NON-CODING RNA PROFILES OF DENGUE VIRUS 1-INFECTED *Aedes albopictus* CELLS

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NON-CODING RNA PROFILES OF DENGUE VIRUS 1-INFECTED *Aedes albopictus* CELLS

by

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CHAPTER 1: INTRODUCTION

Aedes albopictus (*Ae. albopictus*), a competent vector of dengue virus (DENV) and Chikungunya virus, is a highly invasive species . It is considered as a public health threat throughout the world because of its high adaptability in both tropical and temperate regions of the world, aggressive spread, and vector competence (Chen et al., 2015). Despite being categorized as less efficient vector of DENV than *Aedes aegypti* (*Ae. aegypti*), *Ae. albopictus* is the principal vector that caused dengue epidemics (Cancrini et al., 2003). Therefore, comprehensive studies on *Ae. albopictus* is important for understanding the mechanism underlying its aggressive spread, high adaptability, and vector competence.

Numerous studies over the past several years have focused on *Ae. aegypti*, primarily due to the availability of genome information (Nene et al., 2007). The availability of genome sequence coupled with transcriptome data, facilitate the process of studying this species in great details (Nene et al., 2007; Akbari et al., 2013). Genetics and molecular studies done in *Ae. albopictus* are very limited due to the lack of genome sequence and transcriptome. Recently, a complete genome sequence and transcriptome data of *Ae. albopictus* have been released; thereby, allowing extensive genetic and molecular analyses of this particular species (Chen et al., 2015).

Ae. albopictus was shown to have a large genome; in fact, it is the largest among mosquitoes genomes sequenced. The size of *Ae. albopictus* (Foshan strain) genome is 1,967 Mb, while *Ae. aegypti* is 1,376 Mb, *Culex quinquefasciatus (C. quinquefasciatus)* and *Anopheles darlingi (An. darlingi)* are 540 Mb and 174 Mb respectively (Nene et al., 2007; Arensburger et al., 2010; Marinotti et al., 2013; Chen et al., 2015). The large size genome is principally due to high abundance of repetitive DNA elements. Besides, it was discovered that *Ae. albopictus* genome exhibits huge expansions of the number of genes, and interestingly, it also harbors significant number of insertions of DNA copies derived from RNA viruses. It was postulated that the observed gene expansion and the high number of integration of flavivirus-like genome in *Ae. albopictus,* may contribute to the success and adaptability of this species as an important vector (Chen et al., 2015).

While infection of viruses such as DENV and Chikungunya cause diseases in humans, infection in mosquito such as *Ae. albopictus* generally does not lead to severe pathology. Therefore, *Ae. albopictus* serves as a persistent reservoir for the viruses such as DENV and Chikungunya to thrive, and at the same time, this mosquito can transmit the viruses to humans (Miesen et al., 2016). Insect immune system plays important role in vector competence observed in *Aedes* mosquitoes. Although protein-coding genes have been the central focus, many reports have indicated that non-coding RNA (ncRNA) genes also play important roles in host-virus interaction and antiviral immunity studies in mosquitoes (Miesen et al., 2016).

One of the most important immune responses in insects is antiviral RNA-interference (RNAi) system that make use of three major conserved small RNAs - microRNA (miRNA), small-interfering RNA (siRNA), and PIWI-interacting RNA (piRNA). Beside small RNAs, another class of ncRNA called long intergenic non-coding RNA (lincRNA) which is longer than 200 nucleotides (nt) and lack open-reading frame have been shown to be involved in host-virus interaction especially in *Aedes* mosquitoes (Etebari et al., 2016). Therefore, ncRNAs are very important in many biological processes, and they have been shown to intimately involve in viral infection.

Dengue virus serotype 2 (DENV2) has been the center of attention in studies involving DENV, despite the fact that other serotypes are similarly as important as serotype 2. In this study, dengue virus serotype 1 (DENV1) was chosen, aiming to observe the effect on ncRNAs in *Ae.* albopictus cell (C6/36) upon infection. In this study, profiles of miRNA, piRNA and lincRNA following DENV1 infection were investigated. siRNA was not studied because C6/36 cell was shown to be unable to produce siRNA. This is due to the dysfunctional of *dcr2* gene which is responsible for producing siRNA in C6/36 cell (Brackney et al., 2010). Taken together, studying ncRNAs and their responses following DENV1 infection is crucial in understanding the mechanisms underlying host immunity, viral pathogenesis, and it may provide valuable insights in developing novel strategies in controlling vectors and viral replication.

