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Specific Inhibition of Transient and Stable EGFP Gene Expression by Double Stranded RNA Interference in Mouse Preimplantation Embryos

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Abstract: *Double stranded RNA (dsRNA) interference is a useful tool for interfering with gene function by promoting the sequence-dependent degradation of targeted mRNA in several organisms. In the present study, in order to confirm and improve the effect of dsRNA, we investigated an inhibitory effect of dsRNA on both transient and stable gene expression of enhanced green fluorescent protein (EGFP) in mouse preimplantation embryos. In the transient expression system, the rates of fluorescent embryos were significantly decreased by co-injection of EGFP dsRNA and EGFP expression vector fragment into the pronucleus of zygotes. In the stable expression system, EGFP expression in transgenic embryos was significantly decreased by injection of EGFP dsRNA into both the pronucleus and cytoplasm of zygotes, but, cytoplasmic injection caused a more significant EGFP inhibition than pronuclear injection. In quantitative PCR analysis, the expression of the EGFP gene was also inhibited by dsRNA injection, whereas the endogenous gene expression was not affected. These data suggest that dsRNA can inhibit the specific gene expression without affecting the development and expression of other genes.*
Key words: *RNAi, dsRNA, EGFP, Gene expression, Mouse embryos*

Double-stranded RNA (dsRNA) inhibits gene expression in a sequence-specific manner by triggering the degradation of targeted mRNA. This effect, called as RNA interference (RNAi), has been studied initially in *Caenorhabditis elegans* [1, 2] and *Drosophila melanogaster* [3, 4]. Recently, dsRNA-mediated inhibition of gene expression has also been widely studied in mammalian cell systems. In mammalian embryos, it has been reported that microinjection of dsRNA into cytoplasm of mouse embryos resulted in the specific inhibition of both maternally and zygotically expressed proteins [5, 6]. Wianny and Goetz showed the specific inhibition of three different genes (*c-mos*, E-cadherin, GFP) in early embryos [5]. In the case of the two endogenous mouse genes, the gene expression patterns inhibitied by dsRNAs were similar to those of null mutants. Also it was reported that transfection of dsRNA into mouse embryo teratocarcinoma (EC) P19 and F9 cells resulted in a sequence specific decrease in the level of protein expressed from either exogenous or endogenous genes [7]. But, gene specific interference with dsRNA has not been fully studied in mammalian preimplantation embryos. So far, cytoplasmic injection of dsRNA has showed effective interference [5, 6], but there is no clear information on the effect of other injection sites. In the present study, in order to establish the system for RNAi and to confirm the effect of dsRNA in mouse preimplantation embryo, we investigated 1) the effect of dsRNA on the gene expression of enhanced green fluorescence protein (EGFP) by using a transient and stable EGFP expression system, and 2) an effective injection site for dsRNA.

Materials and Methods

Preparation of EGFP expression vector fragment and dsRNA

The expression vector was prepared by modification of EGFP-inserted plasmid by addition of the promotor and poly A sequence. The expression vector was then linearized by digestion with restriction enzyme.

EGFP dsRNA was essentially synthesized according to the procedure described by Wianny and Goetz [5]. After amplification of the EGFP coding region in the EGFP expression vector, then PCR product (600 bp) was cloned into a plasmid by using TA cloning vector (Invitrogen, Carlsbad, CA) that has a dual RNA polymerase promoter for Sp6 and T7 priming sequences. After cloning, the purified plasmid DNA containing the EGFP fragment was used for the dsRNA synthesis. Then PCR was carried out with an M13 forward and reverse primer pair to amplify the fragment containing the EGFP, SP6 and T7 regions. The sense and antisense single-stranded RNAs were synthesized with the PCR product containing the SP6 or T7 polymerase with SP6 or T7 RNA polymerase (Wako, Tokyo). DNA templates were then digested by DNase (Wako) treatment. The single-stranded RNA products were extracted with phenol/chloroform followed by ethanol precipitation. The RNAs were then dissolved with annealing buffer (10 mM Tris, pH7.4, 0.1 mM EDTA) and annealed for an initial heat denaturation at 68°C for 10 min followed by incubation at 37°C for 3–4 h. Formation of dsRNA was confirmed by migration on an agarose gel: for each dsRNA, the mobility on the gel was shifted compared to the single stranded RNAs.

