Interplay of Hemolymph (HL) miRNA in The Regulation of Aging

Swastik Mukherjee[1], Sandip Pal[2] and Dalia Mukhopadhyay[3]

1. Asutosh College, Department of Zoology

2. Barrackpore Rashtraguru Surendranath College, Department of Zoology

3. Asutosh College, Department of Zoology

Abstract - The aim of this study is to show that the microRNAs circulating in the hemolymph (HL) of Drosophila are in complete correlation with the microRNAs circulating in the body fluids of mammals (for example, humans) thereby regulating aging. Both Hemolymph (HL) miRNA and human miRNAs circulate in vivo in stable form and are nuclease resistant. Homologs of Drosophila hemolymph (HL) miRNAs are present in human body. Among them, miRNA184 is an important one. The miRNA184 regulates lifespan in both Drosophila and humans through IIS (Insulin/Insulin like growth factor pathway). In both Drosophila and humans, inhibition of IIS pathway leads to overexpression of miRNA184. This miRNA184 leads to activation of autophagy specific genes, Atg8 (Autophagy related gene8) in Drosophila and MAP1LC3B (Microtubule Associated Protein IA/IB Light Chain-3), homolog of Atg8, in humans. Proper autophagy increase lifespan or longevity. Similarly, reduced expression of miRNA184 leads to inactivation of autophagy specific genes leading to impaired autophagy that further leads to reduced longevity. This impaired autophagy is mainly responsible for Epilepsy in children that show congenital aging thereby producing an aged phenotype at an early age. These HL miRNAs can be used as a biomarker to control/monitor the pathways regulating aging. These HL-miRNAs can be used in the study of human disease related research.

Keywords: Drosophila, hemolymph microRNA, body tissue microRNA, humans, aging and biomarker.

I. INTRODUCTION

a) Over the years it has been known that purely body tissue microRNAs circulate in the *Drosophila* body. Now it has been investigated that some specific body tissue microRNAs accumulate in the hemolymph forming a complete unique microRNA known as Hemolymph microRNAs (HL). These hemolymph miRNA accumulates in young and adult group in an age dependent manner thereby regulating aging. These miRNAs are unique to *Drosophila* in the respect of showing correlation with mammalian (for example Humans) miRNA that circulate inside their bio fluids. In *Drosophila*, hemolymph circulates in direct contact with internal organs that are analogous to the blood plasma circulating in humans.

b) MicroRNAs (miRNAs) belong to a class of endogenous 22-nucleotide noncoding RNAs that regulate posttranscriptional gene expression for the inhibition or activation of targeted protein translation, depending on the gene complementarity. Within the complicated regulatory networks of mRNAs, multiple miRNAs can target a single mRNA, while a single miRNA can impact multiple mRNAs (Li et al., 2013). Because of this, miRNA plays an important role in various stages of growth and development.

c) Aging is defined as the process of temporal loss of physiological integrity and function resulting in disrupted healthy cellular behaviour (López-Otín et al., 2013). This aging is associated with altered levels of circulating miRNA (reviewed by Dhahbi, 2016).

II. MATERIALS AND METHODS

2.1 COLLECTION OF HEMOLYMPH AND BODY TISSUE miRNA

2.1.1 COLLECTION OF HEMOLYMPH (HL)

To obtain pure, disease free *Drosophila* for the experimental analysis, stocks of Oregon R-C *Drosophila Melanogaster* was collected from the Bloomington Drosophila Stock centre. They were anesthetized under light CO_2. A total of 12 bottles were prepared, each bottle containing 50 virgin males. Four fly bottles that were randomly selected were assigned as the young group after 2 days. This group is used as the four replicates to collect hemolymph and body tissue. Again after 25 days, remaining 8 bottles were selected and assigned as the old group to use it as eight replicates for collection of hemolymph and body tissues. Now, *Drosophila* from both young and old groups is collected. With a contaminant free tungsten needle, a puncture is created in the head and thorax portion of the drosophila. With a slight thumb pressure, the head and thorax portion is pressed to let out the hemolymph. This process of hemolymph collection is known as bloodletting. This process helps in minimizing the contamination with gut contents that occurs during decapitation technique (Reviewed by Dhahbi, 2016). Collected hemolymph is then transferred to contaminants free Eppendorf tubes with the addition of phosphate buffer

saline to make a final volume of 200microlitre preventing it to drying off (Figure 1).

