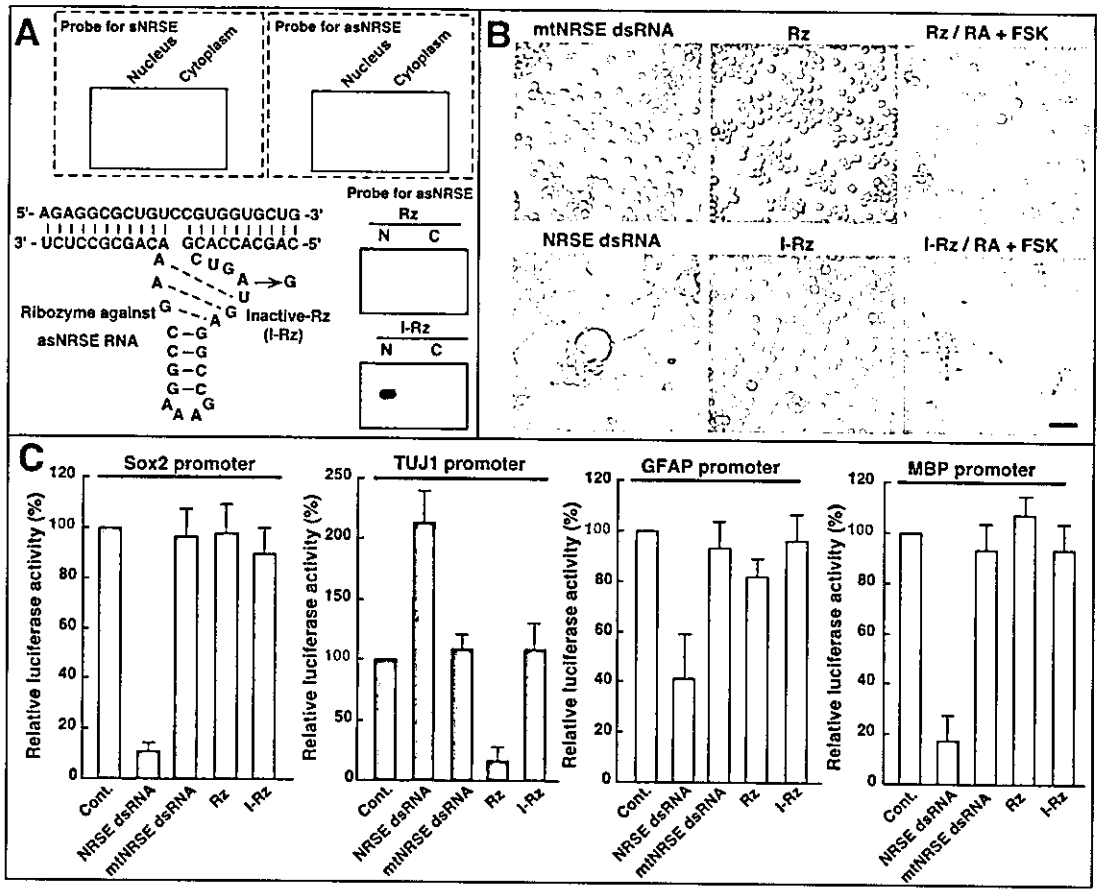

Figure 5. Loss of *NRSE* dsRNA Blocks Neuronal Differentiation

(A) Nuclear localization of the *NRSE* dsRNA was confirmed by Northern blot analysis. The ribozyme (Rz) sequence was designed to cleave the *asNRSE* RNA, and the effect was assessed by Northern blot using fractionated RNAs extracted from 4-day RA+FSK-treated HCN cells. Nuclear fraction, (N); cytoplasmic fraction, (C).

(B) Cells with Rz cleaving antisense *NRSE* RNA showed strong antidifferentiation effects. Scale bar is equal to 10 μm.

(C) The effect of Rz on the function of *NRSE* dsRNA in each lineage. Cell type-specific promoter-based reporter assay was performed in HCN A94 cells. The results shown are the averages of results from three sets of experiments.

also TUJ1-(yellow) positive (red arrows). Conversely, cells expressing *NRSE* RNAs at the lowest levels were TUJ1-negative (blue arrows).

Interaction between NRSF/REST and the *NRSE* dsRNA

To examine the potential interaction between NRSF/REST protein and *NRSE* dsRNA, we incubated the cell extract with biotin-labeled *NRSE* dsRNAs. We also prepared biotin-labeled *NRSE* dsDNAs as a positive control. To assess the specificity in the interaction, negative controls with the partial sequence of the multicloning site (MCS) in pBlueScript II SK+ were prepared. Bound proteins were "pulled down" and analyzed by Western blot (Figure 6B). The immunoblot revealed that both the *NRSE* dsDNAs and dsRNAs bound NRSF/REST, demonstrating an interaction between NRSF/REST and *NRSE* dsRNA.

To compare the affinity between *NRSE* dsDNA and *NRSE* dsRNA to NRSF/REST protein, we carried out electrophoretic mobility shift assay (EMSA). NRSF/REST with cMyc-tag was expressed in 293T cells and immunoprecipitated with anti-cMyc antibody. After purification, the protein was incubated with either *NRSE* dsDNAs or dsRNAs. We tested a range of NRSF/REST protein concentrations; the highest one tested produced a shift in dsDNA migration, whereas a 16-fold lower concentration of NRSF/REST protein was enough to produce a shift in dsRNA (Figure 6C). Surprisingly, these data revealed that the affinity of NRSF/REST to the *NRSE* dsRNAs was much higher than the affinity to *NRSE* dsDNAs. Binding of NRSF/REST to the sequence of the MCS control dsRNAs or dsDNAs was not observed. Furthermore, no apparent band-shift was observed in samples of bovine serum albumin (BSA) incubated with *NRSE* dsRNAs or dsDNAs. This highly specific binding between *NRSE* dsRNA and NRSF/REST protein may contribute to a functional switch of the NRSF/REST machinery from transcriptional repressor to activator.

Expression of NRSF/REST Protein and *NRSE* dsRNA in the Adult Hippocampus

We next examined the expression of NRSF/REST mRNA and *NRSE* dsRNA in the adult mouse hippocampus by in situ hybridization. Although NRSF/REST mRNA was expressed in nonneuronal glial cells (data not shown), the mRNA was highly expressed in hippocampal neurons (Figure 7), suggesting that NRSF/REST is playing

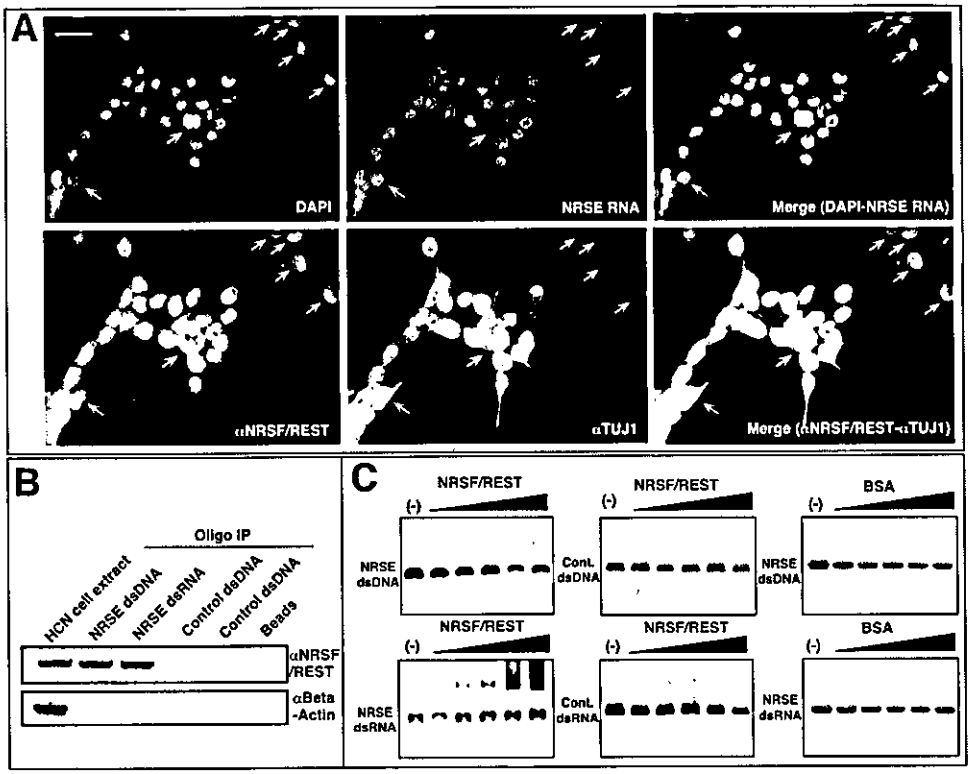

Figure 6. Localization of *NRSE* dsRNAs in the Nucleus of Neuronal Cells and Their Interaction with NRSF/REST Protein

(A) Nuclear localization of *NRSE* dsRNAs and NRSF/REST. Scale bar is equal to 10 μm.

(B) Binding of *NRSE* dsRNAs to endogenous NRSF/REST proteins. Proteins that had bound to the biotin-labeled oligonucleotides were "pulled down" with streptavidin beads and were analyzed by Western blot.

(C) EMSA of NRSF/REST protein against *NRSE* dsRNA and dsDNA. While the concentration of each nucleotide was fixed as 20 μM, protein amount was increased 2-fold by each lane depending on arrow direction.

not only a role as a transcriptional repressor in nonneuronal cells but also a role in neurons in vivo. To verify the specificity of the in situ experiments, we did additional experiments with two negative control probes: a probe with the same nucleotide content containing a scrambled sequence and a probe with the same sequence in the reverse direction. There was no detectable signal with either negative control probe (data not shown). An enlarged view of NRSF/REST and *NRSE* dsRNA expression is shown in Figure 7B. Similar expression patterns of NRSF/REST have been previously documented (Kallunki et al., 1998; Palm et al., 1998; Timmusk et al., 1999). Interestingly, the expression of *NRSE* dsRNA was highly restricted in the subgranular region of dentate gyrus, in a region where adult neurogenesis is continuously occurring (van Praag et al., 2002; Kempermann, 2003) (Figure 7), supporting the in vitro data that *NRSE* dsRNAs function at an early stage in neuronal differentiation (Figures 1 and 2).

These results suggest the existence of interactions between proteins (NRSF/REST complex) and dsRNAs (the *NRSE* dsRNA) in addition to dsDNAs (*NRSE/RE1* element). After the participation of dsRNAs in cells at early stages of neurogenesis, the NRSF/REST complex alters binding partners from repressors to activators to initiate transcription of neuron-specific genes. Derepression events might include global changes in cells, but at least the key players—proteins, dsDNAs, and dsRNAs—could recognize each other within the nucleus in order to direct neurogenesis.