Oocyte collection and experiment

Matured female F1 (DBA × C57Bl/6J) mice (Charles River Japan Inc.) were superovulated by intraperioneal injections of 5 IU of pregnant mare's serum gonadotrophin (SEROTOROPIN, Teikokuzoki) followed 48 hrs later by 5 IU human chorionic gonadotrophin (hCG; PUBEROGEN, Sankyo). Then the female mice were mated with male ICR mice (Charles River Japan Inc.) for experiment 1 or a transgenic strain of homozygous male mice that express EGFP under the control of the ubiquitous CAG promoter for experiment 2. Fertilized one-cell embryos were collected from fallopian tubes 20 hrs after hCG injection.

Experiment 1

In order to investigate the inhibitory effect of dsRNA on transient expression of the EGFP gene, the linearized EGFP expression vector fragment and dsRNA were coinjected into zygotes. The small amount (~2 pl) of EGFP expression vector fragment (10 μg/ml) was microinjected into each male pronucleus of zygotes with or without a 600 bp EGFP dsRNA (10 μg/ml) by means of a cell injector (CIJ-1, Shimazu). As a control, zygotes were injected with TE buffer. After the injection, embryos were then cultured in M16 medium (Sigma) for 4 days at 37.5°C in a 5% CO_2 atmosphere. Expression of EGFP in the embryos was observed by fluorescence microscopy (Nikon).

Experiment 2

In order to investigate the effective injection site of dsRNA and the inhibitory effect of dsRNA on the expression of the EGFP gene, we used a stable EGFP expression system with EGFP transgenic mouse. To avoid the effect of maternal gene expression, we used heterozygous embryos in which the transgene is only expressed from paternally derived genes. After superovulation and mating of F1 females with EGFP transgenic males established in our laboratory, collected zygotes were microinjected with EGFP dsRNA (70 μg/ml) into the pronucleus or cytoplasm. Although the EGFP expression in our system starts from the 2-cell stage, the fluorescence was too weak for the analysis by the 8-cell stage in the transgenic strain used for the experiments. Therefore we evaluated the EGFP expression at the morula and blastocyst stages. Expression of EGFP in the embryos was observed by fluorescence microscopy. In some embryos, the intensity of EGFP was captured and analyzed with image analyzing software (NIH image, NIH, USA).

Quantitative Reverse Transcriptation-Polymerase Chain Reaction (Quantitative RT-PCR)

After injection of EGFP dsRNA into the cytoplasm, embryos developed to the morula stage were collected and used for RT-PCR analysis. As a control, embryos developed to morula after injection of TE were analyzed. For quantitative RT-PCR assay, total RNAs were extracted by using a QIAGEN RNeasy mini kit (QIAGEN, Hilden, Germany) with DNase treatment according to the manufacturer's protocol. Oligo-dT primed reverse-transcription with MMLV-reverse transeciptase (Promega, Madison, WI, USA) was carried out with total RNAs from 6–10 morulae. The cDNA was treated with RNase and purified with a PCR purification kit (QIAGEN). Approximately 1/30 to 1/10 of total cDNA products were used for each PCR amplification. Quantitative RT-PCR was carried out

Table 1. Effect of dsRNA on EGFP expression in mouse embryos with co-injection of EGFP expression vector fragment

Treatment	No. of ova used	No. of replications	% of cleaved	% of morulae	% of blastocysts	%of FL M/M	% of FL BL/BL
control	57	4	88.8 ± 7.9	87.8 ± 10.5	84.6 ± 7.1	0	0
EGFP	164	4	90.5 ± 4.1	85.9 ± 3.9	56.0 ± 7.3*[a]	54.7 ± 37.6**	59.5 ± 21.5**[c]
EGFP+dsRNA	145	4	86.6 ± 7.3	45.9 ± 9.1**	25.3 ± 5.4**[b]	18.3 ± 11.7**	3.7 ± 3.7**[d]

Significant difference within each column. *: P<0.05, **: P<0.01 compared with control, a vs. b: P<0.01, c vs. d: P<0.05, FL M/M: fluorescence expressed as morulae/total morulae, FL BL/BL: fluorescence expressed as blastocysts/ total blastocysts.

