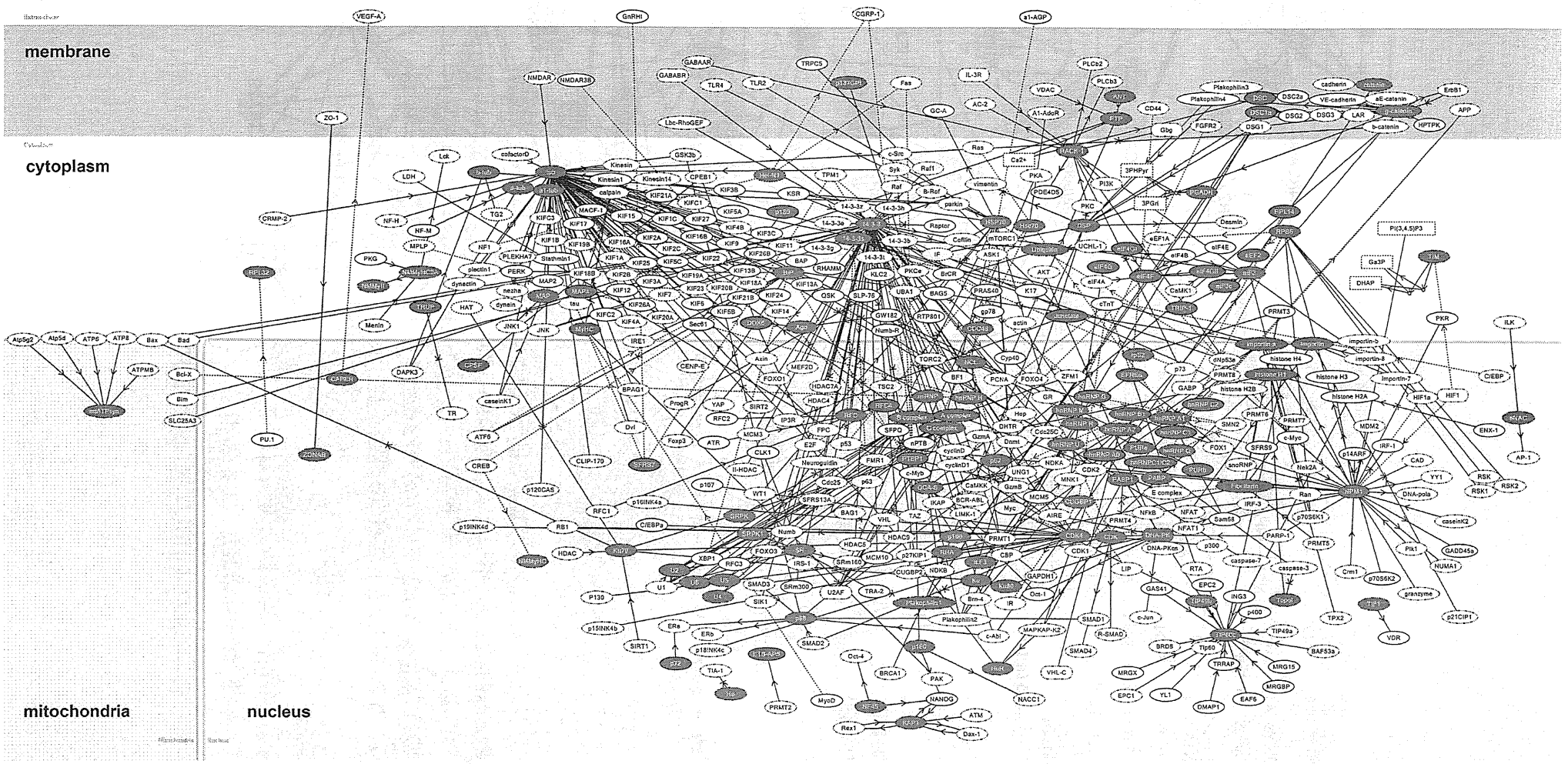
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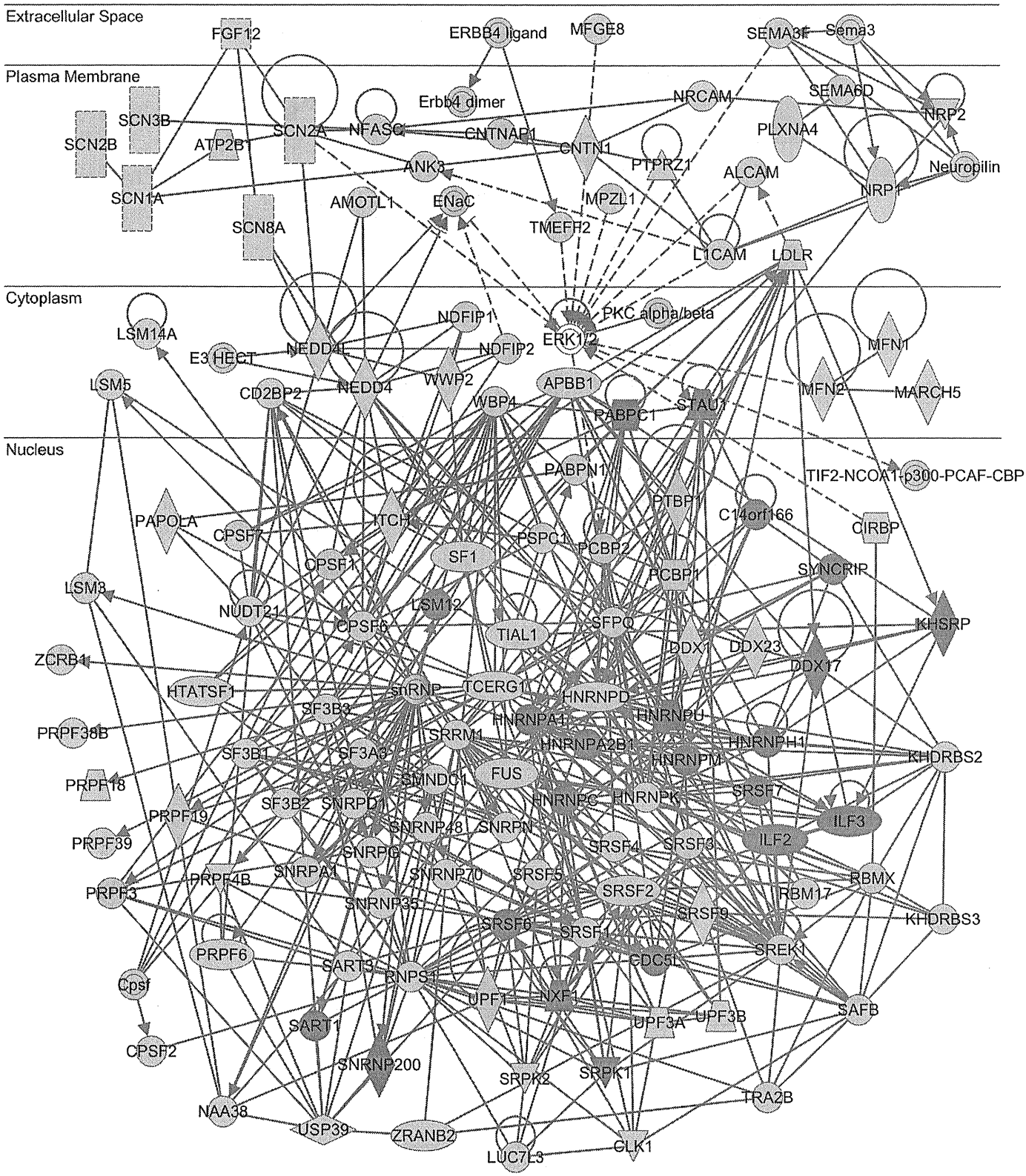
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ENaC
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ERK1/2
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SRPK2
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ZRANB2
CLK1
LUC7L3

