REVIEW PAPER |

RNAi: Applications and possibilities in next generation ageing research

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Abstract

Ageing is characterized by a general decline in physiological function that leads to morbidity and eventually, mortality. This conserved process is related to so many genes in living organism those are directly or indirectly involved to regulate ageing. RNA interference (RNAi) is a kind of genetic tool that has ability not only to screen specific gene but also to silence gene expression. The search for genetic mechanisms affecting lifespan and ageing represents an important part of ageing research. The feasibility of genome wide RNAi screens potentially much simplifies the identification of novel ageing related genes. In this review the applications of RNAi in ageing research were discussed. Besides the purposes of the study were to highlight the current and future impacts of this technology in the field. In particular, how the flexible control of RNAi induction can support the study of ageing through specific experiments has become highlighted. Since RNAi has emerged as a powerful tool for the study of ageing it has possibility to allow the further characterization of the roles of specific genes in the ageing process as well as the efficient identification of new genes implicated. Corresponding genes with age related diseases also be possible to screen and study by RNA interference (RNAi). Specific RNAi time course experiments provide an opportunity for the analysis of high resolution gene expression in ageing relevant processes. In near future RNAi tool box will considerably contribute to the next steps in researching the genetics of ageing and age related diseases.

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Introduction

Ageing is the accumulation of changes in a person over time. It is also called the process of growing older. It is revealed from the Medical Dictionary that ageing includes a reduction in strength, endurance, speed of reaction, agility, metabolism, sexual activity, hearing acuity. Ageing of any organism is partly controlled by its genome. Ageing is characterized by a general decline in physiological function that leads to morbidity and eventually mortality (Jae *et al.*, 2010). In a broad sense, ageing is defined as the gradual biological impairment of normal function, probably as a result of changes made to cells (mitotic cells, such as fibroblasts and post mitotic cells, such as neurons) and structural components (such as bone and muscle). These changes would consequently have a direct impact on the functional ability of organs (such as the heart, kidney and lungs), biological systems (such as the nervous, digestive and reproductive system) and ultimately the organism as a whole. It is very much essential to know the underlying genetic mechanisms for in-depth understanding of ageing (Nadege *et al.*, 2010). There are several theories of ageing and which depict that life span was under the control of many genes those act late in life or beneficial in early life (Ljubuncic *et. al.,* 2009). In this work the importance and impact of RNAi in molecular ageing research was reviewed. The overall work plan is given in Fig. 1.

Interfering ribonucleic acid (RNAi)

Interfering Ribonucleic Acid (RNAi) or RNA interference was first recognized by Rich Jorgenson and his team in plants (Napoli *et. al*; 1990). Further work was found by Fire and his colleagues. They were investigating the efficiency of injecting single stranded antisense RNA as a method of gene silencing. They observed the ability of nematodes to hybridize with endogenous mRNA and inhibit translation (Fire *et al.,* 1998). In 2006, the American researcher Andrew, Fire and Craig C. Mello were awarded the Nobel Prize in physiology and Medicine for participating of RNAi in the elucidation of gene silencing (Natália *et. al.,* 2010).

RNAi in short is a messenger RNA (mRNA) degradation process in eukaryotes. The inherent naturally occurring intracellular mechanism of RNAi causes sequence specific post transcriptional gene silencing (Henny *et al.*, 2007). It is one of the series of natural processes by which short RNA molecules influence gene expression in living cells, RNAi does not inactivate a target gene itself but destroys its mRNA (T. A. Brown, 2007). There are several forms of RNAi such as micro (mi) – RNA, piwi-Interacting (pi RNA), short-Hairpin (sh) RNAs and small modulatory(sm) RNAs (Henny M. *et al.*, 2007). Naturally, in eukaryotes, primary mi RNA (pri-mi RNA) undergoes processing to form preliminary mi RNA (pre- mi RNA) and then transport out of the nucleas. In cytoplasm, pre- mi RNA further processed into short interfering RNA (sh RNA) and ultimately play role in gene silencing. (Michaela and Matthias, 2007). The underlying mechanism of RNAi biogenesis is given in Fig. 2. When synthetic si RNA is constructed and incorporated into host cytoplasm via different ways (e.g. expression vector, soaking methods etc.), the construct assemble with RNA induced silencing complex (RISC). Synthetic Si RNA contains two stands called passenger stand and guide strand (Bernstein *et al.*, 2001). Synthetic siRNA construct releases two strand by the activity of endonuclease Dicer (an RNAse III family enzyme) (I Bantounas *et. al.,* 2004). Actually guide strand is complementary to target mRNA (Markus *et al.*,2006). siRNAs usually bind with high complementarity to coding sequences and induce mRNA degradation whereas miRNAs usually imperfectly hybridize to untranslated regions and prevent translation or reduce mRNA stability for example by accelerating deadenylation (Wu L *et al.*, 2006). Guide strand assemble with RISC bind to target mRNA as well as either reduce mRNA stability or plays role in mRNA cleavage (Fig. 2) (Michaela and Matthias, 2007).