Table 2. Effect of injection site of dsRNA on EGFP expression in EGFP transgenic mouse embryos

Injection site	No. of ova used	No. of replications	% of cleaved	% of morulae	% of blastocysts	%of FL M/M	% of FL BL/BL
control	71	4	100	100	88.4 ± 9.3	100	100
pronucleus	124	4	100	92.9 ± 2.4	81.9 ± 2.3	59.8 ± 9.8**[a]	80.8 ± 8.4*[c]
cytoplasm	122	4	100	95.3 ± 3.3	87.1 ± 4.9	37.4 ± 8.9**[b]	70.9 ± 10.4**[d]

Significant difference within each column. *: P<0.05, **: P<0.01 compared with control, a vs. b: P<0.01, c vs. d: P<0.05, FL M/M: fluorescence expressed as morulae/total morulae, FL BL/BL: fluorescence expressed blastocysts/ total blastocysts.

using the Light Cycler FastStart DNA Master SYBR Green I (Roche, IN, USA) with a LightCycler instrument (Roche). Gene-specific primers were designed based on published sequences: GFP sense, 5'-TAAACGGCCACAAGTTCAGCGTGT-3'; GFP antisense, 5'-TTCTCGTTGGGGTCTTTGCTCAG-3'; Oct3/4 sense, 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3'; Oct3/4 antisense, 5'- CTCGAACCACATCCTTCTCT-3'. The reaction conditions of each gene were carried out followed according to the instruction manual.

Statistical analysis

Statistical analysis of each experiment was carried out by ANOVA. When p<0.05, the difference was considered as significant.

RESULTS

Experiment 1: Effect of dsRNA on EGFP expression in the transient EGFP expression system

Table 1 shows the effect of dsRNA on development and expression of EGFP in embryos coinjected with EGFP vector fragment. There were no significant difference in the cleavage rate between the experimental groups and the control. The rates of morula were significantly decreased (P<0.01) by coinjection of EGFP expression vector with dsRNA (45.9 ± 9.1%) compared with the two control groups (87.8 ± 5%) and the EGFP expression vector-injected embryos (85.9 ± 9.1%). The rates of blastocyst

formation were significantly decreased by injection of both EGFP expression vector (56.0 ± 7.3%, P<0.05) and EGFP expression vector with dsRNA (25.3 ± 5.4%, P<0.01), when compared with the control (84.6 ± 7.1%).

More than half of the embryos which developed to the morula and blastocyst stage showed the fluorescence expressed from the injected EGFP vector (54.7 ± 5.4% and 59.5 ± 21.5%, respectively). At the blastocyst stage, the coinjection of EGFP dsRNA still significantly inhibited the fluorescence (3.7 ± 3.7%, P<0.05) compared with embryos which were injected with only EGFP expression vector (59.5 ± 21.5%).

Experiment 2: Effect of dsRNA injection site on EGFP expression in EGFP transgenic embryos