1.1 Project aims and objectives

This study aims to comprehensively characterize the profiles of ncRNAs in *Ae. albopictus* cell (C6/36), and investigate transcriptomic changes at genomic scale following DENV1 infection. Specific objectives of this study are as follows:

1. To investigate the profiles of miRNA, piRNA, and lincRNA in *Ae. albopictus.*
2. To investigate the expression profiles of miRNA, piRNA, and lincRNA in C6/36 cells following DENV1 infection.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of non-coding RNA

Non-coding RNAs (ncRNAs) are essential in regulating various biological mechanisms inside cells, including cellular transcription, translation, and RNA processing ((Lai 2002; Lewis et al., 2003; Etebari et al., 2016). While there are many ncRNAs, such as small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), this study will focus on two categories of ncRNAs – small RNAs and lincRNAs. Small RNAs have been shown to mediate gene expression in eukaryotes at epigenetic and post-transcriptional levels (Mallory & Vaucheret, 2010). Small RNAs in metazoa can be categorized into three distinct groups based on their biogenesis and mechanism of action. The three categories are miRNA, siRNA, and piRNA (Lai 2002; Lewis et al., 2003; Bartel 2009; Siomi et al., 2011).

2.2 miRNA

miRNAs, around 22 nucleotide (nt) in length, function in the regulation of gene expression in both animals and plants by targeting cognate messenger RNAs (mRNAs) via imperfect base-pairing, resulting in either mRNA cleavage or translational repression (Mallory & Vaucheret, 2010). These two modes of actions (mRNA cleavage and translational repression) will subsequently silence the expression of certain genes. miRNA was shown to base-pair with target mRNA within its ‘seed’ sequences - typically around nucleotide 2 to 8 (Bartel 2009). Recent studies in humans and *Drosophila melanogaster* (*D. melanogaster*), however, suggest that the real seed sequence within miRNA is strikingly much smaller, which is only three nucleotides (Herzog & Ameres 2015). miRNAs have been implicated in many biological processes such as development, immunity, pathogenesis, and infection (Bartel 2009; Gottwein & Cullen, 2010).

miRNAs are produced from specific loci in the genome that encode transcripts containing hairpin loop domains, known as primary miRNAs (pri-miRNAs). Biogenesis of miRNAs begin with the transcription of pri-miRNA by RNA polymerase II (Pol II). These nascent transcripts of pri-miRNAs have stem loop domains, and undergo modification such as 7-methyl-guanosine cap, and a poly-A tail (Kim 2005). Then, pri-miRNAs are processed by the microprocessor complex, which is composed of an enzyme Drosha with its partner, Pasha (DGCR8 in the vertebrates), resulting in 60-70 nt-long RNA hairpins known as precursor miRNAs. These precursor miRNAs are then translocated into the cytoplasm by Exportin5 (Bartel 2004).

In the cytoplasm, precursor miRNA undergoes cleavage mediated by Dicer-1 and and its protein partner, Loquacious-PB (Loq-PB), to generate small RNA duplex. After unwinding the duplex, one strand is preferentially incorporated into Argonaute-1 (Ago1), while the other strand (passenger strand) is degraded (Kim 2005; Ender et al., 2008) . Once the selected strand (mature strand) is loaded into Ago1 to form RNA-induced silencing complex (RISC), miRNA can execute its function to post-transcriptionally silence gene expression (Aleman et al., 2007; Azlan et al., 2016) . An outline of miRNA biogenesis pathway is illustrated in Figure 2.1.

