutation spectrum in p53 from human cancers is similar to that in aging yeast (*17*, *18*, *26*). These results raise the possibility that GH and IGF-1 signaling may promote mutations and cancer not only by preventing apoptosis of damaged cells but also by increasing DNA damage in both dividing and nondividing cells. To test this hypothesis, we incubated confluent human mammary epithelial cells (HMECs) in medium supplemented with 15% serum from either GHRD subjects or their relatives based on previously described methods (*47*, *48*) for 6 hours and then treated them with H_2O_2 for 1 or 24 hours, followed by comet analysis to detect DNA strand breaks. The medium did not contain any additional growth supplements during the 6-hour incubation period. Because cells were incubated to greater than 90% confluence, cell growth during the preincubation and H_2O_2 treatment periods was minimal. Comet analysis indicated that cells incubated in serum from GHRD subjects had fewer DNA breaks after treatment with 700 μM H_2O_2 for 1 hour (Fig. 3, A and B) or 24 hours (Fig. 3, A and C) compared to cells incubated in serum from relatives. This suggests that serum from GHRD subjects can protect against oxidative DNA damage independently of cell division. We also incubated confluent HMECs in medium supplemented with serum from relatives, GHRD serum, or GHRD serum supplemented with IGF-1 (200 ng/ml) for 6 hours (normal levels of IGF-1 in Ecuadorian adults range from 96 to 270 ng/ml) (*36*). Treatment with 700 μM H_2O_2 resulted in higher cytotoxicity in cells incubated in GHRD serum than in control serum (Fig. 3D). This effect was completely reversed by the addition of IGF-1 (200 ng/ml) to GHRD serum (Fig. 3D). HMECs also displayed higher caspase activity in response to H_2O_2 treatment when incubated in GHRD serum than in serum from relatives (Fig. 3E), in agreement with the proposed role of IGF-1 signaling in increasing cancer incidence by preventing apoptosis (*27*). We also observed increased cytotoxicity in mouse embryonic fibroblast (MEF) cells in response to H_2O_2 after incubation in GHRD serum (Fig. 3F).



J

Gene	Fold	z ratio	P value
SOD2	1.27	3.84	0.0004
FBXO32	1.16	5.35	0.0016
DDB1	1.18	2.28	0.0005
PDK4	2.00	8.27	0.0044

Fig. 3. Effect of reduced IGF-1 signaling on DNA damage and apoptosis of damaged cells. (**A** to **C**) Comet assay to analyze DNA damage in HMECs incubated in serum from relatives/GHRDs and treated with H_2O_2 for 1 or 24 hours. (A) Representative micrographs of cells treated with 700 μM H_2O_2. (B and C) Tail olive moment in cells treated with H_2O_2 for (B) 1 hour or (C) 24 hours. Data represent means ± SEM. At least six serum samples were tested per group, and 100 to 200 cells were analyzed per sample. (**D**) LDH activity in HMECs incubated in serum from relatives/GHRDs and treated with H_2O_2 for 24 hours. Data represent means ± SEM. Six serum samples were tested per group in triplicates. (**E**) Activation of caspases in HMECs incubated in serum from relatives/GHRDs and treated with H_2O_2. Data are calculated as percentage of untreated control and represent means ± SEM. Six serum samples were tested per group. (**F**) LDH activity in MEFs incubated in serum from relatives/GHRDs and treated with H_2O_2 for 24 hours. Data represent means ± SEM. Six serum samples were tested per group in triplicates. (**G**) Tail olive moment to measure basal DNA damage in R+ (IGF-1R overexpression) or R− (IGF-1R–deficient) MEFs. Data represent means ± SEM. One hundred to 200 cells were analyzed per sample. (**H**) Representative Western blot showing phosphorylation status of Akt (Ser^{473}) and FoxO1 (Ser^{256}) in R+ and R− cells. (**I**) FoxO activity in R+ and R− cells transfected with a luciferase reporter plasmid. (**J**) List of FoxO target genes significantly up-regulated in HMECs incubated in GHRD serum versus serum from relatives. *P < 0.05; **P < 0.01; ***P < 0.0001.

