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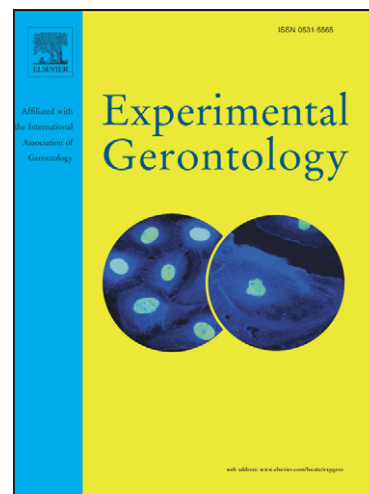
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# Novel modulators of senescence, aging, and longevity: small non-coding RNAs enter the stage

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## 1. Abstract

During the last decade evidence has accumulated that the aging process is driven by limited allocation of energy to somatic maintenance resulting in accumulation of stochastic damage. This damage, affecting molecules, cells, and tissues, is counteracted by genetically programmed repair, the efficiency of which thus importantly determines the life and 'health span' of organisms. Therefore, understanding the regulation of gene expression during cellular and organismal aging as well as upon exposure to various damaging events is important to understand the biology of aging and to positively influence the health span. The recent identification of small non-coding RNAs (ncRNAs), has added an additional layer of complexity to the regulation of gene expression with the classes of endogenous small inhibitory

RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), QDE1-interacting RNAs (qiRNAs) and microRNAs (miRNAs). Some of these ncRNAs have not yet been identified in mammalian cells and are dependent on RNA dependent RNA polymerases. The first mammalian enzyme with such activity has only now emerged and surprisingly consists of the catalytic subunit of telomerase (hTERT) together with RMPR, an alternative RNA component. The so far most studied small non-coding RNAs, miRNAs, however, are now increasingly found to operate in the complex network of cellular aging. Recent findings show that (i) miRNAs are regulated during cellular senescence *in vitro*, (ii) they contribute to tissue regeneration by regulation of stem cell function, and (iii) at least one miRNA modulates the life span of the model organism *C. elegans*. Additionally, (iv) they act as inhibitors of proteins mediating the insulin/IGF1 and target of rapamycin (TOR) signalling, both of which are conserved modulators of organism life span. Here we will give an overview on the current status of these topics. Since little is so far known on the functions of small ncRNAs in the context of aging and longevity, the entry of the RNA world into the field of biogerontology certainly holds additional surprises and promises. Even more so, as miRNAs are implicated in many age-associated pathologies, and as RNAi and miRNA based therapeutics are on their way to clinics.

## 2. Introduction

The fast progress of deep sequencing technologies has led to the discovery of a surprisingly large variety of non-coding RNAs that by now complement the long-known, canonical non-coding RNAs like ribosomal RNA (rRNA), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), RNA component of human telomerase (hTERC), or the RNA contained in the signal recognition particle. In total, around

95% of the human genome have been found to be transcribed, probably resulting in a million different individual ncRNAs in human cells (Birney et al., 2007; Mattick and Makunin, 2006) The physiological roles of most ncRNA classes identified in mammalian organisms like endogenous siRNAs (endo-siRNAs), or piRNAs are still only emerging, and other classes like trans-acting siRNAs (tasiRNAs), cis-acting siRNAs (casiRNAs) or exogenous siRNAs (exo-siRNAs) (reviewed in (Ghildiyal and Zamore, 2009)), as well as qiRNAs (Lee *et al.*, 2009) have so far only been detected in plants or *C. elegans*.

In contrast, we observe exponential growth in knowledge about the physiological roles of miRNAs in many of the most important processes of life from birth to death, ranking from regulation of pluripotency of embryonic stem cells, development, cell cycle regulation, differentiation, energy metabolism, tumor suppression, to apoptosis. Due to the multiple targets of each single miRNA that are estimated up to even several hundred, miRNAs might resemble transcription factors that influence global cellular responses (Ambros, 2003, 2004; Carthew and Sontheimer, 2009). Thus, it is hard to imagine that regulatory gene networks will be found, where miRNAs are not involved as prominent regulators or fine-tuners.

Since most advances in our understanding of ncRNAs have been made in the field of miRNAs, we will here primarily summarize the knowledge on the role of miRNAs in cellular senescence and their role in modulation of organismal life span. However, since we are convinced that individual members of less well characterized classes of ncRNAs will emerge as modulators of aging, we have also added a short overview speculating on their possible influences on aging of cells and organisms.

### **3. Small non-coding RNAs: miRNAs are among the best understood so far**

MicroRNAs are a class of recently identified small non-coding silencing RNAs of around 22 nt of length (reviewed by (Ghildiyal and Zamore, 2009)). MicroRNAs are mainly transcribed as pri-miRNAs by RNA polymerase II (Lee *et al.*, 2004). However, examples exist where they arise from introns, that after being spliced out as lariats, are debranched, and form the necessary hairpin structure for entering the miRNA biogenesis pathway (Berezikov *et al.*, 2007). This pathway consists of nuclear processing of the pri-miRNA by the Microprocessor complex Pasha/DGCR8 and Drosha (Gregory *et al.*, 2004) generating pre-miRNA. Regulation of miRNA maturation can be inhibited at this step by a post-transcriptional uridylation (Heo *et al.*, 2008; Heo *et al.*, 2009).

After transcription, miRNAs are exported to the cytoplasm, where dicer cuts the stem loop region producing small double stranded RNA (dsRNA). This dsRNA is then loaded onto the RNA induced silencing complex containing the RNA endonuclease Ago1, and unwound. Thermodynamic stability of the 5'-end of the duplex determines, which of the two strands is retained in the RISC complex as guide strand, and which is cut and degraded. Finally, the guide strand confers specificity to the RISC complex that now recognizes mRNA targets that are in turn either degraded or translationally repressed (Bhattacharyya *et al.*, 2006), depending on a variety of not completely understood factors ranging from degree of mismatches, cellular status, or transcriptional history of the mRNA transcript (Carthew and Sontheimer, 2009). Surprisingly, specific cellular conditions can turn miRNAs from silencers to translational activators (Orom *et al.*, 2008; Vasudevan *et al.*, 2007).