Table 2 shows the effect of the dsRNA injection site on the expression of EGFP in EGFP transgenic embryos. Almost all control embryos derived from the transgenic mice strongly expressed EGFP in morulae (Fig. 1A and B). Injection of dsRNA into the pronucleus or cytoplasm did not inhibit the embryonic development to morula and blastocyst (Table 2). A significant decrease in EGFP expression was observed in morulae after injection of dsRNA into the pronucleus (59.8 ± 9.8%, P<0.01) and cytoplasm (37.4 ± 8.9%, P<0.01) (Figs. 1B and D, respectively). Between the two dsRNA-injected groups, EGFP expression was significantly decreased in morulae when dsRNA was injected into the cytoplasm (37.4 ± 8.9%) rather than the pronucleus (59.8 ± 9.8) (P<0.05). Expression of EGFP
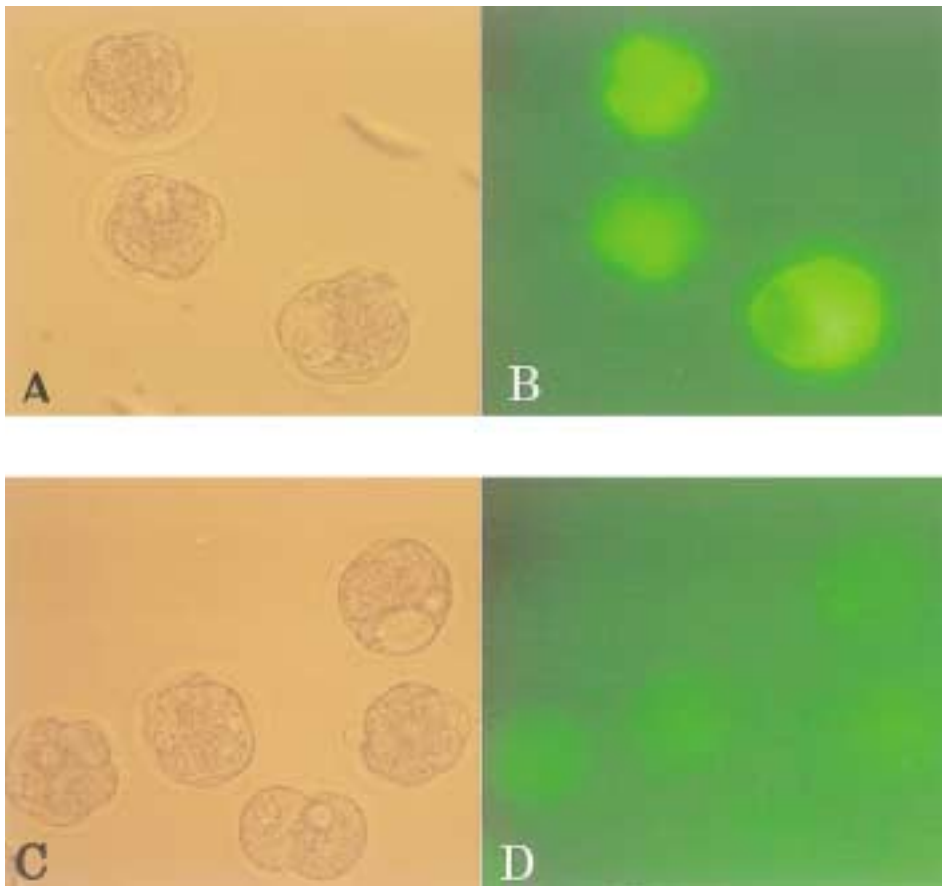
Fig. 1. EGFP expression in EGFP transgenic embryos after injection of dsRNA at the morula stage. A, B: Control embryos (A: Light microscopy; B: Fluorescence), C, D: Embryos injected with EGFP dsRNA (C: Light microscopy; D: Fluorescence). × 200.
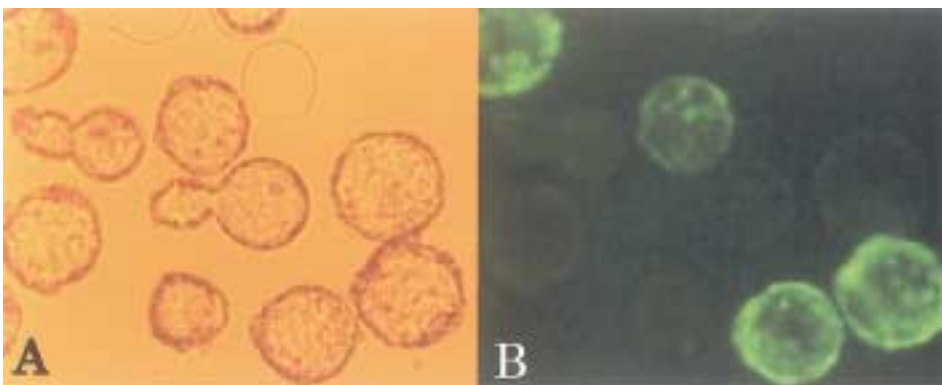

Fig. 2. EGFP expression in EGFP transgenic embryos injected with EGFP dsRNA at the blastocyst stage. A: Light microscopy; B: Fluorescence. × 100.

was gradually restored at the blastocyst stage (70.9 ± 10.4 vs 80.8 ± 7.4%) (Fig. 2).

Quantitative analysis showed a significant decrease in the intensity of EGFP in the morula and blastocyst stage embryos caused by cytoplasmic injection of dsRNA compared with control embryos (Fig. 3A and B).
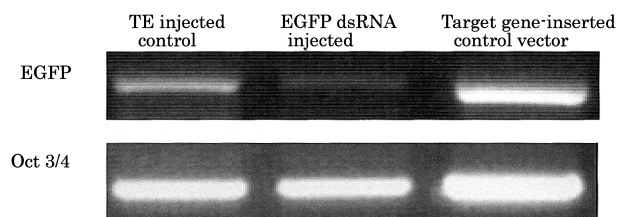
A