To test whether IGF-1 signaling was responsible for the sensitization of cells to oxidative damage, we analyzed DNA damage in MEF cells lacking the IGF-1R (R− cells) or overexpressing the human IGF-1R (R+ cells) (*49*). R+ cells had higher basal DNA damage than did R− cells (Fig. 3G). Western blot analysis confirmed the anticipated increase in phosphorylation of Akt (Thr^{308}) and FoxO1 (Ser^{256}) in R+ cells compared to R− cells, indicating that Akt was activated, whereas FoxO1 was inactivated in the R+ cells (Fig. 3H) (*50*–*52*). The very low level of total FoxO1 protein in R+ cells may be due to the Akt-mediated phosphorylation of FoxO, which results in its ubiquitination and proteasomal degradation (Fig. 3H) (*53*). Reduced FoxO activity in R+ cells was also confirmed by a

FoxO-driven luciferase reporter (Fig. 3I). Because FoxO transcription factors protect against oxidative stress as well as promote apoptosis (*51*, *54*, *55*), we hypothesize that increased FoxO activity could account, in part, for the protective effects observed in HMECs incubated in GHRD serum. In support of this, microarray analysis of HMECs incubated in either control or GHRD serum showed that of 44 genes that were significantly up-regulated in the GHRD serum–treated group, 4 genes, including *SOD2*, were FoxO targets (Fig. 3J).

Protective effects of reduced pro-growth signaling in yeast and mammals

A complete list of genes with significant differential expression in HMECs incubated in either control or GHRD serum is shown in table S2. Ingenuity pathways analysis (IPA) of global gene expression patterns revealed significant differences in pathways involved in cell cycle regulation, gene expression, cell movement, and cell death, among others (fig. S1). IPA also indicated that genes regulating apoptosis were up-regulated (fig. S2), whereas Ras, PKA, and TOR signaling were down-regulated in cells incubated in GHRD serum (fig. S3). Reverse transcription–polymerase chain reaction (RT-PCR) analysis confirmed a 30% higher mRNA level of mitochondrial MnSOD (manganese superoxide dismutase) (SOD2) in cells incubated in GHRD serum, and a 70, 50, and 20% reduction in N-Ras, PKA, and TOR expression, respectively (Fig. 4A).

Ras, PKA, and TOR/S6K are central regulators of pro-aging and disease-promoting pathways, and SOD2 is a key mediator of cellular protection against oxidative stress in organisms ranging from the unicellular yeast to mammals (*2*, *17*, *18*, *46*, *56*–*59*). To further test the role of these genes in age- and oxidative stress–dependent DNA damage, we generated a yeast triple-mutant strain lacking *RAS2*, *TOR1*, and *SCH9*. Our previous studies have shown that yeast *sch9*Δ mutants exhibit lower age-dependent genomic alterations than wild-type cells in part due to reduced error-prone Polζ-dependent DNA repair (*18*). We observed a fourfold lifespan extension in the *ras2*Δ*tor1*Δ*sch9*Δ triple mutants compared to wild-type cells (Fig. 4B). We analyzed age-dependent DNA genomic instability in the *ras2*Δ*tor1*Δ*sch9*Δ and wild-type cells by measuring the mutation frequency of the *CAN1* gene. Mutations that inactivate the arginine permease encoded by *CAN1* allow cells to grow in medium containing the normally toxic arginine analog canavanine (*60*). The frequency of age-dependent mutations in the *CAN1* gene—which are mainly point mutations, including a high frequency of G to T (transversion) and C to T (transition) base substitutions (*17*)—was much higher in wild-type cells compared to the *ras2*Δ*tor1*Δ*sch9*Δ mutants (Fig. 4C). Whereas wild-type cells were susceptible to H_2O_2 treatment, the *ras2*Δ*tor1*Δ*sch9*Δ mutants were almost unaffected at the concentrations tested (Fig. 4D) and were highly protected against oxidative stress–induced mutations (Fig. 4E).