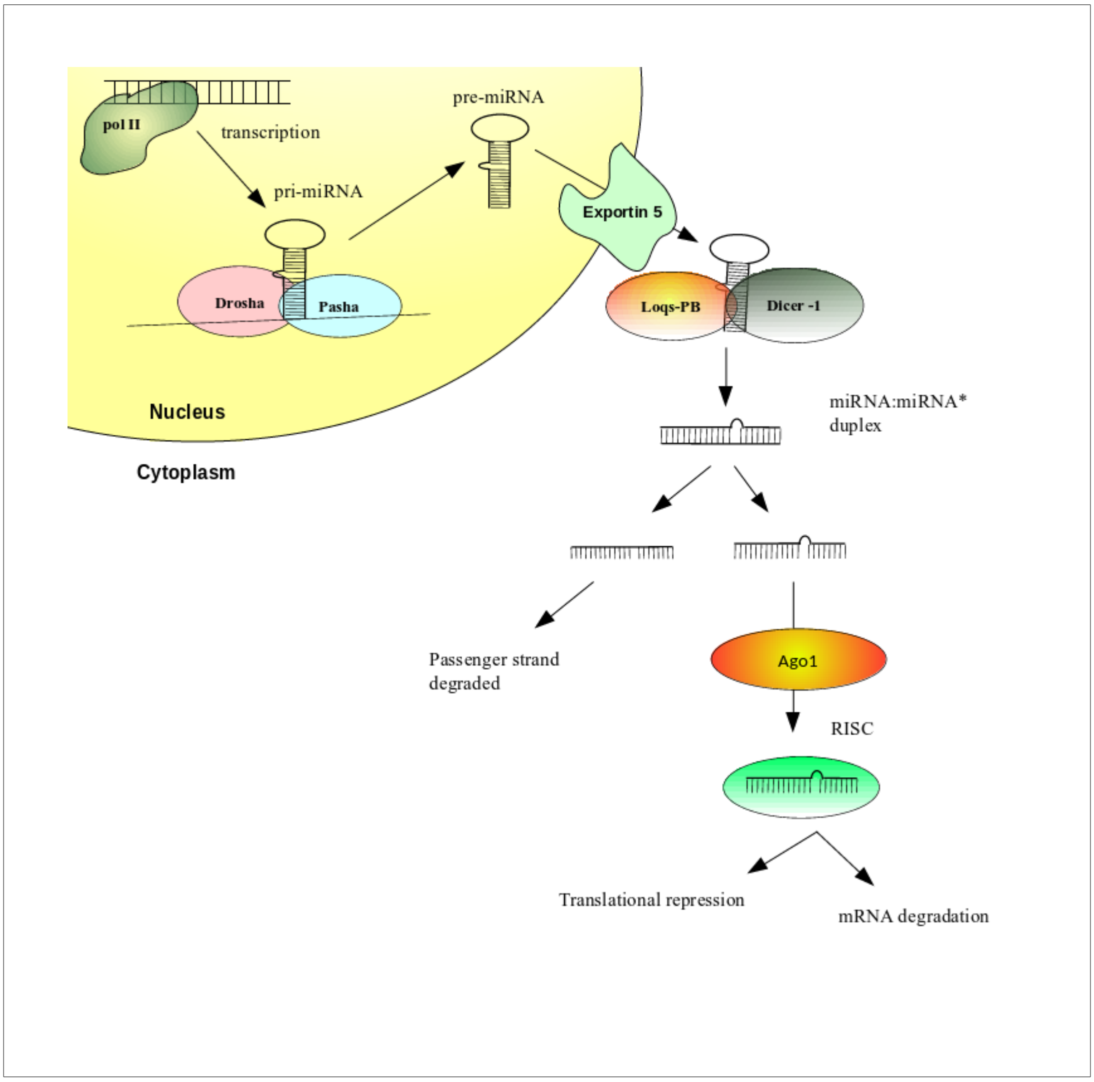

Figure 2.1 Biogenesis of miRNA. The illustration depicted here is based on miRNA biogenesis in *D. melanogaster.* (Adapted from Azlan et al., 2016)

2.3 siRNA

siRNAs are around 21 to 24 nt RNAs in length that originate from long double-stranded RNA (dsRNA) or hairpins, both of which can be encoded endogenously in the genome or can be exogenously introduced into the cells. Originally, siRNA was found to be involved during transgene and exogenous virus silencing in plants (Mello & Conte, 2004). Later, it was found that transposable elements (TE), centromeres, and repeats sequences are among the endogenous sources of siRNAs (Lippman & Martienssen, 2004). Thereafter, other endogenous sources of siRNA are discovered, namely sense-antisense pairs mRNA, pseudogene-derived antisense transcripts, and RNA hairpins (Allen et al., 2005; Golden et al., 2008). Therefore, it has become apparent that foreign RNAs are not the sole sources of siRNAs, but host genome can give rise to siRNAs as well.

In *D. melanogaster*, siRNA is cleaved from long dsRNA into siRNA duplex by the action of Dicer-2 and Loquacious-PD (Loq-PD). The duplex is then unwound and the selected strand is loaded into Argonaute-2 (Ago2) to form RNAi-silencing complex (Matranga et al., 2005; Tomari & Zamore 2005). Similar to miRNA pathway, RISC assembly is required for siRNA to execute its function. However, in contrast to miRNA, siRNA represses its target by annealing to its target mRNA via complete complementarity. resulting in mRNA degradation (Carthew & Sontheimer, 2009). An outline of siRNA biogenesis pathway is illustrated in Figure 2.2.