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PART I Systems Biology in Cancer

Title: Human MicroRNA Targetome Indicates a Specialized Role of MicroRNAs in Regulation of Oncogenesis

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targetome

ZEB

Abstract

MicroRNAs (miRNAs), a class of endogenous small noncoding RNAs, mediate posttranscriptional regulation of protein-coding genes by binding to the 3' untranslated region of target mRNAs, leading to translational inhibition, mRNA destabilization or degradation. A single miRNA concurrently downregulates hundreds of target mRNAs, and thereby fine-tunes gene expression involved in diverse cellular functions, such as development, differentiation, proliferation, apoptosis and metabolism. However, it remains unknown whether the set of miRNA target genes designated "targetome" regulated by an individual miRNA constitutes the biological network of functionally-associated molecules or reflects a random set of functionally-independent genes. To address this question, we studied the molecular network of the whole human miRNA targetome. Among 1,223 human miRNAs derived from miRbase Release 16, Diana-microT 3.0, a target prediction program, predicted reliable targets from 273 miRNAs. Among them, KeyMolnet, a bioinformatics tool for analyzing molecular interactions on the comprehensive knowledgebase, successfully extracted molecular networks from 232 miRNAs. In miRNA targetome networks, the most relevant pathway was transcriptional regulation by RB/E2F, important regulators of oncogenic transformation, the disease was adult T cell lymphoma/leukemia, and the pathological event was cancer, indicating that the human miRNA system termed "miRNAome" plays a specialized role in regulation of oncogenesis. The predicted targets derived from approximately 20% of all human miRNAs construct biologically meaningful molecular networks, supporting the view that the miRNA targetome generally constitutes the biological network of functionally-associated molecules in human cells.

Contents

Abbreviations

EMT	Epithelial-Mesenchymal Transition
HPRD	Human Protein Reference Database
IPA	Ingenuity Pathways Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
miTG	microRNA-Targeted Gene
MRE	MicroRNA Recognition Elements
PPI	Protein-Protein Interaction
RISC	RNA-Induced Silencing Complex
3'UTR	3' Untranslated Region

1 MicroRNAome and MicroRNA Targetome

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs conserved through the evolution. They mediate posttranscriptional regulation of protein-coding genes by binding to the 3' untranslated region (3'UTR) of target mRNAs, leading to translational inhibition, mRNA destabilization or degradation, depending on the degree of sequence complementarity (Guo et al. 2010). During the biogenesis of miRNAs, the pri-miRNAs are transcribed from the intra- and inter-genetic regions of the genome by RNA polymerase II, followed by processing by the RNase III enzyme Drosha into pre-miRNAs. After nuclear export, they are cleaved by the RNase III enzyme Dicer into mature miRNAs consisting of approximately 22 nucleotides. Finally, a single-stranded miRNA is loaded onto the Argonaute-containing RNA-induced silencing complex (RISC), where the seed sequence located at positions 2 to 8 from the 5' end of the miRNA serves as an essential scaffold for recognizing the target mRNA (Bartel 2009).

Currently, more than one thousand of human miRNAs are registered in the miRNA database named miRBase Release 17 (April 2011; www.mirbase.org). In general, the 3'UTR of a single mRNA is targeted by several different miRNAs, while a single miRNA at one time reduces the production of hundreds of target proteins that constitute "targetome" (Selbach et al. 2008). Such redundant interactions between miRNAs and their targets are responsible for the complexity of miRNA-regulated gene expression. Furthermore, certain miRNAs activate transcription and translation of the targets (Vasudevan et al. 2007; Place et al. 2008). Consequently, the whole human miRNA system termed "miRNAome" regulates greater than 60% of all protein-coding genes (Friedman et al. 2009). By targeting multiple transcripts and affecting expression of numerous proteins, miRNAs regulate diverse cellular functions, such as development, differentiation, proliferation, apoptosis and metabolism. Therefore, aberrant regulation of miRNA expression is deeply involved in pathological events that mediate cancers (Blenkiron and Miska 2007; Garzon et al. 2010) and neurodegenerative disorders (Shioya et al. 2010; Wang et al. 2011).

Recent advances in systems biology have made major breakthroughs by illustrating the cell-wide map of complex molecular interactions with the aid of the literature-based knowledgebase of molecular pathways (Viswanathan et al. 2008). The logically arranged molecular networks construct the whole system characterized by robustness, which maintains the proper function of the system in the face of genetic and environmental perturbations (Kitano 2007). In the scale-free molecular network, targeted disruption of limited numbers of critical components designated hubs, on which the biologically important molecular interactions concentrate, efficiently disturbs the whole cellular function by destabilizing the network (Albert et al. 2000). Therefore, the identification and characterization of hub molecules located in the center of the miRNA targetome network would help us to understand biological and pathological roles of individual miRNAs. A recent study determined the human miRNA-regulated protein-protein interaction (PPI) network by utilizing the Human Protein Reference Database (HPRD) and the miRNA target prediction program TargetScan (Hsu et al. 2008). They found that an individual miRNA often targets the hub gene of the PPI network, although they did not attempt to clarify functionally relevant pathways, diseases, and pathological events that play a central role in the miRNA targetome network.

