Generating microRNA-overexpressing recombinant retrovirus for
analysis of B cell functions
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Abstract

Identification of wild-type lin-4 and let-7 genes in Caenorhabdititis elegans marked
the first milestone of miRNAs discovery. Functional characterization of these prototype
miRNAs suggests the presence of similar non-coding RNAs in animal genomes. In recent
years, emerging studies have increasingly revealed more insights into the significance
and mechanisms of miRNAs-mediated gene regulation in organisms. Essentially, these
short non-coding RNAs play diverse functional roles of post-transcriptional gene
regulation in various developmental stages, differentiation processes and hematopoiesis.
The novel discovery of miRNAs involvement in the development of hematopoietic cell
demonstrates their central role in modulating immune response. Therefore, there is great
potential for advancing our current understanding of immune system regulation with
further understanding of the functions of miRNAs and its mediated molecular networks.

Introduction to miRNA transcription

A majority of primary miRNAs (pri-miRNA) is transcribed from the human genome
by RNA polymerase II. It has also been recently reported that mammalian miRNAs can
be encoded within intronic sequences of messenger RNAs (mRNAs). The pri-miRNAs
undergo an endonucleolytic cascade to eventually derive the mature miRNAs (Bushati
and Cohen, 2007). Within the pri-miRNA, more than one miRNAs may be encoded.
Internal complementary base-pairing occurs along the pri-miRNA sequence to produce
hairpin structures of ~70 nucleotides (nt) with imperfect base pairing in the stem endings.
These hairpin loop structures are also known as the precursor-miRNAs (pre-miRNAs).
The Microprocessor, a multimeric protein complex consisting of Drosha and DGCR 8, a
dsRNA-specific Rnase III enzyme and a dsRNA-binding protein is responsible for
cleaving the pre-miRNAs from the pri-miRNA sequences (Kai and Heissmeyer, 2008).
The resulting 2nt 3’overhang of pre-miRNAs are bound by Exportin-5 to be exported into
the cytoplasm via a Ran-GTP dependent mechanism. In the cytoplasm, the pre-miRNAs
are further processed into ~22nt mature miRNAs duplexes by a cytoplasmic dsRNA-
specific endonucleolytic complex consisting of Dicer and TRBP, a Rnase III enzyme and
a dsRNA-binding protein respectively. The assembly of RNA induced silencing complex
(RISC) in human cells is initiated by a trimeric complex consists of TRBP, Argonaute
protein (Ago2) and Dicer. A single functional miRNA strand from the mature RNA
duplex is preferentially loaded into RISC. It is hypothesized that miRNA target sites in
3’UTR comprises of a ‘core sequence’ responsible for complementarity base-pairing with
the ‘seed nucleotides’ located ~7nt from the 5’ ends of the miRNAs.

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Modes of miRNA-mediated repression

Ongoing research on miRNA-mediated repression suggests two most plausible mechanisms of repression via either target mRNAs degradation or translational repression of target mRNAs (Bushati and Cohen, 2007). The mature miRNA bound by Ago2 in RISC directs the complex to target mRNAs with complementary regions in the 3’ untranslated regions (3’UTRs). The mechanism of target mRNAs degradation is more often observed for mRNAs with 3’UTR that has near-perfect complementarity to the mature miRNA sequence bound in RISC. However, since most animal miRNAs has imperfect base-pairing with the targeted 3’UTRs, translational repression of mRNAs is more commonly observed than mRNAs degradation. The targeted mRNAs have reduced half-life due to destabilization by deadenylation and subsequent decapping. There are substantial evidences to show that miRNAs can block either translation initiation by interfering with the recognition of the 7 methylguanosine or elongation of mRNAs as the association of miRNA with translating polysomes disrupt ribosomal movement along mRNAs (Lodish et al., 2008). Regardless of the mode of repressions on target mRNAs sequences, both mechanisms eventually result in a reduced expression of the target proteins (Chen and Rajewsky, 2007).

Physiological roles of miRNA regulation

miRNA-mediated gene regulation are involved in diverse physiological processes such as during life developmental stages, differentiation processes and hematopoiesis. miRNAs can function as developmental switches and mRNAs involved in developmental processes have comparatively longer 3’UTR containing more miRNA target sites than those involved in basic cellular processes (Bushati and Cohen, 2007). lin-4 of C. elegans is an example of a miRNA capable of regulating developmental process by repressing protein expression from lin-14 mRNA (Lee et al., 1993). The absence of lin-4 retards the transition of C. elegans from the first to second larval stage due to differentiation defects. During differentiation processes, miRNAs has a plausible role of defining cell types by repressing the expression of unwanted mRNAs transcripts remaining from developmental transitions. An example of miRNA with such a role is miR181 in terminal differentiation of myoblasts (Naguibneva et al., 2006). It enhances differentiation process by repressing the expression of differentiation inhibitor, HOX-A11.

