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Chapter 9

ROLES OF *LET-7* FAMILY MIRNAS IN DEVELOPMENT AND DIFFERENTIATION

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1. ABSTRACT

MiRNAs (miRNAs) are a class of 18 to 25-nucleotide, short, non-coding RNAs that regulate gene expression post-transcriptionally. The lethal-7 (*Let-7*) gene is one of the founding members of the first two miRNA families identified in *C. elegans*, and the first miRNA known to be found in humans. *Let-7* family miRNAs are expressed from early development through maturity in both invertebrates and vertebrates. The majority of miRNAs are not essential for viability or development in *C. elegans*. However, *Let-7* family miRNAs are critical regulators of development that control both early and late developmental timing decisions, and were elucidated by extensive genetic and molecular analysis using *C. elegans* mutants as well as *Drosophila* mutants. In contrast to invertebrates, our understanding of the functional roles of *Let-7* family miRNAs in vertebrate development is still limited because there are many redundant *Let-7* family miRNAs, and thus experiments to clarify *in vivo* functions of *Let-7* family miRNAs using *Let-7* family miRNA mutants in vertebrates are practically difficult. However, indirect but significant *in vivo* evidence has suggested that *Let-7* is essential for gene regulation in mouse development. In addition, ectopic expression of *Let-7* in zebrafish embryos leads to early down-regulation of endogenous *Let-7* targets, resulting in severe embryonic developmental abnormalities, and suggesting that *Let-7* plays a significant role in zebrafish development. Although studies on vertebrate *Let-7* family miRNAs are still in the early stages, these studies have provided us with evidence of important biological roles for *Let-7*. Based on past and recent findings, we review the expression patterns, biological roles, and functional mechanisms of *Let-7* family miRNAs in normal development of both invertebrates and vertebrates. In addition, we describe *in vitro* analysis on the molecular roles of *Let-7* family miRNAs underlying cellular

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differentiation. Future studies on *Let-7* family miRNAs will provide us with not only important clues to understanding cell differentiation in development, but also useful tools for diagnosis and/or therapies in the medical field.

2. INTRODUCTION

Development requires an intricate machinery of cell proliferation, differentiation and morphogenesis. Understanding how developmental events are coordinated in developmental schedule needs identification of the genes that regulate developmental timing. The heterochrony, a term that describes changes in the relative timing of developmental event between a mutant and wild-type organism or between distinct species (Pasquinelli and Ruvkun, 2002). Mutations in heterochronic genes cause misregulations of developmental events. In 2000, *Let-7* was identified as a heterochronic switch gene from the study of a genetic screen for mutations that suppress the synthetic sterile phenotype of strain bearing LIN14 (n179) and egl-35 (n694) in *Caenorhabditis elegans* (Reinhart et al., 2000). The adult mutants die by bursting their vulva, therefore named lethal-7 (*Let-7*). Following identification of lin-4 as the first small temporal RNA (stRNA), *Let-7* next turned out to encode stRNA. Suppression of target mRNA by stRNAs was believed to be specific event to *C. elegans*. However, it was also found that the *Let-7* sequence is well conserved across species and *Let-7* is expressed in various human tissues (Pasquinelli et al., 2000). Furthermore, the stRNA synthesis mechanism was also common across species, which was discovered from studies on Dicer (an RNA-processing enzyme containing RNase III activity) knockout (KO) in *Drosophila melanogaster* and targeted disruption of Dicer mRNA in human cultured cell line, HeLa cells. These observations showed that Dicer plays a central role in stRNA generation as like in siRNA synthesis (Hutvágner et al., 2001). Nowadays, *Let-7* is known as a miRNA.

MiRNAs are encoded on genomes, and transcribed by RNA polymerase II and III like long RNAs and processed by Drosha (an RNA-processing enzyme containing RNase III activity) and DGCR8 (known as Pasha) in the nucleus (Lee et al., 2003; Han et al., 2004, Wang et al., 2007; Carthew and Sontheimer, 2009). The pre-miRNAs are further cleaved by Dicer to generate mature miRNAs in the cytoplasm. MiRNAs are incorporated into a multi protein complex, known as the RNA induced silencing complex (RISC). The RISC post-transcriptionally regulates mRNA expression by translational inhibition and/or degradation of target mRNAs through the interaction between target mRNAs and its miRNA (Carthew and Sontheimer, 2009). The base paring much between a stretch of 6-8 nucleotides at the 5' end of miRNA, called a "seed" region, and 3'UTR of mRNA is important for targeting of mRNA by miRNA (Lewis et al., 2005; Grimson et al., 2007). It has been shown that miRNAs which have similar seed sequence target same genes. MiRNAs which contain a similar seed region are categorized as family miRNA. The human *Let-7* miRNA families consist of 13 discrete genetic loci that resolve into 10 unique mature miRNA sequences, *Let-7*a, *Let-7*b, *Let-7*c, *Let-7*d, *Let-7*e, *Let-7*f, *Let-7*g, *Let-7*i, miR-98, and miR-202. Only the human miR-202, not the other organisms, is classified as a *Let-7* family miRNA. The *C. elegans* genome encodes 9 copies of *Let-7* family miRNAs, *Let-7*, miR-48, miR-84, miR-241, miR-265, miR-793, miR-794, miR-795, and miR-1821. Among them, *Let-7*, miR-48, miR-84, and miR-241 are experimentally detectable *Let-7* family miRNAs (Rouse and Slack, 2008). Interestingly from *Let-7* functional analysis, developmental timing and differentiation have been focused not

only in invertebrates, but also in vertebrates. Despite *Let-7* was identified as the second miRNA after lin-4, its high evolutional conservation through animal species from *C. elegans* to human led to findings that miRNAs are general regulators of gene expression in various species. Here we review advanced research in understanding the contribution of *Let-7* and *Let-7* family to development and differentiation in invertebrates and vertebrates.