At present, it remains unknown whether the miRNA targetome regulated by an individual miRNA generally constitutes the biological network of functionally-associated molecules or simply reflects a random set of functionally-independent genes. To address this question, we attempted to characterize the molecular network of the whole human miRNA targetome. We found that the miRNA targetome constitutes the biological network of functionally-associated molecules in human cells (Satoh and Tabunoki 2011). Furthermore, functional annotation of the miRNA targetome suggested that the human miRNAome plays a specialized role in regulation of oncogenesis. Importantly, we identified a collaborative regulation of gene expression by transcription factors and miRNAs in cancer-associated miRNA targetome networks.

2 Molecular Network of MicroRNA Targetome

2.1 MicroRNA Target Prediction Programs

First of all, we downloaded the complete list of 1,223 human miRNAs from miRBase Release 16 (September 2010; www.mirbase.org). In general, miRNAs regulate gene expression by forming energetically stable Watson-Crick base pairs with target mRNAs. In most occasions, the seed sequence conserved through evolution located at positions 2 to 8 from the 5' end of the miRNA serves as an essential scaffold for recognizing the target mRNA. The thermodynamic rule makes it possible to fairly accurately predict miRNA target mRNAs by using computational approaches (Bartel 2009). Since open source miRNA target prediction programs, such as TargetScan 5.1 (www.targetscan.org), PicTar (pictar.mdc-berlin.de), miRanda (www.microrna.org) and Diana-microT 3.0 (diana.cslab.ece.ntua.gr/microT), are armed with own unique algorithms, the set of predicted targets often vary among distinct programs utilized (Boross et al. 2009). Furthermore, the lists of predicted targets are mostly cell and tissue-type non-specific, and they inevitably have a risk for containing numerous false positive ones. Recently, to overcome these problems, the miRTarBase (mirtarbase.mbc.nctu.edu.tw) has been established, which represents the largest collection of more than 3,500 manually curated miRNA-target interactions from 985 articles, all of which are experimentally validated by luciferase reporter assay, western blot, quantitative RT-PCR, microarray experiments with overexpression or knockdown of miRNAs, or pulsed stable isotope labeling with amino acids in culture (pSILAC) experiments (Hsu et al. 2011).

We searched the target genes of individual 1,223 miRNAs on the Diana-microT 3.0 target prediction program, which was selected because of the highest ratio of correctly predicted targets over other prediction programs (Maragkakis et al. 2009). Diana-microT 3.0 calculates the miRNA-targeted gene (miTG) score that reflects the weighted sum of the scores of all conserved and non-conserved miRNA recognition elements (MRE) on the 3'UTR of the target mRNA. The miTG score correlates well with fold changes in suppression of protein expression. To optimize the parameter of miRNA-target interaction, we considered the target genes with a

cutoff of the miTG score equal to or larger than 20 as the highly reliable targets, because we found that the targets with the miTG score < 20 exhibited the significantly lower precision score, an indicator of correctness in predicted interactions, compared with those having the score ≥ 20 ($p = 2.78E-08$ by Mann-Whitney's U-test) (Satoh and Tabunoki 2011).

2.2 Molecular Network Analysis Tools

To identify biologically relevant molecular pathways from large-scale data, we could analyze them by using a battery of pathway analysis tools endowed with a comprehensive knowledgebase, such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.kegg.jp), Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, www.ingenuity.com), and KeyMolnet (Institute of Medicinal Molecular Design, www.immd.co.jp). KEGG is an open-access database, while both IPA and KeyMolnet are commercial ones, all of which are updated frequently.

KEGG includes manually curated reference pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes, and human diseases (Kanehisa et al. 2010). Currently, KEGG contains 146,294 pathways generated from 406 reference pathways. IPA is a knowledgebase that contains approximately 2,500,000 biological and chemical interactions and functional annotations with definite scientific evidence, curated by expert biologists. KeyMolnet is a tool for analyzing molecular interactions on the literature-based knowledgebase, composed of the contents on 137,300 molecular relationships among human genes, miRNAs, proteins, small molecules, diseases, pathways and drugs. The core contents are collected from selected review articles and textbooks with the highest reliability, curated by expert biologists. The KeyMolnet library contains a panel of human canonical networks constructed by core contents, which represent the gold standard of the networks, composed of 430 pathways, 885 diseases, and 208 pathological events (Satoh et al. 2009; Satoh 2010; Satoh and Tabunoki 2011).

