Systematic investigation of aging-related molecular factors

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Abstract. Aging is an irreversible process that negatively impacts vitality and health. Investigation of aging on a molecular level in vertebrates revealed key regulatory genetic pathways. However, previous studies have been focused on one pathway. Moreover, the functions of small molecules taken from diet were almost neglected. Thus, current knowledge about aging lacks comprehensiveness, and most aging-related factors remain unknown. In addition, the inter- and intra- regulations of different pathways were still unclear. In this paper, we summarized regulatory pathways of aging on a molecular level, such as insulin/insulin-like growth factors signaling (IIS), target of rapamycin (TOR) pathway and telomerase-related pathways. In summary, reduced IIS and TOR signaling are anti-aging. However, in the case of telomer, which was previously shown to be associated with aging, the underlying mechanism was not well understood. Hence, we proposed design of experiments for deconvolution. Furthermore, we investigated the diet dependency of aging at a molecular level, such as how it relates to fasting, carbohydrate, and fat. Plus, we searched for biological techniques that can be used for the discovery of new aging-related pathways and the inter-/intra regulation between known genetic pathways. Our paper provided insights into the unbiased systematic investigation of aging.

1. Introduction

Aging is the progressive decline in functional integrity and homeostasis, culminating in death. Old age is accompanied by an increase in diseases that are rare in young individuals. Once thought to be an inevitable outcome of life, aging is in fact regulated by both genetic pathways and environment, such as diet [1, 2]. Much of our current understanding of aging originates from model organisms such as yeast, C. elegans and flies. Currently, genetic pathways that are heavily studied include the insulin/insulin-like growth factor (IGF) pathway, target of rapamycin (TOR) pathway, telomerase pathway and sirtuin pathways [3-6]. Investigations of these genetic pathways have yielded many unprecedented results. However, they lack comprehensiveness and only focus on one or a few aspects. Moreover, the environmental contributions, such as diet, were mostly neglected. In addition, how known pathways interact and regulate each other was not well understood, not to mention the possibility of potential new genetic pathways. Thus, to gain a holistic understanding of aging, we need to connect seemingly irrelevant pathways and develop methods that allow us to discover new aging-related factors.

In this paper, we summarized previous findings from the investigation of IIS pathway, TOR pathway, and telomerase pathway. We found that despite genes and metabolites that were shown to reduce or accelerate aging from previous literature7, there remains aspects that were associated but neglected. For example, in the case of IIS-dependent longevity, metabolomics analyses were mostly neglected in any model organisms of study, although massive restructure of metabolism in reduced IIS organisms was proposed8. Another example would be in the case of telomerase activity. In an organism that lacks telomerase activity, the impact in terms of downstream changes, such as changes in gene expression, protein translation, and metabolism were not well understood. Proposals were made in this work to understand more about known aging-related genetic pathways.

In addition, we deeply investigated the impact of diet on aging, such as intermittent or periodic fasting, fats, and carbohydrates in the diet. We found that not all fats or carbohydrates were created equal in the context of aging. Lastly, we searched for biological techniques that can be used to study aging. We summarized basic techniques that were used to study omics from the central dogma of biology, which were genomics, transcriptomics, proteomics, and metabolomics. We then summarized methods that allow us to study the interaction between omics, including co-fractionation assays, such as PROMIS, that can be used to study the interactions between aging-related metabolites and their potential protein targets9. Another method is ChIP-seq, that were used to study protein-DNA interactions and can be used to study the genetic regulation of gene expression [10]. Together, we proposed methods that target the investigation of aging as a whole and may provide a basis for future study of aging.