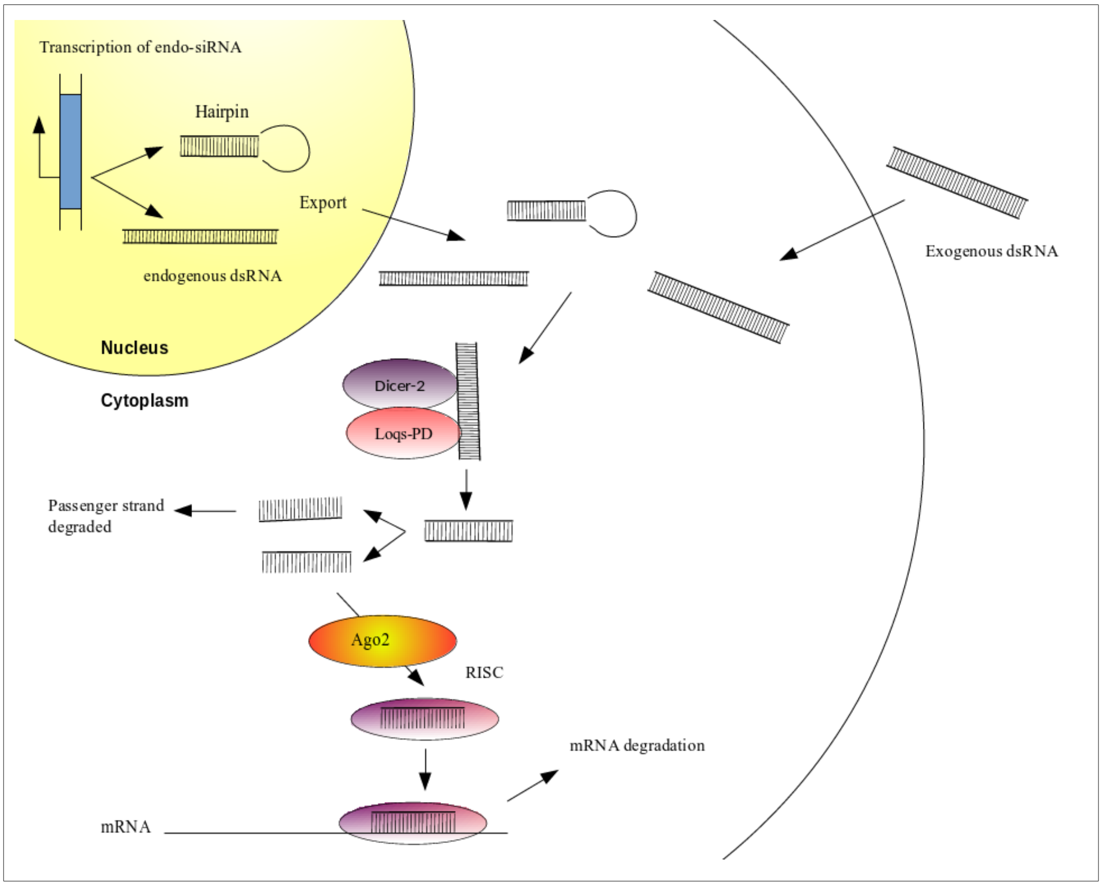

Figure 2.2 Biogenesis of siRNA. The illustration depicted here is based on siRNA biogenesis in *D. melanogaster* (Adapted from Azlan et al., 2016)

2.4 piRNA

piRNAs (24-35 nt in length) are a distinct category of small non-coding RNAs, and one of their canonical tasks is to control the activity of transposition and transcription of transposable element (TE); thereby, ensuring the inheritance of genomic information from one generation to another unscathed (Moazed 2009; Siomi et al 2011). The piRNA pathway regulates transposon activity at both transcriptional and post-trasncriptional level. Due to their restricted expression in the animal germ cells and strikingly diverse sequence identity, piRNAs have been the most enigmatic class of small non-coding RNAs (Siomi et al., 2011).

Similar to miRNAs and siRNAs, piRNAs cannot work in isolation because they require to form RISC in order execute their function. piRNAs require PIWI proteins (one of the subclades of the AGO family) to form RISC. In *D. melanogaster*, there are three PIWI proteins that participate in PIWI/piRNA pathways, namely, Piwi, Aubergine (Aub) and Argonaute-3 (Ago3), while in *Mus musculus* (*M. musculus*), PIWI proteins involved are MIWI2 and MILI (Siomi et al., 2011). Unlike Ago1 and Ago2 (AGO clade proteins), PIWI proteins in both mammal and insect do not display ubiquitous expression pattern, making it harder to dissect the mechanistic details of their functions (Brennecke et al., 2007).

Extensive investigations on the Piwi/piRNA pathway have improved our understanding on the relationship between this pathway and TEs, and its functions in development (Siomi et al., 2011; Handler et al., 2013). Mutations in Piwi/piRNA pathway components resulted in significantly elevated transcription of transposon, leading to higher transposition of transposons in the genome. Mobilization of TEs can lead to mutation; thus, increasing genetic variation within species (Klenov et al., 2007). In line with their function in silencing TE expression, all members of PIWI protein family are loaded with piRNAs that are complementary to the transposon transcripts, and are able to recognize and cleave their target TE mRNAs (Brennecke et al., 2007; Gunawardane et al., 2007).

