Chapter 2
Discovery of Human MicroRNA Precursor Binding to Folic Acid by Small RNA Transcriptomic SELEX

Abstract MicroRNA (miRNA) is about 22 nt single-strand RNA, which is one of the small ncRNAs (Ha and Kim in Nature Reviews Molecular Cell biology, 15 (8):509–524, 2014). miRNAs are loaded into an Argonaute protein to form the RNA-induced silencing complex (RISC) and RISC binds to target mRNA via base paring with miRNA to regulate the expression of target gene. miRNAs are transcribed from genome as part of a long primary transcripts (pri-miRNAs) and then pri-miRNAs are cleaved to produce precursor miRNAs (pre-miRNAs) which form hairpin structure. Pre-miRNAs are exported to the cytoplasm and they are cleaved to mature miRNAs. Biogenesis and functions of miRNAs are regulated by proteins and RNAs in many steps (Ha and Kim in Nature Reviews Molecular Cell Biology, 15(8):509–524, 2014). Recently, regulation of miRNA functions by binding with small molecules was discovered in human (Baselga-Escudero et al. in Nucleic Acids Research 42(2):882–892, 2014). This discovery suggests the existence of RNA aptamer elements binding to small molecules with specific biochemical activities in various human miRNAs. I will report the discovery of human microRNA precursor binding to metabolite and analyses of the function of this RNA. In my master course, tRNA- and rRNA-depleted human small ncRNA library was constructed using tRid technology (Futai et al. in Methods 106:105–111, 2016). Then, SELEX to folic acid was performed to obtain three kinds of RNAs. In my Ph.D., I measured the binding affinity of these RNAs and discovered that precursor microRNA 125a (hsa-pre-miR-125a) are bound to folic acid. Then, further mutation study revealed the essential motif of hsa-pre-miR-125a for binding to folic acid.

Keywords SELEX · Small RNA · MicroRNA · Ribozyme · Folic acid

N. Terasaka, *Applications of Aminoacylation Ribozymes That Recognize the 3′-end of tRNA*, Springer Theses, DOI 10.1007/978-4-431-56517-8_2

2.1 Introduction

Various small noncoding RNAs (ncRNAs) have been discovered in the recent years. Among them, the functions and biogenesis of microRNA (miRNA) have been extensively studied. miRNAs are about 22 nt RNAs pairing to mRNAs to regulate the translation. Canonical pathway of miRNA processing in mammalian is shown in Fig. 2.1 (Winter et al. 2009). An miRNA is initially transcribed as part of a long primary transcript, microRNA (pri-miRNA) by RNA polymerase II or III (Lee et al. 2004; Cai et al. 2004; Borchert et al. 2006). In mammalian, pri-miRNAs are cleaved by Drosha-DGCR8 microprocessor complex in nucleus to produce about 60–80 nucleotide precursor microRNA (pre-miRNA) forming hairpin structure (Lee et al. 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). The pre-miRNA is then exported to the cytoplasm by Exportin-5-RanGTP (Okada et al. 2009; Yi et al. 2003). In the cytoplasm, pre-miRNA is cleaved to about 22 nt mature miRNA duplex by the RNase III enzyme Dicer (Hutvagner et al. 2001) in complex with the double-stranded RNA

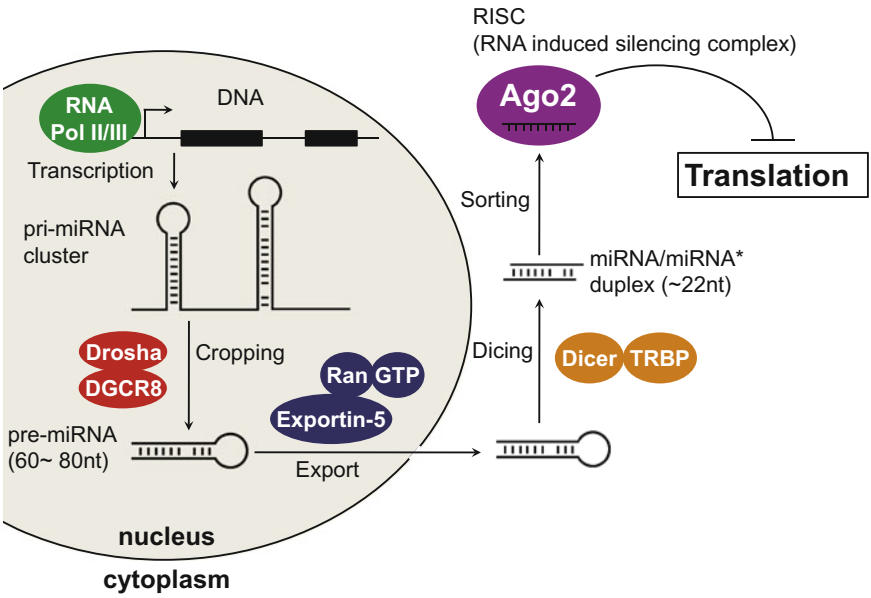

Fig. 2.1 Canonical pathway of microRNA processing. A miRNA is initially transcribed as part of a long pri-miRNA by RNA polymerase II or III. In mammalian, pri-miRNAs are cleaved by Drosha-DGCR8 microprocessor complex in nucleus to produce about 60–80 nucleotide pre-miRNA forming hairpin structure. The pre-miRNA is then exported to the cytoplasm by Exportin-5-RanGTP. In the cytoplasm, pre-miRNA is cleaved to about 22 nt mature miRNA duplex by Dicer in complex with TRBP. One strand of the duplex is loaded into an Argonaute protein (Ago2) to form RISC. RISC binds to target mRNA via base paring with miRNA, whereas Ago2 functions as effectors by recruiting factors that induce translational repression

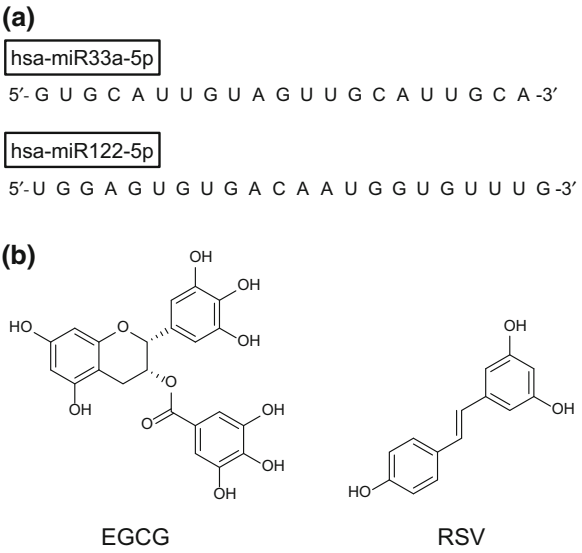

Fig. 2.2 Human miRNAs directly binding to polyphenols. **a** Sequences of hsa-miR-33a-5p and hsa-miR-122-5p which directly bind to specific polyphenols. **b** Chemical structures of epigallocatechin gallate (EGCG) and resveratrol (RSV)

binding protein TRBP (Haase et al. 2005). One strand of the duplex is loaded into an Argonaute protein to form the RNA-induced silencing complex (RISC) and the other strand is discarded (Khvorova et al. 2003; Schwarz et al. 2003; Liu et al. 2004). RISC binds to target mRNA via base paring with miRNA, whereas Argonaute protein functions as effectors by recruiting factors that induce translational repression, mRNA deadenylation and mRNA decay (Huntzinger and Izaurralde 2011).

Biogenesis and functions of miRNAs are regulated by proteins and RNAs in many steps including their transcription, their processing by Drosha and Dicer, their loading onto Argonaute proteins and miRNA turn over, and their dysregulation which are associated with many human diseases including cancer (Ha and Kim 2014; Hansen et al. 2013). Recently, it was reported that direct binding of specific polyphenols (epigallocatechin gallate and resveratrol) to hsa-miR-33a-5p and hsa-miR-122-5p ("hsa" indicates *Homo sapiens*, "5p" indicates mature miRNA derived from 5′-end of pre-miRNA) modulates divergently their levels in hepatic cells (Fig. 2.2) (Baselga-Escudero et al. 2014). This is the first report about post-transcriptional regulation of miRNAs by binding of natural small molecules. In addition to these miRNAs, the RNA elements in human mRNAs binding to ATP or GTP have been discovered by genomic SELEX (Fig. 2.3) (Curtis and Liu 2013; Vu et al. 2012). From these discoveries, I considered that more RNA aptamer elements binding to metabolites with specific biochemical activities exist in other human small ncRNAs. In this chapter, I aimed to discover human small ncRNA including microRNAs and these precursors which bind to metabolite.