The mode of action in silencing depends on the recognition of target mRNAs by either of three mechanisms. Binding may depend on the 'seed' region consisting of nucleotides 2-8 of the miRNA only. This seed can be supported by 3' base-pairing after a short bulge of non-complementarity in the 'canonical' binding model. Finally, a shorter seed of down to 4 nucleotides at the 5' end is still able to silence targets if 3' compensatory complementarity supports miRNA-mRNA binding (Brennecke *et al.*, 2005). Due to this 'loose' specificity, one miRNA is able to regulate up to several hundred mRNA targets and therefore seems to orchestrate a large variety of cellular processes similar to transcription factors (Lim *et al.*, 2005; Stefani and Slack, 2008), but also in concert with transcription factors forming feed forward loops (Shalgi *et al.*, 2007).

While quite impressive knowledge on the biogenesis of miRNAs has been collected, it is surprising to find only two recent studies addressing the question of their degradation, which is directed by the 5'-->3' exoribonuclease XRN-2 in *C. elegans* (Chatterjee and Grosshans, 2009) and the 5'-->3' exoribonuclease SDN1 in *A. thaliana* (Ramachandran and Chen, 2008).

The importance of miRNAs is further underlined by the fact that the loci encoding miRNAs have also been found to contain SNPs and translocations that are correlated with different diseases (Duan *et al.*, 2009; Mishra and Bertino, 2009; Mishra *et al.*, 2008).

## 4. Contribution of miRNAs to aging and longevity of organisms

### 4.1. Conserved longevity pathways modulated by miRNAs

#### 4.1.1. IGF-signalling

Although our knowledge on miRNAs is almost exponentially growing, there is still only one report showing that a single miRNA can modulate the life span of an organism, while several miRNAs might influence the life-span by regulating factors of well known longevity pathways (Fig. 1). One of these well accepted pathways is insulin/IGF1 signalling (Bartke, 2008; Tatar et al., 2003).

Therefore, it is not too surprising that the so far only miRNA that increases life span upon overexpression, is *lin-4*, while its knock-down dramatically shortens the life-span of *C. elegans* (Boehm and Slack, 2005). The target mRNA of *lin-4* has been identified as *lin-14*, which acts in parallel to or upstream of *daf-2* eventually inhibiting *daf-16* (Boehm and Slack, 2006). Thus, the increase in longevity of *lin-4* overexpression seems to be mediated by the insulin-like signalling pathway (Fig. 1A).

In the fly, the insulin receptor homologue InR regulates also dacapo, a p21/p27 homologue, which is targeted by miR-7, miR-278 and miR-309 thereby controlling cell division in germline cells (Yu et al., 2009). Similarly, miRNAs modulate IGF-1 in human cells, since IGF-1 is targeted by miR-1 and miR-206 (Shan et al., 2009), while both, IGF-1 and IGF-1R are targeted by miR-320 in rats (Wang et al., 2009a). Furthermore, let-7a3 inversely correlates with IGF2 and IGFBP3 protein levels (Lu et al., 2007). Downstream of insulin/IGF1, the insulin/IGF1 receptor substrate IRS-1 is also modulated by miR-145 (La Rocca et al., 2009; Shi et al., 2007). Furthermore,



lipid metabolism and insulin secretion have also been found to be regulated by miR-122 (Esau et al., 2006) and miR-375 (Poy et al., 2004), respectively.

IGF-1 signalling is also linked to the widely discussed SIRT1 and resveratrol pathway modulating organismal life-span (Allard et al., 2009; Guarente, 2007; Longo, 2009). Interestingly, inhibition of miR-217 in senescent endothelial cells reduces the percentage of senescent cells by increasing SIRT1 activity (Menghini *et al.*, 2009). Thus, miR-217 inhibition might act similar to resveratrol in prolonging longevity, an idea that still lacks experimental evidence. However, candidate miRNAs regulating this pathway are summarized in Fig. 1A.

#### 4.1.2. Steroid signalling pathways

Besides work on *lin-4*, alterations in miRNA transcript levels during worm aging have been observed as well (Ibanez-Ventoso *et al.*, 2006), but no general screening approach aiming at identifying miRNAs that prolong the worm life span has been published to date. Such a study seems promising, since potential miRNA candidates modulating the life span in worms seem highly plausible. One such candidate might be *let-7* that targets the nuclear hormone receptor DAF-12 in worms. DAF-12 is related to vitamin D and liver X receptors in vertebrates (Grosshans *et al.*, 2005) and controls developmental progression (Bethke *et al.*, 2009). DAF-12 mediates life span extension by steroids/removal of germ line (Broue et al., 2007; Rottiers et al., 2006), regulates longevity in dependence of thermosensitivity (Lee and Kenyon, 2009), and seems to contribute to the differences of lifespans between male and hermaphrodite worms (McCulloch and Gems, 2007).

Similarly to *C. elegans*, interfering with steroid signalling also modulates the life span of *D. melanogaster*. In this case the steroid is ecdysone (Simon et al., 2003)

and since miR-14 has been found to target the ecdysone receptor (Varghese and Cohen, 2007), it also presents a candidate for life-span modulation. However, the effects of *let-7* or miR-14 overexpression and knock-down will be very interesting in regard to the lifespan of these model organisms (Fig. 1B). Other candidates that might modulate longevity can be deduced from their role in signalling pathways that are known to be intricately related to the life-span of organisms.

#### **4.1.3. Target of Rapamycin (TOR) pathway**

Recently, it has been reported that Dicer and dafapo are regulated in dependence of nutrients (Yu *et al.*, 2009). It is intriguing to think that this might represent a connection to TOR signalling, a key pathway of nutrient sensing that also modulates organism life span (Harrison *et al.*, 2009). So far only two reports connect the TOR pathway with miRNAs (Fig. 1C), specifically miR-21 is regulated by TOR and NFkappaB (Vinciguerra *et al.*, 2009), while miR-30a targets beclin-1 mediated autophagy, interfering with rapamycin inhibition of TOR in human tumor cells (Zhu *et al.*, 2009). Autophagy seems key in the life span prolongation by TOR inhibition at least in yeast and worm (Alvers *et al.*, 2009; Toth *et al.*, 2008). Still, experimental evidence for a modulation of life span by these miRNAs is lacking, as much as analysis on miRNAs in the context of calorie restriction.

