Small interfering RNA and gene silencing in transgenic mice and rats

Hidetoshi Hasuwa[a], Kazuhiro Kaseda[b], Thorbjorg Einarsdottir[a], Masaru Okabe[a,*]

[a]*Genome Information Research Center, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan*
[b]*Research Institute for Microbial Diseases, Osaka University, Yamadaoka 3-1, Suita, Osaka 565-0871, Japan*

Abstract After short duplexes of synthetic 21–23 nt RNAs (siRNA) were reported to be effective in silencing specific genes, a vector-based approach for siRNAs was demonstrated in mammalian cultured cell lines. However, the effect of RNA interference (RNAi) on various differentiated cells in live animals remains unknown. In this report, we demonstrate that transgenically supplied siRNA can silence ubiquitously expressed enhanced green fluorescent protein in every part of the mouse and rat body. These results suggest that transgenic RNAi could function as an alternative method of gene silencing by applying homologous recombination to embryonic stem (ES) cells, and should be successful even in species where ES cell lines remain unestablished.
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1. Introduction

Double-stranded RNA (dsRNA)-based gene silencing or RNA interference (RNAi) is an ancient and evolutionarily conserved mechanism for sequence-specific post-transcriptional gene silencing among species from various kingdoms (reviewed in [1,2]). The system probably is the most well-studied in *Caenorhabditis elegans* where long dsRNAs, ingested as part of the food source, are processed into short (19–25 nucleotides) small interfering RNAs (siRNA) by a ribonucleotide–protein complex. The shorter siRNAs then prompt the specific degradation of homologous cellular RNA. Until recently, the use of RNAi to silence mammalian genes was not applicable because the introduction of dsRNAs longer than 30 nt elicits a viral response that is sequence-non-specific [3]. However, the introduction of short duplexes of synthetic 21–23 nt RNAs (siRNA) into mammalian cells have a gene-specific silencing function [4]. The limitation of this method is that the transfected synthetic siRNA works for only a few days in mammalian cells. To circumvent this problem, a vector-based approach for the synthesis of siRNAs which were driven by RNA polymerase III (pol III) promoter was demonstrated to silence various genes in mammalian cultured cell lines [5–11]. Recently, RNAi from an exogenously administered plasmid was shown to silence a concomitantly adminis-

tered transgene expression in adult mouse livers [12]. However, the effect was observed only in a transient expression system and the effect was shown only in the liver. In this report, we establish a transgene-based RNAi system using a transgene containing an enhanced green fluorescent protein (EGFP) siRNA driven by a pol III promoter, suggesting that it may be an alternative method for gene disruption. This system was also successful in silencing EGFP expressed in the rat, indicating that transgenic RNAi could analyze the function of genes in animals where homologous recombination is not possible due to the lack of embryonic stem (ES) cell lines.

2. Materials and methods

2.1. Plasmid construction

The H1 promoter [13] was PCR amplified from genomic DNA of the human 293 cell line, using the following primers: 5′-CCGCTCGAGAAGCTTCTAGACTGCAGTGGTCTCATACAGAACTTATAAGATTCCC-3′ and 5′-GATATCGGATCCGCGGAATTCGACGCTGACGTCATCAACCC-3′. The PCR product was digested with *Eco*RV and *Xho*I, and cloned into the *Xho*I and blunted *Sac*I site of pBluescript II SK$^+$ (pH1). To insert the target sequence that encodes the EGFP siRNA, DNA oligos (5′-CCCAACCACTACCTGAGCACCCAGTTCAAGAGACTGGGTGCTCAGGTAGTGGTTTTTTGGAAA-3′ and 5′-CTAGTTTCCAAAAAAACCACTACCTGAGCACCCAGTCTCTTGAACTGGGTGCTCAGGTAGTGGTTGGGTGCA-3′) were synthesized, annealed and cloned into the *Pst*I and *Xba*I site of pH1 (pH1/siRNA$_{EGFP}$).

CAG/HcRed1 was constructed from the pCX-EGFP [14] and pHcRed1-N1 (Clontech) plasmids. *Not*I and *Xho*I sites were introduced between the CAG promoter and the rabbit β-globin polyA region of pCX-EGFP (pCXNX). The HcRed1 gene was amplified by PCR using primers including *Not*I or *Xho*I sites at their 5′ ends. Both the PCR product and pCXNX were digested with *Not*I and *Xho*I, and were ligated (pRed). To produce the siRNA transgenic animals with red fluorescence as an RNAi transgene marker, pH1/siRNA$_{EGFP}$ was digested with *Bam*HI and *Hin*dIII, and inserted into the corresponding restriction sites of pRed (pGtoR).