Discussion

NRSE dsRNA Stimulates Neuronal Differentiation through an Interaction with NRSF/REST Complex

In this study, a dsRNA with a restrictive silencer element was identified as a functional transcriptional activator. Genes important for neuronal properties contain the *NRSE* sequence, which is recognized by the protein NRSF/REST (Palm et al., 1998; Schoenherr et al., 1996). The maintenance of neuronal gene repression in nonneuronal cells depends on the ability of NRSF/REST to bind the *NRSE* sequence (Chen et al., 1998; Huang et al., 1999; Lunyak et al., 2002). To repress gene expression, NRSF/REST recruits negative transcriptional regulators such as HDACs and methyl-DNA binding proteins (Lunyak et al., 2002; Naruse et al., 1999). The question of how multipotent adult neural stem cells switch from actively repressing neuron-specific genes in the "stem cell state" to actively expressing neuron-specific genes in the "differentiated state" appears to be explained, at least in part, by the *NRSE* dsRNA. The cell that will become a neuron activates transcription of genes marked by the *NRSE*. These cells supply noncoding RNA that forms dsRNA with an *NRSE* sequence. The

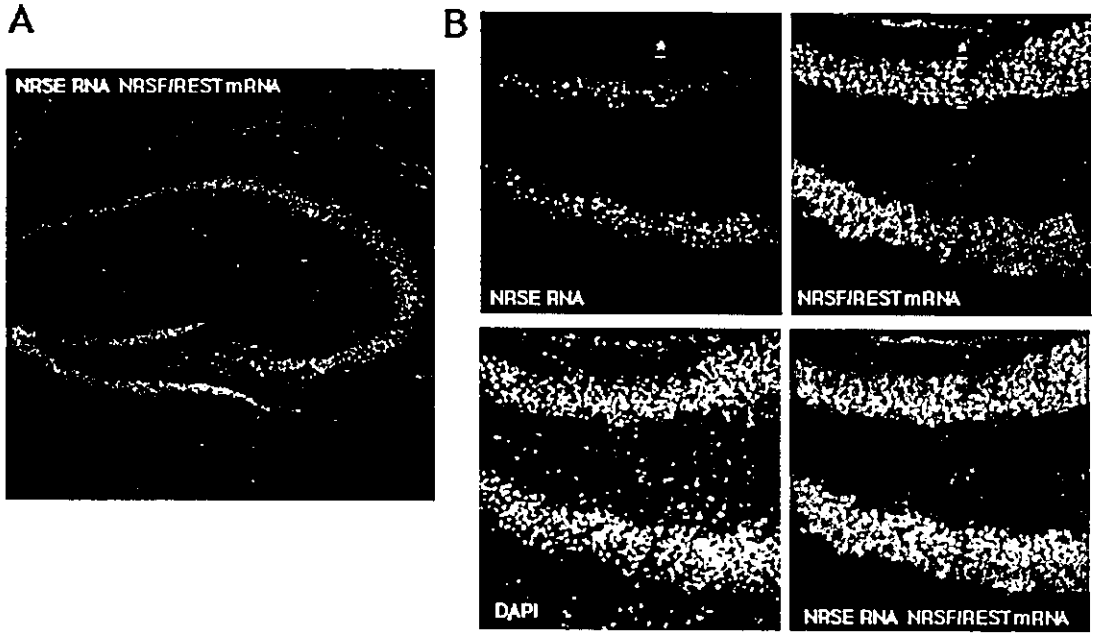

Figure 7. In Situ Hybridization Analysis for NRSF/REST mRNA and *NRSE* dsRNA in Adult Mouse Hippocampus
(A and B) *NRSE* dsRNA expression was highly restricted within the subgranular layer of the dentate gyrus, whereas NRSF/REST mRNA was expressed in a widespread neuronal area in adult hippocampus.
(B) Higher magnification view.

dsRNA interacts with NRSF/REST machinery, resulting in the NRSF/REST switching cofactors from repressors to activators. This intrinsic ability of the NRSF/REST machinery implies that NRSF/REST can function as a flexible mediator of *NRSE* regulatory elements.

Multipotent neural stem cells require a highly selective gene regulation system to achieve uniquely different fates. During the uncommitted stem cell state, the genes required for neural differentiation are repressed (Figure 8), whereas commitment to the neuronal lineage requires repression of stem cell-, astrocyte-, and oligodendrocyte-specific genes and activation of neuron-specific genes (Figure 8). Adult hippocampal neural stem cells would be considered one of the cell types that need selective gene regulation for endogenous fate determination. Noncoding dsRNAs encoding *NRSE* sequences play a unique role in *NRSE* element dependent gene regulation, without alteration of the expression of a key protein player, NRSF/REST, at the transcriptional level. The expression of *NRSE* dsRNA modulates the function of NRSF/REST between activator and repressor of neurogenesis.

We have shown that *NRSE* dsRNAs can act as inducers of neuronal differentiation. Interestingly, introduction of *NRSE* dsRNA alone is sufficient to activate *NRSE/RE1*-containing neuron-specific genes and induce neuronal differentiation. Furthermore, introduction of a ribozyme targeted against the *NRSE* dsRNA has antineuronal differentiation effects, suggesting that *NRSE* dsRNAs are also necessary to induce neuronal differentiation. It should be noted that the NRSF/REST mRNA is highly expressed in adult hippocampal neurons as well as in nonneuronal cells, and the expression of *NRSE* dsRNAs was highly restricted in the subgranular layer of dentate gyrus, one of the neurogenic regions in the adult mammalian brain (van Praag et al., 2002; Kempermann, 2003). These findings imply that *NRSE* dsRNAs also participate in neuronal differentiation in vivo.

Possible Mechanism of *NRSE* dsRNA-Mediated Neuronal Differentiation

NRSE dsRNA binds NRSF/REST as well as the *NRSE* dsDNA (Figure 6B). A simple model of the mechanism of dsRNA-dependent activation is that the dsRNA captures NRSF/REST as a decoy and releases the genome from the repression. However, the ChIP analysis (Figure 4A) indicates that NRSF/REST protein remains stably associated with the *NRSE/RE1* machinery in both the "stem cell state" and the "differentiated states." These results also show that the nature of the *NRSE*-containing chromatin changes from a repressed state (association with HDACs, MBD1, and MeCP2) to an activated state (association with acetylated histones) and does not involve a change in the association of the NRSF/REST protein itself. How can we explain a change in NRSF/REST function, even though NRSF/REST proteins appear to remain physically associated at *NRSE/RE1* sites within different cell stages? We postulate several models (Figure 8). Model A, based on pull-down experiments (Figures 6B and 6C) and mutation analyses (Figure 3D), proposes that there is a physical interaction between the *NRSE* dsRNA and NRSF/REST protein, suggesting a critical sequence dependency between dsRNAs and proteins. Basically, *NRSE/RE1*-containing neuronal genes are actively repressed by the NRSF/REST machinery (through the association of HDACs and methyl-DNA binding proteins). At the onset of neuronal differentiation, the dsRNA interacts directly with *NRSE* dsDNA-NRSF/REST ma-

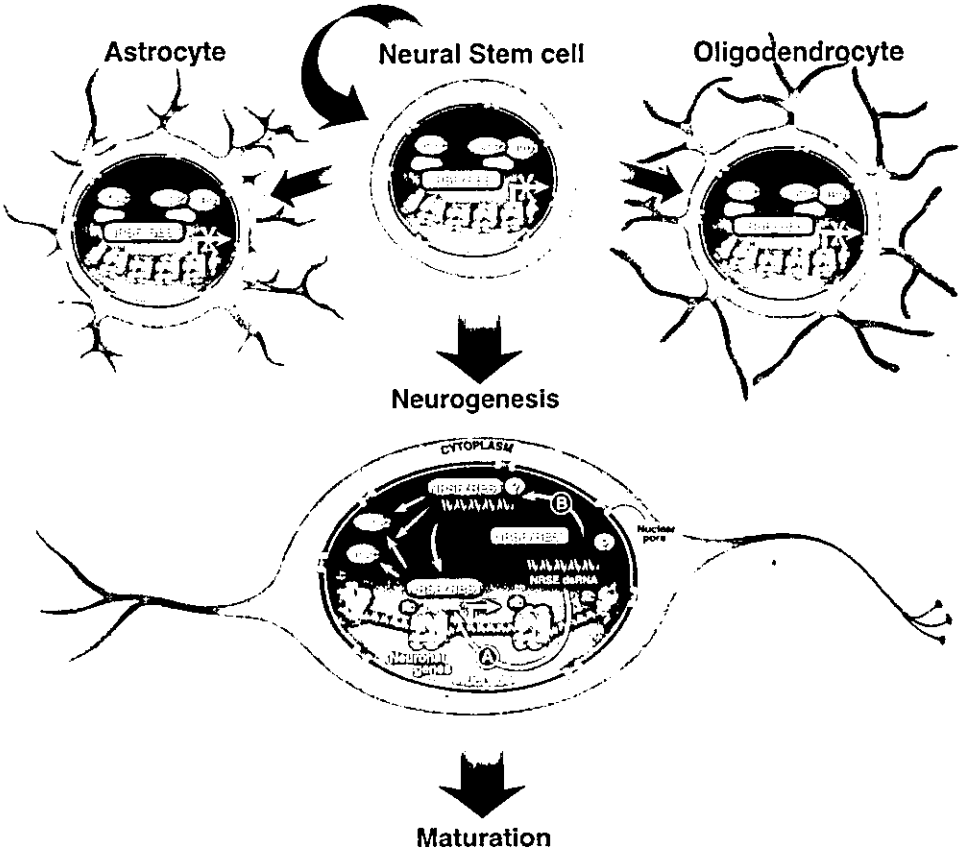

Figure 8. Schematic Representation of Activation Events by *NRSE* dsRNA

NRSE dsRNA can trigger gene expression of neuron-specific genes through interaction with NRSF/REST transcriptional machinery. This interaction results in the NRSF/REST complex no longer binding to HDACs, MeCP2, and MBD1.

chinery within the genome and triggers an organizational change in transcriptional activation (Figure 8, Model A). Another possibility is that the NRSF/REST protein acts as a homodimer. In this case, the *NRSE* dsRNA could bind one monomer of NRSF/REST while the other monomer remains physically associated with dsDNA/chromatin. In Model B, through physical interactions with the NRSF/REST complex, *NRSE* dsRNAs alter NRSF/REST function, possibly by inducing a conformation change in NRSF/REST and/or associated proteins (Figure 8, Model B). In both cases, after an interaction with *NRSE* dsRNAs, the NRSF/REST complex can no longer associate with repressor proteins, such as HDACs, MeCP2, and MBD1.

NRSF/REST is a krüppel family zinc finger protein that contains a DNA binding domain with an eight zinc-finger cluster and one zinc finger at carboxyl terminal (Palm et al., 1998; Shimojo et al., 2001; Tapia-Ramirez et al., 1997). A zinc finger domain includes 2 conserved cysteine and 2 conserved histidine residues in a C-2-C-12-H-3-H (C_2H_2) type motif. Zinc finger domains have been found in numerous nucleic acid binding proteins and interact with nucleotides in the major groove of the nucleic acid. They have the ability to bind to both RNA and DNA. C_2H_2 motif zinc finger proteins represent one of the most common nucleic acid binding motifs found in nature (Hoovers et al., 1992). Proteins containing these motifs are generally DNA binding transcription factors that recognize specific sequences in the context of the B-form helix. However, some zinc finger proteins are also able to bind single-stranded RNA, double-stranded RNA, and RNA-DNA hybrids. Recently, a zinc finger protein was identified that possessed higher binding affinity to A-form dsRNA and RNA-DNA hybrids than to B-form dsDNA helix (Finerty and Bass, 1999). Interestingly, the NRSF/REST protein contains similar features to these RNA binding motifs. Although as mentioned above, Figures 6B and 6C showed possible interactions between NRSF/REST and the *NRSE* dsRNA, it remains to be determined whether NRSF/REST alone is capable of binding to the dsRNA or if this association involves a larger protein complex capable of binding *NRSE* dsRNA. Some proteins may specifically and dominantly recognize the short dsRNA form itself in the nucleus, and this complex interacts with NRSF/REST within the genome at *NRSE/RE1* loci, as a common machinery for the dsRNA-dependent transcriptional regulation (Figure 8, Model B).

Noncoding Small dsRNA Regulates Gene Expression at a Transcriptional Level

In animals, the dsRNA-specific endonuclease, Dicer, produces miRNAs and siRNAs for gene silencing (Bernstein et al., 2001; Hutvagner et al., 2001). miRNA/siRNAs target mRNAs through their sequence homology, leading to gene silencing via the Dicer complex within the

cytoplasm. Posttranscriptional gene silencing by noncoding RNA critically contributes to regulation of developmental timing, spatial patterning of cell fates, and cellular physiology (Eddy, 2001; Fire et al., 1998; Pasquinelli, 2002).