B
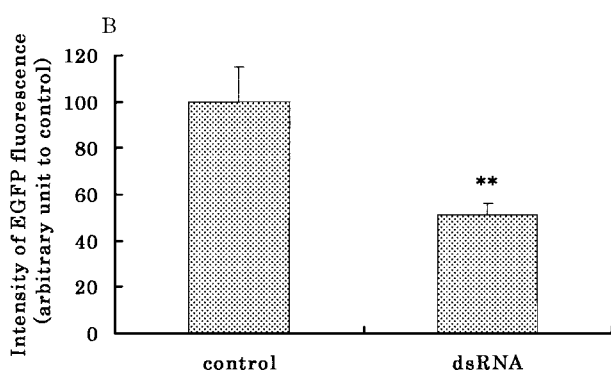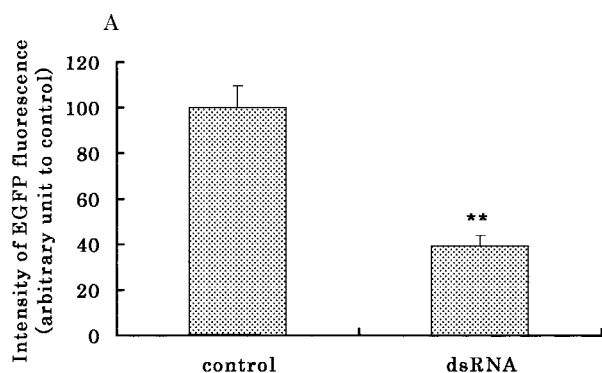
Fig. 3. Relative amount of EGFP fluorescence in morula (A) and blastocyst (B) stage embryos after injection of EGFP dsRNA. Data show the intensity of EGFP fluorescence in embryos relative to the intensity of control embryos as 100 arbitrary units.

Fig. 4. Effect of dsRNA on the expression of EGFP gene in EGFP transgenic embryos at the morula stage. Amount of EGFP mRNA was quantified by quantitative RT-PCR. As a control, the expression of Oct 3/4, a housekeeping gene, was quantified.

Quantitative RT-PCR showed a decrease in EGFP mRNA in molurae injected with EGFP dsRNA, whereas the Oct 4 mRNA was not changed (Fig. 4).

DISCUSSION

In the present study, we have shown that injection of EGFP dsRNA leads to a reduction in the amount of transient and stably expressed EGFP protein in mouse preimplantation embryos. Since the initial finding of RNAi in *C elegans* [1], effective gene silencing with RNAi has been widely used in many species [3–6, 8]. The use of dsRNA has also provided an effective method for studying the gene regulation in mammalian embryo development [5, 6]. In the present study, we tried to establish a transient and stable EGFP expression system in mouse embryos and also tried to investigate the effect of dsRNA in both expression systems. Our present experiments confirmed the previous report that

the injection of dsRNA for E-cadherin and a modified form of GFP (MmGFP) inhibited the expression of mRNA and protein synthesis in mouse embryos [5]. The duration of dsRNA for inhibiting the target mRNA is reported to be up to 6.5 days post-implantation [5]. In the present study, dsRNA almost completely inhibited the EGFP expression by the blastocyst stage in a transient expression system (Table 1). In contrast, fluorescence was fully restored by blastocysts in transgenic embryos after injection of dsRNA (Table 2). The possible reason for the different inhibitory effect of dsRNA in the two EGFP expression systems is the difference in the concentration of the inserted EGFP gene or the different transcriptional systems. The concentration of injected EGFP-expression vector may be sufficient for the inhibitory effect of injected dsRNA in the transient expression system.

In contrast, in the stable expression system, the continuous transcription or the copy number of EGFP gene inserted into the genome might cause the increase in the transcription of EGFP genes. In fact, the intensity of EGFP is higher in EGFP transgenic embryos than in EGFP expression vector-injected embryos (data not shown). Therefore, the number of EGFP genes transcribed or translated to EGFP in the different expression systems should be noted. Besides, the earlier restoration of fluorescence may be caused by the concentration of dsRNA injected into zygotes. We used about 1/20 of the concentration of EGFP dsRNA in the previous report by Wianny and Goetz [5]. This supposition is supported by the results of Svoboda *et al.* who reported that the reduction in the target mRNA caused by dsRNA is both time and concentration dependent [6].