2.2. Animals and DNA injection

The 'green' mouse line, C57BL/6 TgN(act-EGFP)OsbC14-Y01-FM131, and the 'green' rat line, Rat SD TgN(act-EGFP)Osb4, have been established as described previously [14,15]. Fertilized green mouse eggs were prepared by fertilizing wild-type (B6D2F1) eggs in vitro with sperm from homozygous 'green mice'. Fertilized green rat eggs were obtained by mating wild-type (SD) females with heterozygous 'green rat' males. The pGtoR and pRed plasmids, which were digested with *Sal*I and *Bam*HI and purified from an agarose gel, were injected into the pronuclear stage of fertilized eggs. The injected embryos were cultured in kSOM until the blastocyst stage or immediately implanted into pseudopregnant animals to generate transgenic animals.

2.3. Microscopic analysis

DNA-injected embryos and newborn animals were observed on a

*Corresponding author. Fax: (81)-6-6879 8376.
E-mail address:* okabe@gen-info.osaka-u.ac.jp (M. Okabe).

fluorescence inverted microscope IX70 (Olympus) and fluorescence stereoscopic microscope SZX12 (Olympus). The green and red fluorescences were photographed under 510 nm (green, EGFP) and 610 nm (red, HcRed1) band pass filters equipped with a CoolSNAP fx and cx color digital camera (Roper).

2.4. Genotype analysis

Genotypes of all mice and fetuses were determined by PCR analysis on tail or fetal DNA. PCR was carried out using the EGFP (GAAT-TCGCCACCATGGTGAGC, GAATTCTTACTTGTACAGCTCG-TCC) and human H1 promoter primers (GAACGCTGACGTCAT-CAACCC, TGGTCTCATACAGAACTTATAATAAGATTCCC).

2.5. Immunoblot analysis

Total cellular proteins were extracted with 30 mM 3-[(3-cholamido-propyl)-dimethylammonio]-1-propane-sulfonate/phosphate-buffered saline with 1 mM phenylmethylsulfonyl fluoride, separated by SDS–PAGE on 12.5% polyacrylamide gels, and transferred onto polyvinylidene difluoride membranes. EGFP, CD9, and Shc were visualized using an ECL detection system (Amersham Bioscience) after incubation with rat anti-EGFP monoclonal antibody (kindly provided by Dr. Fujita of Mitsubishi-Kasei Institute of Life Sciences), anti-CD9 antibody KMC8.8 (Pharmingen) or anti-Shc antibody c-20 (Santa Cruz Biotechnology). Either a horseradish peroxidase-linked goat anti-rat or rabbit IgG (Chemicon) was used for the secondary antibody. The visualized signals were quantified by the NIH image program.

3. Results and discussion

Although long dsRNAs are thought to be detrimental to mammalian cells [3], this may not be applicable in early embryos. In this regard, when mouse eggs are injected with dsRNA corresponding to GFP, RNA-induced gene silencing was reported [16,17]. However, the silencing effect continued for only a few days. The injection of synthesized siRNA (21-mer) of EGFP into 'green' one cell eggs is also effective in silencing the expression of the transgene, but again only for a limited time period (data not shown). Fluorescence began to re-appear after the blastocyst stage. This was probably due to a dilution of injected siRNA by a rapid increase of embryonic body mass. To circumvent this limitation, we investigated the possibility of a transgene-based gene silencing system to maintain the RNAi effect in animals for an extended period. 'Green' mice [14] and rats [15], i.e. mice and rats that express EGFP in all tissues and cells, were used as a model system. The silencing EGFP transgenic construct consisted of the pol III human H1 promoter [13] with a hairpin precursor sequence for siRNA (Fig. 1a). The transgene also contained HcRed1 driven by a CAG promoter, which was designed to monitor the genomic integration of the transgene by turning the cells 'red' while the dsRNA silences the 'green'. This 'green to red' transgenic construct (pGtoR) was injected into the pronuclei of 'green' mouse eggs prepared by fertilizing wild-type B6D2F1 eggs in vitro with sperm from homozygous 'green' mice. Initially, silencing of EGFP was examined in mouse preimplantation blastocysts. The embryos were cultured for 3 days in vitro in kSOM medium [18] following transgene injection. The blastocysts appeared 'red' due to the expression of the HcRed1 portion of the transgenic construct, indicating injection was successful. Significantly, EGFP fluorescence was greatly reduced. This EGFP silencing was not due to the expression of HcRed1 because when only the pRed (CAG/HcRed1) construct was injected, the intensity of EGFP fluorescence in the blastocysts was not reduced (Fig. 1b).