Pre-miRNAs approximately 70 nt in length are made within the nucleus, and a protein complex(es) recognizes them, exports them to the cytoplasm, and passes them to the next players for various gene silencing events (Lee et al., 2002). The identified 21–25 bp *NRSE* dsRNA is smaller; if there are no molecules to keep them within the nucleus, they should naturally diffuse through the nuclear pore and out into the cytoplasm. Since *NRSE* dsRNAs are clearly localized only in the nucleus (Figures 5A and 6A), there must be molecule(s) involved in sequestering their localization. One major candidate is the NRSF/REST protein, since it can recognize the *NRSE* dsRNA through sequence specificity by the zinc-finger motifs. Other candidates are the above-mentioned specific proteins that recognize the short dsRNA form itself in the nucleus and perhaps act as global regulators, like Dicer for a miRNAs/siRNA regulatory mechanism (Figure 8, Model B).

The dsRNAs so far identified may regulate mRNA expression at a posttranscriptional step in the cytoplasm. The currently reported *NRSE* dsRNAs appear to function exclusively at the transcriptional level, suggesting a novel aspect of noncoding dsRNA function. Even though their functions are different, their nucleotide lengths are almost the same (21–25 bp), enabling them to diffuse in cells without limitation. Therefore, dsRNAs might be sequestered in specific cellular compartments through interaction with their cognate protein partner(s) to mediate effects in a spatial-temporal and sequence-dependent manner of target mRNA, DNA, and proteins. Many questions remain, including the exact mechanism that produces small modulatory dsRNAs within the nucleus, and whether a noncoding *NRSE* gene(s) exists. We believe that the *NRSE* dsRNA defines a class of functional noncoding RNAs that have primary roles in regulating gene expression at the transcriptional level, and we propose that this class be named small modulatory RNAs (smRNAs).

Experimental Procedures

Cell Culture

HCN A94 cells were cultured as described (Gage et al., 1995). For neuronal differentiation, cells were cultured in N2 medium (Invitrogen) containing RA (1 μM, Sigma) and forskolin (5 μM, Sigma). For astrocyte differentiation, cells were cultured with 50 ng/ml BMP-2 (R&D systems), 50 ng/ml LIF (Chemicon), and 1% FCS (HyClone) for 4–10 days. For oligodendrocyte differentiation, cells were cultured in N2 medium after FGF-2 withdrawal for 2–4 days. Cell imaging was performed using microscope (Nikon TE300) with a SPOT camera.

Construction of Plasmids

The *sNRSE* RNA- and *asNRSE*-expressing lentiviral vectors were constructed by using CSC PW, a lentiviral vector. The CMV promoter was digested out. U6 promoter drives each *NRSE* RNA sequence with the terminator at the 3′ end first amplified by PCR. Each U6 cassette was subcloned into CSC PW. Ribozyme-expressing vectors were constructed similarly. The production of lentivirus has been described elsewhere (Pfeifer et al., 2001), and infections were almost 100% (viral titers were >1.5 × 10^4 Tu/ng defined by the P24 assay).

Murine *Sox2* promoter on *Sox2* pBS SK (gift from Dr. Rizzino) was inserted into pNeoLuci at the site of MCS (Clontech). Murine TUJ1-, *GFAP*-, *MBP*-and rat GluR2-ptomoters were cloned by PCR from genomic DNA and each promoter was inserted into pNeoLuci. The mtGluR2-luciferase construct with mutated *NRSE/RE1* substituted with random nucleotides (from TTCAGCACCACGGACAGCGCC to GCATCCGCACCGCTAGCGCAG), was also prepared. The TATA, *NRSE*-TATA and *mtNRSE*-TATA luciferase reporter plasmids were constructed in pGL2-basic plasmid (Promega).

Northern Blotting Analysis

Total RNA was extracted with TRIzol reagent (Gibco-BRL). To prepare cytoplasmic fraction, cells were incubated in digitonin lysis buffer (50 mM HEPES/KOH, [pH 7.5], 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EGTA, and 50 μg/mL digitonin) on ice for 10 min. The lysate was centrifuged at 1,000 × g and the supernatant was collected as the cytoplasmic fraction. The pellets resuspended in NP-40 buffer (20 mM Tris-HCl, [pH 7.5], 50 mM KCl, 10 mM NaCl, 1 mM EDTA, and 1% NP-40) were used as the nuclear fraction. Purified RNA was loaded on a 3.5% NuSieve-Seakem agarose gel (FMC Inc.) and transferred to a Hybond-N nylon membrane (Amersham Biosciences). The membrane was probed with synthetic oligonucleotides that were complementary to the sequences of each *sNRSE* or *asNRSE* that had been labeled with ^{32}P by T4 polynucleotide kinase (NEB). Prehybridization and hybridization were carried out using EazyHyb solution (Clontech) following manufacturer's instructions.

In Situ Hybridization and Immunofluorescence Studies

Cells were fixed in fix/permeabilization buffer (50 mM HEPES/KOH, [pH 7.5], 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EGTA, 2% paraformaldehyde, 0.1% NP-40, and 0.02% SDS) for 15 min. The FITC-/rhodamine-labeled oligodeoxynucleotide probes matching complementary to *asNRSE* and NRSF/REST mRNA were denatured for 10 min at 70°C and chilled. Hybridization buffer, containing 20% dextran sulfate and 2% BSA in 4× SSC, with probes were placed on the cells for 16 hr. Cells were rinsed in 2× SSC/50% formamide and in 2× SSC for 20 min each.

Immunofluorescence studies were performed basically as described (Gage et al., 1995): rabbit anti-β tubulin-III (TUJ1; 1/7500, Covance), guinea pig anti-*GFAP* (1:500; Advanced Immunochemical, Inc.), rabbit anti-NF200 (Advanced Immunochemical, Inc.), mouse anti-RIP (1/250, Immuno), rabbit anti-calbindin (Advanced Immunochemical, Inc.) and DAPI (Sigma). All secondary antibodies were from Jackson ImmunoResearch. Images were analyzed using Bio-Rad Radiance confocal imaging system (Hercules, CA).

Luciferase Assay

Luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The luminescent signal was quantitated with a luminometer (Lumant LB 9501). As an internal control, a plasmid containing Renilla luciferase gene was cotransfected.

Chromatin Immunoprecipitation (ChIP), RT-PCR

ChIP assay was done essentially as described (Takizawa et al., 2001) by using ChIP assay kit (Upstate). We used the monoclonal 12C11 antibody (gift from Dr. Anderson) and NRSF-P18 antibody (Santa Cruz), as the antibody for NRSF/REST. RT-PCR was performed by using total RNA extracted from HCN A94 cells. 1 μg RNA was used for first-strand cDNA synthesis with SuperScript II (GibcoBRL). PCR primer sequences are available upon request.

Pull-Down Assay with Biotin-Labeled Oligonucleotide

Biotin-labeled RNA was synthesized with an AmpliScribe T7 transcription kit (Epicentre Technologies). Streptavidin-agarose beads (Gibco BRL) were washed with binding buffer (20 mM Tris-HCl, [pH 7.5], 60 mM KCl, 2.5 mM EDTA, and 0.1% Triton X-100) and suspended. While the beads were kept on ice, cell extract was mixed with 70 μg of biotinylated RNA. After incubation on ice for 10 min, the total volume was adjusted to 1 ml with binding buffer.

Then the sample was transferred to the tube with agarose beads, and the tube was rotated slowly overnight at 4°C. The beads were washed 5 times with wash buffer (20 mM Tris-HCl, [pH 7.5], 350 mM KCl, and 0.01% NP-40) and resuspended in binding buffer. Proteins were eluted by boiling the beads and were separated by SDS-PAGE.

EMSA

The expression plasmid of cMyc-tagged NRSF/REST (gift from Dr. Anderson) was transfected in 293T cells, and the lysate was incubated with cMyc-antibody in RB buffer (20 mM Tris-HCl, [pH 7.5], 50 mM $MgCl_2$, 10 mM NaCl, and 1 mM EDTA) overnight at 4°C. After 1 hr of incubation with Fast Flow protein agarose beads (Upstate) at room temperature, associated proteins were precipitated. The beads were washed 3 times with wash buffer and resuspended in RB buffer. Proteins were eluted by pH 2.0 elution buffer (Upstate) and were neutralized immediately with Tris-Cl pH 8.5 buffer. The protein solution was purified and concentrated with Millipore Centricon (Amicon). Resultant protein solutions were sequentially diluted and each solution was incubated with preannealed and prestained 20 μM oligonucleotides for 30 min at room temperature. Oligonucleotide prestaining was done with SYBR green I (Molecular Probes) for DNA and SYBR green II (Molecular Probes) for RNA. Samples were loaded on 2% Nusieve agarose gel and the image was developed by Eagle Eye II (Stratagene).

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Induction of DNA methylation and gene silencing by short interfering RNAs in human cells

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Double-stranded RNAs (dsRNAs) induce post-transcriptional gene silencing in several species of animal and plant[1–2]. In plants, dsRNAs targeted to CpG islands within a promoter can also induce RNA-directed DNA methylation[3–8]; however, it remains unclear whether gene silencing mediated by DNA methylation can be induced by dsRNAs in mammalian cells. Here, we demonstrate that short interfering RNAs (siRNAs; 21–25-nucleotide RNA molecules) induce DNA methylation and histone H3 methylation in human cells. Synthetic siRNAs targeted to CpG islands of an E-cadherin promoter induced significant DNA methylation and histone H3 lysine 9 methylation in both MCF-7 and normal mammary epithelial cells. As a result, these siRNAs repressed expression of the E-cadherin gene at the transcriptional level. In addition, disrupting the expression of either one of two DNA methyltransferases (*DNMT1* or *DNMT3B*) by specific siRNAs abolished the siRNA-mediated methylation of DNA. Moreover, vector-based siRNAs targeted to the *erbB2* (also known as *HER2*) promoter also induced DNA methylation in MCF-7 cells. Thus, siRNAs targeted to CpG islands within the promoter of a specific gene can induce transcriptional gene silencing by means of DNA-methyltransferase-dependent methylation of DNA in human cells, and might have potential as a new type of gene therapeutic agent.

Double-stranded RNAs induce RNA interference (RNAi)-mediated post-transcriptional gene silencing in animals and plants[1–2]. In this system, siRNAs are generated by the RNase III Dicer enzyme and are incorporated into the RNAi-induced silencing complex (RISC)[9,10]. The siRNA–RISC complex then promotes degradation of cytoplasmic messenger RNAs[11–16].