Many 145 miRNAs involved in the control of a variety of carcinogenesis mechanisms, were modulated by natural agents, including vitamins, oligoelements,

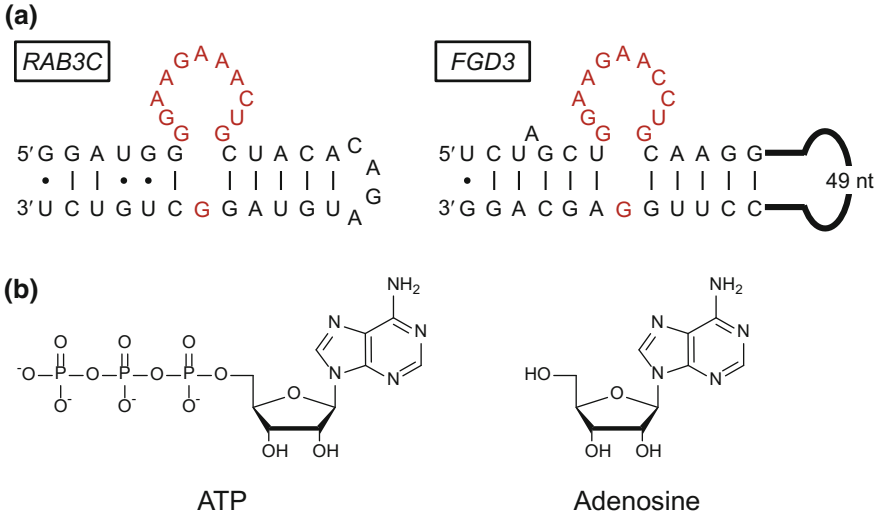

Fig. 2.3 Adenosine aptamers discovered in human transcripts. **a** Secondary structures of the regions containing adenosine aptamers of human *RAB3C* gene and human *FGD3* intron (Vu et al. 2012). *Red* bases are essential motifs binding to adenosine. **b** Chemical structures of adenosine 5′-triphosphate and adenosine

polyphenols, isoflavones, indoles, isothiocyanates, phospholipids, saponins, anthraquinones, and polyunsaturated fatty acids (Izzotti et al. 2012). Although the detailed mechanisms of how to modulate expression of miRNAs by these molecules are unknown, there is a possibility that some of these molecules directly bind to miRNAs or those precursors to regulate the functions. Among these natural agents, I focused my attention on folic acid (vitamin B9), which is one of the water soluble vitamins. The term "folic acid" refers to the synthetic form of the vitamin whereas "folate" refers to the natural forms, such as those present in food (Melse-Boonstra et al. 2004). Folate includes oxidized, reduced, polyglutamylated, methylated, and formylated forms of folic acid (Selhub 1989). Deficiency of dietary folate has been linked to developmental anomalies (Mulinare et al. 1988) as well as increased risk for a number of cancers (Jiang et al. 2003). In addition, folic acid deficient culture medium changes the expression level of many human miRNAs and folic acid supplementation in the culture medium restored miRNA levels, which indicate that modulation of miRNAs by folate is reversible (Marsit et al. 2006). From these results, I chose folic acid as a candidate molecule binding to miRNA.

In order to discover the certain RNAs interacting with small molecules or proteins, pull down method, computational prediction (Barrick et al. 2004) and genomic SELEX (Singer et al. 1997) have been widely used. These approaches have been very successful, but they have some limitations. By classical pull down method, although RNAs expressing in vivo can be discovered, it is difficult to detect RNAs with low abundance in vivo. Computational predictions rely primarily on conservation and structural stability as signal for an active molecule, thus constraining the

range of possible predictions (Zimmermann et al. 2010; Dinger et al. 2008). Genomic SELEX is one of the SELEX methods, where libraries are derived from genomic DNA. This methodology enables to identify new RNA aptamer elements even if the RNA is less abundant. However posttranscriptionally processed RNA sequences [e.g., splicing, nontemplated addition of nucleotides (Koppers-Lalic et al. 2014) and circular permutation (Pan and Uhlenbeck 1993)] cannot be obtained by genomic SELEX because the library was derived from genome. In addition, obtained sequence may not be transcribed from genome in vivo. To overcome these problems, I decided to perform SELEX using library prepared from natural transcript, which is called as cDNA-SELEX (Chen et al. 2003; Dobbelstein and Shenk 1995) or transcriptomic SELEX (Fujimoto et al. 2012). This method enables to discover the small molecule-binding RNAs transcribed in vivo even if the level of expression is very low.

In the small RNA fraction (shorter than 200 nt), tRNAs and rRNAs are much abundant (Lindberg and Lundeberg 2010). Therefore, it is necessary to remove tRNAs and rRNAs from small RNAs in order to construct a cDNA library of high quality, which enables every potential small ncRNAs binding to metabolite equal candidacy for selection. Although several kits to remove the rRNAs using hybridization probes or ribonuclease are commercially available, the reported methods to deplete tRNAs require each sequence to be individually identified and prepared about 30 different probes (Yuan et al. 2003; Liu et al. 2009). In my master course, tRNAs in the small RNA fraction were labeled with biotin-conjugated amino acids using flexizymes and were depleted, which is called as tRid (Futai et al. 2016). From this small RNA library, cDNA library for SELEX was constructed and then selection to folic acid was performed to obtain three kinds of RNA sequences. To my knowledge, this is the first example of transcriptomic SELEX using small RNA library and this method of SELEX was named as small RNA transcriptomic SELEX.

In this chapter, the study during my master course will be briefly summarized and I will report binding analyses of RNAs obtained from small RNA transcriptomic SELEX performed during my Ph.D.

2.2 Results and Discussions

2.2.1 Summary of Master Course Study

In my master course, tRNA-depleted small RNA library was constructed using tRid technology (Futai et al. 2016) and some RNAs binding to folic acid were discovered using small RNA transcriptomic SELEX. First, I briefly summarize the study during my master course.

Because the expression level of miRNAs and precursors are totally different among the cells and conditions, eight human cell lines (BeWo, HuH-7,

Table 2.1 Human miRNA precursor profile [adapted from Landgraf et al. (2007)] and cell lines used in this chapter

Organ systems and cell types	Numbers of miRNA precursors	Corresponding cell lines used in this chapter
hsa_Osteosarcoma-U2Os-uninduced	168	HuO-3N1
hsa_Placenta	152	BeWo
hsa_renal_carcinoma-DH1-diff-3d	159	VMRC-RCW
hsa_Teratocarcinoma-NT2-norm	111	NEC14
hsa_Hepatoma-PLC	139	HuH-7
hsa_Breast-adenocarcinoma-MCF7	100	MCF7
hsa_Cervix-HeLa-IFNa	120	HeLaS3
hsa_Burkitt-patient3	128	RAJI

VMRC-RCW, NEC14, HuO-3N1, RAJI, MCF7 and HeLaS3 cells) were chosen based on the expression levels of miRNAs to include various miRNAs into the library (Table 2.1) (Landgraf et al. 2007). After extracting small RNAs from these cell lines, rRNAs were removed by hybridization with complementary nucleotide probe (Ribominus) and tRNAs were depleted by tRid (Fig. 2.4) (Futai et al. 2016). Consequently tRNA-/rRNA-depleted small RNA library was constructed. Next, tRNA-/rRNA-depleted small RNA library was converted to cDNA library. RNA library was ligated with 3′- and 5′-adaptors containing promoter sequence for T7 RNA polymerase. Then, RNA library ligated with adaptors was reverse transcribed to cDNA and amplified by PCR (Fig. 2.4). In comparison with the crude fraction of small RNAs, the population of non-rRNA/tRNA small RNAs was suitably enriched, with only 24 and 9% of tRNAs (including non-CCA tRNA fragments and pre-tRNAs) and rRNAs, respectively, remaining in the final fraction (the total number of sequences was 89, Fig. 2.5). This small-RNA-enriched library was deemed to be applicable to smaRt-SELEX against a target molecule.

Folic acid (Fig. 2.6a) was immobilized onto the magnetic beads via ester bond, whose surface is covered by hydroxyl group. Small RNA transcriptomic SELEX using the folic acid-immobilized beads and the library described above was performed four rounds. PAGE analysis of RNA library indicated the fractions of small RNAs bound to the folic acid beads were enriched over the background and several discrete bands appeared (Fig. 2.6b and c). The RNA pool at the fourth round was then cloned and sequenced and three RNA sequences (hsa-pre-miR-125a, FA1 and FA2) were enriched (Table 2.2). FA1 is antisense sequence to mitochondrial mRNA of ND1 (NADH dehydrogenase 1) bearing additional poly-A/C sequence at the 3′-end, which is not coded in genome. FA2 is 5′-fragment of $tRNA^{Gly}_{GCC}$, whose 3′-fragment was discovered as small RNA (tRF3006) (Lee et al. 2009).

Because 3′- and 5′-adaptors were ligated with naturally transcribed RNA sequences for reverse transcription and PCR during SELEX, it was possible that these adaptors are essential for binding to folic acid. The secondary structures predicted using mfold software (Zuker 2003) are shown in Fig. 2.7. In the case of FA1 and FA2, ligation of adaptors dramatically changed the secondary structure