Fig 1: (After Joseph M.Dhabi et al 2016)

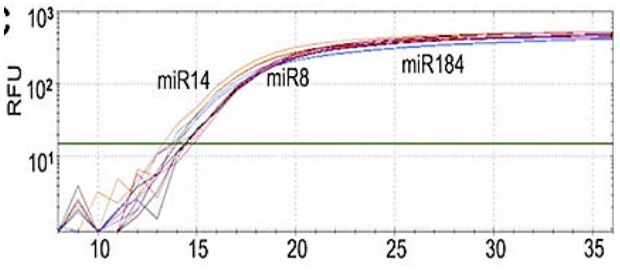
2.1.2 COLLECTION OF BODY TISSUE (BT)

After collection of hemolymph from the *Drosophila* specimens, the entire body devoid of hemolymph is purely body tissue. This body tissue is completely flash frozen and grounded to fine powder in liquid nitrogen. This powdered body tissue is then preserved at -80 degree Celsius.

2.2 COLLECTION OF HL-miRNA AND BT-miRNA

Total RNA concentration including miRNA was extracted from both hemolymph and body tissues by Qiagen miRNeasy Serum/Plasma kit. After extracting the RNA, 3' and 5' adapters were ligated to the end of RNA (both miRNA and mRNA) and subjected to reverse transcription. The reverse transcribed copy that is the cDNA is then amplified by PCR. For cDNA copies of miRNA, readily available primers of miRNA14, miRNA184, let-7, bantam and *Caenorhabditis elegans* cel-mir-39 were used. Similarly, for cDNA copies of cellular mRNA, primers for Tubulin, Actin and Gapdh were used. The purpose for using these cellular mRNAs was to determine whether hemolymph miRNAs were carryover from tissue debris or hemocytes.

2.2.1 REAL-TIME QUANTITATIVE PCR



Amplification with readily available primers like mir14, miRNA184 and miRNA8 showed plots after 10 cycles of amplification. Amplification with cel-mir-39, let-7 and bantam primers also showed amplification plots after 10 cycles of amplification. Now, determination of presence of Tubulin, Gapdh and Actin mRNA was performed in both hemolymph and body tissues (S2 cells). It has been found that considerable amount of these mRNAs present in the tissues but not in the hemolymph. Where S2 cells showed the presence of these mRNAs after 20 cycles of amplification, there the hemolymph showed minute amount of these mRNAs after 30 cycles of amplification. Due to absence of cellular mRNAs in hemolymph, it has been accepted that hemolymph miRNAs are completely different from those that accumulate in body tissues. They are not leftovers of dead or lysed cells. Rather these miRNAs accumulates separately and circulate in the hemolymph (Figure 2).

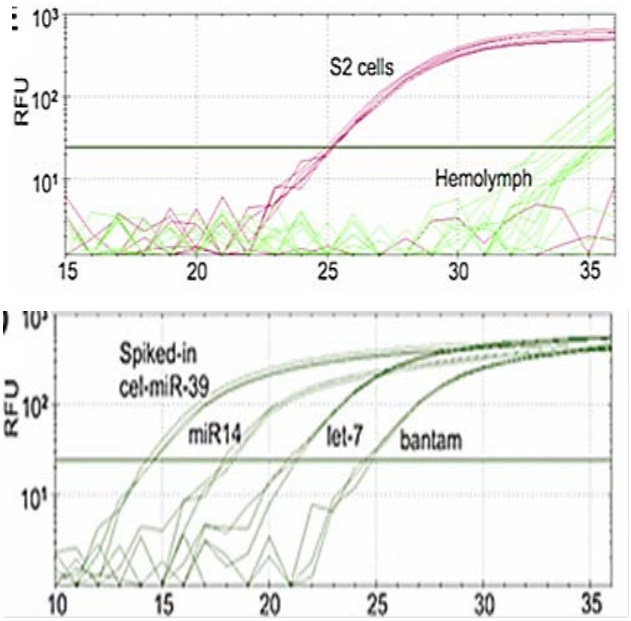
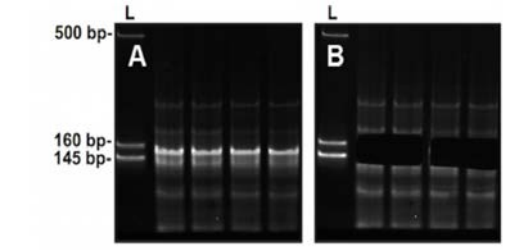