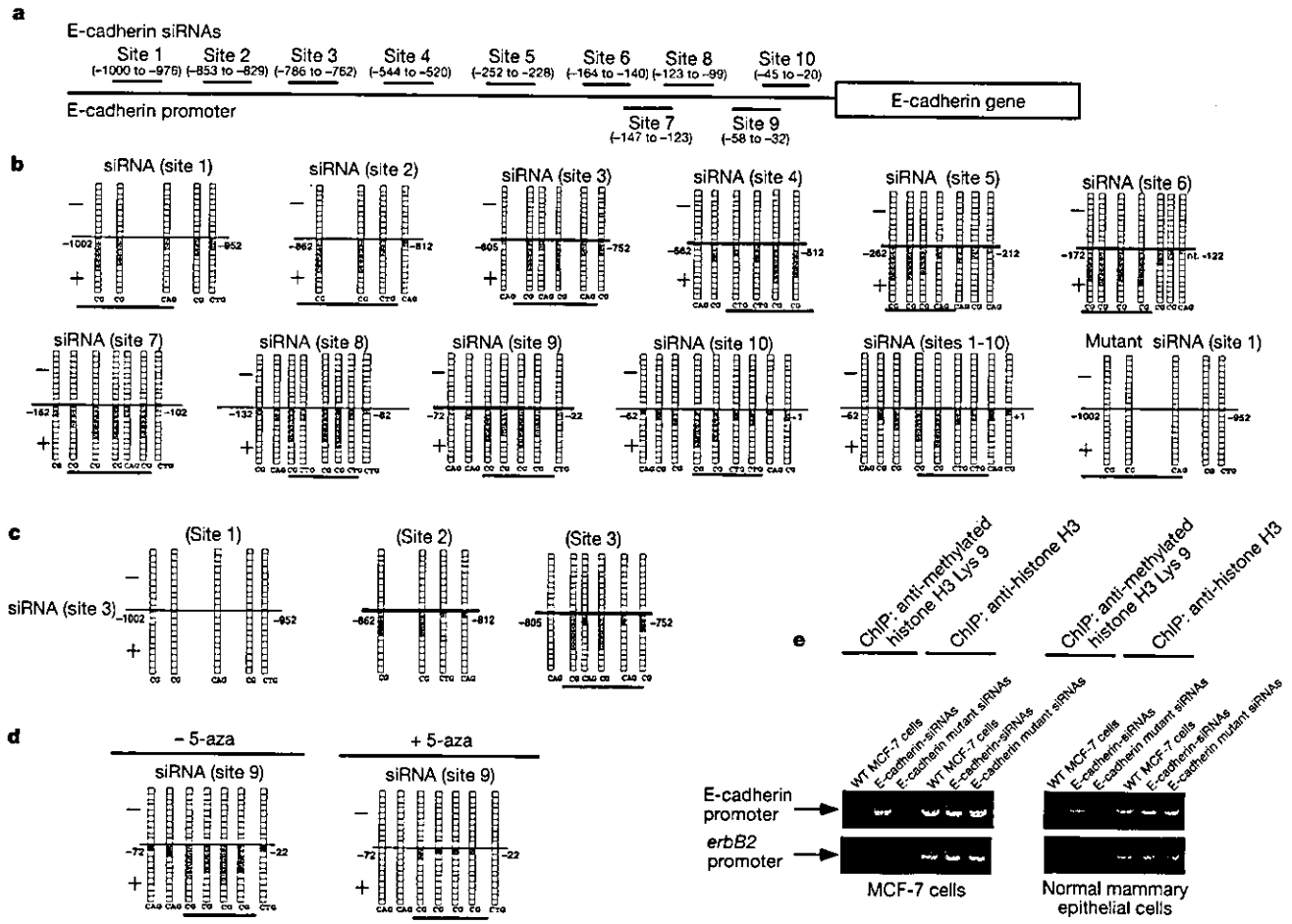

Figure 1 DNA methylation of the E-cadherin promoter by siRNAs in MCF-7 and normal breast epithelial cells. **a**, The ten target sites for siRNAs in the E-cadherin promoter. Nucleotides are indicated in parentheses. **b**, Induction of DNA methylation within the E-cadherin promoter in MCF7 cells by siRNAs, as determined by bisulphite sequencing (see text for details). The number (out of a possible ten) and position of methylated cytosines are indicated by grey squares; underlining indicates the target sequences of siRNAs. **c**, Details of extended methylated regions within the E-cadherin promoter when siRNA was targeted to site 3 within the E-cadherin promoter in MCF7 cells, as determined by bisulphite sequencing. **d**, Relative levels of DNA methylation of the E-cadherin promoter at site 9 in MCF7 cells in the presence or absence of 5-aza, as determined by bisulphite sequencing. **e**, Detection of methylated histone associated with the E-cadherin promoter in cells that were treated with a combination of E-cadherin siRNAs (site 1–10). Methylated histone H3 at lysine 9 was detected using a chromatin immunoprecipitation (ChIP) assay with specific antibodies for methylated histone H3 at lysine 9. Histone H3 antibody is a positive control. WT, wild type.

To determine whether synthetic siRNAs might induce DNA methylation in mammalian cells, we synthesized siRNAs targeted to CpG islands of the E-cadherin promoter (E-cadherin-siRNAs), as E-cadherin can be silenced by aberrant methylation of the promoter in several lines of tumour cells[17–19]. Moreover, as CpG sites in the E-cadherin promoter are unmethylated in MCF-7 cells[17,18], we chose MCF-7 cells for this study. We selected ten CpG sites (sites 1–10) in the E-cadherin promoter as targets of siRNAs (Fig. 1a). Each E-cadherin-siRNA (100 nM) was introduced into MCF-7 cells and, 96 h later, total DNA was collected and genomic DNA was isolated. We then performed bisulphite sequencing to determine the methylation status of the E-cadherin promoter. We treated genomic DNA with bisulphite using a CpGenome DNA-modification kit (Intergen). We amplified the bisulphite-modified E-cadherin promoter by polymerase chain reaction (PCR) and cloned the product of PCR using a TA cloning kit (Clontech). We picked ten independent colonies for each target site and analysed the cloned sequences by direct sequencing.

As shown in Fig. 1b, we found methylated DNA in MCF-7 cells that harboured each respective E-cadherin-siRNA (sites 1–10) and cells harbouring all E-cadherin-siRNAs (sites 1–10; the number of grey squares indicates the number of methylated cytosines). By contrast, a mutant form of the E-cadherin-siRNA directed against site 1 that had eight point mutations in both its sense and antisense strand failed to induce methylation. Moreover, a combination of all E-cadherin-siRNAs together failed to induce DNA methylation within a non-targeted *erbB2* promoter (Supplementary Fig. S1), highlighting the specificity of the E-cadherin-siRNAs. We next examined the possibility of methylation beyond the target site in the absence of RNA-directed RNA polymerase (RdRP) in mammalian cells. Notably, siRNAs targeted to site 3 of the E-cadherin promoter induced methylation not only at that site but also at the adjacent site (site 2), at least to some extent (Fig. 1c). However, site 1, located ~200 nucleotides upstream of site 3, was unmethylated.

Aberrant methylation is often detected in cancer cells. Therefore, we next examined the induction of DNA methylation by siRNAs in normal breast epithelial cells. We detected methylation of CpG sites in normal breast epithelial cells that had been treated with E-cadherin-siRNAs (testing methylation at sites 6 and 10; Supplementary Fig. S2). Furthermore, the extent of methylation in MCF-7 cells that had been treated with a combination of all ten E-cadherin-siRNAs was reduced in the presence of 1 μM 5-aza-2′-deoxycytidine (5-aza), an inhibitor of DNA methylation (Fig. 1d).

As methylation of histone H3 at lysine 9 is induced by RNAi in plants, fission yeast and *Drosphila*[8,20,21], we examined whether our set of siRNAs could induce its methylation in mammalian cells using a chromatin immunoprecipitation assay with specific antibodies for methylated histone H3 at lysine 9. Each cell lysate from MCF-7 cells and normal mammary epithelial cells in the presence or absence of the E-cadherin-siRNAs (sites 1–10) was incubated with specific antibodies for methylated histone H3 at lysine 9 or antibodies for histone H3, and the DNA interacting with the methylated histone H3 was immunoprecipitated. The precipitated DNA was then amplified using specific primers for the E-cadherin promoter. As shown in Fig. 1e, the precipitated DNA from the cell lysate of E-cadherin-siRNA-treated MCF-7 cells produced a band corresponding to the E-cadherin promoter region. By contrast, we did not detect the corresponding band when the same procedure was performed in the absence of E-cadherin-siRNAs or in the presence of mutant siRNAs. Similar results were obtained using cell lysate from normal mammary epithelial cells. In addition, it has been reported that an RNA component is involved in maintenance or stabilization of a higher-order structure at pericentric heterochromatin in mammalian cells[22].

Taken together, these results suggest that siRNAs targeted to the E-cadherin promoter can induce not only DNA methylation but also histone H3 methylation at lysine 9 in human cancer cells and normal cells, and that the effects of siRNAs can spread to nearby regions even in the absence of RdRP.

To examine whether the induction of DNA methylation by E-cadherin-siRNAs was correlated with expression of the E-cadherin gene, we performed northern blotting analysis with a probe specific

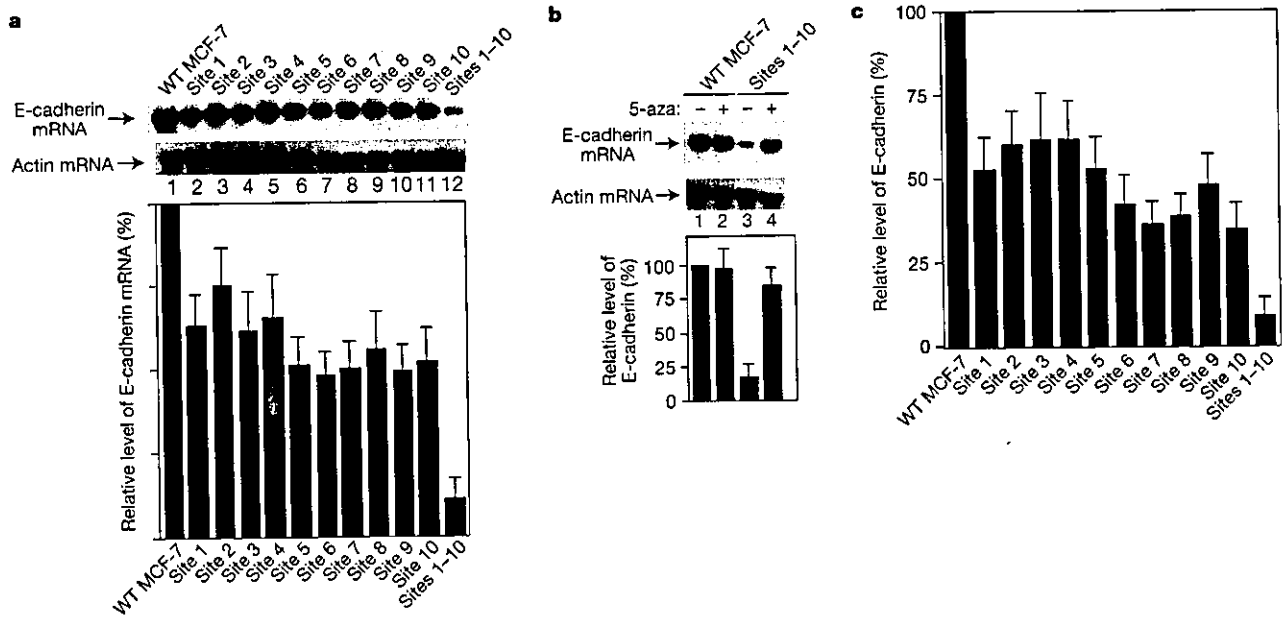

Figure 2 Effects of E-cadherin-siRNAs targeted to the E-cadherin promoter on E-cadherin mRNA expression. **a**, Levels of E-cadherin mRNA expression in cells transfected with E-cadherin-siRNAs. Total RNA from each line was analysed by northern blotting analysis. Actin mRNA was used as the endogenous control. Values are means ± s.d. **b**, Relative levels of E-cadherin mRNA expression in cells transfected with E-cadherin-siRNAs and grown with and without 5-aza. **c**, Levels of E-cadherin expression in cells transfected with E-cadherin-siRNAs. E-cadherin in each line was detected by western blotting with specific antibodies. Relative levels of E-cadherin were analysed densitometrically. Values are means ± s.d.

for E-cadherin mRNA. As shown in Fig. 2a, the level of E-cadherin mRNA expression in each line of MCF-7 cells harbouring a specific E-cadherin-siRNA was lower than in wild-type MCF-7 cells (lanes 1–11). Notably, the level of E-cadherin mRNA expression in MCF-7 cells that had been treated with a combination of all E-cadherin-siRNAs (lane 12) was significantly lower than in MCF-7 cells transfected with a single siRNA, clearly demonstrating the additive effects of the siRNAs. Treatment with 5-aza almost completely abolished the effect of the E-cadherin-siRNAs (Fig. 2b, lane 4). Western blot analysis with an E-cadherin-specific antibody also revealed similar reductions in the amount of E-cadherin (Fig. 2c). Thus, siRNAs targeted to the E-cadherin promoter act as gene silencers at the transcriptional level, and induction by siRNAs of DNA methylation within the E-cadherin promoter is inversely correlated with the level of gene expression and is additive.