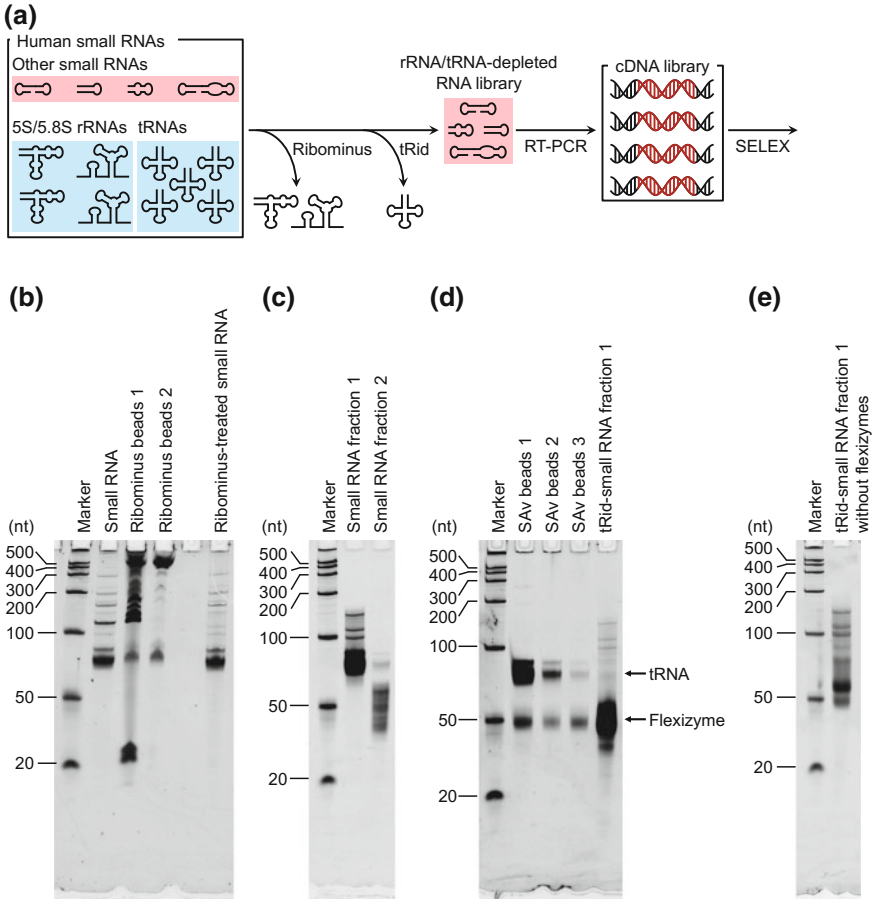

Fig. 2.4 Construction of a human tRNA-depleted small RNA library. **a** Schematic illustration of small RNA transcriptomic SELEX. From the small RNA fraction, the 5S and 5.8S rRNAs were removed using probes complementary to these rRNAs (RiboMinus, Life technologies), and then tRNAs were depleted using the tRid method. The rRNA-/tRNA-depleted small RNA library was converted to cDNA by RT-PCR and SELEX was performed. **b** RiboMinus™ treated human small RNA. Lanes marked Ribominus beads 1 and 2, indicate magnetic beads after RiboMinus treatment which were directly loaded onto the polyacrylamide gel. **c** Separation of rRNA-depleted small RNA into fraction 1 (lager than about 60 nt) and 2 (smaller than about 60 nt). **d** Depletion of tRNAs from small RNA fraction 1 by tRid. In the lanes marked streptavidin (SAv) beads 1, 2, and 3, the magnetic beads after the tRid treatment were directly loaded onto the polyacrylamide gel. **e** Removal of flexizymes after the tRid treatment

(referred as FA1 + adp and FA2 + adp). On the other hand, the structure of hsa-pre-miR-125a was retained when adaptors were ligated (named as hsa-pre-miR-125a + adp). Therefore, it was necessary to investigate whether these RNAs can bind to folic acid without adaptor sequences.

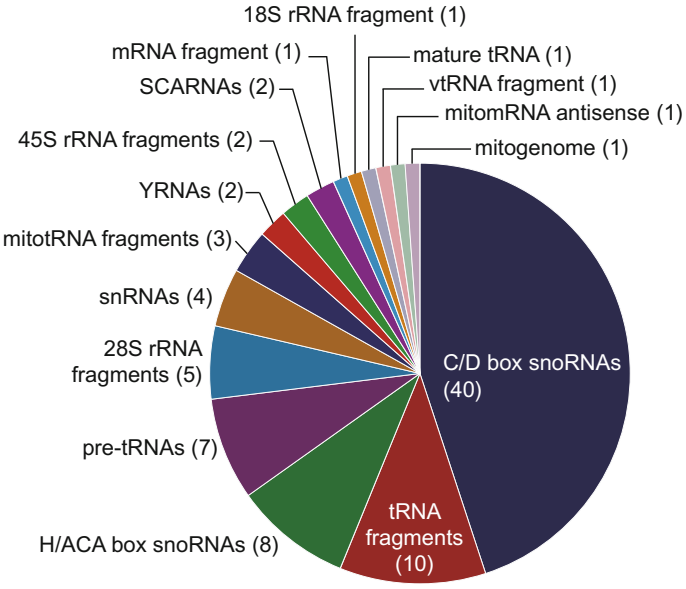

Fig. 2.5 The proportion of rRNA-/tRNA-depleted human small RNA library. Total sequence number was 89. The numbers after the name of RNAs indicate the number of sequences

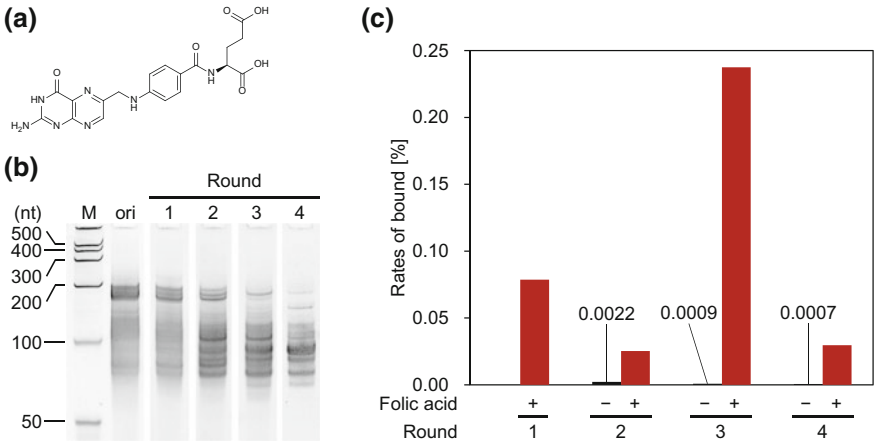

Fig. 2.6 Isolation of folic acid binding small RNAs using small RNA transcriptomic SELEX. **a** Chemical structure of folic acid. **b** Polyacrylamide gel analysis of the RNA library from each round of smaRt-SELEX. M indicates the RNA marker, "ori" indicates the RNA library before smaRt-SELEX. **c** Bead-binding activity of RNAs from each round of the smaRt-SELEX. Proportional binding was determined by normalizing bound quantities to input quantities of RNA. *Black bars* indicate binding of RNA to hydroxyl-beads that were not conjugated to folic acid, and *red bars* indicate binding of RNA to folic acid-immobilized beads. Hydroxyl-beads were not used in the first round

Table 2.2 RNAs obtained from SELEX

Sequence (5′ to 3′)	Remarks	Frequency
TTTTATGGCGTCAGCGAAGGGTT GTAGTAGCCCGTAAAAACCAAAAAAACC	FA1 (Antisense of MT-ND1 mRNA)	20/40
GCATTGGTGGTTCAGTGGTAGAA TTCTCGCCTGCCACGCGGGAGGCCCGGGT	FA2 (5′-fragment of $tRNA^{Gly}_{GCC}$)	7/40
TCCCTGAGACCCTTTAACCTGTGAGG ACATCCAGGGTCACAGGTGAGGTTCTT GGGAGCC	Hsa-pre-miR-125a	3/40
TCCTCTTTAGTATAGTGGTGAGTATC CCCGCCTGTCACGCGGGAGACCGG	5′-fragment of $tRNA^{Asp}_{GTC}$	1/40
CCTGTCACGCGGGAGACCGGGGTT CGATTCCCCGACGGGGAGCCA	3′-fragment of $tRNA^{Asp}_{GTC}$	1/40
TCACTGACTGTCTTGGGAGGAGGGG CTGGGTGTGGCACACAGTGA	Chromosome 22 Antisense of MYH9 gene	1/40
GGGAGGGTGCCTGGAGGAGTGGAG GGATTGGATTTACACCCTCTTA	Chromosome 2	1/40
CAGCCGCAAGGGAGGCTGGGAAG TACAGTCATGCCTTCAGGTAGCCGT GAGCCCTG	Chromosome 1	1/40
GAAAAAGTCATGGAGGCCATGGGGT TGGCTTGAAACCAGCTTTGGGGGGTTCGAT TCCTTCCTTTTTTTGCCA	$tRNA^{Ser}_{UGA}$	1/40
TACGGACTCACTATAGGGAGCTATCG ACGTAACGCTGGGGATTGTGGGTTCGTCCCA TCTGGGTCGCCA	Similar to $tRNA^{Arg}_{CCG}$	1/40
TTTTGGGGTTTGGCAAAAACCAAA AAAACCAACCCAAACCCACCAAAAACCAA	No match in the databases	1/40
TTGGCTGAGGATGCGGAGGGGAGGAG GCTGAGTA	Chromosome 11 Intron of TAF6L gene	1/40
TGAGGATGGTGGTCAAGGGACCCCTATCACAC CACCACCACCAA	Mitochondrial genome	1/40

2.2.2 K_D Determination and Mutation Study Using Biolayer Interferometry

During my doctor course, binding interaction between folic acid and RNAs obtained from SELEX was investigated. Three RNAs ligated with adaptors were prepared by in vitro transcription and three RNAs without adaptors which were chemically synthesized were purchased from Gene design Inc. Dissociation constant (K_D) values were measured using biolayer interferometory (BLI). In order to immobilize folic acid onto the biosensors, ethylenediamine was immobilized onto