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2. Summary of findings

2.1. Insulin/Insulin-like growth factor 1 (IGF-1)

Insulin/Insulin-like growth factor 1 (IGF-1) signaling (IIS) is highly conserved across the animal kingdom and plays a central role for development and aging, by regulating cell cycle progression, apoptosis, and protein translation of proteins [1, 2, 11, 12]. Mutations in many insulin and IGF-1 pathway genes change the metabolism and extend the lifespan of mammals and several have been linked to human longevity [13]. Many of these effects appear to be mediated by signaling cascades that involve altered metabolism1. Using the genetically tractable system, C. elegans, to study the function of IIS has produced many insights into the functions and regulations of IIS. In C. elegans, mutants with decreased activity of DAF-2, the IGF-1 receptor homolog, display a range of phenotypes, most notably, dramatically increased lifespan (Fig. 1) [1, 8, 14]. Using C. elegans as a model, previous research investigating the mechanisms underlying daf-2 dependent longevity relied on with transcriptomic and proteomic analyses [7, 8, 15]. Recently, it was also shown that changes in protein posttranslational modifications may explain IIS-dependent longevity to some extent [16]. However, the true mechanism of reduced IIS extending lifespan and slowing down aging is still unclear. The most important aspect that needs to be investigated is the changes of metabolism with reduced IIS, which is easily achieved with metabolomic studies using C. elegans, a model organism that was previously extensively used to study metabolism [17-22]. Since many previous studies were performed on C. elegans, a literature search revealed identification of key pathways that were altered in daf-2 mutants that were seemed to be responsible for longevity in C. elegans [23, 24], including food restriction pathways and lipid metabolism. In addition, transcriptomic analyses of daf-2 mutants revealed more than a thousand genes that were expressed differentially7. Any one of these genes can be responsible for longevity, which needs to be further investigated.

Other than the model organism C. elegans, yeast, flies and mice were also used to study IIS-dependent longevity. However, a literature search revealed little information in terms of transcriptomic, proteomic and metabolomic changes with reduced IIS in these organisms. Thus, to investigate IIS in an unbiased manner, multi-omics analyses will be needed to be performed on these organisms.

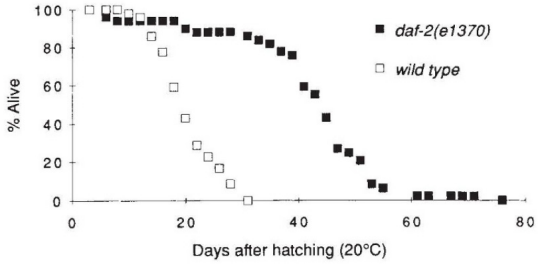

Fig. 1. Lifespan extension of daf-2(e1370) cultured continuously at 20 °C. Figure was referred from previously published work [14].

2.2. Telomere and telomerase

In the absence of telomerase, standard DNA polymerase fails to replicate the complete linear chromosome, resulting in the production of chromosomes with shortened telomeres [25, 26]. Telomeres consequently lose the ability to bind enough telomere-capping proteins, which activates a DNA damage response (DDR) that arrests proliferation27. However, these short telomeres bind just enough telomere-capping proteins to inhibit DNA repair and avoid fusions, which reinforces an ongoing DNA damage signal that continues a permanent DNA damage-induced proliferative arrest [27]. Telomere shortening and damage are, therefore, believed to be causes of cell senescence. Normal aging is also precipitated by accelerated telomere dysfunction. Although telomere shortening cannot explain senescence in non-proliferating and quiescent cells, telomere-associated DDR foci have been reported to age post-mitotic cells, such as neurons, osteocytes, osteoblasts, adipocytes, and cardiomyocytes. Activation of DDR at telomeres (tDDR) also occurs at long telomeres when DNA damage occurs within telomeric repeats (tDD). Such damaged telomeres induce a senescence-like phenotype. In addition, oxidative DNA damage in telomeres is known to inhibit telomerase and disrupt the recognition by telomere-binding proteins which causes telomere uncapping. It is suggested that there is a ‘telomere-centric’ mechanistic rationale for aging-associated processes as it connects to mitochondrial dysfunction, impaired autophagy, epigenetic dysregulation, and altered nutrient sensing [27].

It was recently reported that thymidine nucleotide metabolism controls human telomere length [27]. In this study, a CRISPRi screen was performed on two actively dividing cell types, K562 and HEK293 cells. However, many primary cells were not actively dividing as them. Hence, a CRISPRi or RNAi screen on non-actively dividing primary cells could be performed to screen for genetic pathways that extend telomere length (Fig. 2). Furthermore, how DNA damage regulates cell aging remains largely unclear. One way of studying these would be to perform multi-omics analyses between wildtype cells and TERC-null cells.

Fig. 2. Scheme of RNAi screening strategy for genes affecting telomere length. Figure was referred from previously published work [27].

2.3. TOR pathways

The target of rapamycin (TOR) is a highly conserved serine/threonine kinase that is found in two structurally and functionally distinct complexes named TOR complex 1 (TORC1) and TOR complex 2 (TORC2). Mammalian TOR (mTOR) is believed to regulate whole-body homeostasis by playing an essential role in metabolic organs such as the liver, muscle, and adipose tissue in humans and mice. Having mTOR as a nutrient

and growth factor sensor, its inhibition by chemicals such as rapamycin delays the onset of age-related disease and extends lifespan in mice.

