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Review

miRNAs and aging: A genetic perspective

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ABSTRACT

A growing body of evidence shows that microRNA expression changes with age in animals ranging from nematode to human. Genetic studies of microRNA function in vivo provide the means to move beyond correlation and to explore cause-effect relationships. Genetic studies in *Caenorhabditis elegans* and *Drosophila* have identified cellular pathways involved in organismal aging. Here, we review the evidence that microRNAs act in vivo as regulators of aging pathways, with emphasis on *Drosophila*.

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1. Introduction: miRNA expression and aging

microRNAs (miRNAs) are small non-coding RNAs that function in post-transcriptional gene silencing (reviewed in Ameres and Zamore, 2013). The ability of miRNAs to regulate multiple genes simultaneously offers the potential to regulate biological processes at multiple levels. This potential multiplicity of targets may offer advantages in regulation of the many different cellular and organismal homeostatic processes that are impacted during aging.

Expression profiling studies show that numerous miRNAs are modulated during aging (de Lencastre et al., 2010; Ibanez-Ventoso et al., 2006; Inukai et al., 2012; Liu et al., 2012; Mercken et al., 2013; Noren Hooten et al., 2013). miRNAs have also been implicated in age-related disorders such as neurodegeneration in humans, mice and flies (Delay et al., 2012; Gascon and Gao, 2012; Hebert et al., 2009; Karres et al., 2007; Kim et al., 2007a; Liu et al., 2012; Mouradian, 2012).

At the organismal level, changes in expression of specific miRNAs correlate with differences in lifespan. Long-lived Ames dwarf mice have increased levels of *miR-27a* in the liver and *miR-470*, *miR-669b* and *miR-681* in the brain (Bates et al., 2010). These miRNAs are reported to function via the insulin/IGF-signaling pathway (Liang et al., 2011), which has profound effects on organismal lifespan. A mouse model of Hutchinson–Gilford progeria syndrome, exhibiting premature aging, contains higher levels of *miR-1* in multiple tissues, and has been also shown to function via the insulin/IGF pathway (Marino et al., 2010).

Another likely mode of action for miRNAs is in control of cellular senescence – accumulation of senescent cells can accelerate aging (Baker et al., 2011; Campisi, 2005). Multiple miRNAs have been implicated in regulating the p53 and retinoblastoma pathways

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Table 1
A qualitative comparison between organisms being used in aging research.

Organism	Lifespan	Generation time	Genetic tools	Genetic background
S. cerevisiae	~26 cell divisions	~100 min	Excellent	Controllable
C. elegans	25–30 days	~4 days	Excellent	Controllable
D. melanogaster	60–70 days	2 weeks	Excellent	Controllable
M. musculus	800–1000 days	~3 months	Excellent	Limited control
Non-human primates	~20–50 years	~3–10 years	Limited	NA

during cellular senescence (Martinez et al., 2011; Menghini et al., 2009; Yamakuchi and Lowenstein, 2009). Loss of miRNA biogenesis using *Dicer* loss-of-function mutations induces premature senescence in primary fibroblasts, indicating that miRNAs in general have protective functions in these cells (Mudhasani et al., 2008). However, other miRNAs can promote senescence: over-expression of *miR-34a* induces endothelial cell senescence and suppresses cell proliferation (Ito et al., 2010). Regulation of homeostatic processes, including those whose failure leads to cellular senescence, is likely to involve miRNAs acting both positively and negatively to ensure balance.

2. Model organism genetics: identification of aging-related pathways

Genetics has provided the opportunity to systematically explore cellular pathways related to aging in model organisms including *Drosophila* and *Caenorhabditis elegans*. These invertebrate models are at the forefront of multi-cellular organism aging research because of their short lifespan and ease of genetic manipulation through powerful tools to identify relevant cellular and molecular pathways (Table 1). Additionally, findings from these models can be successfully applied to mammalian models – genetics of aging seem to be conserved over large evolutionary distances. *Drosophila* also has widely significant natural variation for longevity, and quantitative trait loci (QTL) mapping can help identify allelic variation at individual loci (Paaby and Schmidt, 2009). These characteristics have made fruit flies a useful model organism for aging research.