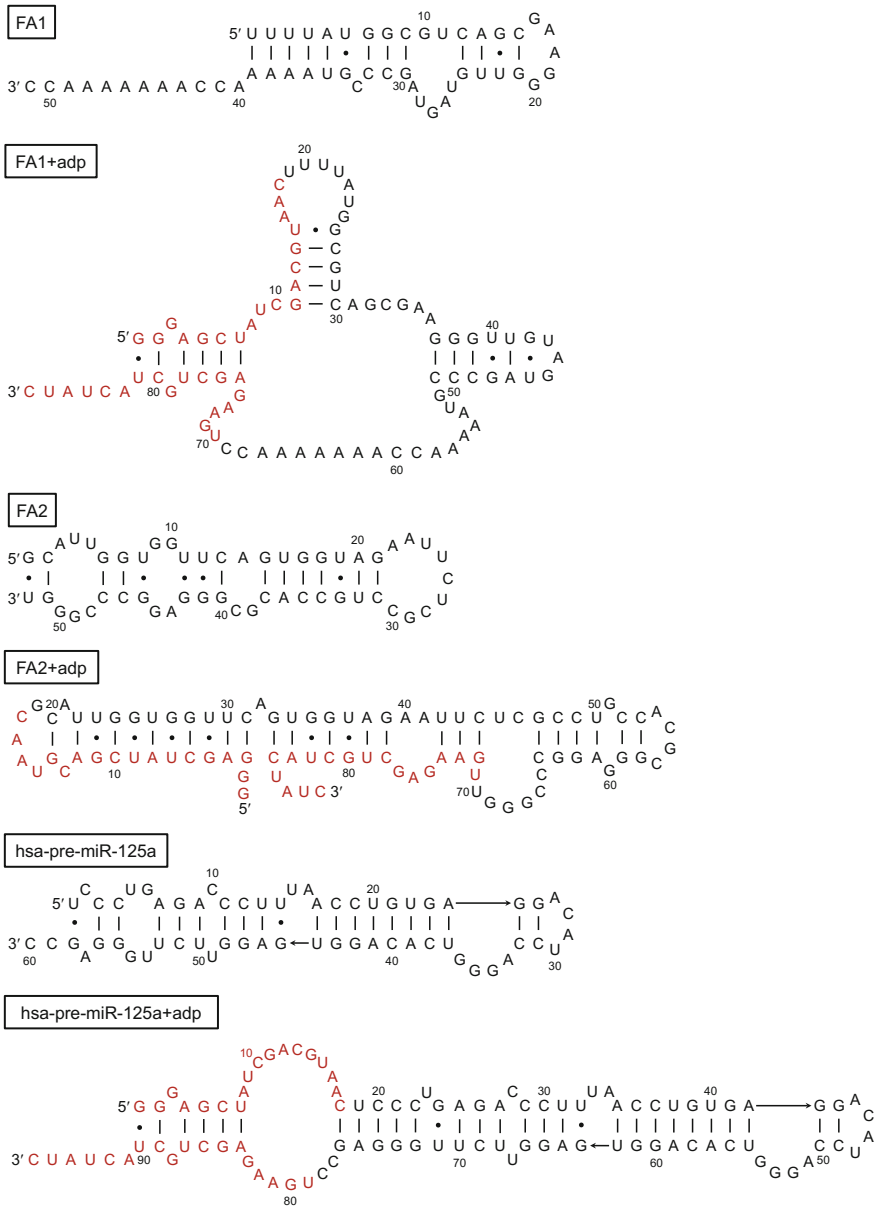

Fig. 2.7 Predicted secondary structure of RNAs obtained from SELEX to folic acid. Secondary structures were predicted using mfold software (Zuker 2003). *Red* bases indicated adaptor sequences and *numbers* indicate the nucleotide positions. *Lines* denote the Watson–Crick base pairs and *bullets* denote wobble base pairs

Table 2.3 Dissociation constant of RNAs obtained from SELEX

RNA	K_D (μM)	R^2
FA1 + Adp	3.8	0.98
FA2 + Adp	ND	
FA1	ND	
FA2	ND	

ND indicates "not determined" and R^2 value is derived from curve fitting in the steady-state analysis

AR2G (Amine Reactive Second-Generation) biosensors whose surface bears carboxyl group. Then, folic acid was conjugated with this sensor via amide bond. Six to eight different concentrations of RNA were used for steady-state analysis to determine the K_D values.

The results of binding analysis about FA1 and FA2 were shown in Table 2.3. FA1 was bound to folic acid only when adaptors were ligated (K_D value was 3.8 μM). This indicates that ligation of adaptors is essential for FA1 to bind to folic acid, which is consistent with the difference of predicted structure between FA1 and FA1 + adp (Fig. 2.6). On the other hand, FA2 did not bind to folic acid with or without adaptors. This result indicates that it was false positive when FA2 sequence was obtained by SELEX to folic acid. During SELEX, cDNAs are amplified by PCR. However, each cDNA is not uniformly amplified at the same efficiency by PCR, which means that the sequences whose PCR efficiency is higher could easily be amplified and therefore such sequences are obtained from final round as false positive (Tsuji et al. 2009).

The results of binding analysis about hsa-pre-miR-125a were shown in Table 2.4. In contrast to FA1 and FA2, hsa-pre-miR-125a can bind to folic acid with or without adaptors, and both hsa-pre-miR-125a and hsa-pre-miR-125a + adp showed the almost the same binding affinity (K_D values were 2.8 μM and 1.9 μM each). These results indicate that adaptors do not affect the binding affinity of hsa-pre-miR-125a to folic acid.

To identify the essential motif of hsa-pre-miR-125a to bind to folic acid, mutants were prepared by in vitro transcription and K_D values of those mutants were measured. Because 5′-end base of the wild-type hsa-pre-miR-125a is not G, the efficiency of in vitro transcription by T7 RNA polymerase was too low. Therefore, U1G/G58C mutant hsa-pre-miR-125a whose predicted secondary structure was the same as that of wild-type was prepared to enhance the T7 transcription efficiency, and U1G/G58C mutant showed the almost the same K_D value as that of wild-type (2.6 μM) (Fig. 2.8 and Table 2.4). Therefore, I introduced the same mutations (U1G/G58C) into other mutants except for m1 mutant (Fig. 2.8 and Table 2.4). First, as a negative control hsa-pre-miR-21 U1G mutant was prepared. The binding affinity of hsa-pre-miR-21 U1G was too low to determine the K_D value. Next, the

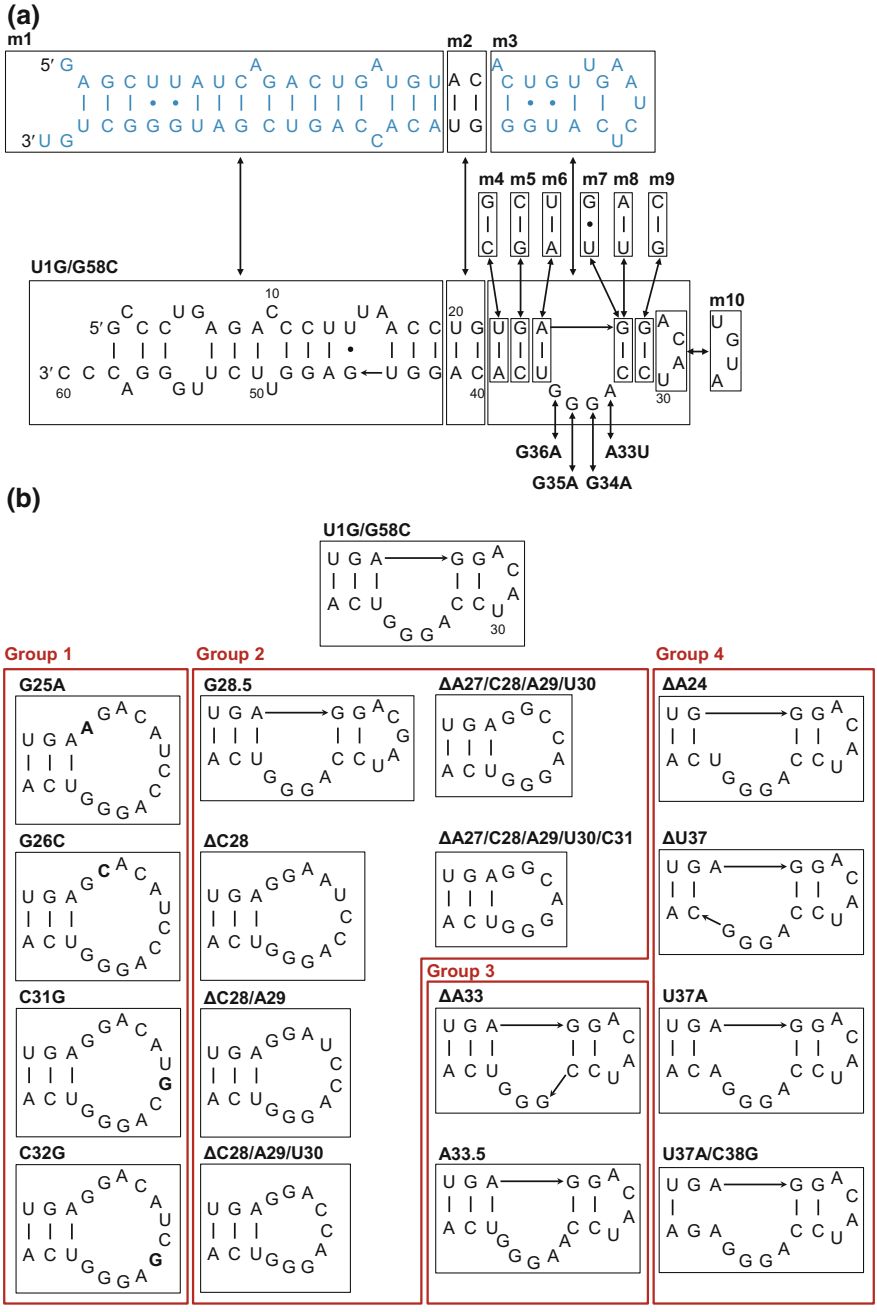

Fig. 2.8 Mutants of hsa-pre-miR-125a. *Lines* denote the Watson–Crick base pairs and *bullets* denote wobble base pairs. Secondary structures were predicted using mfold software (Zuker 2003). **a** Roughly mutated hsa-pre-miR-125a mutants. *Cyan* bases denote corresponding regions to hsa-pre-miR-21 used as negative control. **b** Loop region mutants were divided into four groups. *Bold* bases in group 1 are mutated bases