#### **4.2. Tissue aging and miRNAs: few tissues, few functions**

So far only few tissues have been analyzed in regard to differential miRNA expression during aging. Among them are human (Lukiw, 2007) and mouse brain (Li *et al.*, 2009a) as well as normal mouse liver (Maes *et al.*, 2008) and Ames dwarf mouse liver (Bates *et al.*, 2009). Contrasting results are reported in aging mouse

lung, where either no changes (Williams *et al.*, 2007) or changes are found (Izzotti *et al.*, 2009). Furthermore, members of the miR-17-92 cluster have recently been reported as downregulated in skin, bone-marrow derived mesenchymal stem cells and T cells of old versus young donors (Hackl *et al.*, in press). Still, functional data are missing in these contexts.

#### **4.3. Tissue regeneration, stem and progenitor cells**

Recently, the formidable importance of miRNAs for stem cell biology has emerged, as they are involved in reprogramming of inducible pluripotent stem (IPS) cells, where miR-291-3p, miR-294 and miR-295 (Judson *et al.*, 2009), or miR-145 alone (Xu *et al.*, 2009) modulate reprogramming by Oct4, Sox2 and Klf4. Downstream, the miR-302-367 cluster acts after Oct4, Sox2 and Nanog to maintain stemness of human embryonic stem cells (Barroso-del Jesus *et al.*, 2009). In addition, miR-17-92 member miR-92b induces p57 mediated cell cycle arrest in G1/S in human embryonic stem cells (Sengupta *et al.*, 2009) and is also involved in early stem cell differentiation in mouse embryos *in vivo* (Foshay and Gallicano, 2009).

In regard to aging, especially adult stem and progenitor cells are of high relevance. Replicative decline and premature senescence of somatic stem cells and other self-renewing cells e.g. due to DNA damage contributes to the age-associated functional decline of tissues and organs (Nijnik *et al.*, 2007; Rossi *et al.*, 2007). For example, let-7b is involved in decline of neuronal stem cell self renewal during aging by reducing HMGA2 levels in old but not in young mice (Nishino *et al.*, 2008). Furthermore, neuronal stem cell differentiation is co-regulated by miR-9 and miR-124 targeting the chromatin remodelling factor BAF (Yoo *et al.*, 2009). Finally, miR-27b is

involved in regulating differentiation of muscle stem cells by targeting Pax3/7 (Crist *et al.*, 2009).

## 5. Cellular senescence and miRNAs

### 5.1. Cellular senescence and its *in vivo* implications

Replicative senescence was described more than four decades ago when Hayflick and colleagues showed that normal human cells had a limited potential to proliferate in culture (HAYFLICK, 1965). This irreversible growth arrest is triggered by critically short, unprotected telomeres that induce a DNA damage like signal (Palm and de Lange, 2008) and executed by either of the two important cell cycle inhibitors, p21 or p16 (Ben-Porath and Weinberg, 2005). The senescent phenotype is characterized by a combination of changes in cell behaviour, structure and functions. This includes, alteration in gene expression (Campisi and d'Adda di Fagagna, 2007), protein secretion (Kuilman and Peeper, 2009), and inducibility of apoptosis, which increases in senescent fibroblasts (Wang, 1997) and decreases in endothelial cells (Hampel *et al.*, 2004).

Besides exhaustion of proliferative potential, a senescent phenotype is also induced by various physico-chemical stressors that induce DNA damage and chromatin disruption as well as by oncogenic signals (Cabrera *et al.*, 1992; Maruyama *et al.*, 2009). By now, the presence and age-related accumulation of senescent cells *in vivo* has become well accepted (Campisi and d'Adda di Fagagna, 2007; Campisi and Sedivy, 2009; Wang *et al.*, 2009b) and it is well established that senescent cells *in vivo* contribute to age-associated diseases like atherosclerosis (Erusalimsky, 2009; Erusalimsky and Skene, 2009; Minamino and Komuro, 2007) or

kidney diseases (Melk, 2003). Moreover, it was shown that cellular senescence limits the extent of fibrosis following liver damage and underscore the interplay between senescent cells and the tissue microenvironment (Krizhanovsky et al., 2008).

In fig. 2 we summarize potential effects of cellular senescence on aging of organisms. Since senescent cells never re-enter the cell cycle, cellular senescence is suggested to prevent malignant transformation of potentially mutated cells and thus to contribute to tumor suppression. However, some cell types persist within tissues when senescent and are not eliminated by apoptosis or the immune system, such that their altered functional profile might alter tissue microenvironments in ways that can promote both cancer and aging phenotypes (Krtolica and Campisi, 2002; Rodier et al., 2007).

Recently, it was shown that premalignant mammary epithelial cells exposed to senescent human fibroblasts in mice irreversibly lose differentiated properties, become invasive and undergo full malignant transformation (Parrinello et al., 2005).

The senescent secretome of fibroblasts has been well established by identification of various secreted factors - covered by the term 'senescence-messaging secretome (SMS)' - that contribute to senescence like IGFBP3 (Muck *et al.*, 2008), IGFBP7 (Wajapeyee et al., 2008), key components of the Wnt signalling pathway, IGF1, transforming growth factor- $\beta$  (TGF $\beta$ ), and plasmin (Kuilman and Peeper, 2009) as well as interleukin-6 (Kuilman and Peeper, 2009) and interleukin-8 (IL-8) receptor (Acosta et al., 2008a; Acosta et al., 2008b). Similarly, high levels of IL-8 in supernatants of senescent versus early passage endothelial cells were found which might be involved in destabilization of atherosclerotic plaques (Hampel et al., 2006) and the inflammatory response during atherosclerosis (Lindeman *et al.*, 2008).