DNA methyltransferases (DNMTs) are responsible for all DNA methylation in the cell[23–25]. To determine whether DNMTs participate in siRNA-mediated DNA methylation in human cells, we tried to suppress the expression of genes for DNMTs using siRNAs targeted to the respective mRNAs. We synthesized three separate siRNAs that targeted to *DNMT1*, *DNMT2* and *DNMT3B* mRNAs, respectively. As controls, we used mutant *DNMT1*-, *DNMT2*- and *DNMT3B*-siRNAs with four point mutations in both the sense and antisense strand. We introduced *DNMT*-siRNA or mutant *DNMT1*-siRNA at 100 nM into MCF-7 cells using Oligofectamine, and examined the amount of DNMTs by western blotting. Amounts of DNMT1, DNMT2 and DNMT3B in MCF-7 cells transfected with *DNMT1*-siRNA, *DNMT2*-siRNA or *DNMT3B*-siRNA, respectively, but not the corresponding mutant *DNMT*-siRNAs, were significantly lower than those in wild-type MCF-7 cells (Fig. 3a).

To examine whether reduced expression of DNMT genes affects siRNA-mediated DNA methylation, we introduced a combination of E-cadherin-siRNAs (sites 1–10) into MCF-7 cells transfected with *DNMT1*-, *DNMT2*- or *DNMT3B*-siRNAs. As shown in Fig. 3b, the extent of DNA methylation induced by E-cadherin-siRNAs in MCF-7 cells transfected with *DNMT1*- and *DNMT3B*-siRNA was significantly lower than in wild-type MCF-7 cells or in MCF-7 cells transfected with mutant *DNMT*-siRNA. By contrast, disruption of *DNMT2* expression did not significantly affect siRNA-mediated DNA methylation. Thus, *DNMT1* and *DNMT3B*, but not *DNMT2*, seem to be necessary for siRNA-mediated DNA methylation in human cells.

We next examined the effects of E-cadherin-siRNAs on the expression of E-cadherin in MCF-7 cells transfected with *DNMT*-siRNA. As shown in Fig. 3c, siRNAs targeted to the E-cadherin promoter did not alter E-cadherin gene expression in MCF-7 cells that contained siRNAs targeted to *DNMT1* or *DNMT3B* mRNA (lanes 4 and 16). By contrast, E-cadherin-siRNAs suppressed

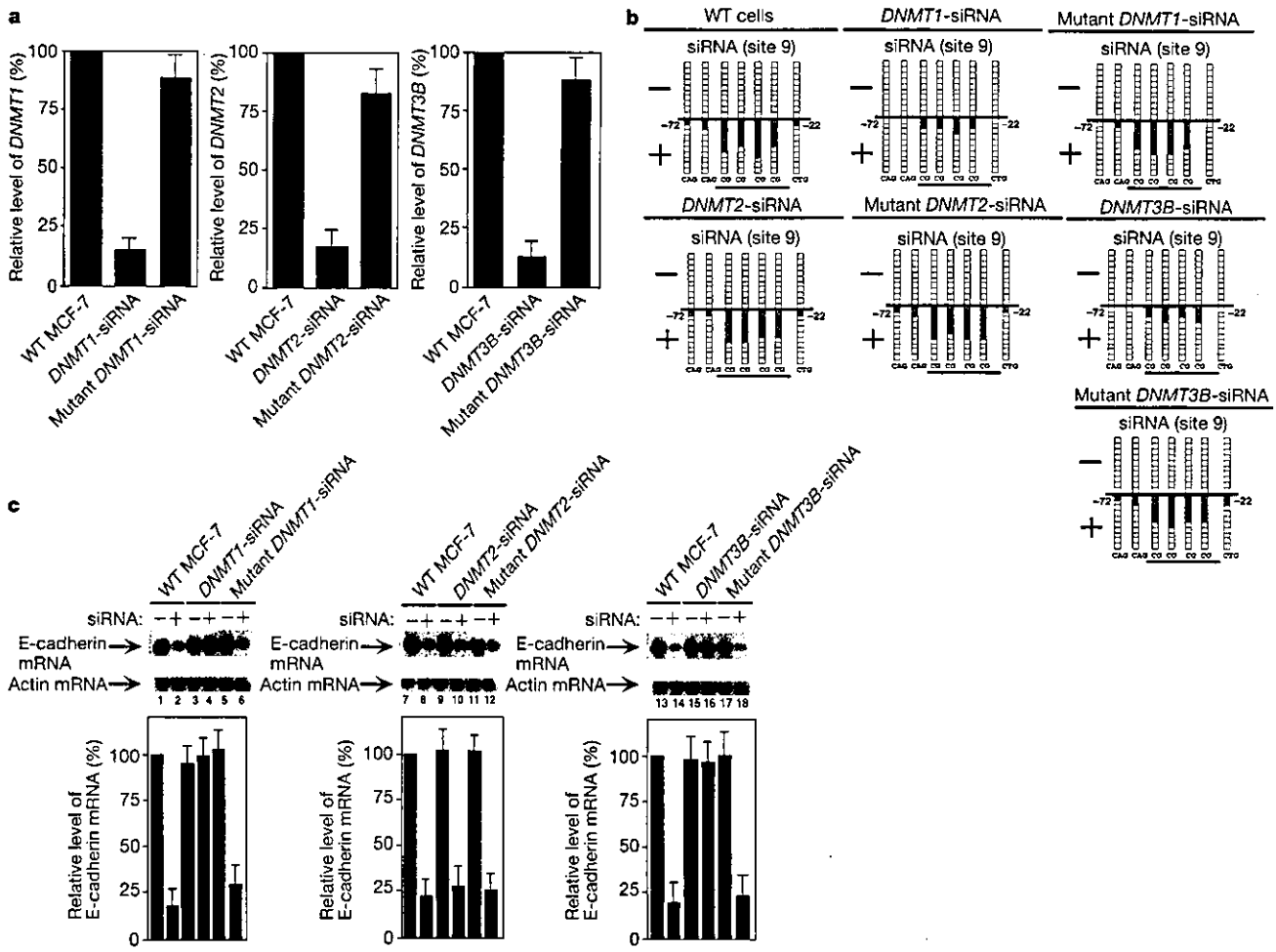

Figure 3 Effects of E-cadherin-siRNAs targeted to the E-cadherin promoter in DNMT1-, DNMT2- and DNMT3B-knockdown cells. **a**, Amount of DNMTs in MCF-7 cells in the presence or absence of *DNMT*-siRNAs. The amount of DNMT1, DNMT2 and DNMT3B was detected by western blotting. Values are means ± s.d. **b**, Level of DNA methylation of the E-cadherin promoter in MCF-7 cells in the presence or absence of E-cadherin-siRNA and various *DNMT*-siRNAs. DNA methylation was detected by bisulphite sequencing. **c**, E-cadherin mRNA expression levels in MCF-7 cells in the presence or absence of E-cadherin-siRNA (sites 1–10) and various *DNMT*-siRNAs. Total RNA from each line was analysed by northern blotting with specific probes. Values are means ± s.d.

E-cadherin gene expression in MCF-7 cells that contained *DNMT2*-siRNA or mutant *DNMT*-siRNAs (lanes 6, 10, 12 and 18), demonstrating that *DNMT1* and *DNMT3B* are necessary for the transcriptional gene silencing that results from the induction of DNA methylation by E-cadherin-siRNAs.

To examine the possibility for gene therapy via the control of DNA methylation by siRNAs, we constructed expression vectors for short hairpin RNAs (shRNAs) targeted to an *erbB2* promoter. The *erbB2* gene is overexpressed and unmethylated in several lines of tumour cells, such as MCF-7 cells, whereas it can be silenced by methylation of the promoter in several lines of normal cells[26]. To express shRNAs in cells, we used the well-characterized $tRNA^{Val}$ promoter system[16]. We have demonstrated previously that $tRNA^{Val}$-driven shRNA induces siRNA-mediated gene silencing in human cells[16]. We selected five CpG islands (sites 1–5) within the *erbB2* promoter as targets of shRNAs. Then, we introduced these shRNA expression plasmids transiently into MCF-7 cells. We confirmed by means of northern blotting that appropriate processing and production of siRNAs had occurred in cells that expressed tRNA-shRNAs (Fig. 4b, lanes 1–5), and then we examined levels of DNA methylation within the *erbB2* promoter. We isolated genomic DNA from each cell line and performed bisulphite sequencing. As shown in Fig. 4c, we detected DNA methylation within the *erbB2* promoter in all lines of MCF-7 cells that expressed a tRNA-shRNA or a combination of all tRNA-shRNAs (sites 1–5). However, a mutant tRNA-shRNA (site 1) with nine point mutations in both the sense and antisense strand did not induce DNA methylation within the *erbB2* promoter. In addition, tRNA-shRNA did not induce DNA methylation within the non-targeted E-cadherin promoter (Supplementary Fig. S3). We obtained similar results with U6 promoter-driven shRNAs (data not shown), demonstrating that induction of sequence-specific DNA methylation by vector-based siRNAs in human cells is a general phenomenon.

To determine whether the vector-based shRNAs had suppressed the expression of *erbB2*, we examined levels of *erbB2* mRNA expression by means of northern blotting. As shown in Fig. 4d, the expression level of *erbB2* mRNA in cells that expressed each respective tRNA-shRNA was lower than in wild-type MCF-7 cells.