Ensembl Gene IDs of target genes retrieved by Diana-microT 3.0 were converted into the corresponding Entrez Gene IDs by using the DAVID Bioinformatics Resources 6.7 program (david.abcc.ncifcrf.gov) (Huang et al. 2009). Non-annotated IDs were deleted. Then, Entrez Gene IDs of miRNA target genes were uploaded onto KeyMolnet. We utilized the neighboring

network-search algorithm that selects the set of miRNA target genes as starting points to generate the network around starting points within one path, composed of all kinds of molecular interactions, including direct activation/inactivation, transcriptional activation/repression, and the complex formation. By importing the list of Entrez Gene IDs, KeyMolnet automatically provides corresponding molecules and a minimum set of intervening molecules as a node on networks. The generated network was compared side by side with human canonical networks described above. The algorithm that counts the number of overlapping molecules and/or molecular relations between the extracted network and the canonical network identifies the canonical network showing the most statistically significant contribution to the extracted network. This algorithm is essentially based on that of the GO::TermFinder (Boyle et al. 2004). The significance in the similarity between the extracted network and the canonical network is scored following the formula, where O = the number of overlapping molecules and molecular relations for the pathway or overlapping molecules alone for the disease and the pathological event between the extracted network and the canonical network, V = the number of molecules and/or molecular relations located in the extracted network, C = the number of molecules and/or molecular relations located in the canonical network, T = the number of total molecules and/or molecular relations of KeyMolnet, and the X = the sigma variable that defines coincidence.

$$\text{Score} = -\log_2(\text{Score (p)}) \qquad \text{Score (p)} = \sum_{x=O}^{Min(C,V)} f(x) \qquad f(x) = {}_{C}C_{x} \cdot {}_{T-C}C_{V-x} / {}_{T}C_{V}$$

2.3 Molecular Network of MicroRNA Targetome

Among 1,223 human miRNAs examined, Diana-microT 3.0 predicted the targets from 532 miRNAs (43.5%) (Satoh and Tabunoki 2011). Among the 532 miRNAs, 273 miRNAs contained a set of highly reliable targets showing the miTG score $\geq$ 20. Among 273 miRNAs having reliable targets, KeyMolnet successfully extracted valid molecular networks of targetome from 232 miRNAs. They are comprised of 19% of total human miRNAs (miRNAome). Then, the generated network was compared side by side with human canonical

networks of the KeyMolnet library, composed of 430 pathways, 885 diseases, and 208 pathological events. We found that not all 232 miRNAs contained all three categories of canonical networks because several miRNAs comprised only small numbers of targets. When top three pathways, diseases, and pathological events were individually totalized, the most relevant pathway was 'transcriptional regulation by RB/E2F' (n = 39; 6.8% of total), followed by 'TGF-beta family signaling pathway' (n = 32; 5.6%) and 'transcriptional regulation by POU domain factor' (n = 24; 4.2%), the most relevant disease was 'adult T cell lymphoma/leukemia' (n = 68; 12.1%), followed by 'chronic myelogenous leukemia' (n = 65; 11.5%) and 'hepatocellular carcinoma' (n = 51; 9.1%), and the most relevant pathological event was 'cancer' (n = 97; 24.7%), followed by 'adipogenesis' (n = 46; 11.7%) and 'metastasis' (n = 36; 9.2%) (Fig. 1) (Satoh and Tabunoki 2011).

Next, we identified and characterized the large-scale miRNA targetome networks by uploading targets greater than 100 per individual miRNA onto KeyMolnet (Table 1). Fifty-two miRNAs constructing such a large-scale miRNA target network include let-7, miR-9, 17, 19, 20, 26, 27, 29, 30, 32, 92, 93, 96, 98, 101, 106b, 124, 137, 147, 153, 218, 372, 429, 495, 506, 519, 520, 603, and their closely-related family members. The miRNA targetome established highly complex molecular networks, in which the pathways of 'transcriptional regulation by RB/E2F', 'transcriptional regulation by Ets-domain family', and 'transcriptional regulation by p53', the diseases of 'chronic myelogenous leukemia' and 'viral myocarditis', and the pathological event of 'cancer' were notably accumulated (Table 1) (Satoh and Tabunoki 2011).

