In vivo analysis of pri-miRNA processing by the Drosha-DGCR8 complex (Narry Kim's lab)

The function of the Microprocessor can be examined *in vivo* by depleting Drosha or DGCR8 by RNAi. The effect of depletion on miRNA biogenesis is usually determined by RT-PCR and Northern blot analysis. RT-PCR is the easiest choice to detect the pri-miRNA level. The PCR primers are usually designed to bind ~100-nt away from the stem-loop so that the PCR product is 200-300 bp-long. Northern blot analysis is to detect mature miRNA as well as pre-miRNA. Both mature and pre-miRNA levels are reduced after depletion of Drosha or DGCR8. However, it may take more than 6 days to obtain a dramatic effect on mature miRNA because most mature miRNAs have long half-life. For prolonged incubation, it is desirable to transfect twice; split the cells on the third day and repeat transfection on the fourth day.

A. Depletion of Drosha or DGCR8.

Materials

1. Drosha-targeting siRNA duplex (siDrosha) used in our lab (20μM stock);
   SiRNA passenger strand: 5'-CGAGUAGGCUUCGUGACUUdTdT-3'
   SiRNA guide strand: 5'-AAGUCACGAAGCCUACUCGdTdT-3'
2. DGCR8-targeting siRNA duplex (siDGCR8) used in our lab (20μM stock)
   SiRNA passenger strand: 5'-CAUCGGACAAGAGUGUGAUdTdT-3'

SiRNA guide strand: 5’-AUCACACUCUUGUCCGAUGdTdT-3’

3. Lipofectamine 2000 (Invitrogen)

4. DMEM supplemented with 10% FBS

5. Opti-MEM (Invitrogen)

6. HeLa cells

Procedure

1. One day before transfection, split HeLa cells in 6 well plates and incubate in 2 ml of medium so that the cells become 30-40% confluent on the day of transfection. DMEM tissue culture medium is supplemented with 10% FBS.

2. Mix 4 μl of 20 μM siRNA duplex with 250 μl of Opti-MEM.

3. Add 6 μl of lipofectamine 2000 with 250 μl of Opti-MEM and incubate at RT for 5 min.

4. Mix the siRNA solution (step 2) and the lipofectamin 2000 solution (step 3) and incubate at RT for 20 min.

5. Add the final solution to HeLa cells.

6. After 2~3 days, extract total RNA or protein from HeLa cells and analyze the level of Drosha or DGCR8 by RT-PCR or western blotting.

B. RT-PCR for pri-miRNA

Materials

1. 5 μg of total RNA extracted from HeLa cells treated with siRNA

2. Reverse transcriptase (Superscript II, Invitrogen)

3. Oligo-dT primer (Invitrogen)

4. RNase inhibitor (RNasin or equivalent, 40 U/μl)

5. Taq polymerase (rTaq, TAKARA)

6. dNTP mix (2.5 mM each)

7. PCR primers (10 μM)

8. PCR machine (T1 thermocycler, Biometra)

Procedure

1. Synthesize cDNA from HeLa total RNA using Superscript II (Invitrogen) and oligo-dT primer (Invitrogen) according to the manufacturer's protocol.

2. Carry out PCR using 2 μl of cDNA and 1 μl of PCR primers in 50ul reaction. A typical PCR condition is as follows.

Step1: Denaturation at 94℃ for 3 min

Step 2: Denaturation at 94℃ for 30 sec followed by primer annealing for 30 sec at a suitable temperature depending on Tm value of the PCR primers and by elongation at 72℃ for 30 sec. 25-35 cycles.

Step 3: 72℃ for 7 min

3. Analyze the PCR products on 2% agarose gel. The bands of the PCR product will intensify when Drosha or DGCR8 is depleted (Fig. 4B).

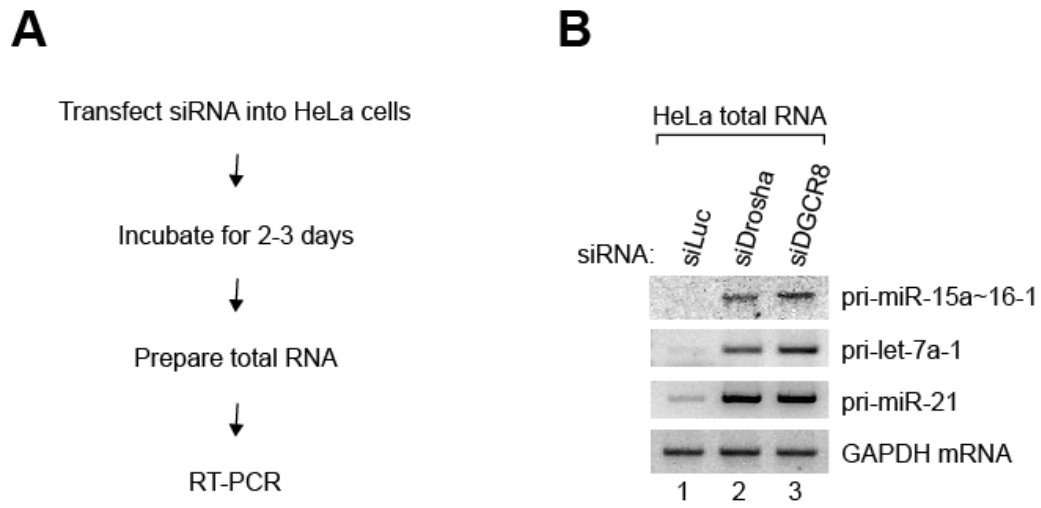

Figure 4. RT-PCR of pri-miRNA. (A) Experimental scheme. (B) Typical results. The siRNA duplex against luciferase (siLuc), Drosha (siDrosha), or DGCR8 (siDGCR8) was transfected into HeLa cells. After 72 hrs, total RNA was prepared and used for RT-PCR.