



## Aging: gene silencing or gene activation?

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**Summary** According to the author's theory of gene silencing, the key process in aging involves reduced expression of a number of genes. Silencing of genes has a complex mechanism, which involves methylation of DNA, histone modification and chromatin remodeling. In addition to deacetylation of the histones and methylation of DNA, recently described RNAi mechanism could initiate formation of silenced chromatin. Hypermethylation of the promoter will silence the gene. Genome-wide hypomethylation will induce genomic instability, amplification of oncogenes and also silencing of the genes through RNAi mechanism. Studies by different groups, conducted in yeast, worms, flies and mice, confirmed substantial changes in gene expression in aging. Among them, the most important was silencing of tumor suppressors and other genes involved in the control of cell cycle, apoptosis, detoxification, and cholesterol metabolism. There was also increased expression of the smaller group of oncogenes and other genes which are associated with typical diseases of old age. Caloric restriction normalizes expression of a substantial percentage of these genes. Animal studies confirmed importance of caloric restriction, which decreases signaling through the IGF-1/AKT pathway and expression of gene *p53*. These studies, however, cannot be directly applied to human aging. It is proposed that age management therapy should attempt to normalize gene expression in the older population to the level typical for young adults. This would require activation of silenced genes and normalization of overexpressed genes. Caloric restriction and exercise are helpful in decreasing the activity of important oncogenes and activation of silenced tumor suppressors, and may have a positive impact, not only on aging, but also on prevention of cancer. Dietary supplements containing phytochemicals should normalize increased expression of oncogenes. Examples are: genistein and EGCG, which effect signaling through the IGF-1/AKT pathway and resveratrol and limonen, which do so through the RAS pathway. A group of amino acid derivatives and organic acids of animal and human origin should activate silenced tumor suppressor genes (Aminocare<sup>®</sup> A10, Aminocare<sup>®</sup> Extra). Among them 3-phenylacetyl-amino-2, 6-piperidinedione intercalates specifically with DNA and protects sequences of tumor suppressor genes, which are vulnerable to the effects of carcinogens. Phenylacetate activates *p53* and *p21* through inhibition of methyltransferase and farnesylation of the RAS protein. Phenylbutyrate activates tumor suppressor genes through inhibition of histone deacetylation. Phenylacetylglutamine decreases genomic instability and expression of oncogenes and promotes apoptosis. The application of DNA microarray techniques to human studies should provide more information about differences in gene expression in different age groups and help design more effective age management regimens.

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## Introduction

Genetic mechanisms may provide the best explanation of the aging process. Two years ago, I had the pleasure of presenting my gene silencing theory of aging [1]. After determination of the sequence of the human genome, it was found that less than 2% of the human genome codes for proteins and only 1/10 of these are active in adult life. This means that approximately 90% of our genes are silenced. The system of biochemical factors named epigenome consist of molecular switches which activate and silence the genes during our life [2]. Methylation of promoter sequences of the genes is the main mechanism for silencing both genes no longer necessary for development and genes which are gradually turned off during the aging process [3,4]. Adult cells have an established methylation pattern in their DNA that is central to the aging program, but, in the very first day of life, that methylation pattern is erased [5]. Most of the genes, which are silenced later, are active during initial embryonal development, then they begin to be blocked through methylation and deacetylation as their expression is no longer needed [6]. The continuous silencing of genes through methylation and deacetylation is a major factor leading to progressive aging, cancer and, ultimately, death [7–10].

Silencing of genes is a complex process, which involves methylation of DNA, histone modification, and chromatin remodeling. A number of excellent reviews and books have recently been written on this subject [5,10–12]. Two biochemical processes, which I described in the previous publication, play a very important part in silencing of the genes: deacetylation of the histones and methylation of DNA [1]. Since then, additional new mechanisms of methylation have been proposed explaining two different issues of DNA methylation in aging cells: (1) site-specific hypermethylation of promoter sequences and (2) genome-wide hypomethylation. It was confirmed recently that genome-wide hypomethylation is inducing genomic instability, amplification of oncogenes and silencing of tumor suppressor genes through RNAi mechanism [13–16].