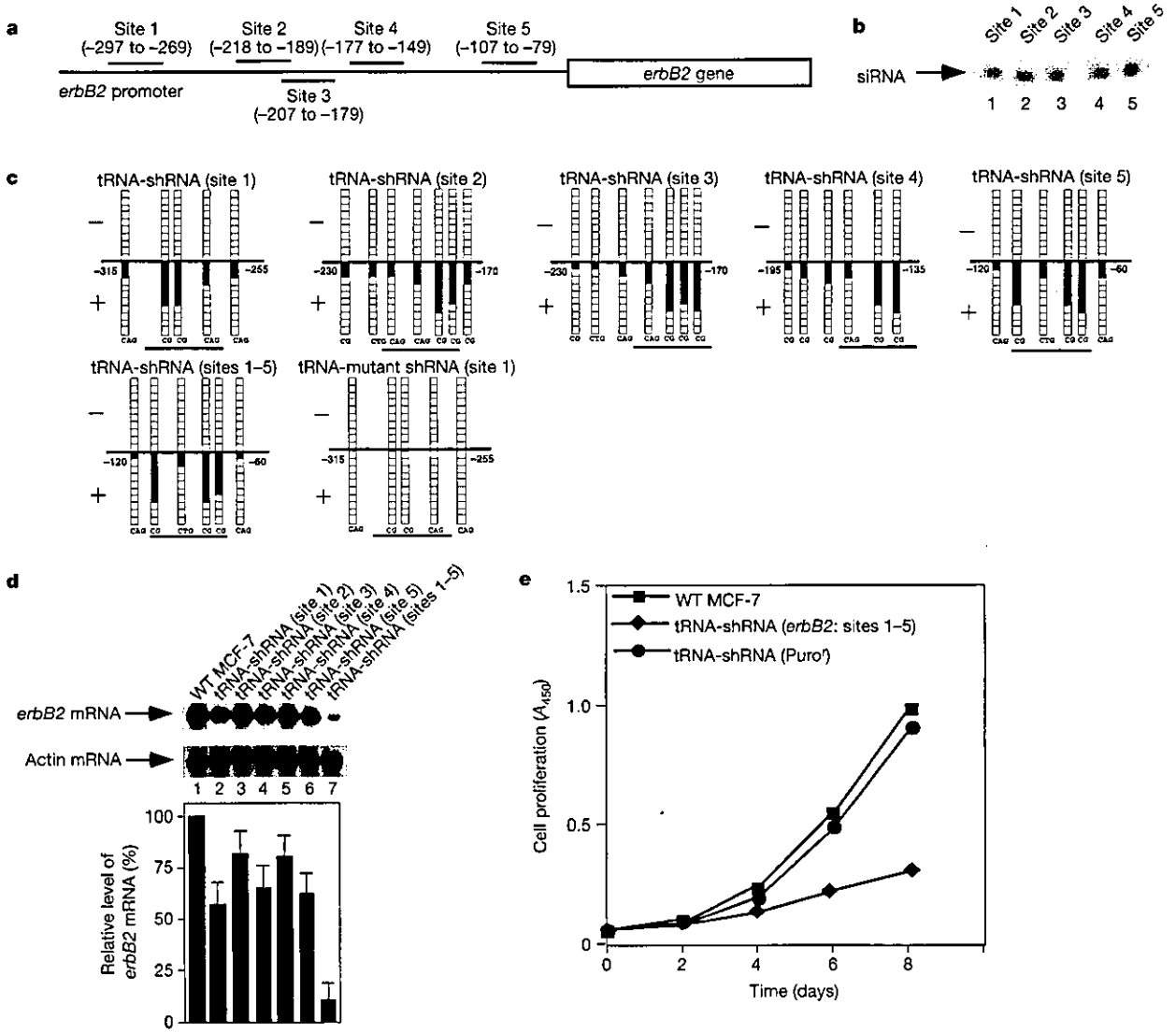

Figure 4 Induction of DNA methylation of the *erbB2* promoter by tRNA-shRNAs. **a**, Five targets for tRNA-shRNAs were selected within the *erbB2* promoter. **b**, Detection, via northern blots, of the expression of tRNA-shRNAs targeted to the *erbB2* promoter. All anticipated siRNAs were detected in cells that expressed tRNA-shRNAs. **c**, Induction by shRNAs of DNA methylation of the *erbB2* promoter, as detected by bisulphite sequencing. **d**, Relative levels of *erbB2* mRNA in cells that expressed tRNA-shRNAs targeted to the *erbB2* promoter. Total RNA from each line was analysed by northern blotting with specific probes. Values are means ± s.d. **e**, Proliferation rates of cells expressing tRNA-shRNAs. tRNA-shRNA ($puro^r$), targeted to a puromycin-resistance gene, was used as a control shRNA.

As observed previously (Fig. 2a), the level of *erbB2* mRNA expression in cells that expressed all tRNA-shRNAs (sites 1–5) together was significantly lower than in wild-type MCF-7 cells and in cells that expressed individual tRNA-shRNAs, demonstrating the additivity of suppression by vector-based shRNAs at the transcriptional level. Transcriptional regulation through the induction of methylation within a promoter by siRNA in mammalian cells seems to be a general phenomenon, as demonstrated by the examples in this study.

To examine the phenotype of cells that expressed tRNA-shRNAs, we analysed the proliferation rates of various cell lines. MCF-7 cells expressing all tRNA-shRNAs (sites 1–5) proliferated significantly more slowly than wild-type MCF-7 cells and cells that expressed tRNA-shRNAs targeted to a puromycin-resistance gene, which we chose as a nonspecific control (Fig. 4e). The reduced proliferation rate of MCF-7 cells that expressed all tRNA-shRNAs (sites 1–5) was correlated with a reduction in the level of *erbB2* mRNA expression in these cells, suggesting that the tRNA-shRNAs targeted to the *erbB2* promoter might have potential utility as therapeutic agents.

The DNA methylation of promoters has an important role in the genesis and development of tumours by regulating the expression of specific genes[27,28]. Many proteins involved in DNA methylation, such as MeCP2, MBD and DNMTs, have been well characterized in human cells. However, it remains unclear how and under what circumstances they are guided to and methylate specific CpG target sites of cognate genes during the genesis or development of tumours.

In plants, long and short dsRNAs can induce sequence-specific DNA methylation, known as RNA-directed DNA methylation[3–8]. In addition, transgenes can also induce sequence-specific DNA methylation[29,30]. These phenomena might reflect the role of these systems as a cellular defence against RNA and DNA viruses. However, it remains to be determined whether similar RNA-directed DNA methylation and transgene-mediated defence systems exist in human cells.

We have demonstrated that synthetic and vector-based siRNAs can induce sequence-specific and *DNMT1*/*DNMT3B*-dependent RNA-mediated DNA methylation in human cells. Our siRNAs induced sequence-specific gene silencing at the transcriptional level. The ways in which siRNAs are guided to and gain access to genomic DNA remain unknown. Synthetic and tRNA vector-based siRNAs are localized predominantly in the cytoplasm, where siRNA-mediated degradation of mRNA also occurs. A small fraction of siRNA–protein complexes might be transported to the nucleus. Alternatively, siRNAs might gain access to genomic DNA during cell division, when the nuclear membrane disappears.

Further investigation of correlations between siRNA-induced DNA methylation and tumour genesis, anti-viral defence and epigenesis in development should provide an insight into small-RNA-induced gene silencing and development. Moreover, it is now possible to control the expression levels of specific genes in mammalian cells using siRNAs not only to disrupt cognate mRNAs but also to interfere with transcription, as demonstrated here. However, it remains to be examined precisely which promoters can be specifically methylated by respective siRNAs. Exploitation of shRNA expression vectors targeted to cognate promoters might even have potential utility in a clinical setting. □

Methods

Preparation of siRNAs

Synthetic siRNAs directed against the E-cadherin promoter (E-cadherin-siRNA) and against DMNT mRNAs (DMNT-siRNAs) were synthesized with a DNA/RNA synthesizer (model 394; PE Applied Biosystems). Sequences of E-cadherin-siRNAs are described in the Supplementary Information. For generation of siRNAs, all synthetic RNAs were annealed by the standard method[13]. We introduced siRNAs into MCF-7 cells using Oligofectamine (Invitrogen) in accordance with the manufacturer's protocol.

Construction of tRNA-shRNA expression plasmids

To construct vectors for expression of tRNA-shRNA targeted to the *erbB2* promoter, we used the pPUR-tRNA plasmid, which includes a chemically synthesized promoter for a human gene for $tRNA^{Val}$ between the *Eco*RI and *Bam*HI sites of pPUR (Clontech)[16]. Sequences (sites 1–5) of shRNAs targeted to the *erbB2* promoter are described in Supplementary Information. Chemically synthesized oligonucleotides encoding *erbB2* promoter-directed shRNAs that included a loop motif were amplified as double-stranded sequences by PCR. After digestion with *Sac*I and *Kpn*I, the fragments were cloned downstream of the promoter of the $tRNA^{Val}$ gene in pPUR-tRNA.

Bisulphite sequencing of E-cadherin and *erbB2* promoters

We extracted genomic DNA from cells by standard methods using proteinase K, phenol and chloroform. We performed bisulphite modifications using a CpGenome DNA modification kit (Intergen) following the manufacturer's instructions. We amplified the bisulphite-modified E-cadherin and *erbB2* promoters using specific primers as follows: for the E-cadherin promoter, forward primer 5′-TCTAGAAAAATTTTTTAAAAA-3′ and reverse primer 5′-CAGCGCCGAGAGGCTGCGGCT-3′; for the *erbB2* promoter, forward primer 5′-CCTGGAAGCCACAAGGTAAAC-3′ and reverse primer 5′-TTTCTCCGGTCCCAATGGAGG-3′. The amplified DNAs were subcloned into the TA-cloning vector. Then we picked ten independent colonies in each case, determined the sequence of the promoter in each plasmid and examined the extent of methylation by determining the number and position of methylated cytosine residues.

Culture and transfection of cells

Human MCF-7 cells were cultured in minimum essential medium (MEM) supplemented with 10% FCS, 1% NEAA and 1 mM Na-Pyr. Human normal mammary epithelial cells (CAMBREX) were grown with mammary epithelial cell medium (CAMBREX). Transfection with pPUR-tRNA-shRNA was performed with the Effectene reagent (Qiagen) according to the manufacturer's protocol. For transfection of MCF-7 cells with various siRNAs, we used Oligfectamine (Invitrogen) according to the manufacturer's protocol.

5-aza-2′-deoxycytidine treatment

For inhibition of DNA methylation by 5-aza-2′-deoxycytidine (5-aza), cells grown to 30–40% confluence were transfected with E-cadherin-siRNAs and then were incubated for 96 h in culture medium that contained a final concentration of 1 μM 5-aza (Sigma).

Northern blotting analysis

Total RNA was purified from MCF-7 cells that expressed tRNA-shRNAs targeted to the *erbB2* promoter with ISOGEN reagent (Wako). Thirty micrograms of total RNA per lane were loaded on a 15% polyacrylamide gel. After electrophoresis, bands of RNA were transferred to a Hybond-N nylon membrane (Amersham). The RNA on the membrane was allowed to hybridize to ^{32}P-labelled probes that were complementary to the sequences of the tRNA-shRNAs. Sequences of synthetic probes were as follows: site 1, 5′-CUCUG CCCCCUCCCCCGGAGUCCGGGAUAA-3′; site 2, 5′-UCCUAGCGCCGGGAAGCU GGGUUGCCUGCA-3′; site 3, 5′-GGUGCGUCCCUCCUAGCGCCGGGAAGCUGG-3′; site 4, 5′-GGUGCGUCCCUCCUAGCGCCGGGAAGCUGG-3′; and site 5, 5′-GAGCAA GCGCGCUCCCAGCUCGCCCCCUCC-3′. For detection of the expression level of E-cadherin, *erbB2* and actin mRNAs, we used cDNA probes of E-cadherin, *erbB2* and actin, respectively.

Western blotting analysis

MCF-7 cells transfected with or without various E-cadherin-siRNAs targeted to the E-cadherin promoter and various tRNA-shRNAs targeted to the *erbB2* promoter were collected. Total protein (20 μg) was fractionated by SDS–polyacrylamide gel electrophoresis (10% polyacrylamide) and bands of protein were transferred to a polyvinylidene difluoride membrane (Funakoshi) by electro-blotting. Immunocomplexes were visualized with an ECL kit (Amersham) after reactions with monoclonal antibodies against E-cadherin (Transduction laboratories), ErbB2 (Oncogene), actin (Chemicon), DNMT1 (Imgenex) and DNMT3B (Imgenex), or with polyclonal antibodies against DNMT2 (Imgenex). Amounts of E-cadherin and ErbB2 were normalized by reference amounts of actin.

Determination of cell proliferation rates

Cell proliferation rates were determined with a Cell Proliferation Kit II (Roche) according to the manufacturer's instructions[16].

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REVIEW

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World of small RNAs: from ribozymes to siRNA and miRNA

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Abstract RNAs, besides bridging genetic information to proteins, the major determinants of bio-structures and functions, serve as active regulators of gene expression. Initiated nearly 20 years ago with ribozymes (the small RNAs with catalytic activity providing fine tuning of gene expression and function, used as molecular scissors and tools for gene discovery), an era of more complex and coordinated gene regulation by small RNAs, siRNA, and miRNA has recently started. Simple nucleotide complementarity results in highly ordered and regulated events, such as assembly of RNA and proteins, resulting in gene silencing either by mRNA degradation or suppression of translation. This article reviews our contributions to the understanding of structure, the function of small RNAs, their use in biotechnology, and the understanding of phenotypes such as apoptosis, metastasis, and differentiation.