2.4.1 piRNA biogenesis

Studies of piRNAs and their protein partners, PIWI proteins, in mice and flies have led to the proposal of two piRNA biogenesis pathways: primary pathway and ping-pong amplification cycle (Brennecke et al., 2007; Gunawardane et al., 2007). The primary biogenesis pathway is thought to contribute to the initial population of piRNAs, and the ping-pong amplification cycle, then amplifies the piRNA pool that targets active TE. The two pathways work together to elicit effective defense against active transposons. Studies in multiple organisms show that these two biogenesis pathways are highly conserved in numerous animal species such as frogs (*Xenopus laevis*), zebrafish (*Danio rerio*), silkworms (*Bombyx mori*), and *Aedes* mosquitoes (Houwing et al., 2008; Kawaoka et al., 2009; Akbari et al., 2013).

2.4.2 The primary piRNA biogenesis pathway

Extensive analysis of piRNA revealed that, while the number of miRNA species is within hundreds, piRNAs can be made up of thousands or millions of individual non-identical sequences. Despite their extremely high sequence diversity, most piRNAs can be mapped to few small regions in the genome known as piRNA clusters. Furthermore, piRNAs exhibit strong bias for uridine (U) at their 5' ends (Aravin et al., 2006; Girard et al., 2006; Aravin et al., 2007; Brennecke et al., 2007). The size of individual piRNA cluster ranges from one to more than 200 kilobases. Furthermore, it was shown that transcription of piRNAs within the piRNA clusters can occur in both genomic strands, and these clusters are called bidirectional clusters (Aravin et al., 2008; Brennecke et al., 2007).

In *D. melanogaster*, primary piRNA biogenesis pathway starts with the transcription of piRNA precursors (long single-stranded RNAs) from piRNA clusters (Girard et al., 2006; Brennecke et al., 2007). Long single-stranded precursor RNAs involved in the primary biogenesis pathway are usually antisense to the transposon mRNAs (Aravin et al., 2007; Brennecke et al., 2007; Aravin et al., 2008). These precursors are then cleaved by a nuclease known as zucchini, defining the 5'ends of the primary piRNAs. The primary piRNAs undergo processing steps, and the mature piRNAs are subsequently loaded into Piwi or Aub to form RISC (Brennecke et al., 2007; Malone et al., 2009). The piRNA RISC (piRISC) produced via this pathway can now target TE transcript for silencing. The knowledge of the mechanistic details on the primary piRNAs processing steps remains obscure.

2.4.3 The ping-pong amplification

In *D. melanogaster*, piRNAs loaded into Piwi and Aub in the primary biogenesis pathway are mostly antisense to the TE mRNAs and display strong bias of U at their 5’ends (Brennecke et al., 2007; Gunawardane et al., 2007). Meanwhile, piRNAs associated with Ago3, a key player in ping-pong cycle, do not have 5’U bias. Instead, they display a preference of adenine (A) at their tenth nucleotide from the 5’ end. It was discovered that huge fractions of piRNAs bound to Aub and Ago3 can form complementary base pairing at exactly their first 10 nt (Aravin et al., 2007; Brennecke et al., 2007). The ability to form such pair is the basis of the ping-pong amplification cycle.

Ping-pong amplification cycle begins with the transcription of piRNA precursors that are both sense and antisense to their target mRNAs. Similar to the primary biogenesis pathway, these long single-stranded precursors are processed by unknown mechanism, and are then loaded into Aub and Ago3. In *D. melanogaster,* Piwi does not participate in the ping-pong amplification cycle (Brennecke et al., 2007; Azlan et al., 2016). While Aub-bound piRNAs are mostly antisense to their targets, Ago3-bound piRNAs, on the other hand, are in sense orientation. Aub bound to antisense piRNA cuts long piRNA precursors in the sense strand, and the resulting shorter RNAs will then be loaded into Ago3. Ago3 associated with sense piRNA cleaves piRNA precursors in the antisense strand, generating the antisense piRNAs that will be eventually bound to Aub. The whole process repeats itself continuously, resulting in the amplification of piRNA species (Brennecke et al., 2007; Azlan et al., 2016) . An outline of piRNA biogenesis pathway is illustrated in Figure 2.3.