## Changes in gene expression in aging

### Studies in nematodes

The initial research of Kenyon [17] indicated that a suppression of a single gene in *Caenorhabditis elegans* doubled its life expectancy. DNA microarray

analysis which followed, revealed that decreased activity of this gene, called *Daf-2*, affects approximately a hundred other genes [18]. The majority of the genes were up-regulated under conditions which block the action of *Daf-2*, which means that they were silenced in aging animals. There were two prominent groups of these genes. The first group contained stress-response genes such as glutathione S-transferase (*Gst4*) and small heat shock protein (*Hsp16.2 genes*). The promotion of longevity by these genes was probably due to prevention or repairing oxidative and other macromolecular damage. The second group of genes encoded antimicrobial proteins. This is not a surprise, since the most common cause of death of *C. elegans* is bacterial infection. It was also found that *Daf-2* increases signaling through AKT pathway and blocks the activity of *Daf-16*. This corresponds to human insulin /IGF-1 signaling pathway.

### Studies in mice

An impressive microarray expression analysis of 11,000 genes in aging livers of mice was published by Cao et al. [19]. The determination of relative levels of expression (mRNA) in young (7 month old) and old (27 month old) mice revealed that 46 known genes (approximately 1% of the total) changed expression during aging. Fifty-seven percent of genes had decreased expression with age and 43% increased. A closer look at the genes, which had increased expression in aging revealed that most of them are associated with typical diseases of old age.

In the group of genes, which were silenced during aging, 23% were involved with the control of the cell cycle. Prominent among them was the tumor suppressor gene *Pten* and *Igfbp1*, which down-regulates *Igf1*. The second group of genes, which are silenced with aging is responsible for xenobiotic metabolism, including *Gsto1*. Among the remaining genes, silencing of *Apoe* is associated with severe atherosclerosis. To summarize, the experiments in mice revealed that the majority of genes changed with aging are silenced and the proportion of genes with decreased expression to increased expression will be much higher if we remove the genes associated with diseases of old age.

### IGF signaling in aging

In the Spindler group experiment, *Igfbp1* was silenced in aging and activated by caloric restriction. *Igfbp1* is one of the factors which down-regulates IGF-1 signaling, has antigrowth activity and, in

mice, is reduced by age. Research by different groups indicated the importance of insulin/IGF-1-like signaling in yeast, nematodes and insects [17–20]. In all of these diverse organisms, caloric restriction extends life span and has a negative effect on the IGF-1 pathway.

IGF signaling begins with binding to IGF-1R, which functions as a typical transmembrane receptor with tyrosine kinase (RTK) catalytic domain [21]. There are two main signaling pathways which lead from this point. One involves phosphoinositol-3 kinase (PI3K)/AKT [22]. The other pathway leads through RAS protein [23]. The PI3K/AKT pathway can be traced to yeast, nematodes and insects; gene *Daf-18* of nematode *C. elegans* is a homolog of *PTEN* and *Daf-16* is coding for a FOXO-family transcription factor. Despite similarity between IGF-1/PI3K/AKT pathways between primitive organisms, mammals and humans, certain specific aspects of human pathways are not replicated even in primates [22].

Mutations which interrupt signaling through this pathway in yeast shift cells into the state of reduced function, which extend the life span. For this extension of life span, yeast requires NAD and sirtuin2 (*Sir2*), which is a histone deacetylase [24]. In *C. elegans* reduced expression of the IGF-1R homolog *Daf-2* or reduced expression of downstream components of the AKT pathway cause dauer formation and life extension [17]. In dauer, the worms are arrested in development, but long-lived. The main factor, which is involved, is *Daf-16*, which has increased activity due to reduced expression of *Daf-2*. Similar effects were observed in *Drosophila*. Mutation, which interrupts insulin/IGF-1 pathway, causes these animals to enter a state of reproductive diapause. They are small in size, produce high levels of fat, are resistant to oxidative stress and live much longer. It was postulated that decreased levels of IGF-1 caused growth arrest and increased resistance to oxidative damage. Examples were mice without pituitary glands, which live a long life as dwarfs, and small dogs, which have low levels of IGF-1 and live longer than large dogs [17,20]. In *C. elegans*, overexpression of *Sir2* potentiates dauer formation and extends life span. In humans *Sir2* inactivates protein p53 through deacetylation. This brought into the picture another important gene: tumor suppressor gene *p53* [24].