3. ROLES OF *LET-7* IN DEVELOPMENT

1) Invertebrates

I) Let-7 and Let-7 Family MiRNA in Developmental Timing in C. Elegans

(A) Discovery and Function of *Let-7*

C. elegans has been serving as a favorable model organism to study cellular and molecular basis underlying various developmental events, because *C. elegans* has a limited number of cells and is genetically well defined. The life cycle of *C. elegans* is 3.5 days (at 20 °C), it develops from egg to adult though four larval stages (L1-L4). By microscopic observation, cell linage in *C. elegans* is completely understood. Therefore, *C. elegans* is an excellent model to study the mechanisms of heterochrony in multicellular organisms (Sulston and Horvitz, 1977). Thus, it is not surprising that lin-4 and *Let-7*, the two founding members of the miRNA family were first identified in genetical analysis of genes regulating developmental timing, or heterochrony, in *C. elegans* (Reinhart et al., 2000; Lee et al., 1993; Moss et al., 2007). *Let-7* was initially found as a stRNA, following the identification of the first found stRNA, lin-4. At the end of larval development *Let-7* is dramatically upregulated with a significant reduction of key heterochronic proteins that promote larval-specific cell fates, thus ensuring the developmental transition into adulthood (Rougvie, 2005; Moss, 2007). In post-hatched larval stages, L1 to L4, hypodermal blast cells, known as seam cells, undergo cell division at each larval stage, and then exit from cell-cycle and differentiate, accompanying the expression of LIN28 protein, a zinc-finger transcription factor, at L4 to adult stages (Ambros and Horvitz, 1984; Rougvie and Ambros, 1995). *Let-7* mutants exhibit heterochronic abnormalities in the seam cell linage. Partial defect of *Let-7* activity results in retarded phenotypes. Their seam cells repeat L4 to adult cell division pattern without cell-cycle exit and undergo abnormal L5 to adult cell-cycle, then the cells exit from cell-cycle, showing that the defect of *Let-7* function retards progression from the juvenile state (Figure 1). In contrast, more complete defect of activity results in lethality by bursting through the vulva at the larval-to-adult transition (Reinhart et al., 2000). These abnormalities seem to arise from overexpression of *Let-7* target genes because it can be partially rescued by knocking down individual *Let-7* target genes (Slack et al., 2000; Abrahante et al., 2003). Further, a *Let-7* overexpression study showed increased dosage of *Let-7* promotes precociously cell-cycle exit and terminal differentiation of seam cells after L3 to L4 cell division, indicating that precocious *Let-7* expression results in premature differentiation of adult cell fates (Figure 1).

(B) *Let-7* Family MiRNA

In *C. elegans* there are other miRNAs which contain similar seed sequences with *Let-7*, including miR-48, miR-84, miR-241, miR-265, miR-793, miR-794, miR-795, and miR1821 (Rough and Slack, 2008). There has been no report about expression patterns and functions of miR-793, miR-794, miR-795, and miR-1821. Similar to *Let-7*, miR-48, miR-84, and miR-241 functions as heterochronic genes to regulate temporal patterning at the transition from L2 to L3 stage (Abbott et al., 2005).

While expression of *Let-7* RNA is detected at about L3 stage, the *Let-7* family miRNAs, miR-48 and miR-241 are observed at about L2 stage, and miR-84 appears from L1, the maximal expression of all *Let-7* family members are L4 (Esquela-Kerscher et al., 2005). Interestingly, single mutants of miR-48 and double mutants of miR-48 and miR-84 undergo extra adult molt. Additionally, miR-48 and miR-241 double mutants and miR-48, miR-241, and miR-84 triple mutants show reiteration of the L2 stage. Further, precocious accumulation of miR-48 accelerates developmental timing of seam cells and the vulva (Abbott et al., 2005; Li et al., 2005). These observations indicate that developmental timing and cell division required *Let-7* family miRNA function (Figure 1).

The loss of function (lf) mutant of *Let-7* causes a reiteration of the fourth larval (L4) cell fate at the adult stage. The gain of function (gf) mutant of *Let-7* exhibits precociously cell-cycle exit and terminal differentiation after the L3 to L4 molt. The miR-84 (lf) or miR-241 (lf) single mutants have no abnormality, but miR-48 (lf) single mutants cultured at 15ºC and the miR-48 (lf); miR-84 double mutants exhibit supernumerary adult molt. The miR-48 (lf); miR241 (lf) double mutants, or the miR-48 (lf); miR-84 (lf); miR-241 (lf) triple mutants promote reiteration of the L2-prolifalative stage. The lin-4 (lf) mutants repeat the L1 cell division pattern. The LIN41 (lf) mutants exhibit precocious cell-cycle exit and maturation. The LIN28 (lf) mutants skip the L2 division pattern. TD, terminal differentiation. Circled arrows indicate repeating the same cell division pattern.

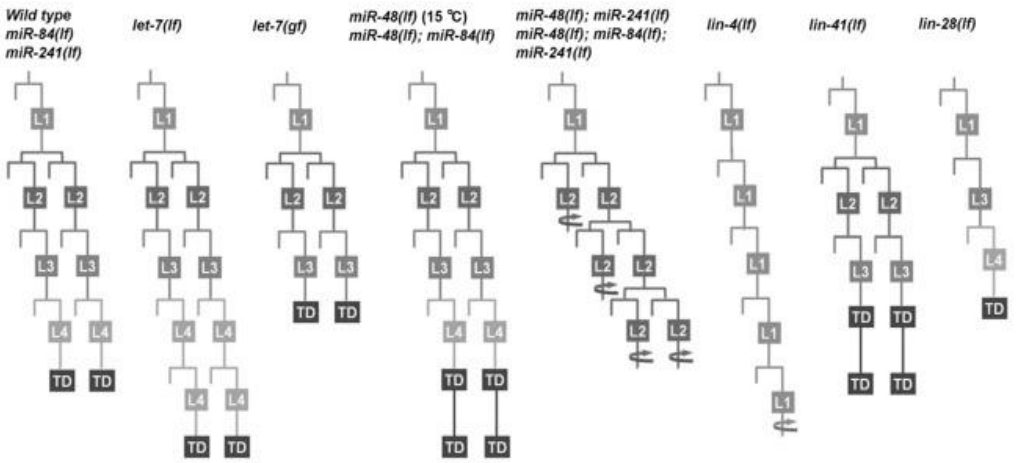

Figure 1. Schematic diagrams of cell lineage in *Let-7*-related *C. elegans* mutants of V1 to V4, and V6 seam cells.