Early work from *Drosophila* shows that senescence can be postponed by natural selection. By selectively breeding older flies, it was possible to isolate fly strains with lifespans up to double that of the starting population (Nusbaum and Rose, 1994). The variants that were under selection included genes that improved stress tolerance (Rose et al., 1992). The oxidative stress pathway was subsequently linked to longevity. Generally, age elicits greater oxidative damage, and lower DNA repair capacity (Kirkwood, 2005). While there exists substantial positive correlative evidence for oxidative damage during aging in *Drosophila*, there is lack of conclusive evidence whether this is a direct cause of aging or its consequence (reviewed in Clancy and Birdsall, 2013). However, genetic interventions that enhanced antioxidant defenses provided some evidence for extension of lifespan. For example, over-expression of human superoxide dismutase (SOD1) in fly motor neurons increased lifespan by ~40% (Parkes et al., 1998). Ubiquitous over-expression of *Drosophila Sod1* in adults also dramatically increased lifespan (Martin et al., 2009). Increasing antioxidant pathway activity by overexpressing *glucose-6-phosphate dehydrogenase* (G6PD) also extended lifespan (Legan et al., 2008). Overexpression of methionine sulfoxide reductase A (*msrA*) (Cirelli, 2006) and overexpression of glutamate-cysteine ligase (Orr et al., 2005) in *Drosophila* also led to extended lifespan by reduction of oxidized methionine residues and by limiting glutathione biosynthesis, respectively.

Genetic studies have identified metabolic processes and nutrient sensing pathways as major lifespan regulators, including dietary restriction, insulin/IGF signaling, and TOR signaling (Fig. 1). Elegant genetic studies in *C. elegans* identified mutations extending lifespan, including *age-1* and *daf-2* (Friedman and Johnson, 1988; Kenyon et al., 1993). Genetic manipulations that extended lifespan were particularly compelling as a starting point to study processes controlling organismal aging. The genes *daf-2*, *age-1* and *daf-16* genes were shown to act in a common genetic pathway (Dorman et al., 1995). Subsequent studies linked these genes to the Insulin/IGF signaling (IIS) pathway. The *age-1* mutant was identified as a PI3Kinase (Morris et al., 1996). *daf-2* was identified as an Insulin-like receptor (Kimura et al., 1997). *daf-16* was identified as a FOXO family transcription factor, whose activity was shown to be regulated by the IIS/PI3K/AKT pathway (Lin et al., 1997; Ogg et al., 1997; Paradis and Ruvkun, 1998). Regulation of FOXO transcription factor activity plays a central role in mediating the effects of IIS pathway on aging in the nematode (Lee et al., 2001, 2003; Lin et al., 2001). Longevity in *C. elegans* can also be extended by dietary restriction (DR), through regulation of IIS pathway activity (Lakowski and Hekimi, 1998). Intriguingly, a lot of genes functioning in DNA repair pathways are required for DR or IIS-mediated longevity – reduced activity of *smk-1*, a gene involved in DNA repair (Wolff et al., 2006) (Kim et al., 2007b), is required for the lifespan extension seen in *daf-2* mutants (Wolff et al., 2006). Attenuation of *p53* activity leads to increased lifespan in a *daf-16* dependent manner in *C. elegans* (Arum and Johnson, 2007).

As in *C. elegans*, the IIS pathway plays a central role in *Drosophila* aging (reviewed in Partridge et al., 2011). Mutations lowering signaling through the IIS pathway such as the *Insulin-like receptor*, the *InR* substrate *chico* extend lifespan (Fig. 1) (Clancy et al., 2001; Hwangbo et al., 2004; Tatar et al., 2001). Likewise, overexpression of *dFOXO*, which is normally inactivated by IIS activity, extends lifespan (Clancy et al., 2001; Hwangbo et al., 2004; Tatar et al., 2001), as does reduced expression of its negative regulator 14-3-3ε (Nielsen et al., 2008). Lifespan can be extended or reduced by manipulating production of insulin-like peptides in the insulin-producing neurosecretory cells (IPC) in the fly brain (Broughton and Partridge, 2009). Ablation of IPCs in *Drosophila* also leads to

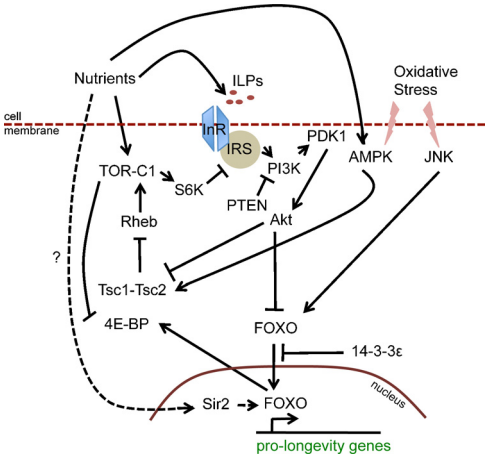

Fig. 1. Network of the major aging pathways in *Drosophila*. ILPs – Insulin-like peptides; IRS – Insulin Receptor Substrate. Solid lines indicate direct links, dashed lines indicate unknown pathways.