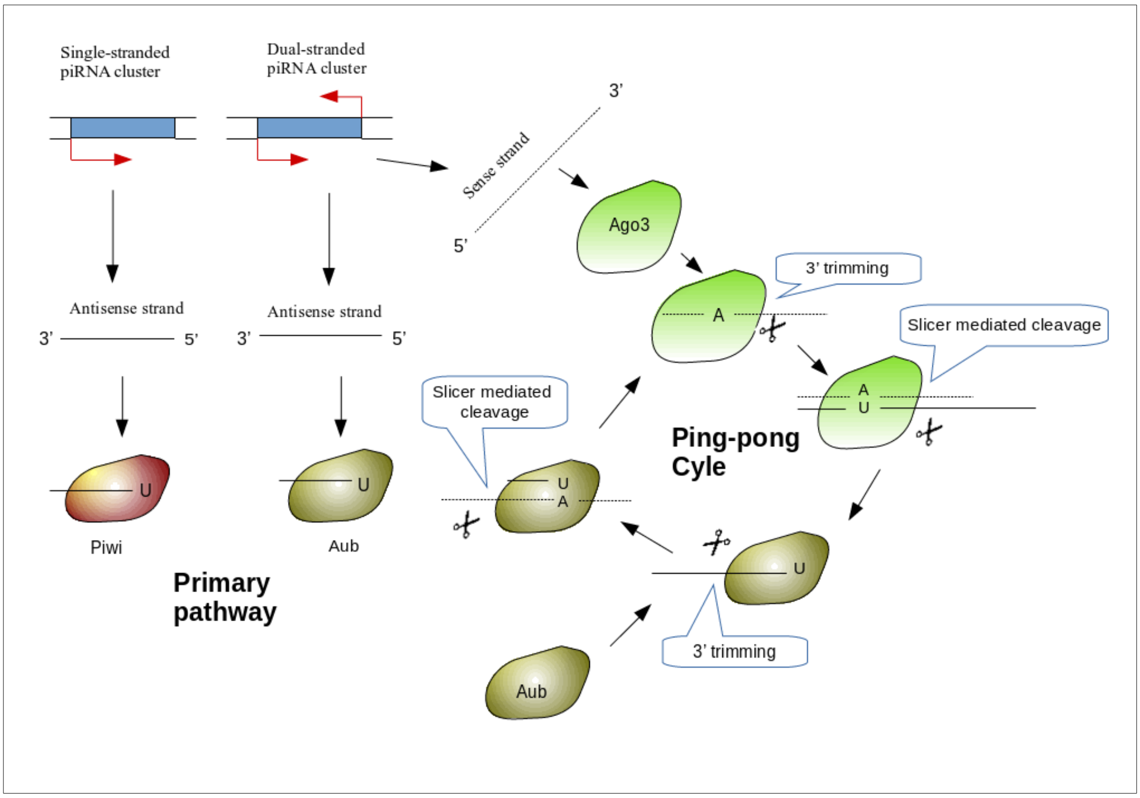

Figure 2.3 Biogenesis of piRNA. The illustration depicted here is based on piRNA biogenesis *D. melanogaster*. (Adapted from Azlan et al., 2016).

2.5 lincRNA

Genome-wide transcriptome experiments have revealed that significant transcriptional activity occurs at the regions between protein-coding genes (intergenic regions) in mammalian genomes (Carnici et al., 2005; Cloonan et al., 2008; Guttman et al., 2010). Transcription within these regions result in long RNA (more than 200 bp) without any capability to code for functional proteins. The discovery of *Xist*, a lincRNA which is essential for X-inactivation in mammalian chromosome (Borsani et al., 1991; Brown et al., 1992), revolutionize the field of lincRNA. Thereafter, thousands of lincRNAs were discovered in other vertebrates and invertebrates (Kim et al., 2010; Ulitsky et al., 2011; Young et al., 2012).

In contrast to most mRNAs, lincRNAs usually exhibit low expression level, many of them do not have significant sequence conservation, and their molecular functions remain largely elusive. Due to these factors, there are considerable debate regarding their biological significance, mechanism of their biogenesis, and mechanism of actions (Guttman et al., 2010; Cabili et al., 2011; Ulitsky et al., 2011; Ulitsky & Bartel, 2013; Young et al., 2012). Although the functions of most lincRNAs are largely unknown, some are shown to regulate gene expression, and play many biological roles inside cells (Ponting et al., 2009).

Several lincRNAs were reported to regulate gene transcription. For example, *Malat1* was shown to post-transcriptionally regulate genes involved in synapses (Bernard et al., 2010). *HOTAIR*, a lincRNA of 2.2 kb in length, represses the transcription of *HOX* genes by recruiting the polycomb complex, resulting in the modification of chromatin state of the genes (Tsai et al., 2010). Beside gene regulation, another potential function of lincRNAs is to serve as precursors for the generation of endogenous small RNAs. For example, in mammals, *BIC*, a proto-oncogene non-coding RNA was later revised and renamed as the precursor miRNA of miR-155 (Lagos-Quintana et al., 2002).

