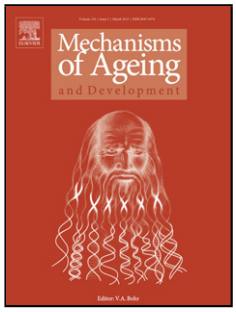

Title: Centenarians maintain miRNA biogenesis pathway while it is impaired in octogenarians

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SHORT COMMUNICATION

MECHANISMS OF AGEING AND DEVELOPMENT

Centenarians maintain miRNA biogenesis pathway while it is impaired in octogenarians.

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Highlights manuscript entitled: Centenarians maintain miRNA biogenesis pathway while it is impaired in octogenarians.

- Over- expression of miRNAs in centenarians versus octogenarians is explained because genes involved in miRNAs biogenesis are up-regulated in centenarians

Abstract

Centenarians but not octogenarians up regulate the expression of miRNAs, as we previously reported. We have looked into miRNA biogenesis. We show that *RNA POL II*, *DROSHA*, *EXPORTIN 5* and *DICER*, are up-regulated in centenarians compared with octogenarians. Furthermore, factors involved in the control of these miRNAs biogenesis genes are also up-regulated in centenarians. Therefore, the up-regulation of miRNA expression in centenarians can be explained in part because miRNA biogenesis pathway is depressed in octogenarians (ordinary aging) while it is maintained in centenarians (extraordinary aging).

This work started as a result of our previous finding that centenarians but not octogenarians up regulate the expression of miRNAs (Serna et al., 2012). We measured the total miRNome in peripheral blood mononuclear cells (PBMCs) of young persons, octogenarians (ordinary ageing) and centenarians (extraordinary ageing) and observed that ordinary ageing results in lowering of the levels of miRNAs. However, individuals displaying extraordinary ageing, i.e. centenarians, overexpress these miRNAs to levels that are superior to those observed in octogenarians. ElSharawy et al., also studied miRNAs in longevity, comparing long-lived persons with adults. Interestingly, they showed 16 miRNAs up-regulated and 64 down-regulated in blood from the long-lived individuals relative to that from the adults.(ElSharawy et al., 2012). These results are interesting as they found different results when comparing centenarians with adults. This might open the hypothesis that the miRNome does not change linearly along aging.

Small non-coding RNAs, and especially miRNAs, are important molecules in the regulation of DNA transcription by RNA silencing (Mourelatos, 2008). They target the majority of protein transcripts and are involved in development, ordinary ageing (Olivieri et al., 2012) and as mentioned above, extraordinary ageing (Serna et al., 2012). miRNAs are 22 nucleotides in length. They are synthesised by RNA polymerases, especially RNA polymerase II and are processed by two enzymes, Drosha and Dicer. Intracellular traffic of molecules is also important for miRNA maturation, especially mediated by exportin 5 (Ha and Kim, 2014).

RNA polymerase II synthesises pri-miRNAs. These are processed by Drosha and Dicer, which are double-stranded RNases that are expressed in all human cells (Conrad and Rauhut, 2002; Cullen, 2004). Drosha converts pri-miRNA into 60-nucleotide pre-miRNA by cleaving an 80 nucleotide loop. This is bound to exportin 5, a protein that induces pre-miRNA export from the nucleus into the cytoplasm. Cytoplasmic pre-miRNA is then processed by Dicer resulting in mature miRNA. Thus Drosha, Dicer and exportin 5 are important in miRNAs biogenesis (Ha and Kim, 2014).

We have now performed studies of the messengerRNA transcriptome in PBMCs and again we have observed that the expression pattern of mRNAs in centenarians is very different from octogenarians and similar to young persons, again showing a successful aging trajectory in centenarians (Borras et al., 2016). Then, by using the whole transcriptome data that we have obtained, we have performed a hypothesis-driven search for such factors that regulate miRNA expression.

The Spanish Centenarian Study Group at RETICEF, began in 2007 as a population-based study of all centenarians living within an area near of Valencia called La Ribera (11^{th} Health Department of the Valencian Community, Spain), which is composed of 29 towns (240.000 inhabitants). Potential subjects were selected from the population data system of the 11^{th} Health Department. We found 31 centenarians of whom 20 met the inclusion criteria. Then we randomly recruited 20 octogenarians of whom 16 met the inclusion criteria and 20 young people of whom 14 fulfilled the inclusion criteria. The inclusion criteria were: to be born within the dates indicated in the study (before 1908 for centenarians, between 1928 and 1938 for octogenarians and between 1968 and 1988 for young individuals), to live in the 11^{th} Health Department for at least the last 6 years

and to sign the informed consent. The exclusion criterion was to be terminally ill for any reason.