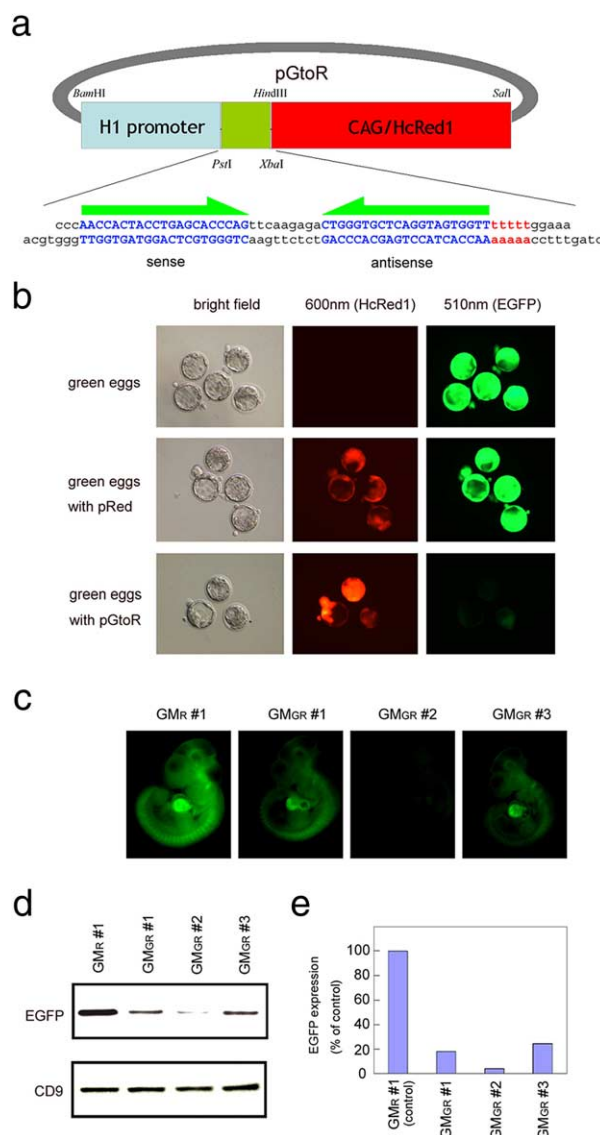Following injection of the transgenes, the mouse eggs were