Table 2.4 Dissociation constant of hsa-pre-miR-125a mutants

RNA	Mutation	K_D (μM)	R^2
hsa-pre-miR-21	U1G	ND	
hsa-pre-miR-125a	WT	2.8	0.98
	Adp+	1.9	0.97
	U1G/G58C	2.6	0.99
hsa-pre-miR-125a U1G/G58C	m1	4.6	0.98
	m2	6.2	0.99
	m3	ND	
	m4	2.1	0.98
	m5	3.2	1.00
	m6	3.2	0.99
	m7	32	0.98
	m8	62	0.96
	m9	48	0.95
	m10	4.2	0.88
	A33U	3.0	0.96
	G34A	38	0.94
	G35A	ND	
	G36A	35	0.99
	G25A	73	0.99
	G26C	62	0.99
	C31G	6.0	0.99
	C32G	11	0.99
	G28.5	5.9	0.98
	ΔC28	1.8	0.96
	ΔC28/A29	1.4	0.94
	ΔC28/A29/U30	1.2	0.93
	ΔA27/C28/A29/U30	5.2	0.97
	ΔA27/C28/A29/U30/C31	12	0.98
	ΔA33	11	1.00
	A33.5	48	0.93
	ΔA24	ND	
	ΔU37	ND	
	U37A	2.1	0.95
	U37A/C38G	ND	

ND indicates "not determined"

some regions of pre-miR-125a U1G/G58C mutant were replaced from pre-miR-21 U1G mutant to prepare m1 and m3 mutant, and the region of pre-miR-125a which is same as hsa-pre-miR-21 was substituted with other bases to prepare m2 mutant (Fig. 2.8a). Mutant m1 and m2 had comparable K_D value but the binding affinity of

m3 was too low to be determined (Table 2.4). In addition, mutations at three base pairs next to the terminal loop did not change the binding affinity (mutants m4, m5 and m6 in Fig. 2.8a). These results indicate that terminal loop region of hsa-pre-miR-125a is important for binding to folic acid. Therefore binding affinity of several mutants whose bases of terminal loop region were substituted with other bases was measured (Fig. 2.8a). Mutants m10 and A33U showed almost the same binding affinity (K_D = 4.2 μM and 3 μM each in Table 2.4) as that of wild-type, but the K_D values of other mutants (m7, m8, m9, G34A, G35A and G36A) increased about 10 to 20 times more than that of wild-type (Fig. 2.8 and Table 2.4).

For further analysis, several point mutants, deletion mutants, and insertion mutants were prepared and divided into four groups (Fig. 2.8b). Each mutant in group 1 has a point mutation in the terminal loop to disrupt a structure of terminal loop. Mutants G25A, G26C, and C32G showed decreased binding affinity but C31G had the comparable binding affinity (K_D = 6.0 μM in Fig. 2.8b and Table 2.4), which indicate that G24, G26, and C32 are important but the predicted structure of terminal loop is not essential for binding. Next, the other bases than important bases for binding observed in mutation study of group 1 were deleted or a base was inserted (mutants in group 2 in Fig. 2.8b). All these mutants except for ΔA27/C28/A29/U30/C31 mutant showed the comparable K_D values, which indicates that minimal length of terminal loop necessary for binding is 8 nt. Both mutants ΔA33 and G34.5 (group 3 in Fig. 2.8b) had weak binding affinity (K_D = 11 and 48 μM each in Table 2.4). This result and the fact that A33U showed the comparable affinity (Table 2.4) indicate that one nucleotide between C32 and G34 is important but the variety of bases is not. Deletion of a base at the next to the terminal loop (ΔA24 and ΔU37 in group 4) and two mispairs next to the terminal loop (U37A/C38G in group 4) abolished the binding affinity but one mispair at the same position (U37A in group 4) did not show the decrease of binding affinity.

From mutation study described above, the essential motif for binding to folic acid was shown in Fig. 2.9. This motif was not observed in other RNAs obtained by SELEX (Table 2.2). By searching the structure of human miRNA precursors registered in the database of microRNA (miRbase (Kozomara and Griffiths-Jones 2014), http://www.mirbase.org/), this motif was discovered only in miR-125a precursors.

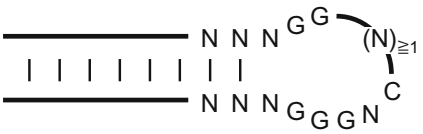

Fig. 2.9 Essential motif for binding to folic acid. N means A/U/G/C. *Black bold lines* indicate RNA and *lines* between bases denote base pairs

2.3 Conclusion

In this chapter, novel interaction between hsa-pre-miR-125a and folic acid was identified using small RNA transcriptomic SELEX. Because the abundance of miRNA precursors including both pri- and pre-miRNAs is too low, the methods to discover the interaction of these RNAs and small molecules have been limited. This small RNA transcriptomic SELEX has great potential to identify the interaction of low abundant small RNAs. In addition, new RNA motif for binding to folic acid was discovered, which is difficult to be discovered by computational analysis.

However, there are still some problems in this method. The first problem is the influence of the ligation of adaptor sequences. Because these adaptors may dramatically change the structure of natural transcript, the potential RNA aptamer elements whose structures are changed by adaptors cannot be identified by this method. To overcome this problem, it is necessary to cleavage the adaptors after PCR or transcription and re-ligate these before RT-PCR (reverse transcription-PCR), or perform multiple selections using different adaptor sets. Second problem is diversity of transcripts extracted from cells. The expression levels of RNAs are different among the cells and conditions of culture. To include all RNAs transcribed in various cells, it is necessary to extract RNAs from various cells or organs including both cancer cells and normal cells.

Analysis of binding affinity of hsa-pre-miR-125a revealed the essential motif in the terminal loop region for binding to folic acid. Because this loop region is common in both pri- and pre-miR-125a, folic acid binds to both precursors. In addition, this motif is conserved in mammalian (Fig. 2.10). It was reported the terminal loop region controls miRNA processing by Drosha and Dicer (Zhang and Zeng 2010). In addition, several regions of pri-miRNAs are important for processing by Dorhsa, apical stem and terminal loop elements of pri-miR-125a have

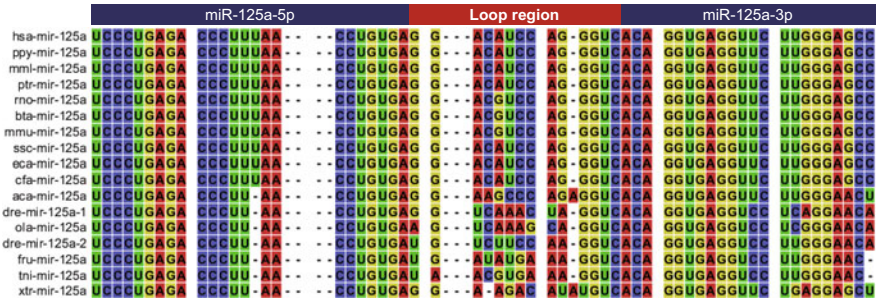

Fig. 2.10 The sequences of pre-miR-125a of various species. These sequences were from miRbase (Kozomara and Griffiths-Jones 2014) (http://www.mirbase.org/). The abbreviations indicate as follows, Hsa: *Homo sapiens*, ppy: *Pongo pygmaeus*, mml: *Macaca mulatta*, ptr: *Pan troglodytes*, rno: *Rattus norvegicus*, bta: *Bos taurus*, mmu: *Mus musculus*, ssc: *Sus scrofa*, eca: *Equus caballus*, cfa: *Canis familiaris*, aca: *Anolis carolinensis*, dre: *Danio rerio*, ola: *Oryzias latipes*, fru: *Fugu rubripes*, tni: *Tetraodon nigroviridis*, xtr: *Xenopus tropicalis*

significant contributions on processing by Drosha (Auyeung et al. 2013). These reports suggested the potential of folic acid binding to terminal loop of miR-125a precursors to regulate the processing. Nonetheless, further studies are necessary to elucidate whether the interaction between folic acid and miR-125a precursors regulate the processing.

In conclusion, I here described the small RNA transcriptomic SELEX methodology, which enabled us to uncover potential aptamers against a metabolite, folic acid in this report, from a pool of small RNAs in a length range of 10–200 nt. This method is certainly extendable to other metabolites as well as various intracellular macromolecules such as proteins for the discovery of potential aptamers derived from naturally occurring small RNAs, including pre- and pri-miRNAs.

2.4 Materials and Methods

Chemical synthesis of α-*N*-biotinyl-phenylalanine cyanomethyl ester (Biotin-Phe-CME)

Biotin-Phe-CME was synthesized as previously described (Saito et al. 2001).

Preparation of RNAs

The Fx3 + N1 and RNAs for BLI analysis were prepared using runoff in vitro transcription with T7 RNA polymerase and purified by denaturing PAGE (8 M Urea, 1 × TBE, %T = 8 and %C = 5). The primers for preparing transcription templates and RNAs are shown in Tables 2.5 and 2.6. All primers were purchased from Eurofins Genomics K.K. (Japan). FA1, FA2, and hsa-pre-miR-125a RNAs were purchased from Gene Design Inc. (Japan). The concentrations of RNAs were determined by absorbance at 260 nm.