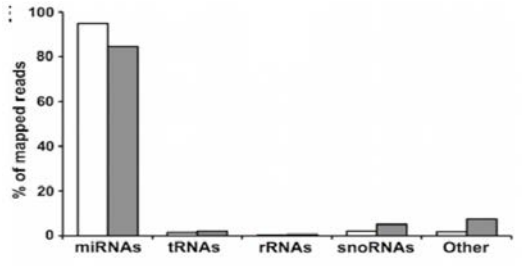
Fig: 2 Y-Axis: Relative Fluorescence Unit (RFU) and **X-Axis:** Cycle of Fluorescence detection. (After Joseph M.Dhabi et al, 2016)

2.3 GEL ELECTROPHORESIS AND SMALL LIBRARY CONSTRUCTION

Amplified cDNAs of both miRNA and mRNA are then subjected to gel electrophoresis and validated for size (Fig 3). The first lane composed of three double stranded DNA fragments of 145, 160 and 500 bp (A). The region between 145 and 160 bps corresponds to the adapter ligated constructs derived from the miRNA. This region was excised from the gel and purified (B). These purified constructs were then used to prepare a library. The library showed both the presence of miRNA and mRNA in hemolymph and body tissues but to varying degree. It showed that more than 80% of the RNA of total 18-28 nucleotides was from both hemolymph (HL) and body tissue (BT) (Fig 3b). Since, hemolymph miRNA originate from hemocytes, it is determined that their relative abundance in hemolymph would be different. Thus, quantification with miRDeep2 revealed three types of hemolymph enriched miRNAs: Frist group contained microRNAs circulating only in young group. Second group of miRNA accumulated in hemolymph of old flies. Third group consisted of miRNAs that were present irrespective of the age of *Drosophila*.



3A



3B

(After Joseph M.Dhabi, 2016)

Fig: **3A:** Bands obtained after cDNAs of miRNA and mRNA subjected to gel electrophoresis. **3B:** Unshaded box: BT-miRNA, Shaded box: HL-miRNA.

III. RESULTS

Mammalian (humans) miRNA that circulate in their bio fluids is in complete correlation with the miRNA that circulate in the hemolymph of *Drosophila*. Like *Drosophila* possess an open circulatory system with hemolymph circulating and flooding the internal organs, humans also possess an open circulatory system with blood plasma washing the internal organs. So, to test the similarity in function of these hemolymph miRNA and blood plasma miRNA, a final stability test was performed. The collected hemolymph with pre added *Caenorhabditis elegans* Cel-mir_39, was subjected to RNase A and DNase I treatment to examine their susceptibility. It has been found that where the Cel-mir-39 was digested and degraded by the RNase A and DNase I, the hemolymph microRNAs i.e., miRNA14, miRNA184 and miRNA8 were resistant to the treatment of either nucleases (Figure 4). This resistance proved that these miRNAs are secreted in a protective form that is similar to the secretory forms observed in higher animals (for example humans). These HL-miRNAs are thus similar to miRNAs circulating in blood plasma of humans with respect to two things – Both circulate in vivo in a stable form and both are resistant to digestion by nucleases. Thus, homologs of these HL-miRNAs in humans are found. In case of old individuals, homolog of dme-miR-100 (*Drosophila)* is hsa-miR-99a, hsa-miR-99b and hsa-miR-100 (Human) and homolog of dme-miR-184 is hsa-miR-184 (this miRNA184 homolog is also present in young too, but in higher concentration than old/adult). In case of young individuals, homolog of dme-miR-14 is hsa-miR-511 and homolog of dme-miR-9a is hsa-miR-9. Some homologs are also present throughout the life of humans like homolog of dme-miR-8 is hsa-miR-8, homolog of dme-miR-125 is hsa-miR-125, and homolog of dme-miR-34 is hsa-miR-34a, b, and c.