### p53 in aging

The studies on extension of life span in yeast through caloric restriction revealed an important

function of the *Sir2* gene. *Sir2* protein is a NAD-dependent histone deacetylase [24]. There are seven analogs of *Sir2* in mammals (SIRT5), which makes this system much more complicated. SIRT1 is the closest relative of yeast *Sir2*. The important function of SIRT1 includes deacetylation of histones and other proteins; among them the p53 protein [25]. In nematodes, *Sir2* works through DAF-16, which in mammals corresponds to four FOXO factors. AKT triggers the release of nuclear FOXOs from their co-activator in the nucleus. This promotes cell cycle arrest and resistance to oxidative stress. Simultaneously, under the condition of cellular stress, SIRT1 deacetylates p53, which also inhibits apoptosis [25]. Based on inhibition of p53 in long-lived organisms, a number of researchers have suggested that p53 promotes aging [10,26,27]. It was proposed that FOXO3 and p53 mediate the effect of the SIRT1, which promotes cell cycle arrest, DNA repair and protection from oxidative stress, but inhibits induction of apoptosis in presence of stress [25]. There are three additional members of the p53 family: p44, p63 and p73 [26–28]. p53 function evolved in higher organisms to prevent neoplastic growth. The apoptotic response to p53 is mostly limited to neoplastic cells [27].

### Inhibition of p53 through IGF-1 signaling

p53 interacts with DNA helicases, such as WRN1, which play an important part in induction of apoptosis. Loss of WRN1 activity promotes aging. IGF-1 signaling counteracts p53 through a number of different mechanisms, involving AKT. Phosphorylation and activation of MDM2 by AKT promote degradation of p53 [22]. A component of the WNT signaling pathway, WSP1 activates AKT and also inhibits p53. Factors activated by p53 which play an important part in apoptosis such as PUMA are inhibited by AKT. NF- $\kappa$ B, which is activated by AKT, inhibits p53 by competing for co-activators, such as PCAF. Due to the opposing activities of p53 and IGF-1, a simultaneous inhibition of IGF-1 and p53, recommended for life extension, is difficult to accomplish.

### Activation of p53 through RAS signaling

Phosphorylation of p53 is necessary for its action and is prevented by WIP1 phosphatase. RAS suppresses expression of *WIP1* and increases phosphorylation of p53 and its ability to induce apoptosis. *WIP1* is a product of the *PPNID* gene, which is amplified and overexpressed in breast and

ovarian cancers and neuroblastoma. *PPNID* forms a feedback inhibition loop to p53 [29,30]. The phosphorylation of p53 through p38 MAP kinase (p38MAPK) is necessary for apoptosis. p38 MAPK is activated through environmental stress. In addition to its effect on p53, it activates growth inhibitors ARF and INK4A. p53 activates gene *WIP1*, which inactivates p38 MAPK which, again, is necessary for the activity of p53. Activation of MEK of RAS pathway induces both p53 and INK4A [23]. RAS and p53 form a fail-safe network and activation of RAS and *WIP1*, in the absence of the function of p53 and p16, leads to neoplastic proliferation [30].

### Aging in animals vs aging in humans

Recently, the studies of Maier et al. [26,31] explained possible regulation of aging in mice by p53. In simple organisms such as nematodes and flies, instead of p53 there is a short isoform p44, which seems to accelerate aging in mice. It incorporates into p53 tetrameres, which results in a hyperactive complex. In these mice, p44 overexpression stimulated transcription of the IGF-1 receptor and resulted in enhanced IGF-1 signaling. The researchers postulated that hyperactive p53 and increased IGF-1 signaling promotes aging [32,33].