Preparation of dial-Fx3

In order to avoid the amplification of Fx3 during the construction of small RNA library, 3′-end of Fx3 + N1 purified by denaturing PAGE was oxidized to dialdehyde group (named as dial-Fx3 + N1) under the following conditions; 20 μM Fx3 + N1 was incubated with 30 mM $NaIO_4$ on ice for 20 min and then 2% (w/v) $LiClO_4$ in acetone was added. The mixture was centrifuged at 15,000 × *g* (room temperature) for 10 min to precipitate dial-Fx3 + N1. The pellet was rinsed with acetone and centrifuged at 15,000 × *g* (room temperature) for 3 min two times, and dissolved in water. The concentrations of RNAs were determined by absorbance at 260 nm.

Cell cultures for construction of small RNA library

BeWo cells (RIKEN Cell Bank, code RCB1644) were grown in Ham's F-12 medium (17458-65, Nacalai Tesque Inc.) supplemented with 100 unit/mL penicillin, 100 μg/mL streptomycin (15140-122, Thermo Fisher Scientific Inc.) and

Table 2.5 Primers and adaptors used in this chapter

Name	Sequence (5′ to 3′)	Remarks
Primer Fx3-1	TAATACGACTCACTATAGGATCGAAAGATTTCCG CAGGCC	Transcription of Fx3 + N1
Primer Fx3-2	NACCTAACGCCAATACCCTTTCGGGCCTGCGGAA ATCTTT	Transcription of Fx3 + N1
Primer Fx3-3	GGCGTAATACGACTCACTATAGG	Transcription of Fx3 + N1
primer Fx3-4	NACCTAACGCCAATACCCTT	Transcription of Fx3 + N1
Primer seq-1	GCCTCTTCGCTATTACGCCAGC	Sequencing
Primer seq-2	TGTTGTGTGGAATTGTGAGCGG	Sequencing
3′-adaptor	*A*pp-TGAAGAGCTGCTACTATC-ddC	Construction of the cDNA library
5′-adaptor	*GGCGUAAUACGACUCACUAUAGGGAGCUAUCGACGUAAC*	Construction of the cDNA library
Primer sele-1	GGCGTAATACGACTCACTATAGGGAGCTATCGACGTAAC	Construction of the cDNA library and selection
Primer sele-2	GATAGTAGCAGCTCTTCA	Construction of the cDNA library and selection
Primer FA1 + Adp-1	GGGAGCTATCGACGTAACTTTTATGGCGTCAGCGA AGGGTTG	Transcription of FA1 + Adp
Primer FA1 + Adp-2	GGTTTTTTTGGTTTTTACGGGCTACTACAACCCTTC GCTGACGCC	Transcription of FA1 + Adp
Primer FA1 + Adp-3	GATAGTAGCAGCTCTTCAGGTTTTTTTGGTTTTTA CGGGCTAC	Transcription of FA1 + Adp
Primer FA2 + Adp-1	GGGAGCTATCGACGTAACGCATTGGTGGTTCAGTG GTAGAATTC	Transcription of FA2 + Adp
Primer FA2 + Adp-2	ACCCGGGCCTCCCGCGTGGCAGGCGAGAATTCTACC ACTGAACCACC	Transcription of FA2 + Adp
Primer FA2 + Adp-3	GATAGTAGCAGCTCTTCAACCCGGGCCTCCCGC	Transcription of FA2 + Adp

(continued)

Table 2.5 (continued)

Name	Sequence (5′ to 3′)	Remarks
Primer hsa-pre-miR-125a + Adp-1	GGGAGCTATCGACGTAACTCCCTGAGACCCTTTAACCTG	Transcription of hsa-pre-miR-125a + Adp
Primer hsa-pre-miR-125a + Adp-2	GGCTCCCAAGAACCTCACCTGTGACCCTGGATGTCCTCAC	Transcription of hsa-pre-miR-125a + Adp
Primer hsa-pre-miR-125a + Adp-3	GATAGTAGCAGCTCTTCAGGCTCCCAAGAACCTCACC	Transcription of hsa-pre-miR-125a + Adp
Primer T7 + Adp	GGCGTAATACGACTCACTATAGGGAGCTATCGACGTAAC	Transcription of FA1/FA2/hsa-pre-miR-125a + Adp
Primer T7	GGCGTAATACGACTCACTATAG	Transcription of pre-miRNAs
pre-miRNA template 1	ACAGCCCATCGACTGGTGTTGCCATGAGATTCAACAGTCAACAT CAGTCTGATAAGCTCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 2	GGGCTCCAAGAACCTCACCTGTGACCCTGGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 3	ACAGCCCATCGACTGGTGTTGTGACCCTGGATGTCCTCACAACAT CAGTCTGATAAGCTCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 4	GGGCTCCAAGAACCTCACCACTGACCCTGGATGTCCTCAGTGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 5	GGGTCCCAAGAACCTCACCTGCCATGAGATTCAACAGTCAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 6	GGGCTCCAAGAACCTCACCTGGGACCCTGGATGTCCTCCCAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 7	GGGCTCCAAGAACCTCACCTGTCACCCTGGATGTCCTGACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 8	GGGCTCCAAGAACCTCACCTGTGTCCCTGGATGTCCACACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs

(continued)

Table 2.5 (continued)

Name	Sequence (5′ to 3′)	Remarks
pre-miRNA template 9	GGGCTCCAAGAACCTCACCTGTGACCCTAGATGTCCTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 10	GGGCTCCAAGAACCTCACCTGTGACCCTAGATGTCTTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 11	GGGCTCCAAGAACCTCACCTGTGACCCTGCATGTGCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 12	GGGCTCCAAGAACCTCACCTGTGACCCTGGTACACCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 13	GGGCTCCAAGAACCTCACCTGTGACCCAGGATGTCCTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 14	GGGCTCCAAGAACCTCACCTGTGACCTTGGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 15	GGGCTCCAAGAACCTCACCTGTGACTCTGGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 16	GGGCTCCAAGAACCTCACCTGTGATCCTGGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 17	GGGCTCCAAGAACCTCACCTGTGACCCTGGATGTCTTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 18	GGGCTCCAAGAACCTCACCTGTGACCCTGGATGTGCTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 19	GGGCTCCAAGAACCTCACCTGTGACCCTGCATGTCCTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 20	GGGCTCCAAGAACCTCACCTGTGACCCTCGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 21	GGGCTCCAAGAACCTCACCTGTGACCCTGGATCGTCCTCACAGG TTAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs

(continued)

Table 2.5 (continued)

Name	Sequence (5′ to 3′)	Remarks
pre-miRNA template 22	GGGCTCCAAGAACCTCACCTGTGACCCTGGATTCCTCACAGGTTA AAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 23	GGGCTCCAAGAACCTCACCTGTGACCCTGGATCCTCACAGGTTAA AGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 24	GGGCTCCAAGAACCTCACCTGTGACCCTGGTCCTCACAGGTTAAAG GGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 25	GGGCTCCAAGAACCTCACCTGTGACCCTGGCCTCACAGGTTAAAG GGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 26	GGGCTCCAAGAACCTCACCTGTGACCCTGCCTCACAGGTTAAAGG GTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 27	GGGCTCCAAGAACCTCACCTGTGACCCGGATGTCCTCACAGGTTA AAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 28	GGGCTCCAAGAACCTCACCTGTGACCCTTGGATGTCCTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 29	GGGCTCCAAGAACCTCACCTGTGACCCTGGATGTCCCACAGGTTA AAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 30	GGGCTCCAAGAACCTCACCTGTGCCCTGGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 31	GGGCTCCAAGAACCTCACCTGTGTCCCTGGATGTCCTCACAGGT TAAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs
pre-miRNA template 32	GGGCTCCAAGAACCTCACCTGTCTCCCTGGATGTCCTCACAGGTT AAAGGGTCTCAGGGCTATAGTGAGTCGTATTACGCC	Transcription of pre-miRNAs

N means A/T/G/C, italic types indicate ribonucleotides, ddC indicates dideoxyribocytidine