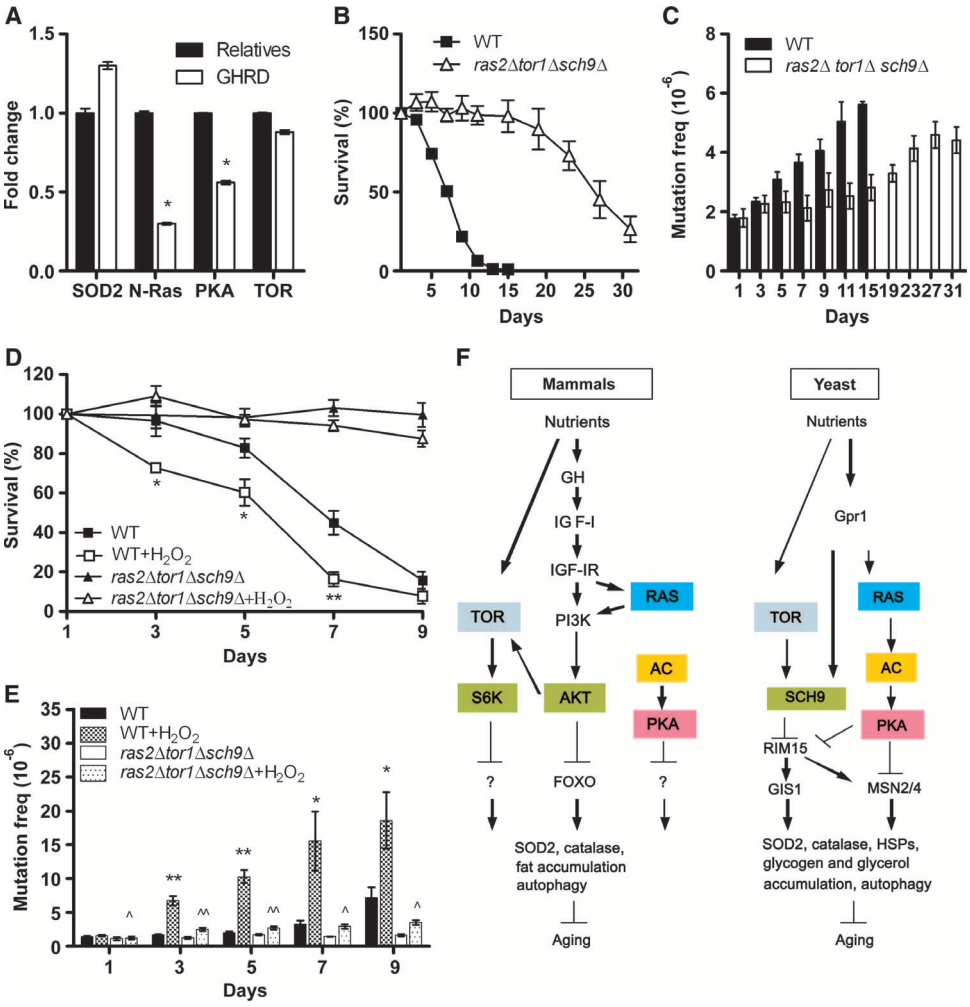


Fig. 4. Protective effects of reduced pro-growth signaling in yeast and mammals. (**A**) RT-PCR indicating up-regulation of SOD2 and down-regulation of N-Ras, PKA, and TOR in HMECs incubated in GHRD serum compared to cells incubated in serum from relatives. (**B**) Chronological survival of wild-type (WT) yeast cells and *ras2*Δ*sch9*Δ*tor1*Δ mutants cells. (**C**) Mutation frequency over time in the *CAN1* gene (measured as *Can*^{r} mutants per 10^6 cells). (**D**) Chronological survival of WT yeast cells and *ras2*Δ*sch9*Δ*tor1*Δ triple mutants treated with H_2O_2. (**E**) Mutation frequency over time in the *CAN1* gene (measured as *Can*^{r} mutants/10^6 cells) in H_2O_2-treated yeast cells. Data represent means ± SEM ($n = 5$). *$P < 0.05$; **$P < 0.01$, compared to untreated WT cells; ^$P < 0.05$; ^^$P < 0.01$, compared to untreated *ras2*Δ, *sch9*Δ, and *tor1*Δ triple mutants. (**F**) Conserved growth factor signaling pathways in mammals and yeast. AC, adenylate cyclase; PI3K, phosphatidylinositol 3-kinase.

DISCUSSION

Our finding that human GHRD subjects are protected against age-related pathologies is consistent with the elevated cellular protection in both yeast and human cells with reduced expression of specific pro-growth genes and with the effect of

serum from GHRD subjects in lowering their expression (Fig. 4F). The results from the human cohort also show similarities with those from GHRD- and GH-deficient mice, which display lower incidence (49%) or delayed occurrence of fatal neoplasms and increased insulin sensitivity (*21*, *22*, *29*–*32*, *34*). Furthermore, the delayed cancer occurrence in GH-deficient mice is associated with a lower mutation frequency in various tissues (*23*).

Unlike model organisms with similar mutations, human GHRD subjects did not live longer lives. The lack of life-span extension in GHRD subjects may be explained in large part by the high proportion of deaths (70%) caused by convulsive disorders, alcohol toxicity, accidents, liver cirrhosis, and other non–age-related causes. The lack of cancer