The specificity of interference by dsRNA is important for the evaluation of RNAi. In the present study, the rates of development were not decreased in the EGFP

transgenic embryos by injection of EGFP dsRNA. These results support the previous report stating that dsRNA did not inhibit genes other than the target gene [5, 6]. And the injection of TE solution into zygotes instead of dsRNA did not inhibit development of the transient EGFP expression system (Table 1) and stable expression system (unpublished data). In addition, the fact that the expression of oct 3/4 was not affected by EGFP dsRNA in transgenic embryos also strongly supports the specificity of dsRNA. To confirm whether dsRNA has a toxic effect on embryonic development, the result obtained in experiment 1, i.e. the decrease in development caused by co-injection of EGFP expression vector and dsRNA, needs to be carefully discussed. In comparison with the lower toxic effect of dsRNA injection on the development of transgenic embryos in experiment 2, the cause of the toxic effect might be other factors such as contaminants involved during the purification of vectors or dsRNA. These possibilities are supported by the previous report [5] that the injection of a 20 times higher concentration of dsRNA (2 mg/ml) did not inhibit the development in comparison with the concentration of dsRNA (70 μg/ml) used for the co-injection in our study.

The results obtained in the present study showed that the injection of dsRNA into cytoplasm was more effective than pronuclear injection. Nevertheless, messenger RNA is synthesized from the DNA template in the nucleus, and these molecules are actively exported from the nucleus and rapidly transported through the pores into the cytoplasm [9–11] where they are translated [12, 13]. The reason for the difference in the inhibitory effect of dsRNA on the different sites is not clear, but, it is possible that some of the injected dsRNA is retained in the nuclear region. Recent studies have revealed that the initial procedure of RNAi by dsRNA is the degradation of dsRNA by a dicer, a kind of bidentate ribonuclease in cytoplasm [7, 14]. For this reason, some dsRNA might not be degradated for the latter interference in the EGFP gene. Since the finding of the accumulation of short 25 nt RNA fragments in Drosophila [14], plant [15], and mouse cells [7], the short RNA fragment has become an important key to mRNA degradation [3]. Recently specific interference with short dsRNA was reported in mammalian cells [7, 16, 17]. These reports strongly suggest similar mechanisms related to a short dsRNA fragment in mammalian embryos including mouse embryos.

Further studies are necessary to clarify and regulate the specific gene expression in mammalian embryo development.

References

1) Fire, A., Ku, S., Mongomery, M.K., Driver, S.E. and Mello, C.C. (1998): Potent and specific genetic interference by double stranded-RNA in *Caenorhabditis elegans*. Nature, 391, 806–811.
2) Mongomery, M.K. and Fire, A. (1998): Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. Trends Genet., 14, 255–258.
3) Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000): An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell. Nature, 404, 293–296.
4) Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P. and Sharp, P.A. (1999): Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev., 13, 3191–3197.
5) Wianny F. and Zernicka-Goetz, M. (2000): Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell Biol., 2, 70–75.
6) Svoboda, P., Stein, P., Hayashi, H. and Schultz, R.M. (2000): Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development, 127, 4147–4156.
7) Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowitz, W. (2001): Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc. Natl. Acad. Sci., 98, 14428–14433.
8) Wargelius, A., Ellingsen, S. and Fjose, A. (1999): Double-stranded RNA induces specific developmental defects in zebrafish embryos. Biochem. Biophys. Res. Commun., 263, 156–161.
9) Harpold, M.M., Evans, R.M., Salditt-Georgieff, M. and Darnell, J.E. (1979): Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequence. Cell, 17, 1025–1035.
10) Hastie, N.D. and Bishop, J.O. (1976): The expression of three abundance classes of mRNA in mouse tissues. Cell, 9, 761–774.
11) Miller, O.L. (1981): The nucleus, chromosomes and visualization of genetic activity. J. Cell Biol., 91, 15s–27s (A review).
12) Watson, J.D. (1963): Involvement of RNA in the synthesis of proteins. Science, 140, 17–26.
13) Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M. and Dernell, J.E. (1981): Transcriptional control in the production of liver-specific mRNAs. Cell, 23, 731–739.
14) Bernstein, E., Caudy A.A., Hammond, S.M., Hannon, G.J.

(2002): Role of a bidentate ribonuclease in the initiation step of RNA intereference. Nature, 409, 363–366.

15) Hamilton, A.J., and Baulcombe, D.C. (1999): A species of small antisense RNA in posttranscriptional gene silencing in plants. Science, 286 (5441): 950–952.

16) Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001): Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 6836, 494–498.

17) Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T. and Weber, K. (2001): Identification of essential genes in cultured mammalian cells using small interfering RNAs. J. Cell Sci., 114, 4557–4565.