Key words ribozymes · siRNA · miRNA · regulation of gene expression · neuronal differentiation

The world of small RNA inaugurated by the ribozyme: from gene silencing to gene discovery

RNA was previously viewed as a mere bridge between DNA (reservoir of all genetic information) and protein (determinants of biological structure, function, and integrity). Its perception as a simple vehicle for deciphering the genetic code to a translatable readout changed about 20 years ago when RNA with catalytic activity, ribozyme—RNA molecule with enzymatic properties—were discovered. Hammerhead ribozymes (because of the resemblance of their two-dimensional structure to a hammerhead) are the smallest ribozymes that are used as molecular scissors in molecular biology and biotechnology to elucidate and eliminate gene functions (Haseloff and Gerlach, 1988; Rossi, 1999). The RNA is induced to fold into its active conformation by the binding of metal ions. It forms two domains: the scaffold (domain 2), on which the ribozyme is built, and the active center (catalytic domain called domain 1) of the ribozyme. Over the last two decades, mechanisms of action of hammerhead ribozymes describing the requirement of divalent metal ions, definition of catalytic domains, and sequence specificity (usually referred to as target site specificity) have been largely demonstrated (Minoshima et al., 2003).

Ribozyme activity *in vivo* critically depends on its effective level of expression, specificity, intracellular stability, target co-localization, and accessibility to the target site. These technical issues impose a major difficulty in the use of ribozymes *in vivo*. Various modifications of plasmids to express ribozymes in cells have thus come into play. It was shown that the ribozymes expressed under the control of a modified RNA polymerase III promoter ($tRNA^{Val}$ promoter) were efficiently expressed, highly stable, and exported to the cytoplasm (Koseki et al., 1999; Kato et al., 2001; Kuwabara et al., 2001a, 2001b). Such expression improved ribozyme activity *in vivo* by many fold. However, efficiently expressed and highly stable ribozymes may still lack activity due to their inaccessibility to the target sites that in turn impose a major hurdle because of unforeseeable secondary and tertiary RNA structures. The rate-limiting step for the cleavage of phosphodiester bonds *in vivo* is the association and annealing of the ribozyme with its target site (Kato et al., 2001; Warashina et al., 2001; Kawasaki et al.,

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2002). In a long RNA molecule, with its secondary and tertiary folded structure, a significant number of target sites are inaccessible to the ribozyme. This phenomenon is often a serious problem in attempts to exploit ribozyme activity, in particular *in vivo*. To overcome the problems of accessibility, computer-generated predictions of secondary structure can be typically used to identify target sites that are most likely to be in an open and accessible conformation. However, such predictions are not always accurate because of unpredictable interactions between RNAs and proteins that influence the intracellular structures of RNAs.

A significant recent modification of ribozymes that overcomes their target inaccessibility has been demonstrated. In this modification, a helicase-binding motif that recruits helicase protein and its unwinding activity *in vivo* was linked to the 3′ end of the ribozyme sequence in the RNA polymerase III promoter ($tRNA^{Val}$)-driven expression plasmid (Warashina et al., 2001; Kawasaki and Taira, 2002a; Fig. 1). Such RNA helicase-coupled ribozymes were indeed shown to have substrate-unwinding as well as strong cleavage activity in *in vitro* and *in vivo* assays (Warashina et al., 2001; Kawasaki and Taira, 2002a; Kawasaki et al., 2002) and were effective where conventional ribozymes did not cause any change in gene expression (Wadhwa et al., 2003). Subsequent to this significant modification of ribozymes, it became possible to prepare effective libraries of hybrid ribozymes with randomized binding arms and to examine their effects after being introduced into cells (Kawasaki and Taira, 2002b; Kawasaki et al., 2002; Suyama et al., 2003a, 2003b). This led us to develop a novel approach for cloning functional genes. Briefly, libraries of hybrid ribozymes in which the target arm sequence was randomized were generated. These were introduced into cells by either plasmid or retroviral vectors and were combined with the selection of a loss or gain of a cellular phenotype. Isolation of ribozymes from selected cells, sequence analysis, and identification of genes in databases could lead to the identification of genes involved in these phenotypes (Fig. 2). The application of the ribozyme-based gene discovery system to apoptosis, metastasis, Alzheimer's disease, and muscle differentiation led to the isolation of several genes that are implicated in these phenotypes, confirming the validity and usefulness of this technology (Kruger et al., 2000; Li et al., 2000; Welch et al., 2000; Beger et al., 2001; Kawasaki and Taira, 2002a, 2002b; Kawasaki et al., 2002; Nelson et al., 2003; Suyama et al., 2003a, 2003b; Onuki et al., 2004; R.W. et al., unpublished data). Besides, many novel genes have been isolated, supporting it as an effective tool to isolate new genes and elucidate novel functional pathways. This system has also led to the isolation of small RNAs involved in regulation of neuronal differentiation (T. Kuwabara et al., personal communication).

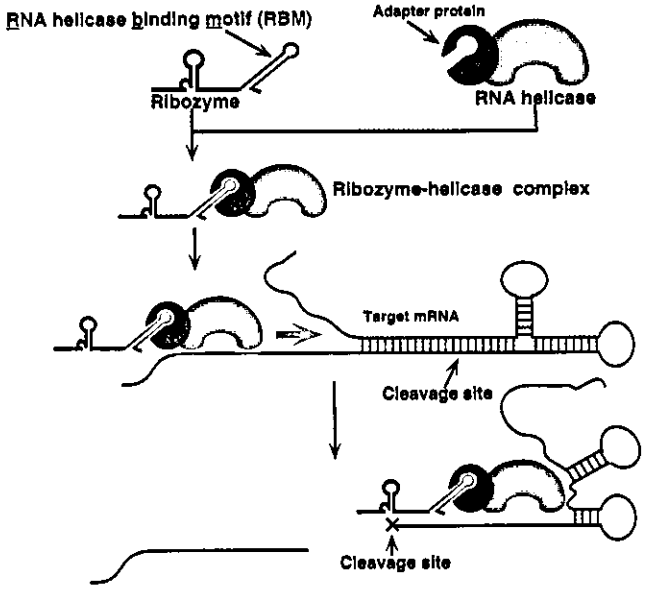

Fig. 1 Schematic representation of cleavage of a normally inaccessible target site by a sliding hybrid ribozyme. The ribozyme is linked to an RNA helicase-binding motif (RBM or connector RNA) that is capable of recruiting a helicase(s) to the target site where it unwinds the substrate structure to expose the cleavage site. The helicase might even be able to slide the ribozyme along the transcript. The coupling of helicase activity to the ribozyme allows suppression of the expression of genes whose transcripts were found previously resistant to cleavage by the conventional ribozyme.

Another significant modification of ribozymes was the construction of allosterically controllable ribozymes that bind to two different target sites (Kuwabara et al., 1999). Such ribozymes were effectively used for suppression of genetic disorders that arise from gene

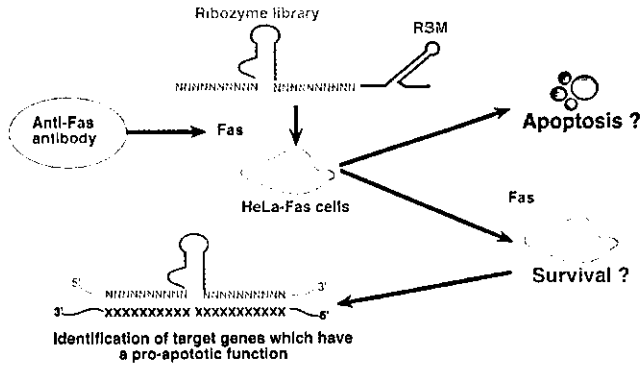

Fig. 2 Schematic presentation of a gene discovery system that can identify functional genes in the Fas-mediated pathway to apoptosis. Randomized RBM-connected ribozyme libraries were introduced into HeLa-Fas cells using the retrovirus expression system. After infection with the randomized RBM-Rz library expressing retrovirus, HeLa-Fas cells that expressed the randomized library were treated with Fas-specific antibody (α-Fas). Surviving clones were picked up and genomic DNA was purified from each clone. Sequences of RBM-connected ribozyme were determined by direct sequencing and the target genes of ribozymes were identified in databases by a BLAST search.

rearrangements. For example, chronic myelogenous leukemia is a hematopoietic malignant disease associated with the expression of a chimeric *BCR-ABL* gene. A ribozyme that can disrupt chimeric *BCR-ABL* mRNA exclusively and is neutral to normal *ABL* mRNA was designed and shown to specifically cleave *BCR-ABL* mRNA (Kuwabara et al., 1998; Tanabe et al., 2000).

The world of small RNA extended to siRNA and miRNA

In the post-genome era, when curiosity in the field of biology centered on "how gene expression and function are regulated," an amazing discovery in biological regulation flashed in science news and made its way to the top scientific journals. The articles, "brave new world of RNA," "a small fortune," "tiny regulators with great potential," "small RNAs: the genome's guiding hand," "something new under the sun," "breakthrough of the year," "small RNAs make big splash," and "big things from little RNA" talked about regulation of gene expression by small RNAs (Ambros, 2001; Couzin, 2002; Moss and Poethig, 2002; Pasquinelli, 2002; Moberg and Hariharan, 2003). These tiny regulators come from the large proportion of non-coding genome referred to as junk DNA and regulate the fate of cells, not by coding for any proteins with functional motifs, but by causing gene silencing by a highly coordinated and sequence-specific mechanism known as RNA interference (RNAi) and/or micro-RNA-mediated regulation. Small RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs), have emerged as the key components of an evolutionarily conserved system of RNA-based gene regulation in eukaryotes. They are involved in molecular interactions, including defense against viruses and regulation of gene expression during development (Cullen, 2002; Carrington and Ambros, 2003). How these 21- to 23-nucleotide (nt) small non-coding RNAs can act as small interfering RNAs (siRNAs) or miRNAs is just beginning to be understood. The two kinds of small RNAs, that is, siRNAs and miRNAs, are produced by the cleavage of double-stranded RNA precursors by Dicer, a member of the RNase III family of dsRNA-specific endonucleases (Bernstein et al., 2001; Provost et al., 2002; Zhang et al., 2002). siRNAs are 21-nt double-stranded RNAs that contain 19 base pairs, with 2-nt, 3′ overhanging ends, that act as functional intermediates in RNAi. These are formed in cells when transposons, viruses, or endogenous genes express long dsRNA or when dsRNA is introduced experimentally. In contrast, single-stranded miRNAs are the products of endogenous, non-coding genes whose precursor RNA transcripts can form small stem loops from which mature miRNAs are produced by Dicer (Ambros et al., 2003; Lim et al., 2003a, 2003b). miRNAs are encoded in genes distinct from the mRNAs whose expression they control. In contrast to siRNA, which can direct RNA destruction mediated by RISC (RNA-induced silencing complex; functions as an siRNA-directed endonuclease), miRNAs suppress protein expression by inhibition of mRNA translation (Fig. 3). Because both siRNAs and miRNAs are found in similar, if not identical, complexes, it is suggested that RISC might be a bi-functional complex that mediates both cleavage and translational control (Mourelatos et al., 2002). Furthermore, recent evidence suggests that siRNAs and miRNAs are functionally interchangeable, in which the target mRNA can be either cleaved or translationally repressed as determined by the degree of complementarity between the small RNA and its target (Hutvágner and Zamore, 2002; Doench et al., 2003; Zeng et al., 2003).