(C) Regulation and Targets of *Let-7* MiRNA

By combination searching for base pairing match between *Let-7* and 3'UTR of heterochronic gene mRNAs and the genetic interaction analysis between *Let-7* and each heterochronic gene mutant, *Let-7* target mRNAs were identified to be LIN41, LIN14, LIN28, LIN42, and DAF12 (Reinhart et al., 2000). In fact, *Let-7* un-regulation at L4 stage down-regulates LIN41 gene, leading to up-regulation of LIN28 transcription factor. The conserved nuclear hormone receptors NHR23 and NHR25 regulate molting from L4 stage to adult. A *Let-7* paralog miR-84 acts synergistically with *Let-7* to promote terminal differentiation of the hypodermis and the cessation of molting in *C. elegans* through regulating NHR23 and NHR25 (Hayes et al., 2006).

Regarding biogenesis of miRNA, it has been demonstrated that RNA Polymerase II (Pol II) is primarily responsible for producing miRNA, while it should be noted that mature miRNAs can be produced from transgenic constructs driven by Pol II and Pol III-specific promoters (Lee et al., 2004; Zhou et al., 2005; Borchert et al., 2006; Zhou et al., 2008). Expression surge of primary *Let-7* (pri-*Let-7*) at L3 during development is critical for *Let-7* function as a heterochronic gene product. However, temporal regulation of *Let-7* transcription is one of the important issues that are still poorly understood. Regulatory region of *C. elegans Let-7* was investigated by transgenic analysis using an upstream sequence of *Let-7* miRNA, containing the putative promoter region, fused with gfp (Johnson et al., 2003). This sequence drove GFP expression to recapitulate the *Let-7* expression during *C. elegans* development. In this *Let-7* promoter sequence a short inverted repeat sequence was shown to be necessary and sufficient to drive *Let-7* expression pattern, however, binding transcription factors remain elusive. Genetic analysis showed that a nuclear receptor DAF12, functions immediately upstream of *Let-7*. However, DAF12 appears not to bind directly to the *Let-7* promoter and thus DAF12 regulation on *Let-7* is considered indirect. Recently, DAF12 with its steroid ligand was shown to directly activate promoters of miR-84 and miR-241, resulting in down-regulation of their target, a transcription factor hunch-back-like-1 (HBL1), allowing L2 to L3 transition (Bethke et al., 2009). DAF12 itself is a target of *Let-7* at later stages, suggesting that feedback loops play a role to regulate stage transitions (Grosshans et al., 2005). Notably, it was recently reported that HBL1 is responsible for inhibiting the transcription of *Let-7* in specific tissues until the L3, indicating that one important function of HBL1 in maintaining larval stage fates is inhibition of *Let-7* (Rouse and Slack, 2009). Thus, this study revealed *Let-7* as the target of the HBL1 transcription factor in *C. elegans* and suggest that a negative feedback loop mechanism for *Let-7* and HBL1 regulation constitute an important machinery to regulate *Let-7* temporal expression. The conserved nuclear hormone receptors NHR23 and NHR25 regulate molting from L4 stage to adult. A *Let-7* paralog miR-84 acts synergistically with *Let-7* to promote terminal differentiation of the hypodermis and the cessation of molting in *C. elegans* through regulating NHR23 and NHR25 (Hayes et al., 2006) (Figure 2).

In addition, interestingly it was found that let-60, encoding the *C. elegans* orthologous of the human oncogene RAS, is a direct target of *Let-7* and miR-84 miRNA *in vivo* (Johnson et al., 2005). Messenger RNAs of three types of human RAS family members, HRAS, KRAS, and NRAS contain multiple *Let-7* family miRNA targeting sites (Johnson et al. 2005). Although daf12 is a *C. elegans*-specific target of *Let-7*, other *Let-7* targets including LIN28, let-60 (RAS), and LIN41 (TRIM71) are conserved through evolution.

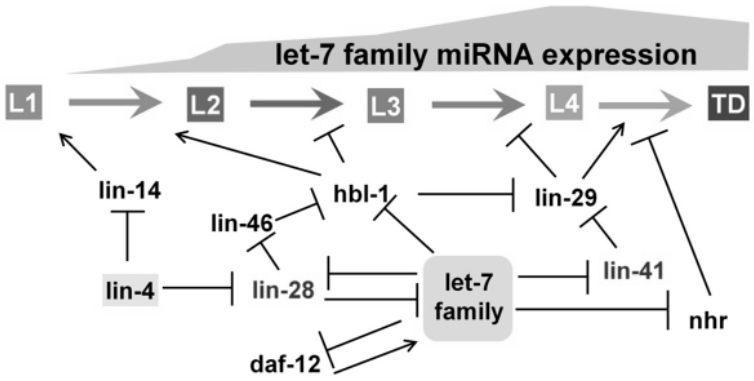

Figure 2. A model of heterochronic gene regulation regarding *Let-7* family miRNAs.