Fig. 1. Transgenic-based gene silencing in mouse embryos. a: A pGtoR (pH1/siRNA$_{EGFP}$-CAG/HcRed1) transgene was constructed. Two oligonucleotides containing sense and antisense 21 nt sequences from the EGFP coding region (blue colored sequence), a 9 nt spacer sequence which provided a loop structure, and a transcription termination signal of five thymidines (red colored sequence) were annealed and inserted downstream of the H1 promoter (pH1/siRNA$_{EGFP}$). b: Green eggs, obtained from wild-type females mated with homozygous 'green' males, were injected with pGtoR, cultured in kSOM for 3 days, and observed for HcRed (middle column) and EGFP (right column) expression by fluorescence microscopy. 'Green' blastocysts with no additional transgene (top row), 'green' blastocysts with the pRed transgene (middle row) and 'silenced' blastocysts with the pGtoR transgene (bottom row). c: Day 10.5 embryos were recovered and their fluorescence observed (from the left, 'green' embryo with pRed GM$_R$ #1, with pGtoR GM$_{GR}$ #1, 2 and 3, respectively). d: Immunoblot analysis of EGFP in day 10.5 embryos. The lysates from E10.5 embryo of one pRed integrated transgenic (GM$_R$ #1) and three pGtoR integrated embryos (GM$_{GR}$ #1, 2 and 3 as shown above) were analyzed by immunoblotting with anti-EGFP (top) and anti-CD9 (bottom) antibody. e: The intensity of the bands in panel d was quantified and normalized to the control EGFP amount.
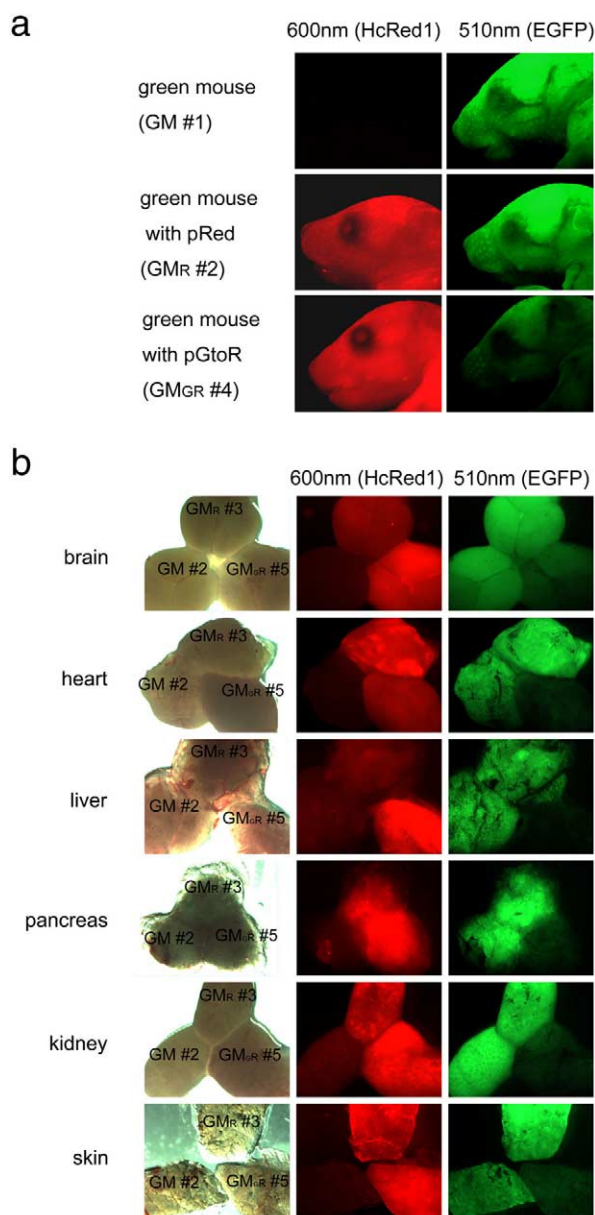
Fig. 2. Transgenic-based gene silencing in newborn mice. Mice were obtained by injecting 'green eggs' with pRed or pGtoR that contains the siRNA$_{EGFP}$ sequence. a: A newborn 'green' mouse without an additional transgene (GM #1), a 'green' mouse containing the pRed transgene (GM$_R$ #2), and a 'silenced green' mouse with the pGtoR transgene (GM$_{GR}$ #4). b: Various organs were removed from newborn founder mice (GM #2, GM$_R$ #3, GM$_{GR}$ #5) containing the genetic backgrounds indicated above and photographed under normal lighting (left), 510 nm (right) and 610 nm (center) band pass filter. The exposure time for each panel was adjusted to obtain optimal images. The absolute fluorescence in muscle and pancreas is brighter than in other tissues.

transplanted to the oviducts of pseudopregnant females. Transgenic founder embryos were recovered at day 10.5 and examined for EGFP silencing. In these embryos, the green fluorescence was diminished in all organs including the heart which showed the strongest expression of EGFP at this stage of development (Fig. 1c). In contrast, when synthesized si-RNA was injected into one cell stage embryos, the EGFP silencing effect disappeared by day 10.5 and all the embryos

showed comparable green fluorescence to normal green embryos (data not shown), indicating that the transgenic approach extended the silencing effect. Proteins from one pRed transgenic embryo GM$_R$ #1 (as a control) and three pGtoR transgenic embryos GM$_{GR}$ #1 to 3 that showed 'red' fluorescence and were positive for human H1 promoter sequence in genomic PCR screening were prepared by homogenizing whole embryos and the amount of EGFP was analyzed by immunoblotting (Fig. 1d). Following quantification, EGFP expression of the three founder mice was reduced to 18, 4 and 24% of the control level, respectively (Fig. 1e). In contrast, the amount of CD9 was not affected by the integration of the transgene (Fig. 1d).