In mammals, mTORC1 appears to play several roles in aging as it controls aging via s6K and 4E-BP to regulate protein synthesis [28]. It is also found that activation of 4E-BP activates FoxO and Nrf, stress-responsive genes, which indicates that mTORC1 may also control aging by modulating stress-responsive genes downstream of 4E-BP (Fig. 3) [29, 30]. In addition, mTORC1 modulates aging through autophagy, in which autophagy acts as a tumor suppressor that extends lifespan [31]. Decreased mTORC1 signaling in a metabolic tissue alone can increase lifespan, whereas the role of mTORC2 in aging remains unclear. Studies of TOR in both mammals and other model organisms showed that TOR is downstream of IIS.

As previously discussed, reduced signaling through the insulin/IGF-1 signaling (IIS) pathway extends lifespan by activating the transcription (Fig. 3) factor FoxO that acts as a tumor suppressor [32]. In C. elegans and mammals, it was demonstrated that increased expression of glutamine synthetase activated by FoxO/DAF-16 inhibits TORC1 activity [33]. Meanwhile, FoxO in mammals and flies induces the expression of sestrin, which in turn inhibits TORC1 signaling, leading to the activation of AMPK, a negative regulator of TORC1 signaling [34]. The above evidence suggests that FoxO may positively affect lifespan and tumor suppression.

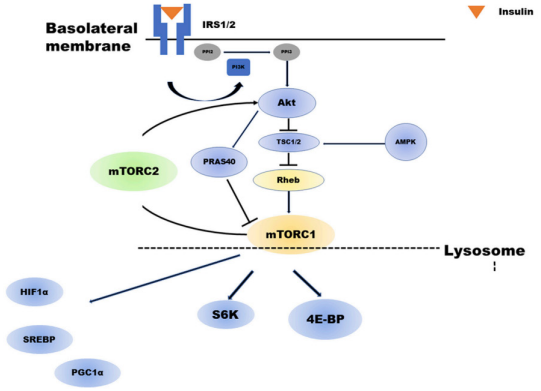

Fig. 3. Overview of the mTOR cascade. Insulin activates both mTORC1 and mTORC2. One component downstream of mTORC1 is 4E-BP. Figure was referred from previously published work [35].

In TORC, many subunits were used to regulate TOR signaling, including GATOR, PROTOR, KICKSTOR and RAPSTOR. Regulation by each subunit is in a unique manner, either activating or deactivating the downstream signaling (Fig. 4). Thus, each subunit can be a target to study aging. For example, inhibitors of GATOR can be used to target aging by regulating TOR signaling [36]. Meanwhile, although transcriptomic and proteomic data are available from current publication, how they are related to aging is still unclear. Also, it is well known that TOR regulates metabolism, but the metabolomic analyses of TOR mutants have not been performed. Hence, looking at downstream genetic pathways and metabolomic pathways may provide more insights into the biological function of TOR signaling and how it is related to aging.

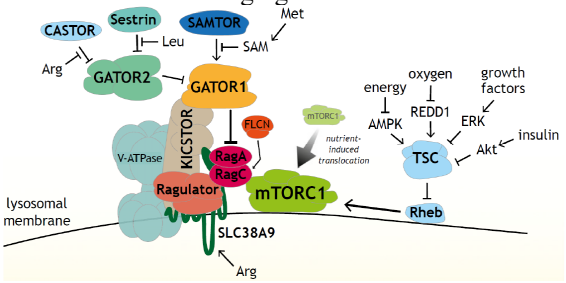

Fig. 4. Schematic showing components of the nutrient-sensing pathway upstream of mTORC1. Figure was referred from previously published work [37].