Finally, if senescent and/or damaged cells are removed from tissues by apoptosis or the immune system, either surrounding cells have to undergo division

and/or neighbouring stem or progenitor cells have to divide and differentiate. In any case this might lead to replicative decline and in consequence to loss of tissue integrity and functionality with increasing age, which results in a vicious circle further accelerating senescence and/or loss of cells within the tissues.

## **5.2. miRNAs in replicative senescence**

The contribution of miRNAs to induction and maintenance of the senescent phenotype might involve regulation of different cellular pathways.

The most obvious is by influencing the cell cycle progression (Fig. 3A), but miRNAs might also lead to differential physiology and secretion of proteins like extracellular matrix or cytokines influencing the SMS (Fig. 3B). In regard to cell cycle regulation, especially the let-7 family of miRNAs inhibits KRAS, HMGA2 and c-MYC. Similarly, miR15a/16-1 cluster and the miR-17-92 cluster are potent regulators of cell cycle progression by targeting CDK6, CARD10 and CDC27 as well as the CDK inhibitor family members p21, p27 and p57 as reviewed recently (Chivukula and Mendell, 2008).

In regard to secretion of cytokines, it is of note that miR-146, which is upregulated in senescent fibroblasts, is an inhibitor of IL-6 and thus might contribute to the protein secretion alterations observed in senescent cells (Bhaumik *et al.*, 2009).

Therefore, it is not surprising that differential miRNA transcription has been found in replicative senescence of fibroblasts (Bhaumik *et al.*, 2009; Brosh *et al.*, 2008; Lal *et al.*, 2008; Maes *et al.*, 2009), and human mesenchymal stem cells (Wagner *et al.*, 2008), as well as in human umbilical vein endothelial cells, T cells, fibroblasts, and renal proximal tubular cells (Hackl *et al.*, submitted).

Thereby, a common downregulation of miR-17, 19b, 20a and miR-106a, members of the miR-17-92 cluster, which was described as the first 'oncomiR' (He *et al.*, 2005) was found not only in senescent cells, but also in cells and tissues derived *ex vivo* from young versus old individuals, as also mentioned above (Hackl *et al.*, submitted). This indicates that this cluster is one additional important player not only in the complex regulatory network of cell cycle and tumorigenesis, but also in aging, emphasising that these processes are intricately interwoven (Campisi, 2003).

### **5.3. Non-coding RNAs, telomerase and telomeres**

Another way to modulate the replicative life span of cells is to alter the telomere length of normal cells. We already have quite some idea about how the telomeric repeat is protected by proteins, especially by the shelterin complex (Palm and de Lange, 2008). However, ncRNA components at the telomeres might exert protective functions as well, since long non-coding transcripts called telomeric repeat-containing RNA (TERRA) are involved in regulating telomerase and chromatin stability (Caslini *et al.*, 2009; Luke and Lingner, 2009). Furthermore, Dicer independent small piRNA-like RNAs, termed tel-sRNAs associate with telomeric heterochromatin in mouse embryonic stem cells (Cao *et al.*, 2009). They share the same specificity for the G-strand as TERRA, contain UUAGGG repeats, and are discussed to act as a sensor for chromatin status and methylation. This is due to the finding that knock-out mice lacking the two histone methyltransferases Suv39h1 and Suv39h2 have aberrantly long telomeres (Garcia-Cao *et al.*, 2004), but drastically lower levels of tel-sRNAs (Cao *et al.*, 2009).

Finally, telomerase itself is a direct target of miRNA dependent post-transcriptional regulation. In thyroid cancer miR-138 (Mitomo *et al.*, 2008) as well as

a novel pre-miR in hepatoma cells, termed RGM249, (Miura *et al.*, 2009), have so far been found to silence hTERT. While this is discussed as a tumor suppressor mechanism, it would be interesting to know if this regulation can also be seen in normal, 'healthy': conditions, e.g. in stem cells, or stimulated, proliferating T cells, where hTERT is physiologically reactivated (Effros *et al.*, 2003).

#### **5.4. DNA-damage, ROS, and oncogene induced senescence**

DNA damage and its repair are largely recognized as modulators of the aging process (von Zglinicki *et al.*, 2005). Especially, mutations in proteins mediating DNA damage signalling (Murga *et al.*, 2009), the nucleotide excision repair (Garinis and Schumacher, 2009; Garinis *et al.*, 2008; Schumacher *et al.*, 2009), and DNA interstrand cross link repair (Grillari *et al.*, 2007) are reported to lead to premature progeroid syndromes. Similarly, long-term survivors of chemotherapy, which in many instances relies on DNA damaging agents, have been reported to prematurely show progeroid phenotypes, for which the term "acquired premature progeroid syndrome" (APPS) has recently been suggested (Grillari *et al.*, 2007).

What is the evidence for effects of miRNAs in these processes? First of all, dicer levels are decreased by diverse cellular stressors including ROS, phorbol esters, interferons, and oncogenic RAS in various cells including fibroblasts (Wiesen and Tomasi, 2009). In addition, H<sub>2</sub>O<sub>2</sub> induced senescence in human fibroblasts and trabecular meshwork cells leads to differential transcription of miRNAs, among them miR-183. Transfection with miR-183 in turn increases the amount of SA- $\beta$ -Gal positive cells (Li *et al.*, 2009b).

Furthermore, RAS is targeted by members of the let-7 family (Johnson *et al.*, 2007; Morris and McManus, 2005). Finally, ROS, etoposide and ionizing radiation



result in global changes of miRNA expression (Simone *et al.*, 2009) and miR-21 protects against ROS induced apoptosis (Cheng *et al.*, 2009).

When looking for individual miRNAs that are involved in DNA repair, only a few miRNAs that target DNA damage repair proteins have been described so far.

#### **5.4.1. ATR as miRNA target**

The DNA damage response pathway involves upstream kinases ATM and ATR that are activated upon DNA damage. ATR, as part of a global response, then induces miR-709 in germ line upon X-ray irradiation, which in turn downregulates BORIS, a negative regulator of DNA methylation (Tamminga *et al.*, 2008) to prevent aberrant hypomethylation in spermatogametes after DNA damage.