mortality and normal life span in GHRD subjects is in agreement with a preliminary study that reported the absence of cancer in a group of 222 patients with congenital IGF-1 deficiencies (*61*) and with the normal life span that was observed in 65 GH-deficient subjects (*45*). In contrast to our study of GHRD subjects with specific mutations and their age-matched relatives, Shevah and Laron (*61*) compared young subjects with IGF-1 deficiencies due to many causes with much older controls, which made it difficult to determine whether cancer incidence was reduced. However, together, these two studies provide strong evidence for reduced cancer incidence in GHR- and IGF-1–deficient subjects and indicate that GHR and IGF-1 are risk factors for age-dependent cancer, at least in specific populations. Our results may also provide a partial explanation for the overrepresentation of partial loss-of-function mutations in the IGF-1R gene among Ashkenazi Jewish centenarians (*62*).

The role of IGF-1 in cancer may involve the well-established pro-growth and antiapoptotic functions of this growth factor (*27*, *63*, *64*). Our studies in yeast and mammals also indicate that pro-growth signaling increases oxidative damage and DNA mutations in nondividing cells (*17*–*19*, *26*, *65*, *66*). In both yeast and mammals, reduction of TOR/S6K, RAS, and AC/PKA signaling renders cells and the organism resistant to age- and oxidative stress–dependent mutagenesis (*2*, *15*, *17*–*19*, *65*, *67*, *68*). This effect appears to depend, in part, on increased activity of stress resistance transcription factors and SOD2 (*18*, *46*, *54*). Mice lacking Cu/Zn SOD or MnSOD are susceptible to increased DNA damage and cancer (*58*). The positive effects of serum from GHRD subjects on cellular changes that promote longevity in model organisms, such as reduced levels of *RAS*, *PKA*, and *TOR* and increased expression of *SOD2* and other FoxO-regulated genes, suggest that the anti-aging and anti-DNA damage mechanisms promoted by reduced growth signaling are conserved from yeast to humans (Fig. 4F) (*7*, *69*, *70*).