Let-7 family miRNAs are expressed from early L1 to terminal differentiated seam cells. The peak of *Let-7* expression is L4. After the L1 stage, individual seam cell lineage is regulated by *Let-7* family miRNAs through their target genes. Lin14 is a target of lin-4 at L1. Lin-4 is another heterochronic miRNA which expressed in L1 stage seam cells. Up-regulation of *Let-7* in the L4 results in down-regulation of LIN41, and consequently up-regulation of the transcription factor LIN28. Lin-46 functions immediately downstream of LIN28, affecting both the regulation of the heterochronic gene pathway. LIN28 and Lin41 are predicted to be important target genes also in vertebrates as well. HBL1 is responsible for inhibiting the transcription of *Let-7* in specific tissues until the L3. DAF12 is a *C. elegans*-specific target of *Let-7*.

(D) Modulation of *Let-7* Function

In addition to temporal regulators of *Let-7* co-factor is another regulator of *Let-7* function. A genetic modifier screen in *C. elegans* identified a ribosomal protein RPS14 as a cofactor of *Let-7* (Chan and Slack, 2009). RPS14 is able to modulate *Let-7* function in *C. elegans*. Reduction of RPS14 gene expression by RNAi suppressed the aberrant vulva and hypodermis development phenotypes of *Let-7* mutant animals and the mis-regulation of a reporter bearing the Lin41 3'UTR, a well established *Let-7* target. Thus, reduction of RPS14 activity leads to the elevation of *Let-7* function. The RPS14 protein co-immunoprecipitated with the nematode Argonaute homolog, ALG1, suggesting that RPS14, may interact with RISC and play roles in miRNA-mediated translational repression.

II) Functions of Let-7 in Drosophila Melanogaster

(A) Expression and Function of *Let-7*

Let-7 gene is not restricted to *C. elegans* but rather evolutionarily conserved throughout animal species up to human (Pasquinelli et al., 2000). In *Drosophila*, *Let-7* also plays an important role in controlling the juvenile-to-adult transition (Caygill and Johnston, 2008; Sokol et al., 2008). Although the potential family miRNAs of *Let-7*, miR-48 and miR-84, in *Drosophila* have been predicted to exist (Lau et al., 2001) and these miRNAs have been undetectable by Northern blots of RNAs from late third instar larvae through pupae (Bashirullah et al., 2003). Similar to *C. elegans Let-7*, it was observed that the expression of

Drosophila Let-7 also begins during the larval-to-adult transition. *Drosophila Let-7* expression is first detected about four hours before puparium formation, the same timing to the end of the third larval instar, and then highly accumulated during pupal development (Hutvágner et al., 2001; Bashirullah et al., 2003; Sempere et al., 2002). The *Drosophila Let-7* is processed from a common RNA precursor that contains two other conserved miRNAs, miR-100 and miR-125 (lin-4) (Bashirullah et al., 2003; Sempere et al., 2002; Sempere et al., 2003). All of these three miRNAs, called as *Let-7*-complex (*Let-7*-C), are coexpressed spatially and temporally. Two research groups investigated the function of *Drosophila Let-7* by generating *Let-7*-C knock out flies. Caygill and Johnston (2008) reported that *Drosophila* mutant lacking *Let-7* and miR-125 led to exhibit widespread phenotypes arising during metamorphosis. The mutant flies showed delay in maturation of abdominal neuromuscular junctions (NMJs). They attributed this phenotype to persistent expression of Abrupt, a *Let-7* target gene, in mutant muscle cells. In addition, they demonstrated that *Let-7* is critical for the appropriate timing of cell-cycle exit of developing wing cells, leading to a small wing phenotype. Sokol et al. (2008) observed that *Let-7*c transcripts are primarily expressed in the pupal and adult neuromusculature. The *Let-7*c mutant flies appear normal but displayed abnormal adult behavior such as severely reduced motility, flight, and fertility. They also found that *Let-7*c molecules are widely expressed in various tissues, including the central nervous system, motor neurons, and muscle cells. Consistent with this observation, the *Let-7*c mutant showed abnormal reorganization of neuromusculature. They found that the dorsal internal oblique muscles (DIOMs), which normally decay within 12 hrs of eclosion (Crossley, 1978; Kimura and Truman, 1990), remains in adult *Let-7*c mutants. It should be noted that they observed that CNS development occurs normally in *Let-7*c mutants.

Sokol et al. (2008) showed that *Let-7* miRNA alone is required for the *Let-7*c-dependent larval-to-adult remodeling of the abdominal neuromusculature through rescue experiment. Taken together, these two studies strongly suggested that *Let-7*c mutant phenotypes are heterochronic ones, because *Let-7*c mutant adults exhibited both juvenile characters such as immature neuromusculature as well as mature adults features such as normal external appearance.

(B) Targets of *Let-7* in *Drosophila Melanogaster*

From the study on *Let-7*c mutant flies, the roles of *Let-7*, as a larval-to-adult remodeling factor of the neuromuscular junction in the abdominal muscle and a cell-cycle exit inducer in the wing during metamorphosis were revealed. Caygill and Johnston (2008) found that the key function of *Let-7* is to suppress of Abrupt gene. Abrupt encodes a nuclear BTB-zinc finger regulatory protein and is broadly expressed during development and required for various functions, including regulating embryonic specificities of the neuromuscular connections, dendrite arborization in embryos (Hu et al., 1995; Li et al., 2004; Sugimura et al., 2004), and development of the fifth longitudinal vein of the wing (Cook et al., 2004). The 3'-untranslated region of the Abrupt mRNA contains at least five *Let-7*-binding sites and is regulated by *Let-7* expression in cultured cell assays (Burgler and Macdonald, 2005). Abrupt protein is expressed throughout the wind disc in late L3 flies (Cook et al., 2004), but its expression is lost early in the pupal stage (Caygill and Johnston, 2008). By using Gal4-mediated misexpression system *in vivo*, they demonstrated that misexpression of UAS-*Let-7* in L3 wing disc cells was sufficient to suppress Abrupt expression prematurely. Interestingly, Abrupt protein continues to be expressed in *Let-7*, miR-125 mutant in pupal stage wing discs.