Relation between ageing research and RNAi

As RNA interference (RNAi) is a technique for silencing gene expression, the feasibility of genome

wide RNAi screens potentially much simplifies the identification of novel ageing related genes (Jose *et al.,* 2004).It is not only helpful to degrade target mRNA involved in ageing but also reduce the stability of mRNA that has involvement in ageing. Although it is a natural process but it is possible to construct RNAi synthetically as well as has possibility to increase life span of several important organisms even human (Jean *et al.,*1996). Specific RNAi time course experiments provide an opportunity for the analysis of high resolution gene expression profiles capturing the dynamics of ageing relevant processes and gene interactions. Research exploiting new avenues opened by the growing RNAi tool box will considerably contribute to the next steps in researching the genetics of ageing and age related diseases (Nadège *et al.*, 2009).

Theories and hypothesis of ageing

At beginning, the process of ageing was thought in the same way that moving parts wear out, machines break down and all things deteriorate slowly over time. After the development of the evolution theory the thinking levels about ageing were modified. Some important theories and hypothesis are discussed here.

August Weismann's Theory:

At the beginning August Weismann developed the theory of programmed death. He also suggested that older members of a species are expected to die of old age by specific death mechanism of natural selection. These natural processes help to eliminate old members of population so that they would no longer compete with younger generations for food and other environmental resources (Weismann , 1882).

Medawar's Theory:

Few years later Medawar another renowned scientist developed another theory. He showed evidence that animals living in harsh conditions in their natural habitat survive for only relatively short period of time compared to those living in the protected conditions of

captivity, which is opposite to what would be expected from the programmed death theory. Besides many advantageous environmental factors, appropriate medical treatment (including vaccinations) may also be an important life-prolonging factor under these circumstances (Predrag Ljubuncic *et al.,* 2009).
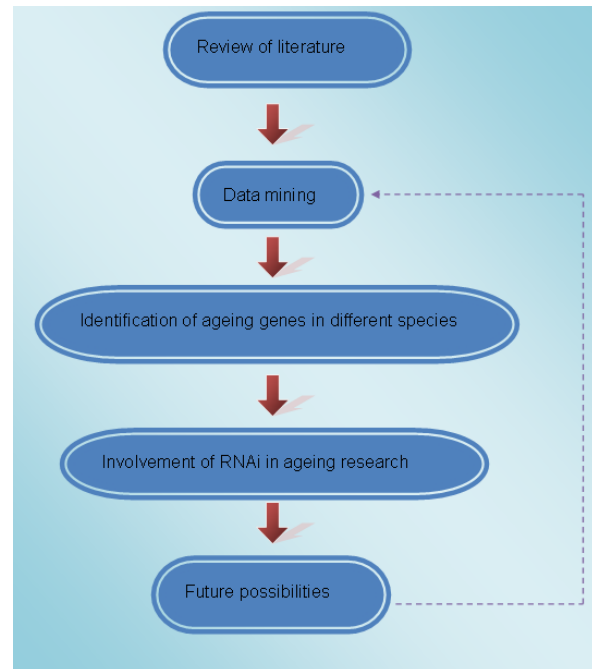

Fig. 1. Overview of the research work.

The Mutation Accumulation Theory:

This theory originates from Peter Medawar's ideas that were related to accumulation of late-acting harmful genes. The basic observations were developed by Peter Medawar. Since all organisms eventually die from different causes (e.g. diseases, accidents, predation), genes beneficial early in life are favored by natural selection over genes beneficial late in life (Medawar, 1952). Consequently, the greatest contribution to create a new generation comes from young, not from old organisms. Thus, the power of natural selection fades with age, making it possible for hazardous late acting genes to exist. This theory considers ageing as a byproduct of natural selection. According to this theory, ageing has no adaptive traits since natural selection does not occur in long lived animals and provides little additional contribution to offspring

numbers effects only in old animals. The probability of an individual reproducing is age dependent. It is zero at birth and reaches a peak in young adults. Then it decreases due to the increased probability of death linked to various external (predators, illnesses, accidents) and internal (senescence) causes. On the other hand, deleterious mutations expressed later in life are relatively neutral to selection because their bearers have already transmitted their genes to the next generation (Charlesworth *et al.*, 2000).
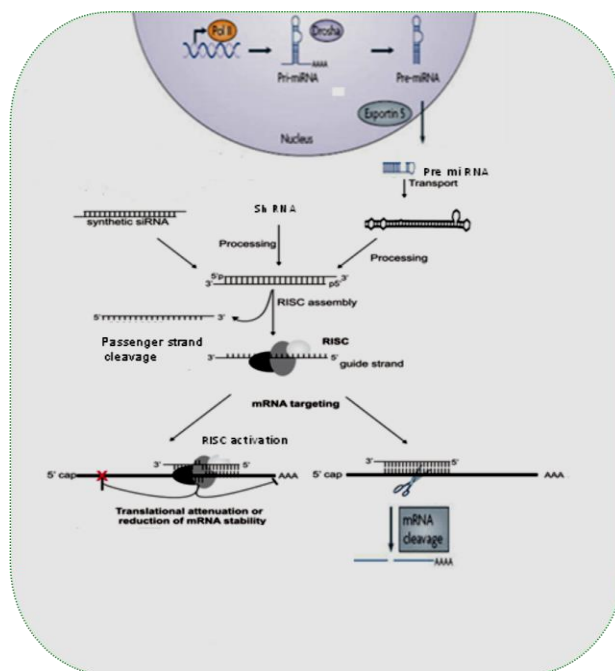

Fig. 2. Mechanism of RNAi.