extended lifespan, altered carbohydrate and lipid metabolism, and enhanced resistance to oxidative stress and starvation (Broughton et al., 2005). There is a complex interplay between systemic and IPC-based IIS activity. Manipulation of the IIS pathway and FOXO in head adipose tissue can influence insulin-like peptide production by the IPC cells (Hwangbo et al., 2004). A recent report has shown that FOXO-dependent expression of *dilp6* in adipose tissue can modulate production of *dilp2* in the brain, which impacts lifespan (Bai et al., 2012). In *Drosophila*, dietary restriction may extend lifespan through regulation of the balance of systemic and IPC IIS pathway activity (Tatar, 2011)

Pathways that interact with the IIS pathway have been examined for effects on aging. The target of rapamycin (TOR) pathway, which links cellular growth to nutritional status, is one example. TOR activity is regulated by cellular energy levels (AMP/ATP ratio) sensed by AMPK and amino acids, as well as by signaling via the IIS pathway. Overexpression of AMPK in metabolic tissues extends lifespan via the TOR pathway (Stenesen et al., 2013). TOR activity controls protein biosynthesis in part through regulation of the translation initiation factor EIF-4E and downstream ribosomal S6 kinase. Inhibition of TOR signaling by modulating TOR pathway genes including dTsc1, dTsc2, dTOR and dS6K extends lifespan (Fig. 1) (Kapahi et al., 2004), and confers resistance to oxidative stress (Patel and Tamanoi, 2006). Interestingly, inhibition of TORC1 by rapamycin also elongates lifespan (Bjedov et al., 2010). In this context, it is worth noting that regulation of protein biosynthetic capacity had been reported to extend lifespan a decade earlier – by introducing an additional copy of the elongation factor EF-1α (Stearns and Kaiser, 1993). A recent study shows that simultaneous inhibition of DAF-2 and S6K, key molecules in the IIS and TOR pathways, respectively, synergistically prolongs lifespan via signaling in the *C. elegans* germline (Chen et al., 2013).

The Jun-N-terminal kinase (JNK) pathway, which is activated in response to stress, counters the effects of the IIS pathway (Fig. 1) (Wang et al., 2005; Biteau et al., 2011). Increased JNK signaling confers tolerance to oxidative stress and elongates lifespan (Wang et al., 2003), by enabling nuclear translocation of *dFOXO*, which leads to expression of heat shock proteins such as *l(2)efl* and *hsp68* (Wang et al., 2005). Over-expressing *Drosophila Plenty of SH3s* (DPOSH) activates *puckered*, a JNK phosphatase and lengthens lifespan (Seong et al., 2001).

Lifespan extension in *Drosophila* has been reported for two histone deacetylases, *rpd3* and *Sir2*, that may also function via mechanisms related to IIS and caloric restriction (Frankel et al., 2011). The *Sir2* family was originally identified in *Saccharomyces cerevisiae* and was shown to increase yeast lifespan (Kennedy et al., 1995). Reducing levels of *rpd3* extends lifespan, and increases *Sir2* expression as well. Direct *Sir2* over-expression also extends lifespan (Rogina and Helfand, 2004; Rogina et al., 2002). These genes are linked to the IIS pathway: Sir2 regulates FOXO activity (Fig. 1) (Partridge et al., 2005), and a reduction in *Sir2* levels precludes lifespan extension by either *rpd3* or dietary restriction (Rogina and Helfand, 2004). However, a recent report re-examining the over-expression of *Sir2* in a more standardized genetic background showed no lifespan extension in either *C. elegans* or *Drosophila* (Burnett et al., 2011). How the deacetylases impact lifespan remains open to question.