Table 2.6 RNAs used in this chapter

Name	Sequence (5′ to 3′)
Fx3 + N1	*GGAUCGAAAGAUUUCCGCAGGCCCGAAAGGGUAUUGG CGUUAGGUN*
FA1 + Adp	*GGGAGCUAUCGACGUAACUUUUAUGGC GUCAGCGAAGGGUUGUAGUAGCCCGUAAAAACCAAAAAAA CCUGAAGAGCUGCUACUAUC*
FA2 + Adp	*GGGAGCUAUCGACGUAACGCAUUGGUG GUUCAGUGGUAGAAUUCUCGCCUGCCACGCGGGAGGCCC GGGUUGAAGAGCUGCUACUAUC*
FA1	*UUUUAUGGCGUCAGCGAAGGGUUGUA GUAGCCCGUAAAAACCAAAAAAACC*
FA2	*GCAUUGGUGGUUCAGUGGUAGAAUUC UCGCCUGCCACGCGGGAGGCCCGGGU*
hsa-pre-miR-21G U1G	*GAGCUUAUCAGACUGAUGUUGACUGU UGAAUCUCAUGGCAACACCAGUCGAUGGGCUGU*
hsa-pre-miR-125a	*UCCCUGAGACCCUUUAACCUGUGAGG ACAUCCAGGGUCACAGGUGAGGUUCUUGGGAGCC*
hsa-pre-miR-125a + Adp	*GGGAGCUAUCGACGUAACUCCCUGAGACCCUUUAACCU GUGAGGACAUCCAGGGUCACAGGUGAGGUUCUUGGGAGC CUGAAGAGCUGCUACUAUC*
hsa-pre-miR-125a U1G/G58C	*GCCCUGAGACCCUUUAACCUGUGAGG ACAUCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
m1	*GAGCUUAUCAGACUGAUGUUGUGAGG ACAUCCAGGGUCACAACACCAGUCGAUGGGCUGU*
m2	*GCCCUGAGACCCUUUAACCACUGAGG ACAUCCAGGGUCAGUGGUGAGGUUCUUGGAGCCC*
m3	*GCCCUGAGACCCUUUAACCUGACUGU UGAAUCUCAUGGCAGGUGAGGUUCUUGGGACCC*
m4	*GCCCUGAGACCCUUUAACCUGGGAGG ACAUCCAGGGUCCCAGGUGAGGUUCUUGGAGCCC*
m5	*GCCCUGAGACCCUUUAACCUGUCAGG ACAUCCAGGGUGACAGGUGAGGUUCUUGGAGCCC*
m6	*GCCCUGAGACCCUUUAACCUGUGUGG ACAUCCAGGGACACAGGUGAGGUUCUUGGAGCCC*
m7	*GCCCUGAGACCCUUUAACCUGUGAGGACAUCUAGGG UCACAGGUGAGGUUCUUGGAGCCC*
m8	*GCCCUGAGACCCUUUAACCUGUGAAGA CAUCUAGGGUCACAGGUGAGGUUCUUGGAGCCC*
m9	*GCCCUGAGACCCUUUAACCUGUGAGC ACAUGCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
m10	*GCCCUGAGACCCUUUAACCUGUGAGG UGUACCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
A33U	*GCCCUGAGACCCUUUAACCUGUGAGG ACAUCCUGGGUCACAGGUGAGGUUCUUGGAGCCC*
G34A	*GCCCUGAGACCCUUUAACCUGUGAGG ACAUCCAAGGUCACAGGUGAGGUUCUUGGAGCCC*

(continued)

Table 2.6 (continued)

Name	Sequence (5′ to 3′)
G35A	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCCAGAGUCACAGGUGAGGUUCUUGGAGCCC*
G36A	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCCAGGAUCACAGGUGAGGUUCUUGGAGCCC*
G25A	*GCCCUGAGACCCUUUAACCUGUGAAGA CAUCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
G26C	*GCCCUGAGACCCUUUAACCUGUGAGCA CAUCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
C31G	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUGCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
C32G	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCGAGGGUCACAGGUGAGGUUCUUGGAGCCC*
G28.5	*GCCCUGAGACCCUUUAACCUGUGAGGA CGAUCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔC28	*GCCCUGAGACCCUUUAACCUGUGAGGA AUCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔC28/A29	*GCCCUGAGACCCUUUAACCUGUGAGGA UCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔC28/A29/U30	*GCCCUGAGACCCUUUAACCUGUGAGGA CCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔA27/C28/A29/U30	*GCCCUGAGACCCUUUAACCUGUGAGGC CAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔA27/C28/A29/U30/C31	*GCCCUGAGACCCUUUAACCUGUGAGGC AGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔA33	*GCCCUGAGACCCUUUAACCUGUGAGGACAUCCGGGUC ACAGGUGAGGUUCUUGGAGCCC*
A33.5	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCCAAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔA24	*GCCCUGAGACCCUUUAACCUGUGGGAC AUCCAGGGUCACAGGUGAGGUUCUUGGAGCCC*
ΔU37	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCCAGGGCACAGGUGAGGUUCUUGGAGCCC*
U37A	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCCAGGGACACAGGUGAGGUUCUUGGAGCCC*
U37A/C38G	*GCCCUGAGACCCUUUAACCUGUGAGGA CAUCCAGGGAGACAGGUGAGGUUCUUGGAGCCC*

N means A/U/G/C, italic types indicate ribonucleotides

10% fetal bovine serum (10437-010, Thermo Fisher Scientific Inc.) in an incubator with 5% CO_2 at 37 °C. HuH-7, VMRC-RCW, NEC14, HuO-3N1, and RAJI Cells (RIKEN Cell Bank, code RCB1942, RCB1963, RCB0490, RCB2104, RCB1647) were grown in RPMI 1640 medium (30264-85, Nacalai Tesque Inc.) supplemented with 100 unit/mL penicillin, 100 μg/mL streptomycin (15140-122, Thermo Fisher Scientific Inc.) and 10% fetal bovine serum (10437-010, Thermo Fisher Scientific

Inc.) in an incubator with 5% CO_2 at 37 °C. MCF7 cells (RIKEN Cell Bank, code RCB1940) were grown in MEM (21442-25, Nacalai Tesque Inc.) supplemented with 100 unit/mL penicillin, 100 μg/mL streptomycin (15140-122, Thermo Fisher Scientific Inc.), 0.1 mM nonessential amino acids (M71455, Sigma-Aldrich Co. LLC.), 1 mM sodium pyruvate (S8636, Sigma-Aldrich Co. LLC.), and 10% fetal bovine serum (10437-010, Thermo Fisher Scientific Inc.) in an incubator with 5% CO_2 at 37 °C. HeLa S3 cells (JCRB Cell Bank, JCRB0713) were grown in DMEM (08458-45, Nacalai Tesque Inc.) supplemented with 100 unit/mL penicillin, 100 μg/mL streptomycin (15140-122, Thermo Fisher Scientific Inc.) and 10% fetal bovine serum (10437-010, Thermo Fisher Scientific Inc.) in an incubator with 5% CO_2 at 37 °C.

Construction of the rRNA-/tRNA-depleted human small RNA library

Once cells arrived to about 20% of confluence, the media were removed and ISOGEN II (311-07361, NIPPON GENE) was added to dissolve cells. Small RNAs (10-200 nt) were isolated according to the attached protocol. The concentrations were determined by absorbance at 260 nm.

Small RNAs extracted from each cell lines were mixed with an equal ratio and 10 μg small RNAs were treated with Ribominus^{TM} Eykaryote kit (A10837-08, Thermo Fisher Scientific Inc.) according to the attached protocol. Ribosomal RNA-depleted small RNAs were separated to 10–60 nt fraction and 60–200 nt fraction by denaturing PAGE (8 M Urea, 1 × TBE, %T = 8 and %C = 5). tRNAs in the 60–200 nt fraction of small RNAs were removed under the following conditions; a mixture of 48 μL of 73.3 ng/μL 60–200 nt fraction of small RNAs and 33.3 μM dial-Fx3 + N1 in 166.7 mM HEPES-KOH (pH 7.5) was heated at 95 °C for 2 min and cooled to room temperature over 5 min. Sixteen μL of 3 M $MgCl_2$ was added, and the mixture was transferred to an ice bath, 16 μL of 25 mM Biotin-Phe-CME in DMSO was added, and the mixture was incubated on ice for 2 h. After 2 h incubation, 8 μL of 3 M NaCl and 220 μL of ethanol were added, and then precipitated. To capture biotin-Phe-tRNAs, the RNA was dissolved with 120 μL SAv binding buffer (5 mM HEPES-KOH (pH 7.5), 500 μM EDTA, 1 M NaCl and 0.1% tween-20) and then mixed with 1.2 mg Dynabeads M-280 Streptavidin (11205D, Thermo Fisher Scientific Inc.) which were prewashed with SAv wash buffer (5 mM Tris-HCl (pH 7.5), 500 μM EDTA and 1 M NaCl) three times before use, SAv solution A (0.1 M NaOH and 50 mM NaCl) two times and 0.1 M NaCl one time. After 15 min incubation using rotary shaker at room temperature, supernatant was recovered and precipitated with ethanol. For the second aminoacylation, the RNA was dissolved in 32 μL of 125 mM HEPES-KOH (pH 7.5) with 0.75 M $MgCl_2$ and incubated at room temperature for 5 min. The mixture was transferred to an ice bath, 8 μL of 25 mM Biotin-Phe-CME was added. After 2 h incubation on ice, 48 μL of 500 mM NaCl and 220 μL of ethanol were added. RNAs were precipitated and dissolved in 80 μL SAv binding buffer. To capture biotin-Phe-tRNAs, the same procedure described above was performed using 800 μg beads. Supernatant was precipitated with ethanol and dissolved in 16 μL of 125 mM HEPES-KOH (pH 7.5) with 0.75 M $MgCl_2$ and incubated at room

temperature for 5 min. The mixture was transferred to an ice bath, 4 μL of 25 mM Biotin-Phe-CME was added. After 2 h incubation on ice, 68 μL of 500 mM NaCl and 220 μL of ethanol were added. RNAs were precipitated and dissolved in 40 μL SAv binding buffer. To capture biotin-Phe-tRNAs, the same procedure described above was performed using 400 μg beads. Supernatant was precipitated with ethanol and dissolved in water. The concentrations were determined by absorbance at 260 nm.

A dial-Fx3 + N1 in the tRNA-depleted small RNA fraction was removed by denaturing PAGE (8 M Urea, 1 × TBE, %T = 8 and %C = 5). All amount of rRNA-/tRNA-depleted small RNA fraction and 10–60 nt fraction were mixed and used for preparation of cDNA library.