Antagonistic Pleiotropy Theory:

George Williams's originates model of antagonistic pleiotropy. It is revealed to this theory ageing evolves due to the pleiotropic effect of genes that are beneficial early in life and then harmful at late ages (Medawar, 1952). He proposed that some genes are beneficial at earlier ages but harmful at later ages. The genes with age related opposite effects are called pleiotropic genes (Williams GC, 1957). Antagonistic pleiotropy theory is based on two assumptions. First, it is assumed that a particular gene may have an effect not only on a feature but also on several traits of an organism (pleiotropy). Second, these pleiotropic effects may affect individual fitness in antagonistic ways.

Telomere Shortening:

It is commonly thought that psychological stress leads to premature ageing and the earlier onset of diseases of ageing (Postnikoff *et al.*, 2012). Several studies correlates between chronic stress and indices of poor health, including risk factors for cardiovascular disease and poorer immune function (Elissa, 2009). Telomeres are one kind of DNA protein complexes. Telomeres and telomerase has crucial roles in cellular ageing and potentially in disease. (Shpiz *et al.,* 2011). They play role to uncap chromosomal ends, as well as promoting chromosomal stability (Imbert *et al.,* 2012).

When cells divide, the telomere is not fully replicated because of limitations of the DNA polymerases in completing the replication of the ends of the linear molecules that ultimately leads to telomere shortening with every replication (Leman *et al.,* 2012). *In vitro*, when telomeres shorten sufficiently, the cell is arrested into senescence. In people, telomeres shorten with age in all replicating somatic cells that have been examined, including fibroblasts and leukocytes (Sofie *et.al.* 2005). Thus, telomere length can serve as a biomarker of a cell's biological age. Whilst telomerase, a cellular enzyme, adds the necessary telomeric DNA (T_2AG_3 repeats) onto the 3'ends of the telomere. Telomerase also has direct telomere protective functions. Cellular environment also plays an important role in regulating telomere length and telomerase activity (Coussens *et al.,* 2010). Most notably, *in vitro*, oxidative stress can shorten telomeres and antioxidants can decelerate shortening. Perceived stress has been linked to oxidative DNA damage in leukocytes in women especially (Marcon *et al.,* 2012). Thus it was hypothesized that chronic psychological stress may lead to telomere shortening as well as lowered telomerase function in peripheral blood mononuclear cells (PBMCs) and to oxidative stress (Elissa *et al.*, 2004).
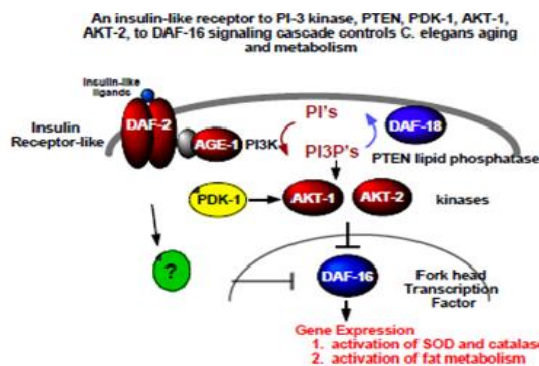
Fig. 3. Insulin signaling pathway (Caleb E. Finch and Gary Ruvkun, 2001).

Mitochondria and Ageing:

Mitochondria is also called power house and the central components of living cells generate the majority of energy from nutrients (Sas *et al.,* 2007). Whilst they generate unstable chemicals that harm both the mitochondrion itself and other components of the cell. This resulting damage is thought to play important role in ageing. Many scientists are developing techniques for attenuating and reversing this damage (Richards *et al.,*2000). Mitochondria usually perform "aerobic respiration"(Gottlieb *et al.,* 2002) which is a process that generates the energy molecule (ATP) from nutrient molecules using oxygen. But it has an unfortunate drawback of aerobic respiration in mitochondria. Sometimes the electrons are not donated to the ETC but instead go on to form unstable molecules called reactive oxygen species (ROS) (Ozawa, 1997). This type of ROS is capable of damaging many types of cellular components. The damaged components may accumulate over time from aerobic respiration as well as it is thought that these components may play a significant role in ageing. As mitochondrial DNA exists in the inner matrix and this is in close proximity to the inner membrane (where electrons can form unstable compounds), so it may say that mitochondrial DNA has possibility to be damaged by ROS (Hayakawa, 1993). It can also occur mutations to mitochondrial DNA that can result in the manufacturing of mutant ETC proteins. It leads to the

leaking of more electrons and more ROS. This type of cyclic problem is also called "vicious cycle". Thus it is hypothesized that vicious cycle that performs in mitochondria plays a critical role in the ageing process (Chomyn and Attardi , 2003).
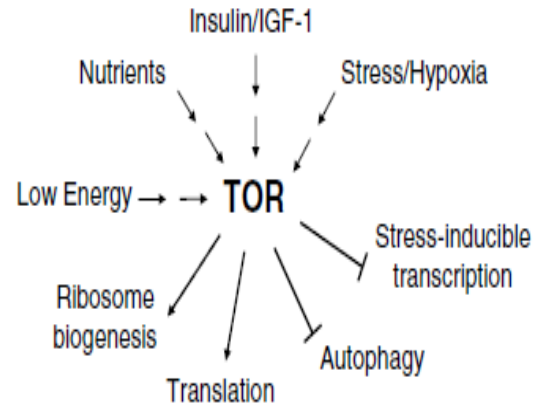

Fig. 4. TOR kinase integrates diverse inputs to modulate gene expression and protein turnover, under condition of stress and during ageing (Wullschleger *et al.,* 2006).