#### **5.4.2. p53: miRNAs up and downstream**

One of the most studied responses to ATR/ATM activity is the phosphorylation and stabilization of p53, which again orchestrates a plethora of effects leading to repair, senescence or apoptosis (Rodier *et al.*, 2007).

Translation of p53 itself is co-regulated by miRNAs, e.g by miR-125b, which is rapidly downregulated upon DNA damage by  $\gamma$ -irradiation allowing for p53 accumulation (Le *et al.*, 2009). Downstream of p53 especially mir-34a, mir-34b, mir-34c are necessary but also sufficient inducers of p53-dependent senescence and apoptosis (Chang *et al.*, 2007; He *et al.*, 2007a; He *et al.*, 2007b; Hermeking, 2007; Kumamoto *et al.*, 2008; Raver-Shapira *et al.*, 2007; Tarasov *et al.*, 2007; Tazawa *et al.*, 2007). These miRNAs are also strongly induced after treating cells with DNA damaging agents like adriamycin (Tazawa *et al.*, 2007), sodium arsenite (Marsit *et*

*al.*, 2006) or tamoxifen (Pogribny *et al.*, 2007). However, the miR-34 effect might be cell type specific, since its overexpression supports proliferation in renal carcinogenesis (Dutta *et al.*, 2007).

In any case, the induction of miR-34 by p53 seems highly conserved during evolution since the mir-34 *C. elegans* homologue is also responsive to *cep-1*, the worm homologue of p53, and miR-34 loss of function mutants resemble *cep-1* mutants in their high sensitivity against radiation (Kato *et al.*, 2009).

Surprisingly, p53 forms a complex with Drosha and p68 and thus plays a direct role in maturation of several specific, proliferation-suppressive miRNAs (Suzuki *et al.*, 2009). On the other hand, inhibition of miRNA biogenesis leads to premature senescence (Mudhasani *et al.*, 2008). It is unclear, if p53 is involved in this step, but it is intriguing to think that the Drosha, p68 and p53 complex disassembly might contribute to increase 'free' p53 to activate cellular senescence.

Since the apoptosis branch of p53 action and the involvement of non-coding RNAs in apoptosis in general has been covered by excellent recent reviews, we refer the reader to those (Baehrecke, 2003; Lynam-Lennon *et al.*, 2009; Yang *et al.*, 2009).

#### **5.4.3. H2AX is regulated by miR-24**

Another event in DNA double strand (dsDNA) break repair and replicative senescence is the phosphorylation of the histone variant H2AX. In turn  $\gamma$ -H2AX is recruited to dsDNA breaks during repair and seems to serve as a 'landing pad' for further DNA repair factors (Kinner *et al.*, 2008), but is also persistently colocalizing in replicatively senescent cells with the telomeres. The formation of such  $\gamma$ -H2AX positive nuclear foci is also widely used as a marker for repair and senescence *in vitro* and *in vivo* (Herbig *et al.*, 2006), although its role during senescence is not clear

so far. Still, H2AX is a direct target of miR-24, and knock-down of miR-24 makes cells hypersensitive against DNA-damage (Lal *et al.*, 2009).

#### **5.4.4. Induction of premature senescence by miRNAs**

Similar to the above described miR-34a, b and c that are able to induce premature senescence in human cells, miR-290 also induces premature senescence in mouse embryonic fibroblasts upon overexpression (Pitto *et al.*, 2009). Finally, suppression of miR-24 in human fibroblasts allows accumulation of its target p16<sup>INK4a</sup>, although it is unclear if this triggers senescence (Lal *et al.*, 2008). p16<sup>INK4a</sup> is an inhibitor of the cyclin-dependent kinases CDK4 and CDK6 that leads to hypophosphorylation of the Retinoblastoma tumor-suppressor protein (RB) eventually resulting in cell cycle arrest.

#### **5.5. Escape of miRNA induced gene silencing during aging?**

Changes in the general use of alternative 3'-untranslated regions (UTRs) allow mRNAs to escape their miRNA regulators (Majoros and Ohler, 2007), similar to alternative retaining of introns that changes target sites (Tan *et al.*, 2007). Indeed such changes are described in quiescent versus proliferating T cells (Sandberg *et al.*, 2008) but also in cancer cells, where in consequence shorter 3'-UTRs arising from alternative cleavage and polyadenylation activate oncogenes (Mayr and Bartel, 2009). No studies have addressed general changes of alternative pre-mRNA splicing or alternative 3'-UTR usage during aging so far.

## 6. Other classes of small non-coding RNAs

### 6.1. Endogenous siRNAs (*endo-siRNAs*)

Many different types of small ncRNAs have been reported to exist in plants and worms. Biogenesis of some of these ncRNAs is dependent on RNA dependent RNA-polymerases (RdRP), which previously had not been found in the mammalian or fly genomes, suggesting that from flies to mammals these classes of ncRNAs do not exist.

However, there is one very recent and surprising exception: the catalytic subunit of hTERT associates not only with its well established hTERC RNA component, but also with the mitochondrial RNA processing endoribonuclease RNA (RMPR). In consequence, the telomerase/RMPR ribonucleoprotein displays RNA dependent RNA polymerase activity producing siRNA like molecules in human cells (Maida *et al.*, 2009).

What is known about *endo-siRNAs* so far? *Endo-siRNAs* in fly and worm derive from various sources like transposable elements or from *cis-natural antisense transcripts*. The *cis-natural antisense transcripts* are formed by bidirectional transcription of the same genomic locus that produces overlapping RNAs. In contrast, *trans-natural antisense transcripts* derive from gene – pseudogene transcripts that may be located distantly within the genome. In any case, overlapping complementary RNAs anneal into RNA duplexes. Functional understanding of *endo-siRNAs* still is scarce (Ghildiyal and Zamore, 2009; Okamura and Lai, 2008), but entry of *endo-siRNAs* into the miRNA silencing pathway seems plausible and would suggest similar mode of action by molecules derived from a different biogenesis pathway. It will be exciting to see if, how and which of them do play a role in aging and age-related processes.