3 Biological Implications of MicroRNA Targetome Networks

3.1 Collaborative Regulation by MiRNAs and Transcription Factors

As described above, the present observations revealed that the human miRNA targetome regulated by an individual miRNA generally constitutes the biological network of functionally-associated molecules. Therefore, it is important to gain deeper insights into biological implications of each miRNA targetome network.

The protooncogene c-myb is a key transcription factor for development of normal hematopoietic cells and neoplasms. Recent studies showed that miR-15a targets c-myb, while c-myb binds to the promoter of miR-15a, providing an autoregulatory feedback loop in human hematopoietic cells (Chung et al. 2008; Zhao et al. 2009). Consistent with these studies, we found 'transcriptional regulation by myb' as the most relevant pathway to the miR-15a targetome network (the score = 602; the score p-value = 7.39E-182) (Fig. 2) (Satoh and Tabunki 2011). These results suggest a scenario that in the miR-15a targetome network, miR-15a synchronously downregulates both c-myb itself and downstream genes transcriptionally regulated by c-myb, resulting in more effective inactivation of the complex molecular network governed by the hub gene c-myb. Thus, a collaborative regulation of gene expression operates at both transcriptional and posttranscriptional levels, which involves coordinated regulation by miRNAs and transcription factors. Therefore, disruption of fine balance of the coordination could lead to development of cancers.

The retinoblastoma protein Rb/transcription factor E2F pathway acts as a gatekeeper for G1/S transition in the cell cycle. The Rb/E2F-regulated G1 checkpoint control is often disrupted in cancer cells. A recent study showed that miR-106b is directly involved in posttranscriptional regulation of E2F1 (Petrocca et al. 2008). E2F1 activates transcription of miR-106b, while miR-106b targets E2F1, serving as a negative feedback loop in gastric cancer cells. Consistent with these findings, we identified 'transcriptional regulation by Rb/E2F' as the most relevant pathway to the miR-106b targetome network (the score = 854; the score p-value = 7.21E-258) (Fig. 3) (Table 1) (Satoh and Tabunoki 2011). Again, it is possible that in the miR-106b

targetome network, miR-106b simultaneously downregulates both E2F family transcription factors and downstream genes transcriptionally regulated by E2Fs, resulting in efficient inactivation of the complex molecular network governed by the hub molecules E2Fs. The relationship between miR-106b and Rb/E2F would serve as another example of coordinated regulation of gene expression by miRNAs and transcription factors.

3.2 Human MiRNAs Act as a Central Regulator of Oncogenesis

A recent study by miRNA expression profiling of thousands of human tissue samples showed that diverse miRNAs constitute a complex network composed of coordinately regulated miRNA subnetworks in both normal and cancer tissues, and they are often disorganized in solid tumors and leukemias (Volinia et al. 2010). During development of cancers, various sets of miRNAs act as either oncogenes named oncomir or tumor suppressors termed anti-oncomir, or both, by targeting key molecules and their networks involved in apoptosis, cell cycle, cell adhesion and migration, chromosome stability, and DNA repair (Blenkiron and Miska 2007; Garzon et al. 2010). Many miRNA gene loci are clustered in cancer-associated genomic regions (Calin et al. 2004). Furthermore, miRNA expression signatures clearly discriminate different types of cancers with distinct clinical prognoses (Lu et al. 2005). All of these observations support the general view that miRNAs act as a central regulator of oncogenesis (Blenkiron and Miska 2007; Garzon et al. 2010).

To prevent oncogenesis in the cells exposed to stressful insults, the transcription factor p53 acts as the guardian of the genome by regulating a battery of target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Deregulation of p53 function is closely associated with oncogenesis (Rivlin et al. 2011). We found 'transcriptional regulation by p53' as the most relevant pathway to the target network of all let-7 family members except for let-7d (Table 1) (Satoh and Tabunoki 2011). It is worthy to note that the tumor suppressor p53 regulates the expression of various components of the miRNA-processing machinery, such as Drosha, DGCR8, Dicer, and TARBP2, all of which have p53-reponsive elements in their promoters (Boominathan 2010). Furthermore, Dicer and TARBP2, along with

p53, serve as a target of the let-7 family miRNAs, suggesting a close link between p53 and let-7 in miRNA biogenesis (Boominathan 2010). The let-7 family regulates the expression of a critical oncogene RAS in human cells (Johnson et al. 2005), and the expression of let-7 family members was greatly reduced in certain cancer cells (Takamizawa et al. 2004).