2.4. Diet

Diet accounts as an environmental factor that play a critical role in aging but was previously mostly overlooked [38]. In this paper, dietary factors were separated into two components, dietary restriction, and dietary intake. It was previously reported that intermittent and periodic fasting promote longevity [39], in a manner that is similar to reduced IIS and reduced TOR signaling, as previously discussed. In terms of dietary intake, the nutritional component of diet also plays a role in aging. For example, it was previously reported that high glucose diet induces obesity and accelerates aging after developmental stage, whereas supplementation of glucose during developmental stage extends lifespan [40, 41]. Such observations led to the idea of all carbohydrates being bad for aging. However, this is not accurate. Carbohydrates such as fiber are beneficial to human and microbiome, resulting in the improvement in immune response, health, and ultimately affects aging [42, 43]. Similar proposals were also found in fat, where it was commonly believed that fat is bad for health. However, different fats have different biological functions. For example, it was reported that unsaturated fatty acids, such as oleic acid, EPA, and DHA, promote human health and slow down aging, whereas others are bad for human health. When it comes to diet, one must keep in mind that not all compounds are created equal. As discussed above, in the case of carbohydrates and fats, every compound is different and may serve different biological functions.

2.5. New approaches towards investigation of aging

When it comes to biology, the central dogma of biology is the most important aspect. The central dogma of biology contains the transformation from DNA to RNA to protein and to small molecule metabolites. Each step of the transformation represents transcription, translation, and enzymatic metabolism, respectively. The study of DNA, RNA, protein, and metabolites is defined as genomics, transcriptomics, proteomics, and metabolomics. As briefly mentioned above, multi-omics analyses can be performed in all biological contexts to study the changes of different omes. For example, genome-wide association study (GWAS) is a genomic

method used to understand the variations between aging phenotypes, such as health span, lifespan, extreme longevity, and epigenetic aging, and is important to develop therapeutics to improve healthy aging [44, 45]. GWAS can be used to identify genotype-phenotype associations by testing hundreds of thousands to millions of genetic variants to find the link between single-nucleotide polymorphisms (SNP) and common diseases. GWAS loci may discover previously unsuspected relevance, which can lead to uncovering new biological mechanisms behind diseases [46]. However, single-phenotype approaches by GWAS often leave shared genetics among aging traits, such as epigenetic age acceleration (EAA) and frailty, unaccounted for. New methods of GWAS—the multivariate GWAS approach—combine univariate GWAS summary statistics to enhance the discovery of biological correlations by increasing effective sample sizes. The method has already discovered novel, unknown variants to be explicitly linked with important aging factors and processes44.

In addition to genomics, transcriptomics and proteomics are also important strategies to study biology. Transcriptomes consist of all RNA transcripts in cells. It is vital for interpreting functional elements of a genome and the development of diseases. RNA sequencing (RNAseq) is useful for studying complex transcriptomes as it reveals the precise location of transcription boundaries, gives information regarding how exons are connected, and demonstrates sequence variations in the transcribed regions. RNAseq also allows the detection of transcripts over a large dynamic range of expression levels, unlike DNA microarray, which is insensitive to genes expressed at low or very high levels. Its high accuracy for quantifying expression levels is also notable using quantitative PCR and spike-in RNA controls. RNAseq, in general, is a high-throughput method that allows the entire transcriptome to be evaluated, offering both single-base resolution and gene expression levels at the genomic scale. RNAseq can be performed to probe gene expression changes in long-lived or short-lived organisms. Identification of such genes potentially reveals new genetical pathways and targets that can be used to study aging and the development of drugs to slow down aging. On the other hand, proteomics is a multi-step technique that studies the interactions, constituents, functions, and structures of proteins and their cellular activities. Proteomics assesses qualitative and quantitative cellular responses related to a certain protein, and the proteomes are measured at post-transcriptional, transcriptomic, and genomic levels. Qualitative proteomics monitors protein mixture composition and protein expression changes and provides information on molecular mechanisms of diseases. Quantitative proteomics can also supply information on disease mechanisms, cellular functions, and biomarker discovery. There are three types of proteomics: expression proteomics, structural proteomics, and functional proteomics. Expression proteomics aims to discover disease-specific proteins and new proteins in signal transduction by studying protein expression patterns in different cells. It also aims to specify protein expression differences between two conditions such as controls and patients. Structural proteomics utilizes nuclear magnetic resonance spectroscopy and X-ray crystallography to determine the three-dimensional structure of functional proteins. Functional proteomics investigates the interactions between an unknown protein with partners from a specific protein complex involved in a particular process.