The lack of type 2 diabetes in the GHRD cohort is particularly interesting considering that obesity is one of the clinical phenotypes of GHRD. The enhanced insulin sensitivity of GHRD subjects, as indicated by reduced insulin concentrations and a lower HOMA-IR index, could explain the absence of diabetes in this cohort. Although increased insulin sensitivity has been associated with a longer life span, some long-lived mice, including fat insulin receptor knockout (FIRKO) mice, exhibit impaired insulin signaling. In this case, however, loss of insulin signaling is restricted to adipose tissue and is not associated with diabetes or glucose intolerance (*71*). Similarly, heterozygous male IGF-1R (IGF 1R +/−) mice show a 16% increase in life span, even though they exhibit impaired glucose tolerance (*6*). The anti-aging benefits of reduced GHR and IGF-1 signaling without the detrimental effects of insulin resistance in GHRD mice may explain, in part, why these mice display reduced disease incidence and a much longer life span than IGF 1R +/− mice.

Our results provide a foundation for further investigation into the role of drugs blocking the GHR and downstream conserved pro-aging pathways to prevent or reduce the incidence of cancer, diabetes, and other age-related diseases, including inflammatory disorders, stroke, and neurodegenerative diseases.

MATERIALS AND METHODS

Subject recruitment

GHRD subjects and their relatives were recruited for the study under protocols approved by the IEMYR in Ecuador and the IRB at the University of Southern California. All participants signed informed consent forms before their participation in the study. Data on deceased GHRD subjects were collected from family members with a detailed questionnaire (table S1). At least two relatives were required to be present at the time of the interview.

Genotyping

Saliva samples were collected with the Oragene OG-250 DNA collection kit (DNA Genotek Inc.) and processed according to the manufacturer's protocol. Genotyping of the E180 mutation was performed with the following primers: forward, 5′-CATTGCCCTCAACTGGACTT-3′; reverse (wild type), 5′-CATTTTCCATTTAGTTTCATTTACT-3′; reverse (mutant), 5′-CATTTTCCATTTAGTTTCATTTAC-3′.

Serum analysis

Serum IGF-1 was measured with an in-house enzyme-linked immunosorbent assay (ELISA) based on paired specific antibodies (R&D Systems) and validated against the commercial kit from Diagnostic Systems Laboratories. The assay was performed after acid-ethanol extraction of the samples and has a sensitivity of 0.2 ng/ml. IGF-2 was measured with paired anti–IGF-2–specific antibodies (R&D Systems). The sensitivity of the assay is 0.2 ng/ml. Fasting glucose levels were measured with a glucose analyzer from YSI Life Sciences, and fasting insulin levels were measured with a human insulin ELISA kit from Millipore. Insulin resistance was assessed with the HOMA-IR index from fasting glucose and fasting insulin values (*44*).

Cell culture

HMECs were purchased from ScienCell Research Laboratories. Cells were cultured in epithelial cell medium (ScienCell) at 37°C and 5% CO_2 on poly-L-lysine (Sigma)–coated culture dishes. The epithelial cell medium consisted of basal medium and a proprietary growth supplement supplied by the manufacturer and no serum. Primary MEFs were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with 15% fetal bovine serum (FBS) at 37°C and 5% CO_2. R+ and R− cells were obtained from R. Baserga and cultured in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO_2. Cells were seeded at a density of 4×10^4 per well for comet and apoptosis assays, 8×10^4 per well for lactate dehydrogenase (LDH) assays, or 2×10^5 per well for microarray analysis and Western blots in 24-, 96-, and 6-well plates, respectively. HMECs and MEFs were incubated in basal medium supplemented with 15% serum from relatives or GHRDs for 6 hours followed by treatment with H_2O_2 for 1 hour (comet and apoptosis assays) or 24 hours (comet and LDH assays). Incubation of cells in human serum was performed in either basal medium for HMECs or DMEM/F12 for MEFs without any additional growth supplements.

For microarray analysis, HMECs were incubated in serum from relatives or GHRDs as above for 6 hours and immediately processed for RNA extraction with TRI Reagent from Ambion.