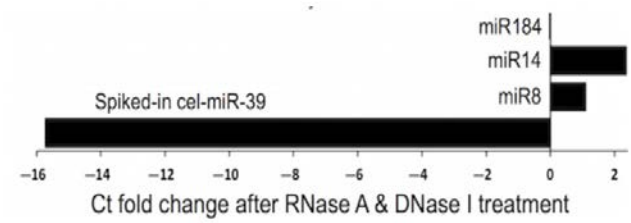


Fig 4: Stability of miRNA by examining susceptibility to Nucleases. (After Joseph M.Dhabi, 2016).

After getting the idea of this similarity between the hemolymph miRNA and human miRNA, now it has been observed whether there is any functional similarity between HL-miRNA and Human miRNA.

Considering miRNA184 that is present in higher concentration in young flies in case of *Drosophila* and young individuals in case of humans, the correlation has been shown in this paper.

3.1 ABUNDANCE OF MIRNA184

It has already been investigated that miRNA184 is predominant in young group of *Drosophila*. It has been found that hemolymph miRNA184 and its human homolog hsa-miR-184 is present in higher concentration in the brain portion compared to the body [4] (Figure 5).

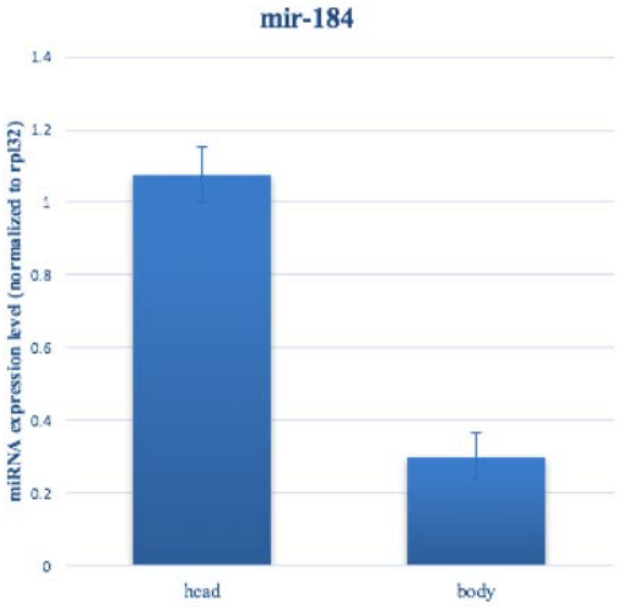


Fig: 5 **Y-axis**: miRNA expression level. **X-axis**: ***First bar*** is Head and ***Second bar*** is Body. (After Bai et al, 2013).

3.1.1 DISCUSSIONS: Regulation of Aging by miRNA184 in *Drosophila* and Humans

In *Drosophila* and humans, miRNA184 regulates aging by interacting with its respective targets for example in this case Activin (In *Drosophila)* , Akt (Protein Kinase B/PKB), mTOR (Mammalian Target of Rapamycin) and

Atg (Autophagy Related Proteins) which are a part of/involved in IIS (Insulin and Insulin like Growth Factor Signalling Pathway). As a result, miRNA184 control IIS pathway. IIS pathway involves binding of insulin and insulin like growth factors thereby leading to activation of the pathway. Insulin and insulin like growth receptors exerts their effects through the phosphorylation and activation of insulin receptor substrates (IRS), which provide docking sites for the interaction and activation of downstream effectors. Due to activation of this pathway, Akt/PKB gets activated. The phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway, then leads to the inhibition of the forkhead box O (FOXO) transcription factors. Another conserved target of Akt is the target of rapamycin complex 1 (TORC1) which is indirectly activated by Akt.