They also showed that the persistent expression of Abrupt protein is due to loss of *Let-7* by using the Gal4-mediated misexpression system, indicating that *Let-7* is both necessary and sufficient for the appropriate temporal repression of Abrupt protein in wing discs. Furthermore, the delay in neuromuscular junction observed in *Let-7*, miR-125 mutant fly was suppressed by a partial loss of Abrupt function, showing that the persistent Abrupt expression in the abdominal muscles of *Let-7*, miR-124 mutants underlies the neuromuscular junction maturation defect. Thus, Abrupt was established as an important *in vivo* target of *Drosophila Let-7*. It should be noted that Abrupt is a target for *Drosophila Let-7*, but not a target for *Let-7* in *C. elegans* or mammals.

(C) Regulation of *Drosophila Let-7* Expression

In *Drosophila*, metamorphosis begins after the third instar larvae to pupae. The steroid hormone 20-hycroxyecdysone (ecdysone) regulates the metamorphosis timing from larval to adult stages. The *Drosophila Let-7* expression is induced in late third instar larvae in precise synchrony with ecdysone and ecdysone receptor (EcR) and highly expressed during pupal development (Pasquinelli et al., 2000; Hutvagner et al., 2001). The Broad-Complex (BR-C) gene functions at the top the hierarchy in the ecdysone pathway and plays an essential role to control expression of downstream target genes (Belyaeva et al., 1981; Zhimulev et al., 1982). BR-C encodes four isoforms of a zinc finger transcription factor. Sempere et al. (2003) examined whether *Let-7* expression is regulated by ecdysone in *Drosophila* using Northern blot analysis in mutant animal, organ cultures and S2 culture cells. They dissected late third instar larva to perform organ culture of imaginal discs, and incubated with or without ecdysone to measure *Let-7* expression. They found that ecdysone is actually required for *Let-7* expression during development. In addition, they also observed loss of *Let-7* expression in BR-C-null mutant animals, indicating that early ecdysone-inducible BR-C is required for *Let-7* expression during metamorphosis. These results showed that ecdysone signaling pathway is closely associated with the heterochronic pathway and implies that hormone-induced expression of *Let-7* plays a significant role in regulation of developmental stage transitions. There is a report that *Let-7* induction occurs independently of either ecdysone or EcR expression, suggesting that *Let-7* expression is not directory regulated by the ecdysone pathway (Bashirullah et al., 2003).

(D) Modulation of *Drosophila Let-7* Function

Parkinson's disease (PD) is a common neurodegenerative disorder and its main symptom is the selective and progressive loss of dopaminergic neurons. During the last decade, several loci were identified to be causative of familial Parkinson's disease. Mutations in Leucine-rich repeat kinase 2 (LRRK2) appear to be the most common genetic cause of a dominant form of PD (Paisán-Ruíz et al. 2004; Zimprich et al. 2004). Gain-of-function mutations of LRRK2 are linked with sporadic forms of PD as well as familial ones. Although molecular mechanism by which LRRK2 mutations cause PD has been unknown, it was recently reported that LRRK2 interacts with the miRNA pathway to regulate protein synthesis (Gehrke et al. 2010). In *Drosophila*, *Let-7* is expressed in dopaminergic neurons, and homozygous *Let-7* mutant showed decreased locomotor activity and a specific decrease in dopaminergic neurons. *Drosophila* e2f1 RNA is translationally repressed by *Let-7*. Gehrke et al. (2010) found that LRRK2 with pathogenic mutations suppressed *Let-7* function and interacts with RISC component Argonaute in *Drosophila*. E2F1 overexpression was sufficient to cause the

degeneration of dopaminergic neurons and decreased locomotor activity. Conversely, increasing *Let-7* expression attenuated pathogenic LRRK2 effects. Thus, this study implies that the regulation of *Let-7* function by LRRK2 plays an important role in survival and maintenance of dopaminergic neurons.

2) Vertebrates

(I) Expression of Let-7 in Vertebrate Development

The human encodes 13 members, the mouse encodes 14 members, the chick encodes 11 members, Xenopus tropicalis encodes 9 members, and the zebrafish encodes 19 members of the *Let-7* family miRNAs. Among 13 human *Let-7* family members, *Let-7*a has identical sequence across various species from *C. elegans* to human. The increase of *Let-7* expression in chick limb development (Lancman et al., 2005; Kanamoto et al., 2006), mouse development (Lancman et al., 2005; Kanamoto et al., 2006), and neuronal differentiation of EC cells was reported (Schulman et al., 2005). Various studies showed that a major role of *Let-7* is to induce cells to exit cell-cycle and promote differentiation of cells. In mice, *Let-7* expression detected by Northern blot analysis increases after embryonic day 10.5 (E10.5) and peaks at E14.5, and then high level of its expression is maintained (Schulman et al., 2005). *Let-7* is undetectable in human and mouse embryonic stem cells, and *Let-7* expression increases upon differentiation (Wulczyn et al., 2007; Thompson et al., 2004). This *Let-7* expression is maintained in various adult tissues (Thompson et al., 2004; Sempere et al., 2004). Interestingly, the reduction of *Let-7* expression is observed in many human cancer cells (Park et al., 2007).

Regardless of several expression studies of *Let-7* in vertebrates, a definitive role of *Let-7* in development has remained elusive, probably due to redundant function by multiple *Let-7* family members in vertebrates. Existence of multiple family members makes *in vivo* direct experiments to draw a clear conclusion very difficult compared with those in invertebrates. Molecular mechanisms underlying the control of developmental timing in vertebrates are poorly understood, although many vertebrate's tissues are temporally regulated during development. Since mammals contain homologues of *Let-7*, a heterochronic gene in *C. elegans*, it has been hypothesized that similar heterochronic genes control developmental timing. Due to difficulty to analyze *Let-7* function *in vivo*, investigation of *Let-7* function in vertebrates has been mainly performed by using culture cell system as we will describe in the following section.