Although lincRNAs lack coding potential, similar to coding mRNAs, they are the products of Pol II, and they undergo polyadenylation, capping and alternative splicing (Ulitsky & Bartel 2013). Due to their mRNA-like features, lincRNAs are usually represented in RNA sequencing (RNA-seq) datasets. Next-generation sequencing allows quick genome-wide identification of lincRNAs including the lowly expressed transcripts, and this technology is independent on complete genome and gene annotation, making it an ideal strategy to detect novel lincRNAs (Ulitsky & Bartel 2013).

2.6 Dengue virus

The alarming increase of dengue fever outbreak in Malaysia and other countries require immediate attention. According to the Ministry of Health Malaysia, there were approximately 20,000 dengue fever cases reported in 2016, including 43 dengue-related deaths (Herriman, 2016). The main cause of this dengue fever is none other than dengue virus, which is transmitted by *Aedes* mosquitoes (*Ae. aegypti* and *Ae. albopictus*). DENV is a single-stranded, positive-strand RNA virus that belongs to the family of *Flaviviridae* (Kuhn et al., 2002). DENV genome is approximately 10.6 kb, and it encodes a single polypeptide composed of three structural and seven non-structural proteins. The three structural proteins are capsid (C), envelope glycoprotein (E), and membrane precursor (prM). Meanwhile, non-structural proteins include NS1A, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Kuhn et al., 2002).

There are four serotypes of dengue virus (DENV1-4) that share around 65-70% homology among each other. The serotypes were categorized based on the variation of antigens present on the viral particles (Kuhn et al., 2002). Despite these variations, infection with each of the dengue serotypes leads to similar range of clinical symptoms (Pang & Loh, 2016). All four serotypes were found to be present and circulated throughout Malaysia (Pang & Loh, 2016).

It was reported that NS5, which contains enzymatic activities required for capping and replication of the viral RNA genome, functions differently across all four serotypes (Hannemann et al., 2013). It was reported that NS5 of DENV2 and DENV3 accumulate in the nucleus during infection. However, NS5 of DENV1 and DENV2 were shown to be predominantly localized to the cytoplasm due to the lack of functional nuclear localization sequence (Hannemann et al., 2013). It was hypothesized that this differences may have auxiliary function in the life cycle of specific DENV serotypes (Hannemann et al., 2013).

2.6.1 Life cycle of dengue virus

Life cycle of DENV begins when the virus binds to the various group of cell surface receptors of the host cells. Viral particle enters the cell via a process known as clathrin-mediated endocytosis. Viral particle binds to the cell surface receptor forming a complex, which will later be captured by clathrin-coated pit. This results in the internalization of viral particle into the cytoplasm (van der Schaar et al., 2008). Upon entry, the vesicles are translocated through endosomes. Acidification inside endosomal vesicles causes the virion to undergo conformational changes, resulting in the trimerization of the E protein. As a result, single-stranded DENV RNA is released into the cytosol (Green et al., 2004; van der Schaar et al., 2008).

Host ribosomes then translate the RNA genome into a single polyprotein, and this process occurs in rough endoplasmic reticulum (ER). The polyprotein is then cleaved by viral and host proteases (Green et al., 2004). Once the viral replication complex is formed, translation of the RNA genome stops, while the synthesis of viral RNA begins. Nascent RNAs are then packaged by capsid forming a nucleocapsid, and this newly formed nucleocapsid buds into the ER lumen, initiating virus assembly. Immature virions are then translocated into the trans-Golgi network, where acidification process takes place, triggering the conformational changes of the virion.

As a result, furin cleavage sites are exposed, enabling the host protease furin to cleave the virion prM protein (van der Schaar et al., 2008). Unlike M protein which is always associated with the virion, pr protein only attached to the virion until the virion is outside the cells. Once the pr proteins are completely detached from the virions, fully mature and infectious DENV particles are formed (van der Schaar et al., 2008).

The extent of prM protein cleavage dictates the level of DENV maturity and infectivity. Complete immature and non-infectious DENV has fully intact prM proteins, whereas, fully mature and infectious DENV has fully cleaved prM proteins. The intermediate forms also exist in which the prM proteins of the virions have not been completely cleaved (van der Schaar et al., 2008).

2.7 *Aedes albopictus*: an important vector of dengue virus

Ae. albopictus can be characterized as a small mosquito with white dorsal stripe and banded legs. Blood hosts for *Ae. albopictus* include humans and other mammals (Lambrechts et al., 2010). Few days after blood feeding, the female mosquitoes lay their egg. It takes around seven to nine days for the mosquito to develop from egg to adult. The life span for adult mosquitoes is around three weeks (Lambrechts et al., 2010). *Ae. albopictus* is a generalist that readily adapts to diverse environmental conditions in both tropical and temperate regions (Chen et al., 2015). Due to its high adaptability to different environment, *Ae. albopictus* is highly spread around the globe.