There is a general consensus that caloric restriction extends life in yeast, nematodes, flies, rodents, monkeys and humans. While studies in animals strongly indicate IGF-1 and p53 signaling pathways are the main regulators of longevity, such relationships are not yet confirmed in humans. Down-regulation of insulin/IGF-like signaling pathways extends longevity in many organisms. Mutations in this pathway simulate starvation conditions [20]. The organisms have reduced activity, improved stress and anti-bacterial resistance, but, at the same time, they also show a number of adverse effects, such as dwarfism, increased accumulation of glycogen and fat. Some of the proponents of life extension by a decrease of IGF-1 signaling provided evidence that dwarfs and small animals live longer [17,20]. This was contradicted by other researchers who found that dwarfs in p44 and p53 experiments lived a short period of time [26,31–33]. The studies on aging in animals were conducted in conditions quite different from their natural environment. It is difficult to imagine that animals which have reduced activity due to caloric restriction would survive longer in their natural habitat, where they would need to fight for survival. Perhaps experiments on animals which

were domesticated by humans centuries ago would give different results. Cats were domesticated approximately 9500 years ago [34]. Observation of cats living in Houston homes compared to feral cats reveals that the animals living as pets at home have 10 times longer survival and are usually overweight.

Growth and aging in humans is characterized by a number of features, which are different compared to animals. The deficiency of growth hormone (GH) in humans leads to a reduced life expectancy and is associated with dwarfism, obesity, hypertension, insulin resistance, and atherosclerosis. Such changes counteract the beneficiary effect of a GH/IGF-1 deficiency in humans. Peripheral insulin resistance causes type 2 diabetes mellitus, which effects over 18 million Americans and shortens their lives [21]. Laron syndrome, in which growth hormone deficiency is caused by a defect in the GH receptor, is associated with dwarfism, obesity, delayed physical and intellectual development, hypercholesterolemia and glucose intolerance. In this syndrome there is interruption of GH signaling and a very low plasma level of IGF-1 [35]. GH treatment is widely used in age management therapy and can increase lean body mass and energy, but it may also produce substantial adverse effects, including development of diabetes and promotion of cancer growth. Therefore, drugs that prevent IGF-1 generation in response to GH may have practical application by reducing plasma IGF-1, but not GH levels. Unfortunately, in experiments with mice, low plasma levels of IGF-1 and elevated GH produced insulin resistance, indicating that reduction of IGF-1 levels is causing adverse effects. A number of researchers indicate that p53 promotes aging. p53, however, has a different mechanism of action in humans and in animals. Inactivation of p53 through deacetylation by Sir2 in yeast may not cause any problems, because yeast do not develop cancer. Blockage of p53 activity in humans will certainly increase the incidence of cancer and will shorten the life expectancy. In human insulin/IGF-1/AKT pathway inhibits p53; therefore, interruption of signaling along this pathway, which promotes longevity, will increase the action of p53 at the same time. On the other hand insulin/IGF-1/RAS pathway promotes p53. Studies in yeast indicate a major part played by AKT and RAS signaling in regulation of longevity, since lack of *sch9* activity extends life three times and blocking *ras* two times.

It is evident from these highly complex relations that in order to control aging, it is necessary to obtain a proper balance between activity of oncogenes and tumor suppressor genes. There is no

doubt that proper levels of activity of p53, p21, ARF, INK4A and PTEN and proper signaling through IGF-1/AKT/NF- $\kappa$ B and IGF-1/RAS is required. High activity along the IGF-1/AKT pathway may promote aging, reduce activity of p53 and cause cancer. Optimal activity of IGF-1/ATK is necessary to prevent diabetes, obesity and provides good muscle strength. Increased activity along the IGF-1/RAS pathway will help replace senescent cells through the action of p53 (promoted by RAS). Overactive RAS and silenced p53, p21, ARF or INK4A will cause cancer and promote aging. Optimal activity of the apoptosis pathway is necessary to assure destruction of senescent, damaged, abnormal, cancerous and useless cells and replace them through optimal activity of the growth factors and oncogenes. Unopposed activity of growth factor/oncogenes will promote cancer. Substantially reduced or markedly increased activity of tumor suppressors may promote aging.