Pathways involved in ageing

There are two pathways that regulate longevity such as Insulin signaling pathway and TOR pathway.

Insulin signaling pathway:

Insulin/IGF-1 signaling pathway is a well conserved, well defined and fundamental pathway to regulate longevity. This insulin-like signaling pathway is part of a global endocrine system that controls whether the animals grow reproductively or arrest at the dauer diapauses stage. The *daf-2, age-1, pdk-1, daf-18,akt-1,akt-2 and daf-16 are the components of* insulin-like signaling pathway (Saltiel *et al.,* 2002). In C. elegans, insulin-like signaling pathway regulates longevity and metabolism. It was investigated that daf-2, age-1, and pdk-1 mutants that are the major components of the C. elegans insulin signaling pathway live 2x to 3x longer than wild type (Matsunaga *et al.,* 2012). According to the Fig. 3, age-1 acts downstream of daf-2and encodes the worm ortholog of mammalian phosphatidylinositol 3-kinase (PI 3-kinase) p110 catalytic subunit. Reduction of function mutations in age-1 causes a two

fold increase in life span. PI 3-kinases generate a membrane-localized signaling molecule, phosphatidylinositol P3drslt (PIP3), which binds to the pleckstrin homology domain of mammalian Akt/PKB and are required for its activation. There are two Akt/PKB orthologs in C. elegans. Using RNA interference, inhibition of both akt-1 and akt-2 activities causes nearly sent parcent arrest at the dauer stage. On the other hand inactivation of either gene alone does not. One of the kinases that phosphorylates Akt/PKB and is required for its activation is 3-phosphoinositide-dependent kinase-1 (PDK1). A loss of function mutation in pdk-1 increases C. elegans life span almost two fold, similarly to a mutation in age-1(Caleb and Gary , 2001).

TOR Pathway:

Another pathway involved in longevity is TOR Pathway. TOR means the target of rapamycin (kinase) that is highly conserved from yeast to humans and functions to integrate multiple nutrient signaling pathways (Laplante *et al.,* 2012). It has functions to integrate multiple nutrient signaling pathways. TOR activity is regulated by four major input signals: nutrient and energy availability, growth factors and stress (Fig. 4) (Wullschleger *et al.*, 2006). TOR interfaces with several cellular processes such as DNA transcription, mRNA translation, protein turnover, autophagy and actin cytoskeletal organization among others (Martin and Hall, 2005). In *C. elegans*, RNAi of TOR produces a longevity phenotype. It is a central regulator of the effects of dietary restriction. TOR signaling is activated by leptin(Wauman *et al.,* 2011), a hormone released from adipose tissue in response to increasing fat levels, and inhibition of TOR activity reduces the anorectic effect of leptin administration (Narasimhan *et al.*, 2009).

Findings from ageing genes research

Actually ageing research in genome level is a very new concept in the modern era of genetic research. It is under study in nematodes, flies, yeast and mice level.

For example, genetic experiments in *Caenorhabditis elegans* have shown that genes that are part of an insulin-related signaling pathway are involved in specifying longevity. Several tissues lose function and structural integrity at different times during ageing. M. Driscoll *et al.* used the microarray expression data to assess changes in tissue specific ageing at the molecular level; they selected genes known to be expressed in specific tissues and then determined whether they showed coordinate age related changes. It was investigated that a total of 46 genes previously known to be expressed specifically in muscle, a total of 88 neuronal specific genes, 508 germline intrinsic genes expressed in both sperm-producing and oocyte-producing animals and a total of 250 oocyte-enriched genes (James *et al.*, 2002). Using a rigorous statistical model with multiple replicates, it was possible to find a small number of genes (only 164). They show statistically significant changes in transcript levels as aging occurs (James *et al.*, 2002). The table 1 was made with the name of some age related genes of several organisms those have been most investigated from beginning.

Table1. List of most investigated genes involved in ageing according to source organism.

Source organism	Genes involved in ageing
C. elegans	daf-2, daf-16, daf-28, age-1, ins-1, ins-7, ins- 18, smk-1, smk-4. (Eleanor M. Maine, 2008)
Drosophilla	dilp-2, dilp-3, dilp-5, sir-1, sir-2. (James Lund *et al.*, 2002)
Flies	skn-1, keap-1. (Johnson *et al.*, 2001)
Mice	nrf-2, sirt-1, clk-1, isp-1. (James Lund *et al.*, 2002)

There were several findings from RNA interference-

mediated gene silencing in various organism leads to early adult-onset mortality and elevated endogenous oxidative stress. In the case of sod gene at first Kim Kirby *et al.* in 2002 constructed a Sod2IR that promotes degradation of *Sod2* mRNA that play role to silence Sod2 gene that impairs Mitochondrial Iron-Sulfur Enzymes. They investigated that Sod2IR has ability to suppress SOD2 protein and destroy SOD2 activity. It was also investigated that RNAi suppression of SOD2 causes rapid mortality in young adults (Kim Kirby *et al.*, 2002). In a recent study, Min *et al.* have shown that *ilp5* was down-regulated in diet-restricted wild type flies. In order to investigate whether dietary restriction increased lifespan by way of *ilp5* down regulation, they tested the effect of dietary restriction on flies with RNAi knockdown of *ilp5* in the medial secretary neurons where *ilp*s are synthesized. Although the level of *ilp5* was now constantly low independent of diet, dietary restriction still increased life span, suggesting that the mechanisms of dietary restriction may be independent of the insulin like pathway (Nadège *et al.*, 2010).