There are two approaches in proteomics: bottom-up and top-down workflows. In the bottom-up method, protein is digested by trypsin into peptides, separated by a specific column, and then its mass is evaluated by mass spectrometry (MS). According to the fractionation step, the bottom-up approach can be separated into two groups: The first approach isolates the proteins from the gel using two-dimension electrophoresis (2-DE) and then digests the proteins into peptides that MS can identify; the second approach, called “shotgun” proteomics, digests the proteins without fractionation, and liquid chromatography (LC) separates the peptides identified by MS. In the top-down method, whole proteins are directly analyzed by MS, having their mass often calculated by electrospray ionization followed by matrix-assisted laser desorption/ionization MS. The application of proteomics in aging is similar to that of RNAseq in different biological contexts.

Another omics that was previously neglected was metabolomics by mass spectrometry. In the case of annotation of the metabolome from living organisms, early efforts focused mainly on using NMR spectroscopy to identify isolated samples of abundant molecules [47-49], as compatible with the low sensitivity of NMR spectroscopy. However, recent advancements of high-resolution mass spectrometry- (HRMS-) based methods greatly accelerated compound discovery by providing sensitivity and high-throughput [21]. For example, untargeted comparative metabolomics based on LC-HRMS enables quick identification of hundreds of small molecules that had remained undetected using NMR-based strategies, due to their low abundances [18, 50]. In the context of aging, comparative metabolomics with LC-HRMS between long-lived and short-lived organisms allows us to quickly detect changes in the metabolome, and thus gaining insights into what small molecule metabolites are aging-associated.

In addition to single omics analyses, new approaches have been proposed to study the interaction between omes. For example, ChIPseq was used to study protein DNA interaction, and co-fractionation assays were used to study protein-protein and protein-small molecule interactions [51-54]. When a metabolite or a protein is identified as a target to study aging. It is important to understand the mechanism of actions. Hence, discovering their potential interacting partners, such as DNA or protein, is important. ChIPseq and co-fractionation assays will provide such information. Together, comparative multi-omics analyses between long-lived and short-lived will reveal information about targets such as genes, proteins, and small molecule metabolites that can be used to study aging, while ChIPseq and co-fractionation assays will provide their

mechanism of actions. Such schemes can be used to investigate aging in a more comprehensive manner.

3. Discussion

The complexity of aging is underscored by the numerous hallmarks of aging, which include deregulated nutrient-sensing, genomic instability, telomere attrition, loss of protein homeostasis, epigenetic alterations, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication, such as inflammation [2]. Genetic modification of the hallmarks of aging can delay aging within vertebrate species. Across vertebrates, genes in these hallmarks have been modified during evolution to result in an incredible diversity of lifespan. Identifying the ensemble of genes that regulate aging in vertebrates, and how they interact, can help delay aging and age-related diseases in humans. Current knowledge about aging-associated genes and genetic pathways most originated from the investigation of IIS, telomere, and TOR pathways. Despite great effort and many unprecedented discoveries, most genes generated from these studies were not deeply investigated, due to their large quantities and lack of good techniques. In this paper, we summarized results from previous studies and proposed what can be added to gain more insights into aging. We also provided methods that can be used to further investigate aging. In the case of IIS-dependent longevity, thousands of genes were generated from previous RNA-seq analyses. Only one or a few were covered. Mutant analyses or RNAi analyses for one or a combination of these genes can be carried out to confirm their association with aging. Furthermore, the metabolomics aspect was neglected. In the case of telomere, little knowledge about the biological effect with low telomerase activity was obtained from previous studies. In addition, studies about TOR pathways primarily focused on inhibition or activation by nutrients or small molecule inhibitors. Thus, the real biological changes associated with TOR were still unknown. Finally, the dietary aspect was almost completely overlooked in previous studies. In this paper, proposals to address these problems were made. Also, techniques that can be used to study aging have been evolving. Techniques in traditional multi-omics analyses have been improved greatly with the development of more powerful instruments, and current techniques, such as protein-metabolite interaction by size exclusion (PROMIS) and ChIP-seq, allow us to study the interaction between different pathways and different omes [9].

With a deeper understanding of aging, personalized medicine approaches may transform strategies to combat aging and help offset potential trade-offs of more generalized interventions. Personalized strategies could incorporate genetic factors and environmental status to slow down aging and age-related diseases. In addition, the aging process is inherently stochastic, which could result in different aging trajectories even when other factors are similar. A crucial step will be to efficiently model genetic variants, as well as other factors, for personalized drug screening. For this, new vertebrate models could open new possibilities for screens for personalized drugs or interventions. The identification of reliable aging biomarkers that provide insights into biological age will also be critical to guide precision medicine approaches.

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