Comet assay

Comet assay was performed according to the method in (*72*) with the comet assay kit from Trevigen. DNA damage was quantified per cell with the Comet Score software. One hundred to 200 cells were analyzed per sample.

LDH assay

LDH activity was assayed in culture medium with the CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega according to the manufacturer's protocol.

Apoptosis assay

Activated caspases were quantified with a fluorescence plate reader with the Fluorescein CaspaTag Pan-Caspase Assay Kit (Chemicon) according to the manufacturer's protocol.

FoxO activity

Cells (50,000 per well) were transfected with 0.2 μg of FoxO luciferase reporter plasmid with the consensus FoxO-binding sequence driving firefly luciferase gene expression in 24-well plates. As an internal control, cells were cotransfected with 0.02 μg of pRL-cytomegalovirus (Promega). Twenty-four hours after transfection, FoxO promoter activity was assayed with the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's protocol.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer, and total protein was assayed with bicinchoninic acid (BCA) assay (Thermo Scientific). Total protein (15 μg) was loaded on denaturing 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Primary antibodies against phosphorylated (Thr ^{308}) and total Akt as well as phosphorylated (Ser^{256}) and total FoxO1 were obtained from Cell Signaling Technologies. Anti–β-tubulin antibody was obtained from Santa Cruz Biotechnology. Secondary rabbit antibody was obtained from Jackson Immunoresearch Laboratories Inc.

Microarray analysis

RNA was extracted with TRI Reagent and hybridized to BD-103-0603 chips from Illumina Beadchips. Raw data were subjected to *Z* normalization and deposited in the Gene Expression Omnibus (GEO) repository (accession number GSE21980). Gene set enrichment was tested with the PAGE method as described (*73*).

Figures S2 and S3 were generated based on the names and descriptions provided by IPA (Ingenuity Systems) and/or Ariadne Pathway Studio 7 (Ariadne Genomics).

Yeast

Wild-type DBY746 (*MATα leu2-3 112 his3Δ,trp1-289 ura3-52 GAL*$^+$) and its derivative *ras2::LEU2tor1::HIS3sch9::URA3*, originated by one-step gene replacement, were grown in synthetic dextrose complete (SDC) medium containing 2% glucose (*2*). Chronological life span in SDC medium was monitored by measuring colony-forming units (CFUs) on YPD (yeast extract peptone dextrose) plates every other day. The number of CFUs on day 1 was considered to be the initial survival (100%) and was used to determine the age-dependent mortality (*74*). Spontaneous mutation frequency was evaluated by measuring the mutation frequency of the *CAN1* (*YEL063C*) gene. Cells were plated onto selective SDC minus arginine plates in the presence of L-canavanine sulfate (60 mg/liter). Mutation frequency was expressed as the ratio of Can^r (canavanine-resistant) colonies over total viable cells (*60*). Resistance to oxidative stress was evaluated in yeast cultures chronically treated with 1 mM H_2O_2 on days 1 and 3.

Statistical analysis

Student's *t* test, two-tailed, was used to analyze insulin, HOMA-IR data, and cellular data from mammalian (comet, LDH, and caspase assays; RT-PCR; and FoxO activity) and yeast experiments (survival and mutation frequency) with GraphPad Prism 5. The proportion from cancer in the GHRD subjects and relatives was compared on the basis of the exact hypergeometric distribution as implemented in StatXact 7 (Cytel Software Corp.).

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/3/70/70ra13/DC1

Fig. S1. Functional clustering of genes with significant differential expression in HMECs incubated in serum from either GHRD subjects or their relatives.

Fig. S2. Ingenuity pathways analysis indicating an up-regulation of apoptosis in HMECs incubated in serum from GHRD subjects.

Fig. S3. Ingenuity pathways analysis indicating down-regulation of Ras, PKA, and RPS6K in HMECs incubated in serum from GHRD subjects.

Table S1. Questionnaire.

Table S2. List of genes with significant differential expression in HMECs incubated in serum from either GHRD subjects or their relatives.

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