Evidence for a central role of the TOR pathway in dietary restriction comes from several species (Dimitroff *et al.,* 2012). In yeast, TOR can be required for the effects of dietary restriction. In *C. elegans*, RNAi of TOR (*let-363*) produces a longevity phenotype that is independent of FOXO/DAF-16. On the other hand, a majority of studies show that reducing TOR signaling in dietary-restricted worms does not further increase lifespan (Nadège *et al.*, 2010).

In 2009, Jeffrey *et al.* reported that RNAi of five genes encoding components of mitochondrial respiratory complexes I, III, IV, and V leads to increased life span in flies. Long-lived flies with reduced expression of ETC genes do not consistently show reduced assembly of respiratory complexes or reduced ATP levels. In addition, extended longevity is not correlated with reduced fertility or increased resistance to the free-radical generator paraquat. Targeted RNAi of two

complexes I genes in adult tissues or in neurons alone is sufficient to extend life span. The data obtained from the research of them suggest that the role of mitochondrial ETC function in modulating animal ageing is evolutionarily conserved and might also operate in humans. Besides their findings suggest that the longer life span of flies with reduced ETC gene expression cannot simply be attributed to reduced energy production leading to decreased "rate of living" (Jeffrey *et al.,* 2009).

Another gene family the sirtuins is recently surveyed by RNAi for its role in ageing. (Marcia *et al.,* 2009). Sirtuins are a highly conserved group of NAD+ dependent deacetylases and this dependence on NAD + suggests that they may be possible mediators of dietary restriction. To increase life span in many organisms *Sir 2* gene is over expressed as well as to decrease life span that gene is down regulated(Burnett *et al.,* 2011). RNAi was used in *Drosophila* to target three *sir* and *sir-like* genes from classes of the large *sirtuin* family that had so far not been examined for their roles in ageing. Similarly to *Sir2*, down regulation of these genes decreased female life-span in all except one line and, moreover, caused total or partial developmental lethality (Li W *et al.,* 2003). Complementary over expression studies are required to demonstrate their potential roles in ageing. It is noteworthy, however, that null mutants for *Sir2* were reported to be viable, in contrast with the results of the RNAi study, in which the deleterious effects might have been due to off-target knock downs (Narasimhan *et al.*, 2009).

Cku-70 and *Cku-80*Ku encode mammalian heterodimer consisting of a 70 and 80 kDa subunit (Ku70 and Ku80) (Bertinato *et al.,* 2001). It binds to ends, nicks, gaps, and hairpins in a DNA sequence-independent manner and in a sequence dependent manner to internal sites convergen (McColl *et al.,* 2005). In addition to its role in DSB repair, Ku functions in telomere maintenance, cell cycle checkpoint arrest, and tumor suppression. Using RNAi

in *C. elegans* to down-regulate one of the two subunits in wild type worms at a time. McColl *et al.* showed that the down-regulation of either subunit triggered an increased sensitivity to methyl-methane-sulfonate but not to UV, two different agents of DNA damage. Moreover, *cku-70* RNAi increased thermo tolerance in wild-type worms and life span in an RNAi-sensitive strain (Hilary *et al.,* 2012). The Ku dimer also seemed to interact with the insulin like signalling pathway, as *cku-70* RNAi amplified the changes in life span and stress resistance in *daf-2* and *daf-16* worms, mutants in the insulin like signalling pathway increasing and decreasing life span, respectively (Narasimhan *et al.*, 2009).

By RNAi treatment always has to be considered in the context of both the level and specificity of the down regulation have achieved by RNAi treatment. Genomic knock down results a phenotype or a lack of it. Depending on the level of knock down, different phenotypes may be observed, as illustrated by the study above. In particular, a phenotype may only be seen at certain knock down levels. A standardization of RNAi protocols and construct design will be critical in obtaining results that can be compared across studies and between laboratories (Gawain *et al.*, 2005).

Not only genes but also transcriptional regulators are known to influence life span. In 2010, Thyagarajan *et al.* established the transcription factor ETS-4, an ortholog of vertebrate SPDEF, as a longevity determinant. Using RNAi, they demonstrated that ets-4 is required post-developmentally to regulate adult life span; thus uncoupling the role of ETS-4 in aging from potential functions in worm intestinal development. They identified seventy ETS-4-regulated genes by gene expression profiling of two distinct ets-4 alleles and analyzed by bioinformatics. Those genes were enriched for known longevity effectors that

function in lipid transport, lipid metabolism, and innate immunity. Some ETS-4-regulated genes function downstream of the FOXO factor, DAF-16 and the insulin/IGF-1 signaling pathway. It was also demonstrated by epistasis and phenotypic analyses that ets-4 functioned in parallel to the insulin/IGF-1 receptor, daf-2 and akt-1/2 kinases. Furthermore, ets-4 required daf-16 to modulate aging, suggesting overlap in function at the level of common targets that affect life span. As ets-4 shares transcriptional targets with GATA and FOXO factors it privides a guideline that overlapping pathways direct common sets of lifespan-related genes (Thyagarajan *et al.,* 2010). According to the research of Thyagarajan B *et al.* ETS-4 is closely related to the human ETS protein SPDEF that exhibits aberrant expression in breast and prostate tumors. Because the genetic pathways that regulate aging are well conserved in other organisms, including humans, their findings could lead to a better understanding of SPDEF function and longevity regulation in mammals (Thyagarajan *et al.,* 2010).