Construction of the cDNA library from rRNA-/tRNA-depleted human small RNA library

The 3′-adaptor (purchased from Integrated DNA Technologies) and 5′-adaptor (purchased from Japan Bio Service) were purified by denaturing PAGE (8 M Urea, 1 × TBE, %T = 20 and %C = 5) before use. For ligation of 3′-adaptor, 6.67 μL mixture of 70 ng/μL rRNA-/tRNA-depleted human small RNA library, 15 μM 3′-adaptor, 25% (v/v) PEG8000, 40 U/μL T4 RNA ligase 2 truncated (M0242S, New England BioLabs), and 1 × reaction buffer attached with T4 RNA ligase 2 truncated was incubated at 16 °C for 12 h. After the incubation, RNAs were extracted with phenol–chloroform mixture and precipitated with ethanol. Nonreacted 3′-adaptor was removed by denaturing PAGE (8 M Urea, 1 × TBE, %T = 8 and %C = 5). RNA library ligated with 3′-adaptor was treated with $NaIO_4$ according to protocol described above, in order to avoid the ligation of contaminated RNA library not ligated with 3′-adaptor in the next step. For ligation of 5′-adaptor, 10 μL mixture of all amount of RNA library ligated with 3′-adaptor, 20 μM 5′-adaptor, 15% (v/v) PEG8000, 30 U/μL T4 RNA ligase 1 (M0204S, New England BioLabs), and 1 × reaction buffer attached with T4 RNA ligase 1 was incubated at 37 °C for 30 min and then at 16 °C for 12 h. After the incubation, RNAs were extracted with phenol–chloroform mixture and precipitated with ethanol. Nonreacted 5′-adaptor was removed by denaturing PAGE (8 M Urea, 1 × TBE, %T = 8 and %C = 5). RNA library ligated with 3′/5′-adaptors was converted to cDNA by reverse transcription and PCR using primers sele-1 and sele-2 and PrimeScript™ One Step RT-PCR Kit Ver.2 (RR055A, TaKaRa), and cDNA was purified by Native-PAGE (1 × TBE, %T = 8 and %C = 5).

Immobilization of folic acid onto the magnetic beads

Magnetic beads whose surface was modified with hydroxyl group (FG-beads, TAS8848 N1120, Tamagawa Seiki) were used for immobilization of folic acid. FG-beads were suspended in *N*,*N*-dimethylformamide (DMF, 10344-00, KANTO CHEMICAL) at 5 μg/μL and washed with DMF three times. FG-beads (5 μg/μL) were incubated at 25 °C for 24 h with 10 mM folic acid (16221-91, Nacalai Tesque

Inc.) in DMF in the presence of 10 mM EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 15022-86, Nacalai Tesque Inc.) and 10% (v/v) triethylamine (202-02641, WAKO). Thereafter, the folic acid-immobilized beads were washed with DMF three times and suspended in DMF at 10 μg/μL.

Small RNA transcriptomic SELEX

cDNA library was transcribed to RNA library by runoff in vitro transcription with T7 RNA polymerase and purified by denaturing PAGE (8 M Urea, 1× TBE, %T = 8 and %C = 5). A 10 μL of 510 ng/μL RNA library and 1× selection buffer (50 mM HEPES-KOH (pH 7.5), 300 mM KCl, 5 mM $MgCl_2$ and 0.05% tween-20) was mixed with of FG-beads whose surface is modified with hydroxyl group which prewashed by 1× selection buffer three times before use, and this mixture was incubated using rotary shaker at 4 °C for 30 min (referred to as "pre-clear" process). After the incubation, the supernatant was added to folic acid-immobilized beads which were washed by 1× selection buffer three times before use, and this mixture was incubated using rotary shaker at 4 °C for 1 h. After the incubation, the supernatant was discarded and the beads were washed with 100 μL of 1× selection buffer (referred to as "wash" process). The beads were suspended in 1 mM folic acid in 1× selection buffer and incubated using rotary shaker at 4 °C for 30 min to elute the RNAs binding to folic acid-immobilized beads. The supernatant was recovered and this elution cycle repeated three times. All of the supernatants were mixed and precipitated with ethanol. The pellet was dissolved in 10 μL of water.

To quantify the amount of recovered RNAs from beads, quantitative RT-PCR was performed as follows: 0.5% of recovered RNAs were mixed with 10 μL of RT mix (500 μM dNTP, 5 μM primer sele-2, 8 U/μL M-MLV Reverse Transcriptase (M1701, Promega K.K.) and 1× reaction buffer attached with reverse transcriptase), and this mixture was incubated at 50 °C for 1 h. After the incubation, 1 μL of reverse transcription reaction solution was mixed with 19 μL of qPCR mix (5 μM primer sele-1, 5 μM primer sele-2 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton-X100, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 1/1,000,000 SYBR® Green I (50512, LONZA), Taq DNA polymerase) and qPCR was performed using LightCycler® 1.5 (Roche Diagnostics K.K.) at 94 °C (20 °C/sec) for 1 min, followed by 35 cycles of 94 °C for 10 s (20 °C/s), 51 °C for 10 s (20 °C/s), and 72 °C for 30 s (0.5 °C/s) to measure C_p values.

The all amount of recovered RNAs not used for qPCR was mixed with RT mix and this mixture was incubated at 50 °C for 1 h. After the incubation, all of the reverse transcription reaction solution was mixed with 200 μL of qPCR mix without SYBR® Green I and DNA was amplified by PCR by C_p + 2 cycle of 94 °C for 10 s, 51 °C for 10 s, and 72 °C for 30 s. DNA was extracted with phenol–chloroform mixture and precipitated with ethanol, and then used for next round of selection.

For the first round of selection, preclear process was not performed, wash process was performed one time and 200 μg beads were used. For the second round of

selection, preclear process was performed one time, wash process was performed one time and 100 μg beads were used. For the third round of selection, preclear process was performed one time, wash process was repeated three times, and 100 μg beads were used. For the fourth round of selection, preclear process was performed one time, wash process was repeated three times and 20 μg beads were used.

Sequencing

The cDNAs of tRNA-depleted human small RNA library and RNAs after SELEX were TA cloned into pGEM®-T Easy Vectors (A1360, Promega K.K.). These vectors were used to transform *E. coli* DH5α cells in LB medium with 100 μg/mL. Inserted cDNAs in the vectors were amplified by colony-PCR using primers seq-1 and seq-2, and sequenced by FASMAC Co., Ltd. (Japan).

To annotate the sequenced RNAs, BLAST search (Altschul et al. 1990) using NCBI human genomic plus transcript database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), fRNA database (Mituyama et al. 2009) (http://www.ncrna.org/frnadb/), genomic tRNA database (Chan and Lowe 2009) (http://gtrnadb.ucsc.edu/), snoRNABase (Lestrade and Weber 2006) (https://www-snorna.biotoul.fr/) and miRbase (Kozomara and Griffiths-Jones 2014) (http://www.mirbase.org/) was performed.

Biolayer interferometry (BLI)

Binding of RNAs to folic acid was measured using the Octet RED96 biolayer interferometry (BLI) instrument (ForteBio, Inc.) and all measurement were conducted at 30 °C and agitation rate is 1000 rpm.

Folic acid was immobilized onto the amine reactive second-generation (AR2G) biosensor from ForteBio as follows; each AR2G sensor was prewet for more than 10 min in water prior to use. Each AR2G sensor was equilibrated in water for 3 min, then activated with EDC/NHS solution (200 mM EDC and 50 mM *N*-hydroxysuccinimide (NHS, A00013, Watanabe Chemical Industries) in water) for 10 min. The activated sensors was modified with amine group by incubating with EDA solution [100 mM ethylenediamine (15020-22, Nacalai Tesque Inc.), 10 mM boric acid buffer (pH 8.5) and 1 M NaCl] for 150 s, then quenched with 1 M ethanolamine (E6133, Sigma-Aldrich Co. LLC., pH 8.5 adjusted by KOH) for 3 min. The amine group-immobilized sensors were equilibrated with 250 mM phosphate buffer (pH 7.0) for 3 min and then folic acid was immobilized by incubating with FA/EDC/NHS/Phos solution [20 mM folic acid, 200 mM EDC, 50 mM NHS and 250 mM phosphate buffer (pH 7.0)] for 20 min. The folic acid-immobilized sensors were incubated with water for 3 min. Reference sensors for the binding analysis were prepared following the same protocols described above using EDC/NHS/Phos solution [200 mM EDC, 50 mM NHS and 250 mM phosphate buffer (pH 7.0)] instead of FA/EDC/NHS/Phos solution.

Binding of RNAs to folic acid was measured as follows: folic acid-immobilized sensors or reference sensors were prewet for more than 10 min in Octet buffer

(50 mM HEPES-KOH (pH 7.5), 300 mM KCl, 50 mM $MgCl_2$, 0.05% tween-20) prior to use. The sensors were equilibrated in Octet buffer for 3 min, then incubated in Octet buffer for 1 min to generate baseline. As an association step, the sensors were incubated in RNA dissolved in Octet buffer. After an association step, the sensors were incubated in Octet buffer as a dissociation step. The sensors were regenerated by incubating in R buffer (20 mM EDTA and 0.05% tween-20) for 10 s 5 times. The ForteBio Data Analysis Octet software was used to perform steady-state analysis. For each RNA, six to eight concentrations were used, from 200 nM to 240 μM (depending on the affinity of each RNAs).

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http://www.springer.com/978-4-431-56515-4

Applications of Aminoacylation Ribozymes That Recognize the 3′-end of tRNA

Terasaka, N.

2017, XII, 89 p. 38 illus., 28 illus. in color., Hardcover
ISBN: 978-4-431-56515-4