(II) A Possible Function of Vertebrate Let-7 during Development

Direct experiments to clarify *in vivo* function of *Let-7* in vertebrate development are practically very difficult as we described, however, there are couple of studies on *Let-7* target genes that may indirectly suggest a possible function of *Let-7* during vertebrate development. The *Let-7* miRNA regulates its targets by binding to complementary site in their 3'UTRs.

LIN28 was first identified in *C. elegans* as a heterochronic gene to regulate developmental timing (Moss et al., 2007; Ambros and Horvitz, 1984). LIN28 is an RNA-binding protein, containing a-cold-shock domain, and retroviral-type CCHC zinc fingers (Moss et al., 1997), and highly conserved through evolution. The mammalian homologs of

LIN28 are LIN28a and LIN28b, which bind to pre-*Let-7* and suppress production of the *Let-7* mature miRNA by inhibiting the pre-miRNA processing by Drosha or the loop cleavage reaction by Dicer (Heo et al., 2009). *Let-7* in turn directly suppresses LIN28 protein expression by binding to the LIN28 mRNA (Rybak et al., 2008; Nimmo and Slack, 2009). In order to investigate the function of LIN28/*Let-7* pathway *in vivo*, Zhu et al. (2010) generated transgenic mice to induce LIN28a expression. Upon LIN28a induction by doxycycline, the expressions of *Let-7*s were reduced in multiple tissues of LIN28-induced mice, and these mice showed increased body size and a proportional increase in organ sizes, and delayed onset of puberty. They found that LIN28a caused increased glucose utilization, a mechanism by which it may drive overgrowth *in vivo* by decreasing expression of *Let-7* family miRNA and increasing expression of *Let-7* target oncogenes such as MYC and RAS.

Another developmentally conserved *Let-7* target gene, Lin41, also known as TRIM71, encodes an RBCC-NHL protein, which has a RING, two B-boxes, and a coiled-coil domain associated with the Asn-His-Leu motif (Kanamoto et al., 2006). In the early embryonic stages of chick and mouse development, Lin41 is expressed in the developing limb buds, and the expression timing of *Let-7* and Lin41 is partially overlapped, suggesting that *Let-7* plays a role in limb development (Schulman et al., 2005). In *C. elegans*, it was proposed that LIN41 is temporally regulated by *Let-7* and lin-4 miRNAs that bind to complementary sites in the LIN41 3'UTR (Lancman et al., 2003). Lancman et al. (2003) reported that 3'UTR of chick Lin41 contains potential miRNA-binding sites for the chick orthologues of both *C. elegans Let-7* and lin-4, suggesting that these miRNAs regulate chicken Lin41. Interestingly, recent study reported that Lin41 functions as an E3 ubiquitin ligase for one of key components of RISC, Argonaute2 (AGO2), in stem cells, indicating the dual control mechanism for regulation of precocious *Let-7* function (Rybak et al., 2009). In mice, loss-of-function mutant of mouse Lin41 produced by gene-trap displayed neural tube closure defect and embryonic lethality, although their underlying mechanisms are unknown (Maller Schulman et al., 2008). As in *C. elegans* Lin41, mouse Lin41 seems to be regulated by *Let-7* and miR-125, a lin-4 homologue, through 3'UTR complementary sites for *Let-7* and miR-125. This fits well with the previously reported result that both human Lin41 and zebrafish LIN41 are regulated by *Let-7* (Lin et al., 2007).

The *Let-7* target sites in LIN41 3'UTR are also conserved in zebrafish. Misexpression of *Let-7* in zebrafish embryos resulted in severe embryonic developmental abnormality, suggesting that ectopic expression of *Let-7* causes precocious down-regulation of one or more endogenous *Let-7* targets (Kloosterman et al., 2004), suggesting that *Let-7* plays a significant role in zebrafish development. Lin et al. (2007) performed silencing experiment of the LIN41 orthologue by microinjection of RNA interference or morpholino in the zebrafish system and demonstrated developmental defects similar to the effects of *Let-7* misexpression experiment in zebrafish embryos reported by Kloosterman et al. (2008).

Fragile X syndrome (FXS) is the most common type of inherited cause of mental retardation, usually due to the loss of fragile X mental retardation protein 1 (FMRP1) encoded on the FMR1 gene. FMRP proteins link to the miRNA function through RISC component protein AGO. Fmrp1 is considered an important factor for synaptogenesis. Interestingly, knock down of *Let-7*c in rat hippocampal neurons lead to abnormal neuronal spine formation, and *Let-7*c is shown to associate with Fmrp1 in the mouse brain (Edbauer et al., 2010), however that function of *Let-7*c is still unknown.

Taken together, vertebrate *Let-7* may play an important functional role in various developmental events, including embryonic growth, limb development, and neural development and function.

4. ROLE OF *LET-7* IN DIFFERENTIATION

1) *Let-7* in Stem Cell Pluripotency and Differentiation

The *Let-7* miRNA was found to function as a heterochronic gene in seam cell differentiation. Both expression and function of mature *Let-7* family miRNAs are controlled in stem cells through *Let-7* target genes. Neuronal induction by retinoic acid (RA) is commonly used for embryonic stem (ES) cell differentiation. The increased expression of mature *Let-7*a is detected in such RA-treated ES cells, embryonic neural stem cells, and the mouse E17 brain, but not in mouse ES cells in which pre-*Let-7*a-1 and pre-*Let-7*a-2 expression is detected (Rybak et al., 2009). Therefore, mature *Let-7* miRNA expression seems to be strictly controlled between at the embryonic stem cell stage and at its differentiation stage. One of the most important genes for regulation of *Let-7* family miRNAs, LIN28 has been known as one of pluripotency factors for induced pluripotent stem cells (iPS) (Yu et al., 2007; Okita et al., 2008), implying that down-regulation of *Let-7* is one of the key events for pluripotency maintenance.