Newborn founder mice which had incorporated the transgenic construct were identified by their 'red' fluorescent appearance and genomic PCR analysis. 'Red mice' that contained the pRed transgene alone showed no decrease in EGFP fluorescence when compared to the original 'green' mice. However, mice containing the pGtoR transgene (e.g. GM$_{GR}$ #4) showed a significant decrease in their 'green' fluorescence (Fig. 2a). To observe the silencing of EGFP in various tissues, brain, heart, liver, pancreas, kidney and skin were collected from the newborn mice with a genetic background of 'green' (GM #2), 'green with pRed' (GM$_R$ #3) and 'green with pGtoR' (GM$_{GR}$ #5). The silencing effect was nearly complete in both the heart and pancreas, two organs that express EGFP intensively (Fig. 2b). Furthermore, all the tis-
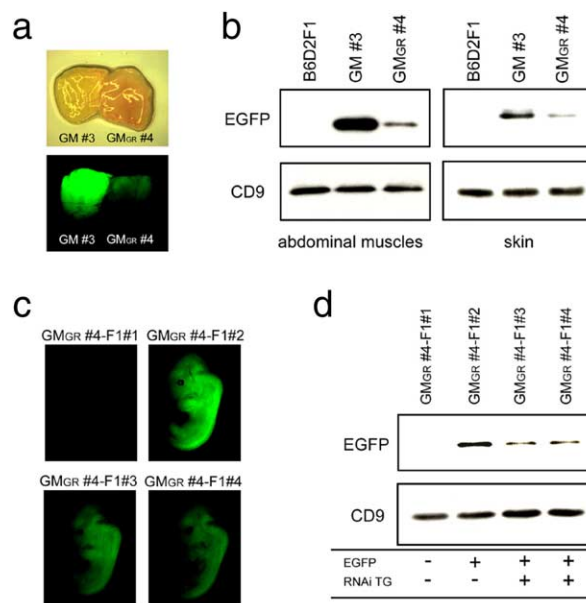

Fig. 3. Transgenic-based EGFP silencing in adult mice and the F1 generation. a: Fluorescent view of abdominal muscle obtained from 8 week old 'green' mouse (GM #3) and from 'green with pGtoR' transgenic mouse (GM$_{GR}$ #4 in Fig. 2a). b: Immunoblot of proteins extracted from abdominal muscle and skin. Eight week old double transgenic mice with a 'green' genetic background and with or without pGtoR integration were compared for EGFP silencing. A B6D2F1 mouse was used to analyze a wild-type mouse. c: Fluorescent view of day 12.5 embryos obtained from B6D2F1 females mated with the GM$_{GR}$ #4 transgenic male from b. Genetic backgrounds of embryos #1 to #4 are as indicated in the table shown in d. d: The embryos shown in c were homogenized and analyzed by immunoblotting. The + and − in the table indicate the result of genotyping the EGFP and H1 promoter sequences by PCR.
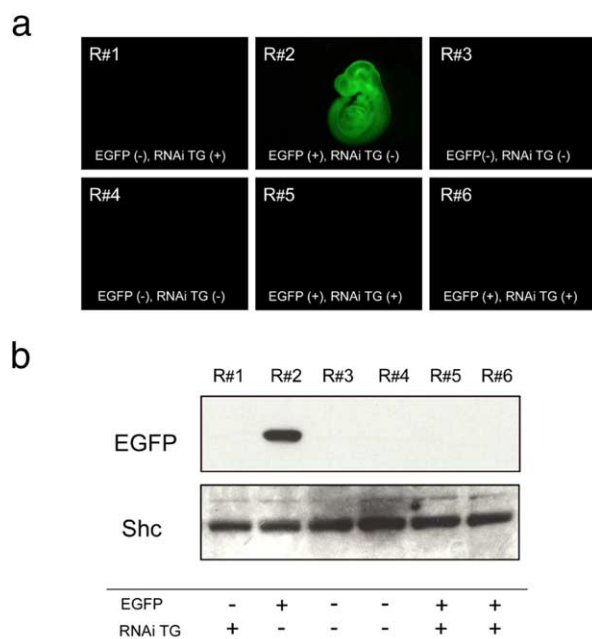
Fig. 4. Transgenic-based EGFP silencing in the rat. a: Fluorescent images of day 11.5 rat embryos containing the pGtoR transgene but without the EGFP transgene (R #1), with the EGFP transgene but without pGtoR (R #2), with neither the EGFP or pGtoR transgenes (R #3 and R #4), and with both the EGFP and pGtoR transgenes (R #5 and R #6). b: Protein extracts from E11.5 rat embryos from R #1 to R #6 were analyzed by immunoblotting with anti-EGFP (top) and anti-Shc (bottom) antibody. Their respective genetic background is indicated.

sues examined, i.e. brain, liver, kidney, skin, showed a decrease in fluorescence in the siRNA transgenic animals, suggesting that the silencing effect was widespread throughout the body. Taken together, these results indicate the transgenic RNAi system enables us to decrease endogenous gene expression in all the organs and at any developmental stage.