All experimental procedures were approved by the Committee for Ethics in Clinical Research of the Hospital de la Ribera, Alzira. All patients or their relatives were fully informed of the aims and scope of the research and signed an informed consent.

We have observed that PBMCs from young persons and centenarians overexpress *RNA POLYMERASE II* when compared with those from octogenarians (see Figure 1). Indeed, it has been shown recently that centenarians possess IgG antibodies to the DNA-directed RNA polymerase II subunit RPB1 with highest frequency (Han et al., 2016). Thus, pri-miRNA must be up-regulated in young individuals and centenarians. This can explain the high miRNA levels in this population.

Then we determined *DROSHA* expression, showing that it is severely downregulated in octogenarians when compared with either young persons or centenarians (see Figure 1b). This may be explained by an increased expression of *GSK3β* in centenarians when compared with octogenarians (15±12 vs 6±1, $p<0.05$). *GSK3β* phosphorylates *DROSHA* and points to a further regulation of miRNA maturation by maintaining its nuclear localisation (see Table 1) (Ha and Kim, 2014).

DROSHA binds to *DGCR8* and we have not observed differences in its expression (results not shown). However, in our Affimetrix gene chip data, we have found an increased expression of a deacetylase *HDAC1* that increases *DROSHA* affinity for pri-miRNA, thus facilitating its RNAase activity (see Table 1). This result, although should be validated by RT-PCR, is in keeping with the increased *DROSHA* expression in centenarians. The results that we report in Figures 1b and Table 1 indicate that the expression and activity of *DROSHA* explains in part the low formation of miRNAs in octogenarians.

We have further looked in our Affimetrix gene chip data the genes that are involved in the cropping activity of *DROSHA*. These are *miR-16, miR-21, HNRNPA1,* and *miR-18a*. Indeed, HNRNPA1 is a multifunctional RNA-binding protein required for processing of several miRNAs such as *miR-18a*. Interestingly, all of these factors are up-regulated in centenarians when compared with octogenarians (Figure Table 1). We only validated by RT-PCR the expression of *miR-21*, confirming the array results (centenarians overexpressed *miR-21* versus octogenarians, 2.97±0.66 vs 0.68±0.12, $p<0.01$) Thus, these results might further support the idea that pre-miRNA synthesis from pri-miRNA is maintained in centenarians and down-regulated in octogenarians. Therefore, we show that some of the mechanisms associated with pri-miRNA processing- are impaired in ordinary aging. This could be one of the reasons for the lower miRNA biogenesis in octogenarians when compared with centenarians and young persons.

Moreover, we have studied the expression of *EXPORTIN 5* and of *DICER*. We have found that *EXPORTIN 5* is significantly up-regulated in centenarians when compared with octogenarians (Figure 1c). This increase in expression of *EXPORTIN 5* may contribute to the increased biogenesis of miRNAs in centenarians, when compared with octogenarians (Serna et al., 2012).

DICER is the final enzyme involved in miRNA maturation. Figure 1d shows that *DICER* is down-regulated in octogenarians when compared with young persons or with centenarians. This is in keeping with the fact that aging is associated to a dysregulation of *DICER* (Mori et al., 2012; Ungvari et al., 2013). Our results also show an increased expression of *miR-21* in centenarians compared with octogenarians (see Figure 1d). This is consistent with the fact that *DICER* can regulate *miR-21* processing. (Ha and Kim, 2014).

We conclude that in centenarians there is an attenuation of the decrease that occurs in miRNome during ordinary aging, because they maintain the miRNA processing machinery activity similar to that found in young individuals. The combined results that we report here are summarised in Figure 2.

Acknowledgments

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Potential Conflicts of Interest: The authors declare no Potential Conflicts of Interest.

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Figure 1. Expression of the enzymes involved in the miRNAs biosgenesis. a. Expression of *RNA POL II* in Young (Y), octogenarians (O) and centenarians (C); b. *DROSHA* in Young (Y), octogenarians (O) and centenarians (C); c. *EXPORTIN 5* in Young (Y), octogenarians (O) and centenarians (C); d. *DICER* in Young (Y), octogenarians (O) and centenarians (C). Results are shown as mean ± SD of n=10-17. Significance is expressed as * $p<0.05$ octogenarians vs. centenarians

Figure 2. Summary of the changes in miRNAs biogenesis in centenarians versus octogenarians.(indicates higher mRNA expression in centenarians)

Gene/miR	C vs Y		O vs Y		C vs O	
	P-value	Fold Change	P-value	Fold Change	P-value	Fold Change
HDAC1	0,03	1,52	0,02	-1,57	1,25E-04	2,38
GSK3B	0,08	1,51	0,01	-1,91	1,96E-04	2,89
HNRNPA1	0,31	1,37	0,01	-2,59	5,86E-04	3,55
miR-16	0,50	1,31	0,05	-2,33	7,95E-03	3,05
miR-18a	0,10	1,77	0,14	-1,72	2,24E-03	3,03
miR-21	4,63E-03	2,62	0,44	-1,31	4,99E-04	3,42