RNAi also play role in ageing research by the screening genes that increase life-span on RNAi knockdown. The first such screens have been discussed and reviewed in detail elsewhere (Lee *et al.*, 2006). It was done specially in invertebrate model organisms. Several genes involved to longevity had been identified in flies, worms and yeast (Erica *et al.,* 2006). With the development of RNAi libraries in worms and the yeast open reading frame (ORF) deletion collection, it has become successful to genome-wide screen. A number of worm screens have now been performed to identify genes whose reduced expression leads to longer lifespan, and two ORF deletion longevity screens have been performed in yeast. Interestingly, these screens have linked previously unidentified cellular pathways to invertebrate ageing (Nadège *et al.*, 2010).

Table 2. Genome-wide searches for ageing mutants (BK Kennedy, 2007).

Report	Method of screening	Genes interrogated	Potential longevity genes identified	Per cent long lived
Caenorhabditis elegans				
Lee *et al*	RNAi screen of genes on chromosome I and II for increased lifespan. RNAi initiated at LI stage	5690	52 on Chr. I of 2663 genes screened	1.8
Hamilton *et al*	Genome-wide RNAi screen for increased lifespan. RNAi initiated at LI stage	16745	89	0.5
Hansen *et al*	Genome-wide RNAi screen for increased lifespan. RNAi initiated from time of hatching	16757	23	0.1
Chen *et al.*	RNAi screen for increased lifespan amongst genes reported to be essential for development.RNAi initiated in old L4 larvae.	57	24	42.1
Curran and Ruvkun	RNAi screen for increased lifespan of genes essential for growth and development. RNAi initiated in L4 larvae.	2700	64	2.4
Kim and Sun	Primary RNAi screen for resistance o ROS amongst genes on Chr. Ill and IV,secondary screen for increased lifespan. RNAi initiated at LI.	6000	84 lead to lifespan increase	1.4
Yeast				
Kaeberlein *et al.*	Screen for yeast deletion strains with increased replicative lifespan	564	13	2.3
Powers *et al.*	Screen of yeast open reading frame deletion collection for increased chronological lifespan.	4800	90	1.9

Almost 150 genes have been identified that increased lifespan when down regulated by RNAi (Samuelson *et al.*, 2007). These spanned a variety of genes, (Table 2) (BK Kennedy, 2007) affecting mitochondrial function, metabolism, signaling, protein-protein interaction, and the regulation of gene expression. Only three earlier identified genes were recovered but not by all of the screens. In general, there was little overlap between genes found in the different screens. RNAi by feeding can yield highly varying levels of down-regulation. Recently, a screen of 16,757 genes with such RNAi-sensitive strains identified 115 that reproducibly increased life-span by at least 10% on knock-down (Samuelson *et al.*, 2007). These genes predominantly

had mitochondrial and metabolic functions. Of these genes, 18 were characterized further, showing that there was no strong association between life-span extension and a slower accumulation of age pigment (lipofuscin) or an increased stress resistance. This highlights the phenotype specificity of the screen. A screen for extended lifespan will thus not necessarily uncover genes playing a role in the ageing process (Samuelson *et al.*, 2007).

RNAi libraries also facilitate large scale interaction screens(Lara and Greg , 2005) Triggering RNAi by feeding in a mutant strain for a gene of interest, Samuelson et al. have screened 15,718 genes for a decrease of life-span in *daf-2* mutants on RNAi knock-down of the candidate. Of the 159 genes identified, many also decreased life-span in wild-type worms and *daf-2; daf-16* mutants, albeit to a lesser extent. Thirty-nine genes shortened life-span specifically in *daf-2* mutants. Tracking general fitness by measuring the decline of activity and age pigment accumulation with age, they could show that only nine genes induced general sickness, whereas the others seemed to specifically accelerate the ageing process. Many of these genes had roles in trafficking and vesicle sorting. Stress resistance has often been correlated with ageing. A screen of more than 6,000 RNAi clones covering the genes on chromosomes III and IV in an RNAi-sensitive strain of *C. elegans* identified 608 clones conferring paraquat resistance (Kim *et al.,* 2007). Out of these, 84 also increased life-span, with only four of them already known from previous studies. The identified genes were involved in mitochondrial function, cell structure and signalling, cell-cell and protein-protein interactions, gene expression, protein turnover, and metabolism. Despite testing RNAi-sensitive strains, RNAi knockdown of only 14% of genes increased both paraquat resistance and life-span, indicating the specificity of RNAi screens for different complementary aspects of ageing, which have to be considered in the compilation of a full picture (Nadège *et al.*, 2010).