DGCR8, DiGeorge syndrome critical region gene 8, encodes an RNA-binding protein required for miRNA biogenesis. The DGCR8-null mice exhibit loss of the production of canonical miRNAs (Han et al., 2004). Interestingly, DGCR8-null ES cells proliferate slowly and accumulate in G1 phase of its cell-cycle (Wang et al., 2007). The acceleration of the G1-S transition is essential for rapid cell-cycle of ES cells (White and Dalton, 2005). Overexpression of the ES cell-specific miRNAs, members of the miR-290 family, rescues the slow proliferation and G1 accumulation of the DGCR8-null ES cells. *Let-7* miRNAs seem to have an opposite role of the miR-294 family regarding cell-cycle maintenance through *Let-7* target genes, C-MYC, N-MYC, LIN28, SALl4, and NANOG (Melton et al., 2010). The C-MYC is one of the first iPS-inducible factors, and LIN28 is one of pluripotency factors for generating iPS cells from human somatic cells without usage of c-MYC (Yu et al., 2007; Okita et al., 2008). GFP (green fluorescence protein) expression in Oct4-gfp knock-in mouse embryonic fibroblasts (MEF) is a pluripotency monitor in iPS cell production. The number of GFP signal-expressing colonies in the OCT4, KIF2, and SOX2 infected MEF cells co-transfected with *Let-7* inhibitor was significantly increased by 4.3 fold comparing with those in a control inhibitor-transfected cells, even without C-MYC co-expression (Melton et al., 2010). These findings indicate that down-regulation of *Let-7* family miRNAs in stem cell plays a critical role in acquirement and maintenance of pluripotency (Figure 3).

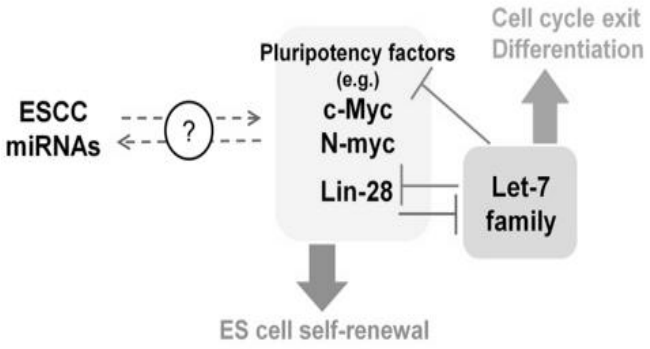

Figure 3. A model of the pluripotency maintenance by down-regulation of *Let-7* family miRNAs.

Embryonic stem cell cell-cycle (ESCC) miRNAs (e.g. miR-290 family) promotes expression of pluripotency factors. *Let-7* family miRNAs target pluripotency factor genes and lead ES cells to cell-cycle exit and differentiation. LIN28, one of the pluripotency factors and *Let-7* target genes, prevents mature *Let-7* family miRNA expression. Therefore, *Let-7* miRNAs play an opposite role against ESCC miRNAs in the ES cell pluripotency maintenance.

2) *Let-7* and Its Target Gene LIN28 in Neural Cell Fate Determination

The neural retina is developmentally a part of central nervous system (CNS) and known as a good model to analyze cell lineage in the CNS. In 1987, Tuner and Cepko (1987) demonstrated that a single neural progenitor cell in the rodent retina is able to generate multiple cell types including both neurons and glial cells. In the developing vertebrate retina, cone photoreceptor cells, ganglion cells, horizontal cells, and part of amacrine cells are generated from early progenitor cells. In contrast, rod photoreceptor cells, bipolar cells, amacrine cells, and Müller glial cells are generated mainly from late progenitor cells (Turner and Cepko, 1987). Similar to the retina, cortical progenitors generate glial cells after generating neurons (Pearson and Doe, 2004). As like ES cells, P19 embryonal carcinoma cells also possess a potential of differentiation along a neuronal-glial lineage by RA induction. Interestingly in these cells, constitutive expression of pluripotency factor LIN28 induces neurogenesis and inhibits gliogenesis (Blazer et al., 2010). In the developing mouse brain, the neurogenesis begins as early as from E10 and that reaches its peak around E12-14. Then after neurogenesis, gliogenesis begins around from E14 and a peak of gliogenesis comes around postnatal day 0 (P0) (Qian et al., 2000). In *C. elegans*, loss of function mutant of LIN28 skips the L2 program and shows premature entering to the L3 program (Figure 1). The gain of function mutant of LIN28 exhibits reiteration of L2 fates and delayed vulval development. These observations indicate that down-regulation of LIN28 in proper developing stages is necessary for proper cell differentiation, and the function of LIN28 in neuron-glia fate determination is well conserved across species.

However, it was recently found that *Let-7* family miRNAs are already expressed in the E9.5 mouse brain (Wulczyn et al., 2007). Furthermore, a recent report about miRNA expression by using deep sequencer indicated that in the E15.5 developing mouse brain *Let-7*

family miRNAs are the most abundant miRNA more than miR-124, which is the most dominant miRNA in the adult brain (Ling et al., 2011), suggesting that *Let-7* is involved in neural cell fate determination rather than neuronal maturation. Additionally, it was reported that the stem cell regulator Tlx (Nr2e1), encoding an orphan nuclear receptor that is expressed in the vertebrate forebrain, and the cell-cycle regulator cyclin D1 are regulated by *Let-7* to control neural stem cell proliferation and differentiation (Zhao et al., 2010). Tlx maintains neural stem cells in an undifferentiated and self-renewable state to repress Tlx downstream target genes, p21 and Pten. Tlx and cyclin D1 are found to be a target of *Let-7*b. *Let-7*b inhibits Tlx and cyclin D1 expression by binding to the 3'UTR sites of their mRNAs. Overexpression of *Let-7*b in neural stem cells showed increased differentiation of both glial cells and neurons by suppressing Tlx expression. Moreover, *in utero* electroporation of *Let-7*b to embryonic neural stem cells resulted in reduced cell-cycle progression. These studies suggested that unknown molecular mechanisms, which are functionally associated between *Let-7* family miRNAs and LIN28, play a role in neuron and glia fate determination during vertebrate development.