Table 1. Affimetrix results of expression of factors involved in the control of miRNAs biogenesis genes. In all cases we found that gene expression for octogenarians was significantly lower than that of centenarians. a. Expression of *GSK3b* in octogenarians (O, n=6), centenarians (C, n=8) and young (Y, n=8); $p<0.001$ centenarians vs. octogenarians. ; b. *HDAC1* in octogenarians (O, n=6), centenarians (C, n=8) and young (Y, n=8); $p<0.001$ centenarians vs. octogenarians. c. *miR-16* in octogenarians (O, n=16), centenarians (C, n=20) and young (Y, n=17); $p<0.01$ centenarians vs. octogenarians; d. *miR-21* in octogenarians (O, n=16), centenarians (C, n=20) and young (Y, n=17); $p<0.001$ centenarians vs. octogenarians. e. *HNRNPA1* in octogenarians (O, n=6), centenarians (C, n=8) and young (Y, n=8); $p<0.01$ centenarians vs. octogenarians. f. *miR-18a* in octogenarians (O, n=16), centenarians (C, n=20) and young (Y, n=17); $p<0.01$ centenarians vs. octogenarians.

Figure 1

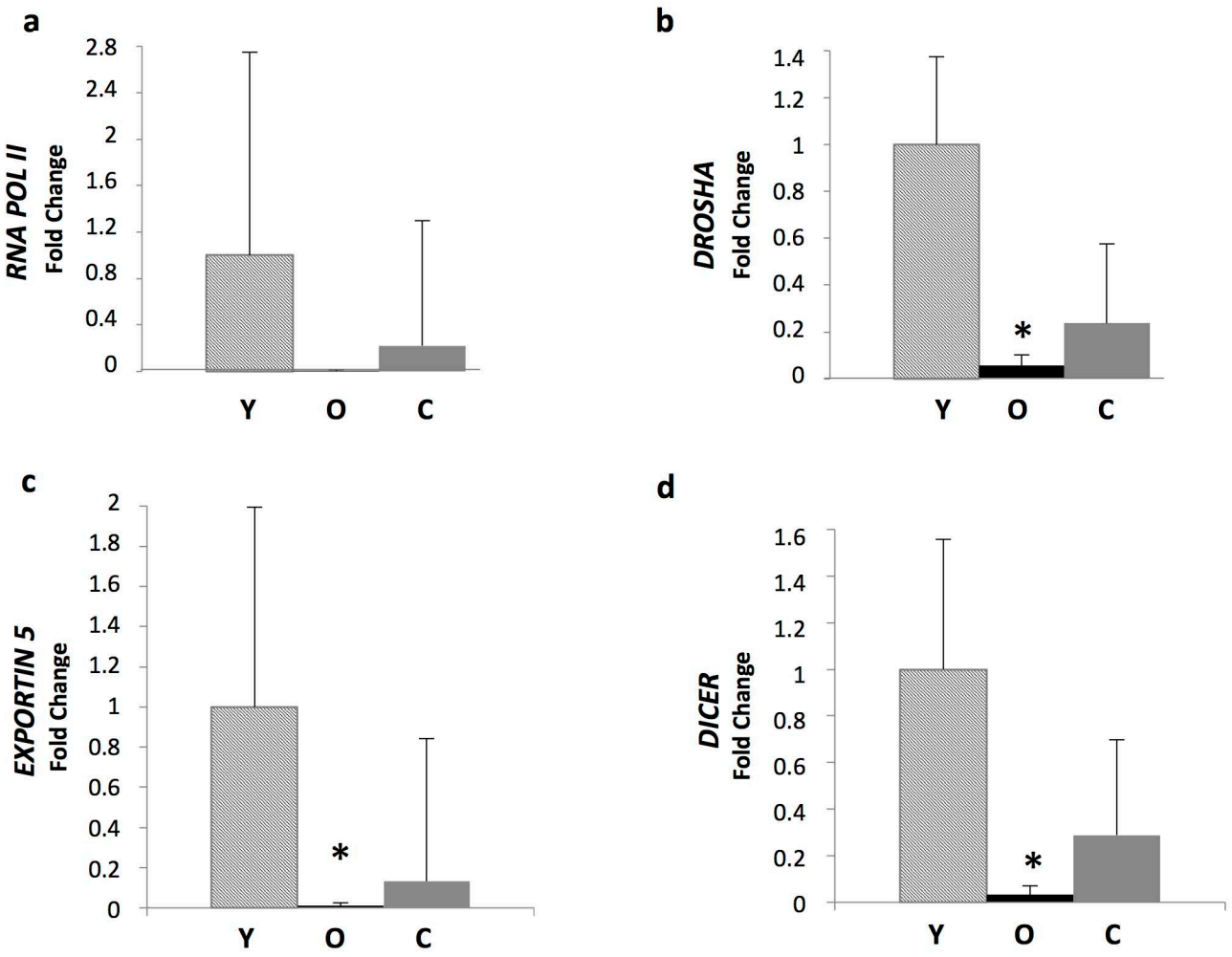

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Figure 2

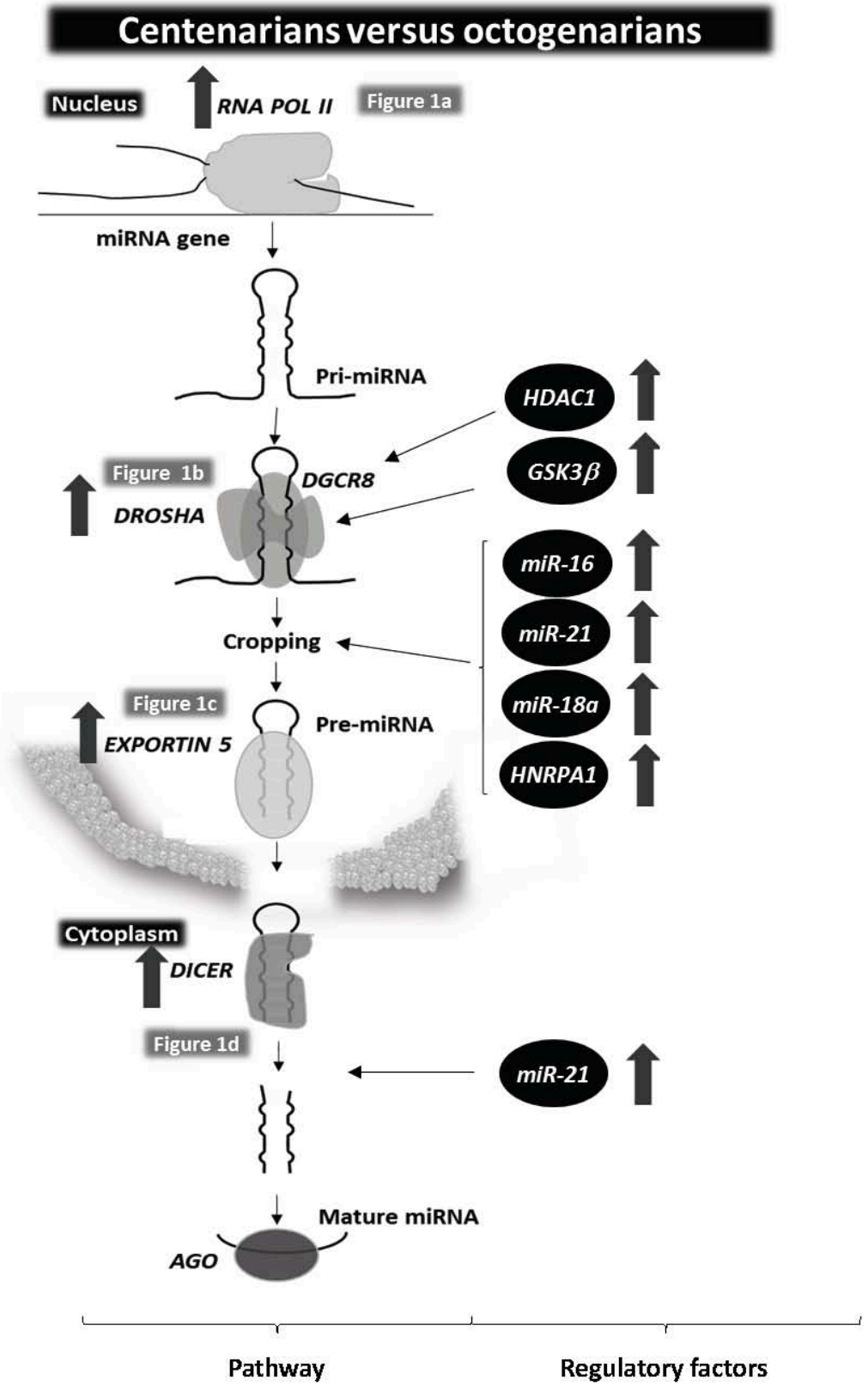

Figure 2. Summary of the changes in miRNAs biogenesis in centenarians versus octogenarians.(↑ indicates higher mRNA expression in centenarians)