Similar to *Ae. aegypti*, upon infection, DENV accumulates and replicates in salivary gland. Salivary gland of the mosquito acts as a reservoir for DENV (Lambrechts et al., 2010). Microscopic studies of DENV2-infected *Ae. albopictus* showed that substantial viral replication occurred in the cells of salivary glands and nervous tissue. Less viral particles were found in the midgut, epidermal cells, fat body and foregut (Sriurairatna & Bhamarapravati, 1977).

2.7.1 ncRNAs in mosquitoes and their relationship with viruses

Several studies have attempted to dissect the mechanism of host-pathogens interaction in *Aedes* mosquitoes over the past few years. Many studies reported that virus infection can consequently modulate immune response, not only through regulating protein-coding gene expression, but also by rewiring the expression landscape of host miRNAs. In mosquitoes, miRNA levels have been reported to be altered in response to infection with chikungunya virus , West Nile virus (WNV), and DENV (Campbell et al., 2014). For instance, a study demonstrated that in *Ae. aegypti* cell lines, infectivity of DENV2 infection was enhanced by miR-375 (Hussain et al., 2011). It was reported that *Anopheles gambiae* (*An. gambiae*) was found to be more susceptible to Plasmodium infection when Dicer1 and Ago1 were knocked down (Winter et al., 2007). Meanwhile, in *Ae. albopictus*, one study reported that following DENV2 infection, most miRNAs were downregulated (Liu et al., 2015).

Recently, there were many studies that provided convincing evidence that Piwi/piRNA pathway is implicated in virus infection including DENV. Unlike miRNAs, most studies put greater attention to virus-derived piRNAs (vpiRNA), rather than host piRNAs upon viral infection (Miesen et al., 2015; Miesen et al., 2016). This is due to the observation that, unlike *D. melanogaster*, *Aedes* mosquitoes are capable of producing virus-derived piRNAs (Petit et al., 2016). This finding indicates that, similar to siRNA pathway, Piwi/piRNA pathway is capable of participating in small-RNA mediated immunity against exogenous viruses (Miesen et al., 2015; Miesen et al., 2016). In fact, knock down of Piwi4 in *Ae. aegypti* cells (Aag2) led to higher replication of Semliki Forest virus (Schnettler et al., 2013).

Most of the host-virus interaction studies focus on the protein-coding genes. However, several studies in human and mouse reported that lincRNAs play roles in host-virus interaction and immunity (Collier et al., 2012). Compared to miRNA, piRNA and siRNA, association of lincRNAs with virus infection in *Aedes* mosquitoes is the least studied. Only one study reported the potential involvement of *Ae. aegypti* lincRNA in DENV infection, while none of such study has been done in *Ae. albopictus* (Etebari et al., 2016).

A total of 3,842 lincRNAs were identified in *Ae. aegypti*, and these newly identified lincRNA showed differential expression following DENV2 infection. dsRNA knockdown of lincRNA_1317, one of the newly identified lincRNA, led to the increase of viral replication in *Ae. aegypti* cells (Etebari et al., 2016). Although the exact mechanistic details of lincRNA interaction with DENV remain elusive, the data from this study will facilitate future experiments on the functions of lincRNAs in *Aedes* mosquitoes.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell culture and virus

3.1.1 Cell culture

Two cell lines were used in this study – *Ae. albopictus* whole larval-derived cells (C6/36, ATCC: CRL-1660) and African green monkey kidney cells (Vero E6, ATCC:CRL-1586). C6/36 cells were cultured in Leibovitz's L-15 medium (Gibco, 41300039), supplemented with 10% Fetal Bovine Serum (FBS, Gibco, 10270) and 10% Tryptose Phosphate Broth (TPB) solution (Sigma, T9157). C6/36 cells were maintained at 27 ºC without CO_2. Vero E6 cells were cultured at 37 ºC in Dulbecco's modified Eagles Medium (DMEM, Gibco, 11995065) supplemented with 10% FBS (Gibco, 10270), and 5% $CO_{2.}$ DENV1, Hawaiian strain, was propagated in C6/36 cells and titered using Vero E6 cells. DENV1 Hawaiian strain was used in this study because it was an established lab strain and cell-culture adaptable. Titration of DENV1 was done in Vero E6 cells because of clearer cytophatic effects (CPE). DENV1 used in this study was a gift from Assoc. Prof. Dr. David Perera, Universiti Malaysia Sarawak.