Autophagy means 'self-digestion '. It is a process in which cellular components, such as organelles, proteins, protein complexes/oligomers, and pathogens, are delivered to the lysosome for degradation and the maintenance of cellular homeostasis (Levine and Klionsky, 2004). To mediate autophagy several autophagy specific genes are required that codes for autophagy related proteins. These genes like *Atg8a* (in Drosophila) and *MAP1LC3B* (Microtubule Associated Protein 1A/1B-Light Chain 3), homolog of Drosophila *Atg8a*, plays an important role to carryout autophagy. If these genes remain inactivated then it leads to impaired autophagy that leads to aging.

In *Drosophila*, overexpression of miRNA184 due to activation of FOXO transcription factor, leads to decrease in IIS pathway, leading to decrease in mTOR. Alternatively, reduction in insulin/IGF-1 and mTOR activates miRNA184. Overexpressed miRNA184 deactivates Activin. Neural insulin secretion is controlled by this muscle Activin. The expression levels of miRNA184 and Activin are inversely reciprocal. Deactivated Activin in turn deactivates the expression of its downstream targets i.e., *Babo* and *Smox* genes. As a result, deactivated activin activates Autophagy-Specific Gene 8a (*Atg8a*). Activated *Atg8a* thereby leads to proper autophagy which in turn increases lifespan or longevity. Similarly, down regulation of miRNA184 leads to activation of IIS pathway. Down regulated miRNA184 activates Activin. This activin in turn inhibits transcription of Autophagy specific gene *Atg8a* by signalling through Smad binding element Smox. As a result, this inhibited *Atg8a* decrease lifespan or longevity (Figure 6).

In humans, mTOR is initiated due to binding of insulin like growth factors to insulin like growth factor receptors. These receptor signals via their tyrosine kinase activities to effectors like the insulin receptor substrates (IRS1 and IRS2), which in turn activate Akt. Akt inhibits the activity of the TSC1/TSC2 (Tuberous Sclerosis Complex) and PTEN (Phosphate and Tensin Homolog), negative regulators of mTOR (in Drosophila too) [8]. As a result, IGF1R signalling gets activated and it activates mTOR and inhibits autophagy. The miRNA184 level in brain regulates concentration of this Akt. Overexpression of miRNA184 leads to reduction in the amount of its target proteins (in this case it is Akt2). Similarly, down regulation of miRNA184 in brain or neurons leads to increase in the amount of its target proteins (Akt2). With the increase in the amount of Akt, autophagy pathway gets hampered. There is no activation of Autophagy specific genes like *MAP1LC3B* (Microtubule Associated Protein 1A/1B-Light Chain 3), homolog of Drosophila *Atg8a*. As a result, there is impairment in autophagy that leads to reduced lifespan or longevity. This pathway is also responsible for Epilepsy in children. This leads to neurodegeneration and produce an aged phenotype at an early age. Due to impaired autophagy pathway, children at a young age show Congenital aging and increased neuronal death. Epilepsy in turn lead to oxidative stress, ATP exhaust and unbalanced ion fluxes thereby leading to increased induction of autophagy that in turn ultimately produce an aged phenotype. With the increase in the amount of Akt, mTOR gets activated. This mTOR in turn leads to impaired autophagy. Impaired autophagy in turn, further reduces lifespan or longevity.

Fig: 6 Regulation of Aging by miRNA184 in *Drosophila* (After Bai et al, 2013).

IV. CONCLUSIONS

Through this project it has been demonstrated that miRNAs circulate in *Drosophila* hemolymph in a similar manner as do mammalian (Human) miRNAs circulate in their bio fluids. Both these miRNAs are nuclease resistant implying they circulate in a stable form.

Both mammalian and HL- miRNA is present in an age dependent manner mediating intercellular communication thereby controlling or regulating aging /lifespan.

V. FUTURE SCOPES

These HL miRNAs can be used as a biomarker to control/monitor the pathways regulating aging.

As we use *Drosophila* as the organismal model for investigation of human disease, the discovery of this new hemolymph (HL) miRNA provides a new direction in disease related research of humans.

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