So far, large-scale screens in *C. elegans* have yielded about 400 genes increasing life-span on RNAi knock-down. While many of these genes could be mapped to known modulators of longevity, e.g., genes of mitochondrial function or involved in metabolism, almost no candidate genes have since been validated in independent studies, and the biological significance of most implicated genes is still unclear. Moreover, with accurate life-span assessments being very labour-intensive, many studies instead employ a rough measure like maximum life span in their primary screens (Otto HE, 2001). While the resulting higher false-negative rates could explain the discrepancies between different screens, even the positive candidates require more detailed scrutiny. On the one hand, it seems surprising that, despite no selection for longevity, so many genes would individually have a strong effect on life span. On the other hand, considering the high false-negative rates, many authors interestingly argue that many more genes increasing life span are to be discovered. It was thought that a lot more effort and time will be required to make sense of the accumulated results of rapidly increasing numbers of large-scale screens in ageing research (Nadège *et al.*, 2010). Improved means for interpreting results from complementary screens will be particularly critical due to the rapid growth of domains accessible to study by large scale RNAi screens that can be expected. For example, future developments of large scale RNAi screens in vertebrates will allow an examination of additional important aspects of ageing, such as mechanisms of brain ageing and the potential role of inflammation (Bruce *et al.,* 2008)

The therapeutic use of RNAi for age related disease

In recent years, invertebrate models have become increasingly prominent in the study of age-related diseases, and associations with genetic disorders (Aubrey and Grey, 2005) The potential therapeutic use of RNAi has early been recognized. Using RNAi, the pathogenic expression of a mutated gene can be

silenced (Quan *et al.,* 2005). In a mouse model of amyotrophic lateral sclerosis, Ralph *et al.* tested a vector particularly suitable for RNAi in the nervous system aiming to down regulate a mutant form of the exogenic human SOD1 causing the disease. Mice injected with a vector containing the RNAi construct had delayed onset and lesser severity of motor impairment, and enjoyed a longer life-span due to the increased survival of motor neurons. A particular strength of RNAi is how well it scales to larger studies. Comprehensive libraries of dsRNA, siRNA and *E. coli* strains synthesizing dsRNA have been established (Jean François *et al.,*2004). Also, viral vector based libraries for mammalian cells are rapidly being developed. These tools potentially allow any gene to be targeted, making efficient large screens possible. RNAi libraries can also be used to screen for candidates interacting with genes involved in age related diseases (Narasimhan *et al.*, 2009).

Bates *et al.* used a *C. elegans* model of Huntington's disease. They investigated the role of histone deacetylases in huntingtin polyglutamine toxicity using mutants and RNAi of corresponding genes. Various histone deacetylases were down regulated that reduce (for *hda-3*) or increase neuro degeneration induced by polyglutamine accumulation (e.g., for *hda-1, hda-4, hda-2*). The employed RNAi constructs had been maximized for specificity to avoid unspecific confounding effects. The unique role of *hda-3* was also confirmed using a deletion mutant (Narasimhan *et al.*, 2009). Expansion of a polyglutamine tract in the huntingtin protein causes neuronal degeneration and death in Huntington's disease patients, but the molecular mechanisms underlying polyglutamine mediated cell death remain unclear. Previous studies suggest that expanded polyglutamine tracts alter transcription by sequestering glutamine rich transcriptional regulatory proteins, thereby perturbing their function. Loss of function alleles and RNA interference (RNAi) were used to examine contributions of *C. elegans* cAMP response element

binding protein (CREB), CREB binding protein (CBP), and histone deacetylases (HDACs) to polyglutamine induced neurodegeneration. Deletion of CREB (*crh-1*) or loss of one copy of CBP (*cbp-1*) enhanced polyglutamine toxicity in *C. elegans* neurons. Loss of function alleles and RNAi were then used to systematically reduce function of each *C. elegans* HDAC. Generally, knockdown of individual *C. elegans* HDACs enhanced Htn-Q150 toxicity, but knockdown of *C. elegans hda-3* suppressed toxicity. Neuronal expression of *hda-3* restored Htn-Q150 toxicity and suggested that *C. elegans HDAC3* (HDA-3) acts within neurons to promote degeneration in response to Htn-Q150. Genetic epistasis experiments suggested that HDA-3 and CRH-1 (*C. elegans* CREB homolog) directly oppose each other in regulating transcription of genes involved in polyglutamine toxicity. *Hda-3* loss of function failed to suppress increased neurodegeneration in *hda-1/ Htn-Q150* animals, indicating that HDA-1 and HDA-3 have different targets with opposing effects on polyglutamine toxicity. Their results suggest that polyglutamine expansions perturb transcription of CREB/CBP targets and that specific targeting of HDACs will be useful in reducing associated neurodegeneration (Bates *et al.*, 2006).

Discussion

Especially RNAi is used for ageing research due to ability of timed knock downs of age related genes. For instance, many genes have crucial roles during development and early interference may even be lethal for test organism (Hamilton *et al.*, 2005). The role of such genes in ageing can still be studied by genetically inducing RNAi at a later developmental stage or by an external trigger. This is especially important for ageing research considering the antagonistic pleiotropy theory (Williams, 1957). These genes were involved in mitochondrial function, protein synthesis, signaling, and gene expression. On the other hand, for 24 genes of 57 that caused larval arrest in constitutive knockdown (42%), an extended lifespan was found in adult specific RNAi knock down (Chen *et al.*, 2007).