Aging is closely linked with the onset of age-associated diseases. Work elucidating interaction between aging pathways and age-related diseases is limited – however, there are some preliminary insights. Dietary restriction (DR) has been shown to promote longevity across diverse species from yeast to primates (Omodei and Fontana, 2011) and also provides protection against multiple age-associated diseases including cancer, diabetes, cardiovascular disease and neurodegeneration in mice and rhesus monkeys (Omodei and Fontana, 2011). In *Drosophila* models of Alzheimer's disease, DR delays aging, but fails to delay neuronal dysfunction (Kerr et al., 2011). Neuro-protective effects of DR on age-related cognitive decline in rodents have implicated components of IIS and sirtuin pathways (Gillette-Guyonnet and Vellas, 2008; Martin et al., 2006). Also, reducing activity of components of the IIS/TOR circuitry can reduce neurotoxicity of misfolded proteins in neurodegenerative diseases models in *Drosophila* (Hirth, 2010). Increasing systemic TOR signaling accelerates senescence of locomotor activity with age in Drosophila (Patel and Tamanoi, 2006), and enhances sensitivity to oxidative damage (Patel and Tamanoi, 2006), indicating that aging and disease susceptibility are interconnected via TOR pathway activity.

3. microRNA genetics: functional links to aging pathways

Given that animal genomes contain hundreds of miRNAs, each with the capacity to regulate hundreds of targets, it is reasonable to expect that miRNAs will play functionally important regulatory roles as modulators of the aging-related pathways described above. Analysis of miRNA mutants in *C. elegans* and *Drosophila* have begun to make these connections (Smith-Vikos and Slack, 2012).

4. IIS pathway

The earliest study directly addressing a link between a microRNA and organismal aging came from *C. elegans*. This study discovered that a *lin-4* loss-of-function mutation leads to decreased lifespan via its target, *lin-14* (Boehm and Slack, 2005). Intriguingly, knockdown of *lin-14* during adulthood was sufficient to increase longevity, suppressing the *lin-4* short-lifespan phenotype. This provided strong evidence for the *lin-4*/*lin-14* relationship in lifespan control. *lin-4*/*lin-14* act via the IIS pathway through effects on the *daf-2* Insulin/IGF-like receptor and perhaps also via regulation of the *daf-16*/FOXO transcription factor. Several other miRNA mutants have been liked to lifespan in *C. elegans*, and in at least two cases to regulation of the IIS pathway (de Lencastre et al., 2010). *miR-239* mutants are long-lived and show greater stress resistance due to reduced IIS pathway activity. Conversely, *miR-71* mutants are short-lived and show elevated IIS pathway activity through upregulation regulation of PI3K and PDK1.

In *Drosophila*, *miR-8* has been shown to regulate IIS signaling in the larval fat body, via its target *U-shaped*, which inhibits PI3K (Fig. 2) (Hyun et al., 2009). *miR-8* mutants show low IIS signaling during larval development with effects on growth. *miR-8* is also expressed in head fat in the adult (unpublished observations), where it might also regulate the IIS pathway. Low IIS activity in head fat should lead to elevated FOXO activity, which has been shown to extend lifespan, acting through *dilp6* on *dilp22* production (Bai et al., 2012; Hwangbo et al., 2004). *miR-8* mutants also show early onset cell death in the brain due to upregulation of Atrophin (Karres et al., 2007), which might be expected to shorten lifespan. These effects may balance out at the organismal level, as *miR-8* was not found to significantly change adult lifespan in an initial screen of *Drosophila* miRNA mutants (Chen et al. unpublished). More in-depth analysis will be needed to determine whether *miR-8* might contribute to lifespan control under specific conditions.

4.1. Ecdysone signaling

Drosophila miR-14 is down regulated with age in the fly brain (Liu et al., 2012), and *miR-14* mutant flies have reduced lifespan and reduced stress resistance (Xu et al., 2003). *miR-14* has been shown to regulate insulin-like peptide production in the IPC neurosecretory cells via its target *sugarbabe* (Varghese et al., 2010). However, the low insulin-like peptide levels in the mutant would be expected