It was important to investigate whether the transgenic RNAi silencing effect was transmitted to the next generation. EGFP expression in abdominal muscle and skin in the 8 week old GM$_{GR}$ #4 transgenic mouse was silenced based on both fluorescence and immunoblotting analysis (Fig. 3a,b). Day 12.5 F1 embryos from this transgenic mouse were also examined. Because the inheritance of the original EGFP transgene and the pGtoR transgene containing the RNAi sequence occurs independently, embryos with various genetic backgrounds were produced. However, when the pGtoR construct was transmitted, the expression of EGFP was diminished (Fig. 3c,d).

We examined the silencing effect of pGtoR in a 'green' transgenic rat line [15]. Fertilized eggs, recovered from wild-type SD female rats which had been mated with 'green' transgenic male rats, were injected with pGtoR as described for the mouse, and the resultant transgenic rats were examined for green fluorescence. The original transgenic rat embryo was ubiquitously green (R #2) (Fig. 4a). However, after incorporation of the second transgene, pGtoR, the fluorescence became almost non-detectable (R #5 and 6). The silencing was specific for EGFP because the Shc protein showed no decrease in its expression (Fig. 4b). While the silencing effect appeared more effective in rats compared to mice, this might be due to the two to three times higher expression of EGFP in the trans-

genic 'green' mice compared to the 'green' rats as measured by flow cytometry (data not shown).

The success of transgenic silencing will bring several important advances in the field of gene function analysis in mammals. First, since the silencing effect is inheritable, the system can provide a new type of gene 'knockout' or 'knockdown' method. While homologous recombination using ES cell lines is a well-established method to study the function of a particular gene, the method is time- and labor-intensive. In contrast, the RNAi approach is relatively rapid. Since a RNAi corresponding to a particular gene functions in 'trans', the phenotypic effect of gene silencing experiments can be examined in hemizygous mice. Second, the results in this paper demonstrate that silencing could be effective in any type of organ and any kind of cell from the early embryo until the adult. Third, silencing in rats indicates that silencing could become a powerful tool to produce genetically modified animals in those species where ES cell lines are not established and homologous recombination has not been demonstrated. Finally, in the future it should be possible to express a particular RNAi in selected organs or at a specific developmental time by using methods such as the Cre/loxP recombination system and/or the tetracycline switch promoter [19,20].

References

[1] Hannon, G.J. (2002) Nature 418, 244–251.
[2] Hutvagner, G. and Zamore, P.D. (2002) Curr. Opin. Genet. Dev. 12, 225–232.
[3] Gil, J. and Esteban, M. (2000) Apoptosis 5, 107–114.
[4] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[5] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Science 296, 550–553.
[6] Miyagishi, M. and Taira, K. (2002) Nature Biotechnol. 20, 497–500.
[7] Paul, C.P., Good, P.D., Winer, I. and Engelke, D.R. (2002) Nature Biotechnol. 20, 505–508.
[8] Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P. and Rossi, J. (2002) Nature Biotechnol. 20, 500–505.
[9] Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Genes Dev. 16, 948–958.
[10] Yu, J.Y., DeRuiter, S.L. and Turner, D.L. (2002) Proc. Natl. Acad. Sci. USA 99, 6047–6052.
[11] Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y. and Forrester, W.C. (2002) Proc. Natl. Acad. Sci. USA 99, 5515–5520.
[12] McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J. and Kay, M.A. (2002) Nature 418, 38–39.
[13] Baer, M., Nilsen, T.W., Costigan, C. and Altman, S. (1990) Nucleic Acids Res. 18, 97–103.
[14] Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997) FEBS Lett. 407, 313–319.
[15] Ito, T., Suzuki, A., Imai, E., Okabe, M. and Hori, M. (2001) J. Am. Soc. Nephrol. 12, 2625–2635.
[16] Wianny, F. and Zernicka-Goetz, M. (2000) Nature Cell Biol. 2, 70–75.
[17] Svoboda, P., Stein, P., Hayashi, H. and Schultz, R.M. (2000) Development 127, 4147–4156.
[18] Liu, Z. and Foote, R.H. (1995) Hum. Reprod. 10, 2985–2991.
[19] Ohkawa, J. and Taira, K. (2000) Hum. Gene Ther. 11, 577–585.
[20] Meissner, W., Rothfels, H., Schafer, B. and Seifart, K. (2001) Nucleic Acids Res. 29, 1672–1682.