## **6.2. Piwi-interacting RNAs (piRNAs)**

A recently discovered class of non-coding RNAs in germline cells are piRNAs that can be found in worms (Batista *et al.*, 2008) (Das *et al.*, 2008) (Wang and Reinke, 2008) up to mammals (Aravin and Hannon, 2008). Their name derives from their binding to the PIWI clade of Argonaute family proteins. In contrast to miRNAs, they are 2'-O-methylated at their 3' termini, a feature that is similar to plant siRNAs. They have been found primarily to contribute to germline stability by silencing transposons. As such they are considered as an innate immune system that discriminates transposons from endogenous genes. Transposon silencing during male germ cell development is executed by sequence specific methylation of cytosines, which makes piRNAs the so far only known guide for directing methylcytosine formation in mammals (Aravin and Hannon, 2008). However, recent data suggest that piRNAs are not only restricted to the germline, but can be operative also in somatic cells (Lin and Yin, 2008; Malone *et al.*, 2009). Since aging is correlated with changes in chromatin structure and methylation status (Adams, 2007; Bandyopadhyay and Medrano, 2003; Fraga and Esteller, 2007; Funayama and Ishikawa, 2007; Liu *et al.*, 2009), and since piRNAs have been found in a complex with RecQ1 (Lau *et al.*, 2006), a DNA helicase necessary for genomic stability (Sharma and Brosh, 2007), it will be exciting to see if and how piRNAs might contribute or cause aging related functional decline.

### 6.3. QDE-2-interacting RNAs (qiRNAs)

A very recently discovered class of non-coding RNAs are qiRNAs, named after their interaction with the argonoute protein QDE-2. So far, they are only described in the fungus *Neurospora crassa*. Most of them derive from the rRNA locus, and their transcription is induced by DNA damage. Since knock-down of QDE-1 leads to higher sensitivity against DNA damage, qiRNAs might be involved in DNA damage repair (Lee *et al.*, 2009).

qiRNA transcription requires the 3 proteins dicer, QDE-1, which is an RNA-dependent RNA polymerase, and QDE-3. Interestingly, QDE-3 is the homologue of *sgs-1* in yeast that has evolved into the two human RecQ family members Werner and Bloom DNA helicase (Lee *et al.*, 2009). Since mutations in the human WRN/BLM genes lead to premature aging syndromes (Martin, 1978; Martin and Oshima, 2000), this class might leave much room for further surprises in the field of biogerontology. Especially, since telomerase has been identified to be the first mammalian RNA dependent RNA polymerase (Maida *et al.*, 2009) which opens a possible path for qiRNA biogenesis also in mammals. Indeed, telomerase overexpression is known to protect fibroblasts (Gorbunova *et al.*, 2002), neuronal cells (Lu *et al.*, 2001) and leukemia cells (Akiyama *et al.*, 2002) against DNA damage induced cell death by a yet unknown mechanism.

## 7. Concluding remarks

Taken together, there are many indications but still scarce evidence that non-coding RNAs are implicated in cellular and organismal aging. Only one miRNA has been reported so far to extend an organism's life-span, while many miRNAs inhibit

important known modulators of aging but have not yet been tested for their influence on 'healthy' aging and longevity.

Age-associated functional decline of tissues and organs, however, predisposes organisms for specific, age-related diseases. Therefore, it is important to note that ncRNAs, especially miRNAs, have emerged as important players in the pathogenesis of a variety of age-related diseases like cancer (Croce and Calin, 2005; Garzon et al., 2009), neurodegenerative diseases (Lukiw, 2007; Nelson *et al.*, 2008), diabetes (Hennessy and O'Driscoll, 2008; Pandey et al., 2009), and of course vascular diseases and atherosclerosis (Mishra et al., 2009a; Rao et al., 2009; Xu et al., 2007). Although miRNAs have been shown to be involved in osteogenic differentiation (Scheideler *et al.*, 2008), nothing is yet known about their involvement in osteopenia and osteoporosis.

Finally, small non-coding RNAs and RNAi have already been introduced as promising novel drugs in preclinical and clinical trials against various diseases (Blenkiron and Miska, 2007; Lopez-Fraga et al., 2009; Mishra et al., 2009b; Nakasa et al., 2009; Takeshita et al., 2009; Zhang, 2009). The power of small ncRNAs and their derivatives as putative therapeutic molecules together with the still scarce functional knowledge about non-coding RNAs in regard to aging gives all reason to expect many surprises for basic and biomedical aging research in regard to non-coding RNAs.

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## 10. Figure legends

**Fig.1: miRNAs involved in pathways known to modulate organism life-span.** (A) insulin/IGF, (B) *C. elegans* steroid signalling (C) TOR signalling and (D) miRNAs involved in stem and progenitor cell function. See text for details and references.

**Fig. 2: Overview on how cellular senescence might contribute to aging of tissues and organisms.** (1) Cells are exposed to DNA damage, reactive oxygen species (ROS), high oncogenic signalling or to telomere shortening due to multiple replications. (2) If no repair or cell cycle arrest checkpoints are operative, cells might undergo immortalization and transformation as first steps of tumorigenesis or (3) cells might undergo cellular senescence or apoptosis. The senescent cells then (4) show an altered secretory phenotype and thus influence signalling, or (5a) they might be removed after undergoing senescence by apoptosis or by the immune system. This in turn (5b) leads to replication/transdifferentiation of neighbouring cells or of replication/differentiation of adult stem and progenitor cells, decreasing their proliferative potential. Finally, (6) the senescent cells display an altered behaviour and physiology in regard to their “daily” tasks within a tissue. All this in turn leads to (7) changes in the microenvironment of tissues and to their functional decline, which in turn (8) enhances the risk of tumor development and (9) accelerates senescence, thus largely contributing to aging of organisms.

**Fig. 3: Overview on how some specific miRNAs might contribute to cellular senescence and the altered phenotype of senescent cells.** (A) miRNAs involved in counteracting or executing various senescence-triggering events. (B)



miRNAs involved in altered cellular behaviour of senescent cells. See text for details and references.