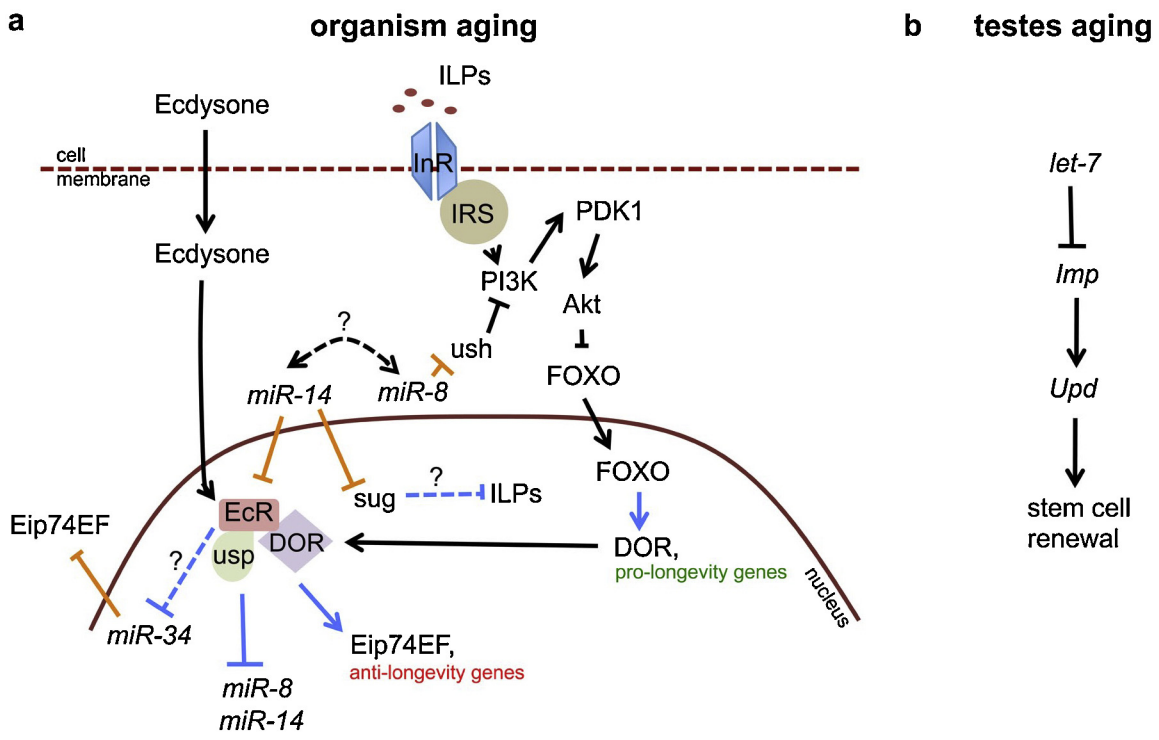

Fig. 2. (a) miRNAs interact with the mutually opposing ecdysone and IIS-mediated aging pathways in *Drosophila* to control longevity. Solid lines indicate direct links, dashed lines indicate indirect links, orange links indicate miRNA regulation of target genes, blue links indicate translational links; (b) *let-7* mediated stem cell renewal pathway in *Drosophila* testes.

to extend lifespan, if the effects on the IIS pathway were dominant. Instead, the *miR-14* mutant is short lived. The lifespan phenotype has been linked to upregulation of a different *miR-14* target, the *Ecdysone Receptor* (Varghese and Cohen, 2007) (Fig. 2).

A second miRNA affecting lifespan also acts via the ecdysone pathway. *miR-34* is upregulated in the brain with age and mutants show reduced lifespan, which may be linked to neuronal cell death (Liu et al., 2012). *miR-34* acts though its target EIP74, an ecdysone-induced transcription factor. Together these mutants indicate that elevated ecdysone pathway activity can lead to neurodegeneration and early death. Interestingly, *miR-8* is transcriptionally repressed by ecdysone signaling (Jin et al., 2012), so the effects of *miR-14* and *miR-34* could potentially be mediated through downregulation of *miR-8* (Fig. 2). This possibility remains to be tested.

The relevance of ecdysone signaling to lifespan is supported by reports that disrupting either the *Ecdysone Receptor* gene, or *DTS-3* which acts in the ecdysone biosynthesis pathway, can extend lifespan (Simon et al., 2003). It would be interesting to identify downstream targets of ecdysone signaling that antagonize lifespan. Thus raising and lowering ecdysone pathway activity can push lifespan in opposite directions, as in the case of the IIS pathway. The two pathways are connected during development. During larval stages, ecdysone production is dependent on the IIS pathway – dInR mutants exhibit decreased ecdysteroid levels, and upregulation of IIS in the prothoracic gland has the opposite effect (Colombani et al., 2005). Additionally, DOR, one of the co-activators of EcR is antagonized by IIS signaling (Francis et al., 2010). It is intriguing that *miR-14* impacts both of these aging-related pathways. The possibility that regulation of ecdysone signaling by *miR-14* and *miR-34* might act via *miR-8* to impact IIS pathway activity is also noteworthy. This raises the possibility that these miRNAs are part of a feedback system that balances the activities of these pathways, with potential implications for aging.