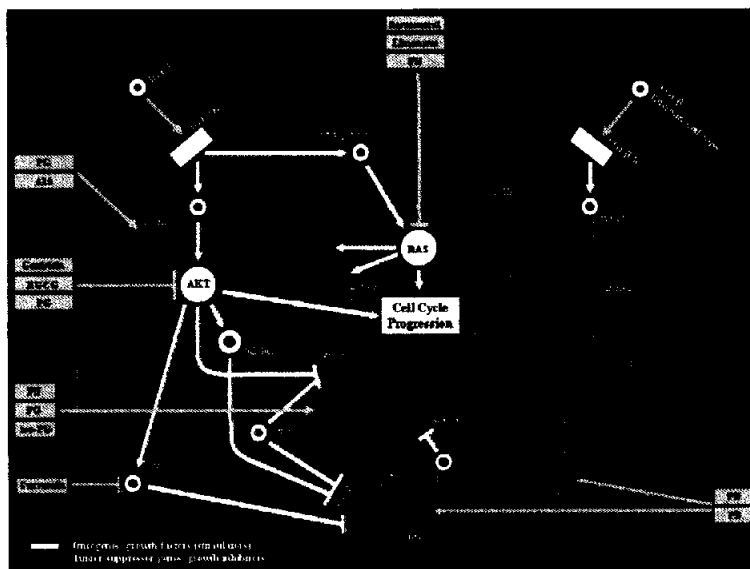
### Restoration of normal gene expression

Based on animal experiments and human observations, the drugs which can restore normal gene expression in aging should inhibit IGF-1/AKT and RAS pathways and provide proper anti-cancer defense through normal activity of tumor suppressors

p53 and p21 [27]. There is no doubt that a proper diet may protect against cancer and extend life expectancy. Ancient Egyptians and Babylonians knew the positive effect of proper nutrition on life extension. While life expectancy of the general population, at that time, was approximately 30 years, the ruling class lived much longer. Ramses II lived 92 years and pharaoh Neferkare ruled for 94 years. One reason for the longer life expectancy of these rulers was a specially selected diet, which is documented in old scriptures and also confirmed by chemical analysis of archeological artifacts.

The use of food supplements, which have a gentle effect on gene expression, may be preferable to the drugs (Fig. 1). A number of phytochemicals play an important part in regulation of oncogenes [36]. Genistein from soybeans and EGCG from green tea can down-regulate AKT pathway [37,38]. Resveratrol from grapes and curcumin from turmeric inhibit NF- $\kappa$ B and RAS signaling [36,39,40]. Unfortunately, resveratrol promotes Sir2, which inactivates p53. Limonene from citrus interrupts RAS signaling through inhibition of farnesylation of the RAS protein [36]. Phytochemicals play a more important part in the regulation of oncogenes than the regulation of tumor suppressor genes.

In this area, however, our team introduced a group of amino acid derivatives and organic acids, which activate tumor suppressors p53, p21, PTEN



**Figure 1** Possible mechanisms of restoration of normal gene expression in aging using dietary supplements. Down-regulation of increased signaling along growth factor/oncogene pathways can be accomplished with phytochemicals. Genistein from soybeans and EGCG from green tea regulate the IGF-1/AKT pathway, resveratrol from grapes and limonene from citrus fruits regulate RAS signaling and curcumin from turmeric regulates NF- $\kappa$ B. PG and isoPG (Aminocare<sup>®</sup> A10 and Aminocare<sup>®</sup> Extra) regulate IGF-1/AKT-2/NF- $\kappa$ B and apoptosis. PN decreases expression of the RAS oncogene. PN and PB activate tumor suppressors p21 and p53 and apoptosis. A10 and PN activate PTEN.