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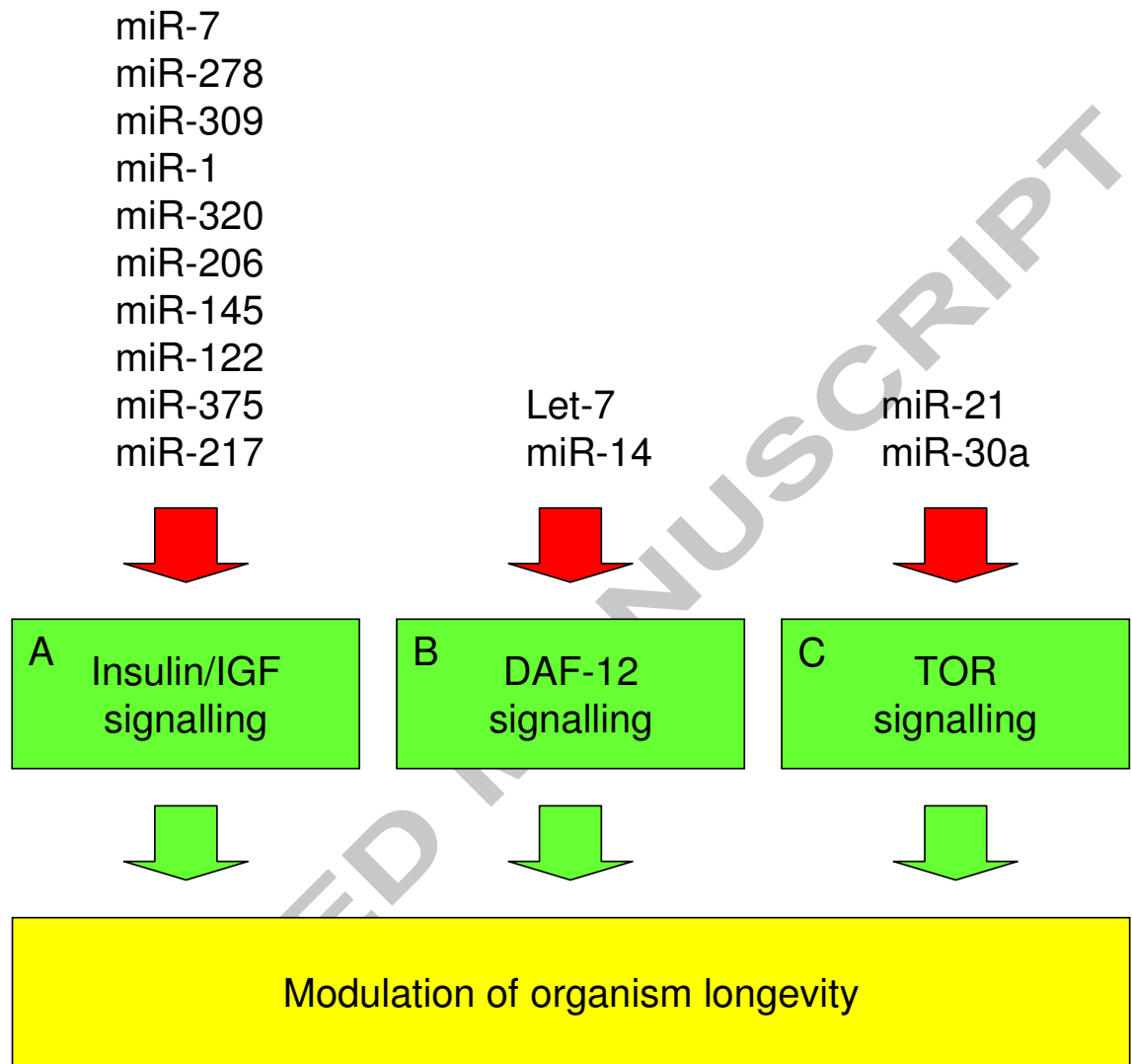


Fig. 1

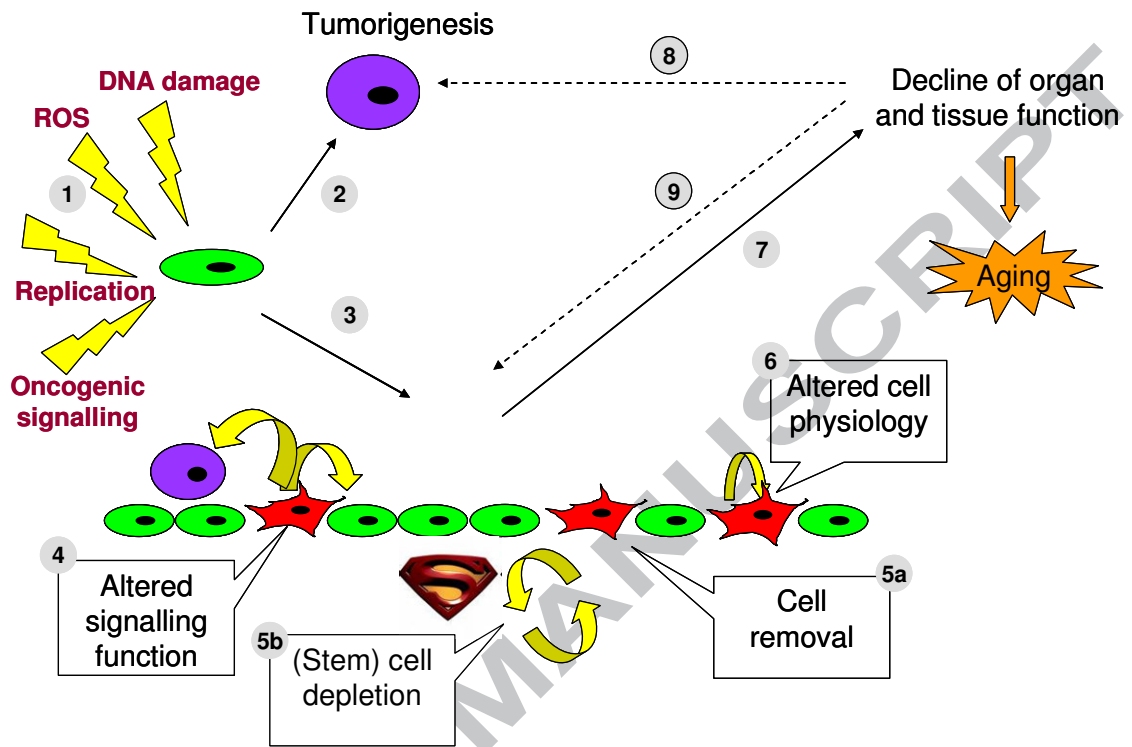


Fig.2:

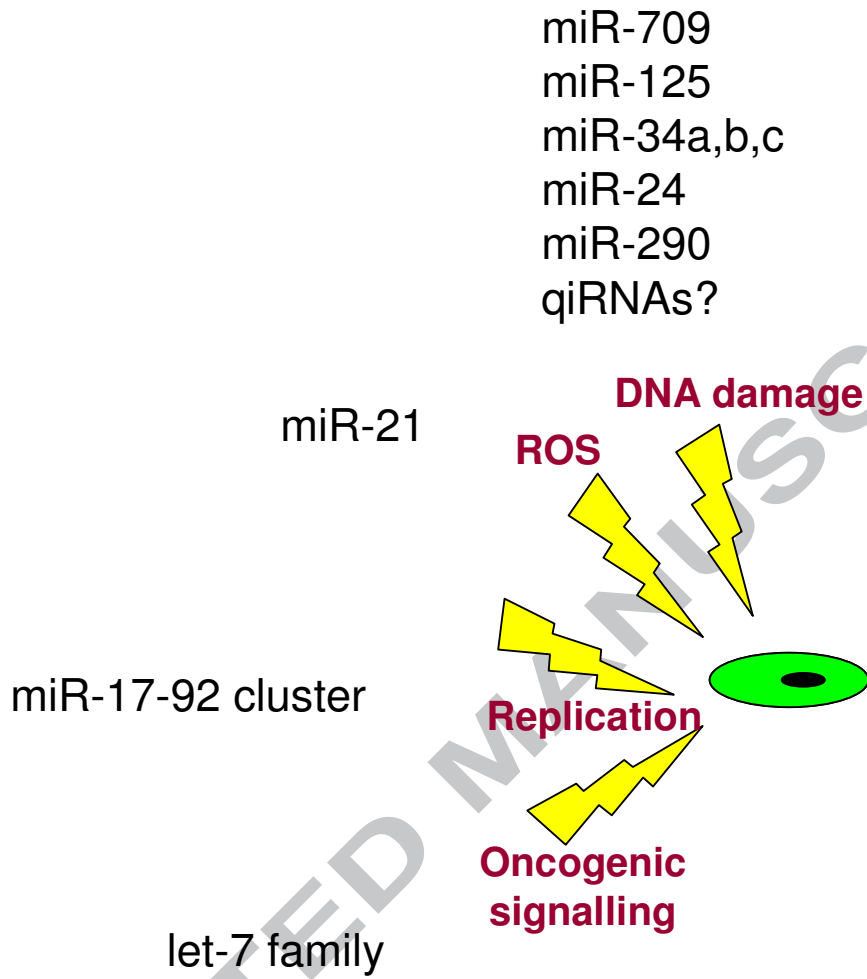


Fig. 3A

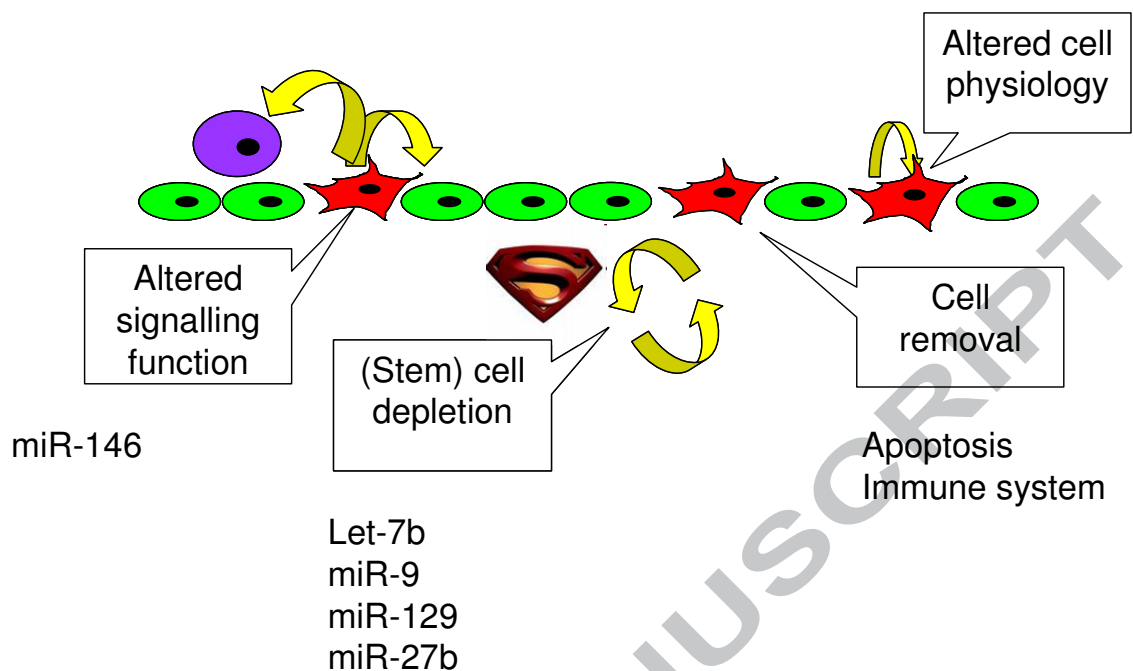


Fig. 3B

ACCEPTED MANUSCRIPT