The identified genes mainly had mitochondrial functions and roles in the regulation of mRNA translation. A considerable number of genes may thus exhibit antagonistic pleiotropy. It is very interesting to explore that RNAi play role for disabling such genes only when they show negative effects. RNAi has ability to trigger such a knock-down at known time points allows the meaningful study of gene expression profile time courses for an analysis of the regulatory interactions and the functional pathways implicated for ageing. In traditional knock-down experiments, only the steady-state long term response of an organism can be seen. Often, initial effects may no longer be visible due to compensatory mechanisms creating redundancy, or the knock-down may be lethal. Timed RNAi knock down permits an analysis of the immediate dynamic regulatory response to the loss of gene function, directly implicating interacting genes and pathways (Hamilton *et al.,* 2005). *Drosophila* is the invertebrate model organism with the highest degree of homology to vertebrates, and the range of observable phenotypes in the fruit fly is larger than in any other system for which genome wide RNAi is available (Dietzl   *et al.,* 2007). This makes the fly a powerful model organism for the study of genes and processes found in vertebrates, including conserved mechanisms of ageing relevant to human age-related disorders. Ageing is complex and can be influenced by many factors. When studying the effect of a gene on life span and ageing, it is thus critical to consider the effects of other genes. Experimental and control groups should thus have the same genetic background. A genome wide library of uniform genetic background consequently facilitates the comparative study of many genes in large-scale screens of genetically identical organisms differing only in the inserted RNAi construct and target knock down. Several age-related disorders, such as cardiac disease are organ specific. A major advantage of *Drosophila* over other existing model systems is that genetic constructs can target RNAi to particular tissues or cell types, allowing appropriately specific studies (Elliott *et al.,* 2008).

The location of the insertion of the RNAi constructs in the lines of the library described in (Dietzl *et al.,* 2007) is largely random. Actually the expression of the transgene and the chance of misregulation of neighboring genes may be regulated by the location of the insertion site. It is therefore important to test the effect of the insertion site. If the phenotype varies across lines, further experiments are required. Transgenic RNAi libraries using this technique are being built and will reduce insertion-site effects (Ni JQ *et al.,* 2008).

The short sized siRNA those are produced from the long hairpin dsRNA arise problem during the RNAi process. These siRNAs can potentially be complementary to sequences of mRNAs of several genes, bind to them and thus trigger RNAi of several genes at a time. This effect is typically responsible for false positives in screens. So the careful design of RNAi constructs and comprehensive target/off-target prediction must be crucially handled in the fields of research (Dietzl *et al.,* 2007). Such kind of off target knock-downs lead to weaken the flies as well as a wrong conclusion (Nadège *et al.,* 2010). A predictable characteristic of RNAi is its efficiency. The efficiency controls the level and speed of down regulation of target gene. It also leads to corresponding differences in phenotype. Sometimes RNAi efficiency can be modulated by technical changes to an experiment. Poor RNAi efficiency can be due to construct insertion at a poorly expressed locus. The level of down-regulation also depends on the strength of the promoter. The expression is thought to be temperature dependent and higher temperatures may thus lead to stronger phenotypes (Ni JQ *et al.,* 2008).

For unknown reasons, it has been reported that RNAi phenotypes tend to be stronger in male than in female flies (Moffat *et al.,* 2007). In contrast, it was observed stronger effects of RNAi on lifespan in females than in males. Finally, while some studies have reported sex specific phenotypes in males, the same effects have

been observed in both sexes by others. Consequently, both sexes should be investigated whenever possible and appropriate (Kusama *et al.,* 2006). Besides there are some problems arise to use RNAi as well as the solutions to overcome those problems. Problems arise to large scale construction of specific constructs with minimal off-target effects or controlling for the effects of insertion sites. It is difficult to recognize the possibility of qualitatively different responses to different levels of down regulation. Hypomorphic mutations of some ageing related gene increase life span, whereas a complete loss of function of the same gene will decrease life-span (Nadège *et al.,* 2010).

Knock down levels must be modulated to avoid these effects. For this there are some technical developments to expand the power of systematic RNAi knock downs. Such as uniform libraries with a common genetic background provide well matched controls for all RNAi lines. Tissue or organ specific highly flexible UAS-GAL4 system offers a unique opportunity for controlling RNAi induction in appropriate site of action. Besides the modern computational techniques control the meaningful study of dynamic data collection, investigation of regulatory interactions as well as the evolvement of ageing related processes over time (Nadège *et al.,* 2010).

Conclusion

RNAi is a powerful tool due to ability of controlled knockdown any gene for the study of gene function and the investigation of interaction among genes. It helps to establish a wide variety of complementary experimental designs. There are different approaches for efficient large scale screening depending on the host organism. A genome wide transgenic RNAi library for *Drosophila* enables systematic screens in an invertebrate model closer to vertebrates that has become very helpful in ageing research. Besides several technical supports may affect outcome as well as using RNAi some alternative aspects of ageing thus have to be examined in complementary studies. RNAi also much facilitates genome wide quantitative perturbance

studies of gene expression, and recent progress in the development of matching computational analysis tools that provides extra advantages in ageing research. RNAi can be used to accelerate the identification of candidate genes affecting ageing and their potential interaction partners, leading to the subsequent further characterization of key players and their relevant interactions, and dynamics in the ageing process.

Future perspectives

Using RNAi for regulation of ageing gene in the case of ageing related diseases will confer long live of human as well as ensure healthy life free from those kind of diseases. In the near future using RNAi it may possible to build up relationships between neurodegeneration, behavioural and cognitive function and lifespan. It will be possible to discover the cellular and biochemical mechanisms of the central nervous system in long lived animals by altering function of related genes using RNAi. The ultimate results will very helpful to identify potential therapeutic targets of functional senescence and neurodegenerative disease. If it is possible to manipulate the RNAi as a right signaling component in the right tissue, it may extend lifespan of adults with few detrimental effects or even improved health and function. However development of more sophisticated and high throughput technologies are required for next generation ageing research and RNAi could be a very effective tool for decoding the ageing in human.

Declaration of conflict of interest:

The author(s) declared that there is no conflict of interest with authorship and publication of the article.

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