and *INI1* and decrease overexpression of *RAS* and *AKT2* and *MYCC* oncogenes [41]. The following substances, present in human blood and food, have been synthesized and found to interact with genes involved in cancer and the aging process: 3-phenylacetyl-amino-2, 6-piperidinedione (A10), phenylacetylglutamine (PG), phenylacetylisoglutamine (isoPG), phenylacetate sodium (PN), phenylbutyrate sodium (PB). A10 specifically intercalates with DNA and protects the sequences, which are vulnerable to the effects of carcinogenes, such as benzo [a] pyrene, urethane, and aflatoxin B<sub>1</sub>. In animal tests, mice and rats were protected from development of breast, lung and liver cancer when they were exposed to benzo [a] pyrene, urethane, and aflatoxin B<sub>1</sub> and were fed a diet containing A10 [42–48]. PG and isoPG are metabolites of A10 [49,50]. In addition, PG is biosynthesized in the liver from glutamine and phenylacetate and was detected in cows milk. PG exhibits antineoplastic activity across a wide array of cancer cell lines. It inhibits the uptake of growth-critical amino acids such as l-glutamine and l-leucine in neoplastic cells. It also normalizes the pattern of genome-wide methylation, stabilizing the genes, decreasing expression of oncogenes such as *AKT-2* and *MYCC* and activating tumor suppressors *PTEN* and *INI1* [51].

PN is present in human blood and in dairy products. It works as a molecular switch, which turns off the electrical signal in the *RAS* pathway and activates *p53* and *p21*. PN inhibits farnesylation of the *RAS* protein and causes down-regulation of *BCL-2* [52,53]. It also binds excessive amounts of l-glutamine, which would have promoted cancer growth [54]. PB exists in lamb and cheese and it is partially converted in the liver to PN and PG. It activates tumor suppressor genes through inhibition of histone deacetylase [55,56].

A10 and PG were formulated, together with selected amino acids and vitamin B-2, into two supplements currently available in the United States and in the European Union. A10 is a component of Aminocare<sup>®</sup> A10 and PG of Aminocare<sup>®</sup> Extra. In addition to cancer preventive effects documented in animals, there were also a number of positive anti-aging effects observed by individuals who have taken these supplements. Positive effects included increased energy, improved healing, reduction of wrinkles and hyperpigmentation spots, reduction of cholesterol concentration in blood, improved cellular immunity, decreased frequency of common viral infections, improvement of benign prostate hypertrophy and a decrease of benign nodules in breasts, as well as antidepressant effects.

## Conclusions

Studies in animals confirm substantial changes in gene expression in aging, the most pronounced being silencing of tumor suppressors and genes responsible for detoxification and prevention of atherosclerosis. A smaller group of genes shows increased expression. Among these are oncogenes and genes associated with typical diseases of old age. Silencing of tumor suppressors may increase signaling through oncogene pathways (for instance, silencing of *PTEN* increases signaling through IGF/ *AKT*). The studies in animals revealed that caloric restriction normalizes expression of numerous genes in aging organisms. It has been postulated that increased IGF-1 signaling and hyperactive *p53* promotes aging. The reversal of such condition extends life in animals, but results in reduced activity and dwarfism. Current animal models used in aging research study these mechanisms, but they are substantially different in humans. Decreased signaling through IGF-1 in animals (which extends their life) will also increase activity of *p53*, which according to these experiments will reduce survival. The function of *p53* and IGF-1 in humans is more complex and somewhat different than in animals. It is clear that different animal models are needed to better represent human aging.

Age management therapy should attempt to normalize gene expression: activate silenced tumor suppressors and decrease overexpression of oncogenes. Diet modification and supplements of plant and animal origin may serve the purpose. It is hoped that further research on gene expression will result in more effective age-management treatments.

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