4.2. Other pathways

A systems biology approach analyzing miRNA and mRNA expression profiles during *C. elegans* aging along with miRNA target prediction algorithms shows that many age-associated miRNAs appear to regulate genes involved in maintaining cellular energy homeostasis and protein quality (Kato et al., 2011). These results along with the examples of *miR-14* and *miR-8* from flies, suggest that miRNAs may work by fine-tuning metabolic genes in the aging process. Many other *Drosophila* miRNAs are predicted to target metabolic pathways, but in most cases nothing is known about their effects on aging.

Apart from organismal aging, miRNAs also regulate tissue and cellular senescence. *Drosophila* testis stem-cell niche is maintained by Unpaired (Upd), a self-renewal factor. However, *upd* undergoes a marked reduction in expression with age, which results in a subsequent decline of niche maintenance in older flies. *upd* RNA is stabilized by Imp, an mRNA binding protein, which is in turn targeted by the miRNA *let-7*. *let-7* expression increases with age, thereby destabilizing *Imp* (Fig. 2). The absence of Imp in older males induces *upd* degradation, which leads to decline of germline stem cells (Toledano et al., 2012). This example illustrates how a miRNA can mediate a programmed progression of tissue-specific aging.

5. Perspectives

miRNAs have intriguing possibilities as biological regulators. They can have multiple targets, and have the potential to influence a biological process through different molecular pathways. Given the widespread changes in miRNA expression associated with aging and age-related pathology, it is tempting to think that miRNAs may prove to be significant modulators of healthy aging and disease. The few directed studies of the functions of miRNAs in aging would seem to support this view. There is a clear need for much

more directed research into the roles of miRNAs in aging. Genetically tractable model systems including *C. elegans* and *Drosophila* may continue to be useful in unraveling functional connections. Genetic knockout collections for *C. elegans* and *Drosophila* miRNAs will provide a key resource (de Lencastre et al., 2010; Miska et al., 2007; Chen et al. unpublished). Moving beyond invertebrate aging models will also be important. Studies in mammalian models that over-express miRNAs or constitutively/conditionally knockout miRNAs will be needed to explore miRNA-regulated aging mechanisms. It will be interesting to learn whether miRNAs involved in aging are conserved across species, or whether conservation is limited to the pathways that they target.

miRNA sponge technology deserves a special mention as a potentially important tool for the study of aging in vivo (Ebert et al., 2007; Loya et al., 2009). miRNA sponges are transgenes containing multiple binding sites that allow sequestration of a miRNA away from its normal targets. Combined with the potential for spatial and temporal control of sponge expression, this approach will allow very precise definition of when and where a candidate 'aging-related' miRNA acts in the animal to impact organismal aging. Some progress has been made in this regard in both *C. elegans* and *Drosophila*.

Short lifespan and ease of genetic manipulation in invertebrate models of aging provides a platform to develop interventions to tackle aging and age-associated disorders. These models provide opportunities for exploring mechanisms of action for pro-longevity compounds. Such findings could potentially be translated to mammalian models. Chemical screens have been successfully used in both *C. elegans* and *Drosophila* and have led to identification of compounds that extend lifespan (Lucanic et al., 2013). Many compounds regulating oxidative stress, protein homeostasis, dietary restriction and the IIS pathway were found to extend lifespan (Lucanic et al., 2013). One promising feature of this approach has been to test drugs already approved for human use in invertebrate models – certain anti-inflammatory, anti-diabetic and anti-depressant drugs were found to extend lifespan, and some of these have been further tested in mammals (Lucanic et al., 2013). Aspirin (2-acetoxybenzoic acid) can increase male lifespan in mice (Strong et al., 2008). In addition, rapamycin (mTOR inhibitor) is able to increase *Drosophila* lifespan (Bjedov et al., 2010) and also that of both male and female mice (Harrison et al., 2009; Miller et al., 2011). However, caution is needed in extrapolating from these results: the effects of natural genetic variation and lab-environment induced variation complicate bench-to-clinic translation (Partridge and Gems, 2007). Major challenges remain in turning these exciting laboratory findings into approved medicines.

Apart from traditional small molecule drugs, the field of miRNA therapeutics has recently received a lot of attention. Current challenges involve developing miRNA specificity and controlled delivery (Lai, 2013; van Rooij et al., 2012). Given the importance of miRNAs in aging processes, and taking a long-term, and admittedly optimistic view, miRNA depletion strategies might serve as a forerunner to therapeutic approaches to age-related degenerative diseases. A better understanding of how miRNAs regulate cellular senescence and organismal aging will provide a foundation on which to base future research on the relevance of miRNAs to modulation and better management of aging and age-related disorders.

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