3) Neural Regeneration and *Let-7*

When the vertebrate retina is injured, retinal Müller glial cells undergo dedifferentiation in the injured retinal region (Fisher and Reh. 2001). However, mechanisms underlying dedifferentiation of Müller glial cells are unknown. The capacity for regeneration of the injured retina seems to be strongest in fish, amphibians and chick. Therefore, zebrafish is one of good model systems to clarify the molecular mechanisms underlying Müller glial cell dedifferentiation. In the injured zebrafish retina, dedifferentiation of Müller glial cells begins 15 hours after injury, and then the Müller glial cells begin to proliferate two days after injury. The cell division becomes at its maximum 4 days after injury, and Müller glial cells stop their proliferation and begin differentiation (Fausett et al., 2001; Fausett et al., 2006). In those retinas, LIN28 expression is induced from at least 6 hours after injury and disappeared until 14 days after injury (Ramachandran et al., 2011). ASCL1A, achaete-scute complex homolog 1, encodes a member of the basic helix-loop-helix (bHLH) family of transcription factors. LIN28 transcription is directory activated by ASCL1A in Müller glial cells of the injured retinal region. The expression of *Let-7* miRNA in the Müller glial cells is down-regulated at least from 15 hours after injury. LIN28 inhibits mature *Let-7* miRNA expression. *Let-7* represses expression of regeneration-associated genes, including ASCL1A, HSPD1, LIN28, OCT4, PAX6B and C-MYC. In the uninjured retina, *Let-7* target genes, C-MYC and KIF4, and HSPD, are expressed in Müller glial cells. *Let-7* may inhibit this expression to prevent premature Müller glial cell dedifferentiation (Figure 4).

Notably, ASCL1A is a reciprocal target gene of *Let-7*, and a recent study shows that direct conversion of human fibroblasts to dopaminergic neuron requires Ascl1 expression (Pfisterer et al., 2011; Caiazzo et al., 2011). It may be interesting to examine if the downregulation of *Let-7* family miRNAs contributes not only to retinal neurogenesis in mammals but also to dopaminergic neurogenesis in the brain, which is related with etiology of Parkinson's disease.

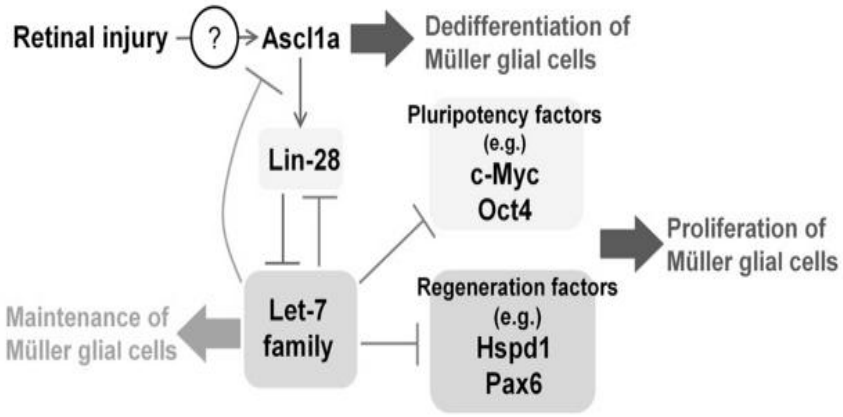

Figure 4. A model of the retinal regeneration by down-regulation of *Let-7* family miRNAs.

The expression of ASCL1A is simulated by retinal injury to repair retinal neurons and begins dedifferentiation of Müller glial cells. ASCL1A activates LIN28 expression and consecutive suppression of *Let-7* miRNA expressions occur in Müller glia cells. After repression of mature *Let-7*, both pluripotency factors and regeneration factors, which are targeted by *Let-7*, are upregulated at the protein level, and then Müller glial cells begin to proliferate. Therefore, the retinal regeneration is required for down-regulation of *Let-7* miRNAs.

CONCLUSION

A frequently-asked question is, "Do heterochronic genes exist in vertebrates, especially mammals?" A variation of this question is "Is mammalian *Let-7* homolog a developmental regulator?" Several recent studies implicated *Let-7* as a regulator of mammalian development. In *C. elegans* and *Drosophila melanogaster*, *Let-7* is a single locus and thus *Let-7* mutant provides us an important clue to reveal *Let-7* function. Studies using the *C. elegans* or *Drosophila* system designate *Let-7* as a heterochronic gene, although target genes of *Let-7* appear not to be same between these two species. On the other hand, the existence of multiple family members of *Let-7* in vertebrates hinders our investigation to clarify *in vivo Let-7* functions during development. At this point, we may still stand at a premature stage to conclusively place *Let-7* to a heterochronic gene in vertebrate development. The accumulation of further observation in the future *in vivo* studies is required to understand precise *Let-7* function in vertebrate development.

Despite *in vivo* loss of function studies in vertebrates are practically of extreme difficulty, functional study at cellular level has been more fruitful. It is of particular interest that mature *Let-7* is absent in ES cells or neural stem cells, however, *Let-7* expression is induced upon differentiation of those pluripotent cells. *Let-7* induces cell-cycle exit which is necessary for cell differentiation in multiple cell types. In accordance with this, it is also interesting that *Let-7* deregulation is observed in certain cancer cells. However, it is still unclear why there are so many *Let-7* family members in vertebrates and whether each of them has a different biological function and/or different target molecule during development. Our knowledge and

understanding on *Let-7* in vertebrate development may be still in an emergence stage and further studies on *Let-7* may provide us not only an important clue to understand cell differentiation in development but also a useful tool for diagnosis and/or therapies in medical field.

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