The micropthalmia associated transcription factor (MITF), a basic helix-loop-helix zipper (bHLH-Zip) transcription factor, acts as not only a master regulator of melanocyte differentiation but also an oncogene promoting survival of melanoma. Recent studies indicate that MITF is a direct target of both miR-137 and miR-148b (Bemis et al. 2008; Haflidadóttir et al. 2010). Again, we identified 'transcriptional regulation by MITF family' as the most relevant pathway to both miR-137 (the score = 339; the score p-value = 1.19E-102) (Table 1) and miR-148b (the score = 40; the score p-value = 3.91E-142) targetome networks (Satoh and Tabunoki 2011).

Zinc finger transcription factors ZEB1 and ZEB2 act as a transcriptional repressor of E-cadherin. A recent study showed that the expression of miR-200b, which targets both ZEB1 and ZEB2, was downregulated in the cells that undergo TGF-beta-induced epithelial-mesenchymal transition (EMT), and was lost in invasive breast cancer cells (Gregory et al. 2008). EMT is a morphological marker of tumor progression, characterized by loss of cell adhesion, repression of E-cadherin expression, and increased cell mobility and invasiveness. We identified 'transcriptional regulation by ZEB' as the third-rank significant pathway (the score = 155; the score p-value = 1.88E-47) (Fig. 4) and 'EMT' as the third-rank significant pathological event relevant to the miR-200b targetome network (the score = 61; the score p-value = 4.15E-19) (Satoh and Tabunoki 2011).

Thus, various miRNAs positively and negatively regulates diverse gene networks associated closely with promotion and prevention of oncogenesis.

4 Concluding Remarks

A single miRNA concurrently downregulates hundreds of target mRNAs by binding to the corresponding 3'UTR of mRNA via either perfect or imperfect sequence complementarity (Selbach et al. 2008). Such fuzzy miRNA-mRNA interactions are responsible for the redundancy of miRNA-regulated targets and their networks. We have addressed the question whether the human miRNA targetome regulated by an individual miRNA constitutes the biological network of functionally-associated molecules or reflect a random set of functionally-independent genes. First, Diana-microT 3.0 identified highly reliable targets from 273 miRNAs out of 1,223 all human miRNAs. Then, KeyMolnet successfully extracted molecular networks from 232 miRNAs, comprising of approximately 20% of the whole human miRNAome. We found that the miRNA targetome regulated by an individual miRNA generally constitutes the biological network of functionally-associated molecules in human cells (Satoh and Tabunoki 2011). Being consistent with our observations, a recent study showed that interacting proteins in the human PPI network tend to share restricted miRNA target-site types than random pairs (Liang and Li 2007). Interestingly, a computational method named mirBridge that assesses enrichment of functional sites for a given miRNA in the annotated gene set showed that many miRNAs coordinately regulate multiple components of signaling pathways and protein complexes (Tsang et al. 2010).

We identified a coordinated regulation of gene expression by transcription factors and miRNAs at transcriptional and posttranscriptional levels in cancer-associated miRNA targetome networks. In mammalian genomes, gene regulatory networks, consisting of positive and negative transcriptional coregulation of miRNAs and their targets, play a crucial role in enhancement of the robustness of gene regulation (Tsang et al. 2007). The protooncogene c-myc directly activates transcription of E2F1, but at the same time limits its translation by upregulating expression of miR-17-5p and miR-20a, both of which negatively regulate E2F1 (O'Donnell et al. 2005). Importantly, a recent study showed that the genes with more transcription factor-binding sites have a higher probability of being targeted by miRNAs and

have more miRNA-binding sites (Cui et al. 2007).

We found that the most relevant pathological event in the whole human miRNA targetome is 'cancer', when top three pathological events were overall cumulated. Furthermore, the highly relevant diseases include 'adult T cell lymphoma/leukemia', 'chronic myelogenous leukemia', and 'hepatocellular carcinoma'. These observations support the general view that the human microRNAome plays a specialized role in regulation of oncogenesis. Therefore, the miRNA-based therapy designed to simultaneously target multiple cancer-associated networks and pathways might serve as the most effective approach to suppressing the oncogenic potential of a wide range of cancers.

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