In the immune system, miRNAs also play a central role in the modulation of the immune response and are actively involved in regulating hematopoiesis. An example is miR17-92 cluster, a polycistronic miRNA gene that encodes six distinct miRNAs possibly sharing similar “seed” sequences and is involved in B cell differentiation (Baltimore et al., 2008). The transition of pro-B cell to pre-B cell is hindered in the absence of miR17-92 and abnormally high apoptotic rate is concurrently observed in the pro-B cell fraction. miR17-92 downstream repression of Bim proteins which are pro-apoptotic factors, may account for its plausible role in lymphoproliferation. The miR146 family, an immune system regulator of response to microbial infection, had been discovered to have distinct patterns of miR146 expression in immune cells, which suggests an importance in the regulation of hematopoietic cell lineage differentiation. Although both miR146a and miR146b genes encode for miR146, the genes are essentially unrelated and located on separate chromosomes. The miR146 genes differ in
their mature sequences by just 2nt at the 3’end (Taganov et al. 2006). The confirmed
downstream targets of miR146a, which include inflammatory signaling receptors such as
IRAK1 and TRAF6, accounts for the proposal of miR146a as a negative regulator of
inflammation and innate immune response (Lindsay, 2008).

Materials and Methods

Common molecular cloning techniques were applied in the construction of
recombinant miRNA-overexpressing retrovirus. Unlike prokaryotic bacterial cells, it is
not possible to directly transform eukaryotic cells with recombinant plasmid vectors for
in vivo expression. Thus, the construction of recombinant retroviruses is extremely useful
for infecting eukaryotic cell lines to study the effects of miRNAs overexpression in
specific cell or tissues culture.

The miR146a sequence was chosen as the miRNA gene of interest in the experiment.
The miR146a primers and conditions for polymerase chain reaction (PCR) were designed
and optimized. The genomic flanking sequences of pre-miRNAs have been shown in
studies to play crucial roles for miRNA maturation in vivo, thus a hundred bases of
flanking sequences upstream and downstream of the pre-miR146a sequence had been
included for PCR amplification. The miR146a inserts were then cloned into pDrive
vector via UA cloning for further amplification and subsequently introduced into the
retroviral vector (MIGR1).

The resulting recombinant miR146a-MIGR1 retroviral vector are eventually
transfected into 293T cells for retrovirus packaging. The recombinant miR146a-MIGR1
retroviruses were then harvested and used to infect fresh 293T cell culture. The
expression of green fluorescence proteins encoded in the MIGR1 retroviral vectors allow
identification of 293T cells that have been transfected by the retroviral plasmid or
infected by the retrovirus successfully. Therefore, the observation of the green
fluorescence in the transfection and infection results concludes the successful
construction of recombinant miR146a-MIGR1 retrovirus.

Results and Discussion

The gradient PCR shows an optimal annealing temperature of 51.7°C for miR146a-
specific primers as characterized by the most distinct band in the gel electrophoresis
result. The successful insertion of miR146a inserts into pDrive cloning vectors was
confirmed via restriction enzyme digestion and gel electrophoresis. Observation of bands
containing fragment sizes of less than 0.5kb implies the presence of miR146a inserts in
the respective plasmid samples. Sequencing results of the miR146a inserts within
recombinant miR146a-pDrive vectors allow us to distinguish the non-mutated
recombinant vectors.

MIGR1 and miR146a-pDrive vectors were double digested with Bgl II and Xho I for
the next ligation procedure and confirmation of incorporation of miR146a into MIGR1
was confirmed via colony PCR and gel electrophoresis. Despite the poor resolution of the
gel electrophoresis results, bands containing fragment sizes of less than 0.5kb that implies
the presence of miR146a inserts were observed.

The recombinant miR146a-MIGR1 vector was co-transfected with plasmids encoding
for viral particles into 293T cells for retrovirus packaging. The green fluorescence protein
(GFP) encoded within MIGR1 allows the visualization of 293T cells transfection results under the fluorescence microscope. Similarly, the successful infections of 293T cells with newly formed recombinant miR146a-MIGR1 retrovirus also emitted green fluorescence when observed.

Conclusion

Retroviruses have great potential as vectors for the delivery of genes of interests into a wide variety of eukaryotic cells for experimental purposes. The production of recombinant miR146a-MIGR1 retrovirus as genetic vectors is valuable for prospective investigations of miR146a-mediated changes in mRNAs expression profiles of primary defense cell lines such as the B-cells to facilitate the understanding of miR146a role in regulating hematopoiesis. Similarly, recombinant retroviruses could also be constructed to over-express other miRNAs to facilitate research on their roles in different cell or tissue cultures. The constructed recombinant retrovirus can also be applied in the field of miRNA target sites prediction. Overexpression of miRNA in the cells followed by subsequent expression profiling and assays for target downregulation would provide researchers with an overview of target mRNAs that are destabilized by the increased miRNAs regulation.

References