MicroRNAs (*miR* genes) are a large family of highly conserved non-coding genes transcribed as short hairpin precursors (~70 nt) that are processed into active 21- to 22-nt RNAs by a ribonuclease, Dicer, which recognizes target mRNAs via base-pairing interactions. The miRNA-miRNP (ribonucleoprotein) complex represses mRNA translation by partial base pairing to the 3′ UTR of target mRNAs. Small RNA of 18–25 bases called microRNA (miRNA) was reported for the first time by the group of Victor Ambros (Dartmouth College) in 1993. They found that miRNA that interacts with the 3′ UTR of *lin-4* mRNA regulates nematode

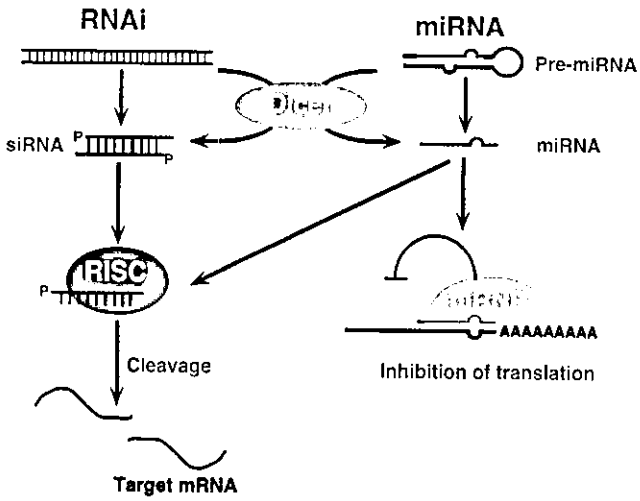

Fig. 3 Diagram to show siRNA and miRNA action. siRNAs are produced from long dsRNAs by ribonuclease Dicer. Then siRNAs are incorporated in RISC and the RISC cleaves the target RNAs. MicroRNAs are expressed as short hairpin RNAs (pre-miRNAs) and are transported to the cytoplasm. Pre-miRNAs are processed to mature miRNAs by Dicer. The miRNAs are incorporated in miRNPs and then miRNPs inhibit translation of target mRNAs by forming partial duplexes with target mRNAs. In addition, miRNA can function as siRNAs in the case of complete matching with target mRNAs.

development. Similarly, miRNA to *let-7* mRNA was identified. Both *lin-4* and *let-7* act as repressors of their respective target genes, such as *lin-14*, *lin-28*, and *lin-41*. Both *lin-4* and *let-7* small temporal RNAs were shown to play a central role in controlling the timing of *Caenorhabditis elegans* cell fate decisions. *let-7* has been conserved through evolution, and its expression correlates with adult development in animals, including *Drosophila* (Bashirullah et al., 2003). A best match for *lin-4* in *Drosophila*, miR-125, is also expressed during pupal and adult stages of *Drosophila* development (Sempere et al., 2003). To date, over 300 miRNAs from diverse eukaryotic organisms have been identified. Annotation of new miRNAs, so that miRNAs can be reliably distinguished from siRNAs, has been proposed along with the establishment of the Rfam database of RNA families (Ambros, 2001, 2003; Ambros et al., 2003; Grad et al., 2003). How these tiny RNAs work is only beginning to be understood; the major hurdle is to identify their target mRNAs. The fact that makes it even more arduous is that miRNA require only a partial homology (in some cases, about 70%). Some of the *Drosophila* miRNAs that align to the K box motif were shown to mediate a negative post-transcriptional regulation of the Hairy *HES* gene family in *Drosophila* (Lai and Posakony, 1997; Lai et al., 1998; Lai, 2002). The human miR-23 containing the antisense sequence to the K box motif was also identified, of which the target gene is not known.

We initiated a study to investigate whether human Hairy *HES* gene is the target of human miR-23 (Kawasaki and Taira, 2003). HES1 is a basic helix-loop-helix (bHLH) transcriptional repressor that is expressed in undifferentiated cells but not in differentiated cells (Kageyama et al., 2000). It participates in the Notch signaling pathway in mammals and acts as an anti-differentiation factor. miR-23 aligns to a coding region of human *HES1* mRNA near the termination codon and to mouse *Hes1* mRNA (nearly the same position as human *HES1* including the stop codon; Fig. 4a). A duplex of *HES1* and miR-23 was also observed using a prediction program of mRNA secondary structure (Mulfold), suggesting that miR-23 may be conserved phylogenetically as a regulator of human and mouse Hes1.

We used NT2 cells as a model for neuronal differentiation. When subjected to retinoic acid (RA)-induced differentiation, NT2 cells showed suppression of Hes1 expression that was independent of the level of mRNA, as detected by Northern analysis. miR-23, on the other hand, was easily detected in differentiated NT2 cells in contrast to undifferentiated cells. Introduction of the synthetic wild-type miR-23, but not the mutant miR-23, into undifferentiated NT2 cells resulted in the reduction of Hes1 protein. Of note, the level of *HES1* mRNA did not change in response to the synthetic miR-23, suggesting that synthetic miR-23

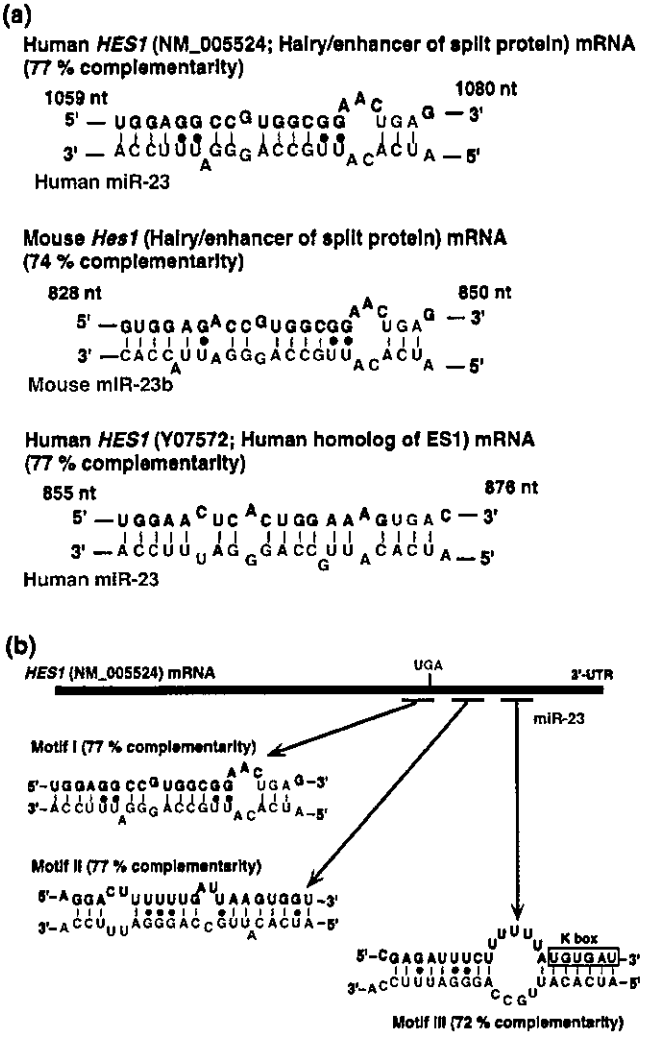

Fig. 4 HES1 is a target of miR-23. (a) The prediction of secondary structures between miR-23 and its target RNAs. The region nearly complementary to human and mouse miR-23 (blue) is located in the coding region, near the termination codon (red) of human Hairy *HES1* (NM_005524), mouse *Hes1*, and human homolog *HES1* (Y07572) mRNAs (top). (b) Human Hairy *HES1* (NM_005524) mRNA has three target regions (motifs I, II, and III) of miR-23 (bottom). Motif III has a K box sequence (black box) that is known, at least in the case of *Drosophila*, to be involved in post-transcriptional negative regulation.

causes gene silencing at the translational level. As expected, targeting of endogenous miR-23 by synthetic siRNA (siRNA-miR-23) indeed resulted in an elevation of HES1 protein, but the mutant siRNA-miR-23 had no effect. These data strongly support that HES1 is a target for miR-23. NT2 cells did not undergo RA-induced neuronal differentiation (as confirmed by cell morphology and differentiation markers such as MAP2) in the presence of siRNA-miR-23, suggesting that miR-23 plays a critical role during RA-induced neuronal differentiation of NT2 cells. The functional regulation of *HES1* by an miRNA mechanism was further confirmed by using two more miRNA motifs (Fig. 4b)

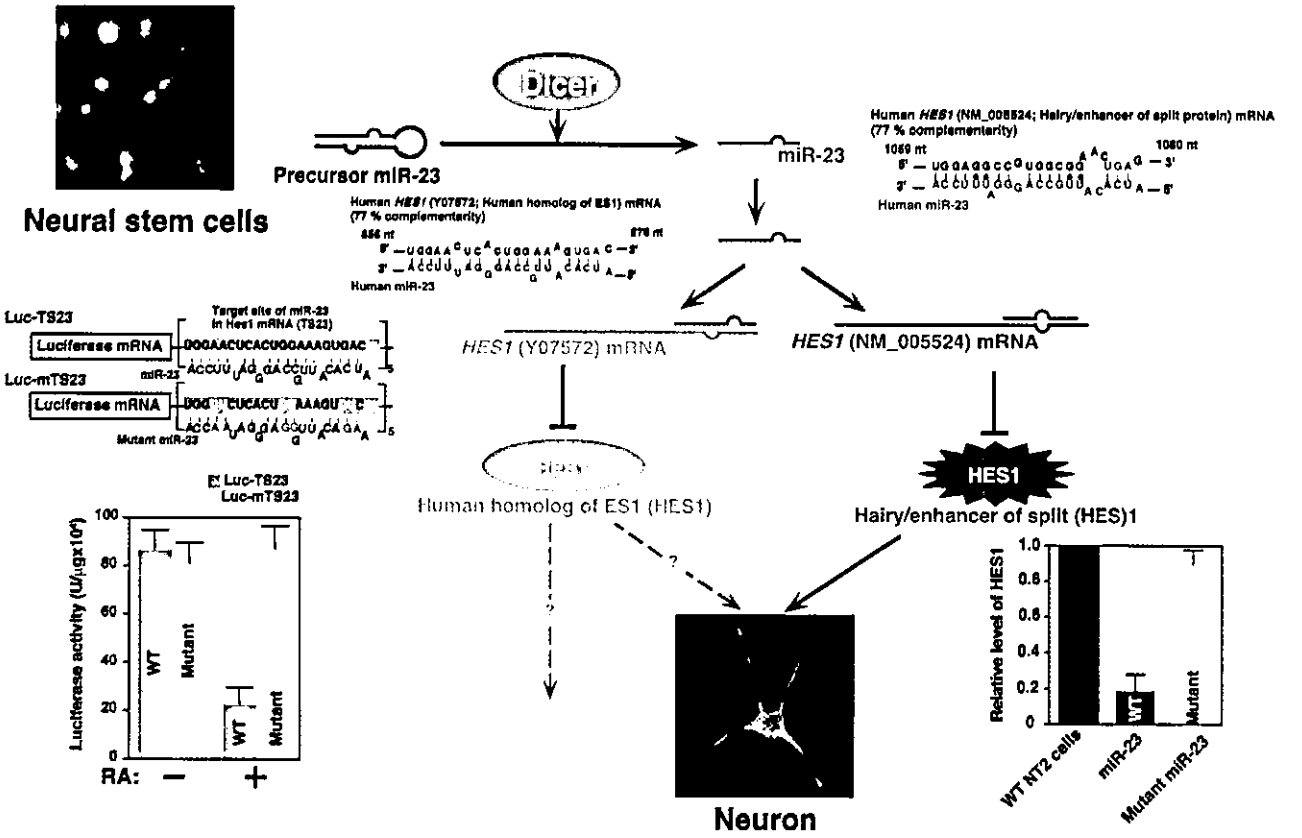

Fig. 5 The model of the function of miR-23 in RA-induced differentiation. When cells were treated with RA, pre-miR-23 is expressed. Then the pre-miR-23 is processed to mature miR-23 by ribonuclease Dicer. The mature miR-23 negatively regulates expression of Hairy *HES1* gene strongly and homolog *HES1* gene weakly. Then these events promote neuronal differentiation of NT2 cells. Left lower panel: sequences of genes for Luc-TS23 and mutant Luc-TS23 (Luc-mTS23). The activity of luciferase, due to the reporter genes, in NT2 cells in the presence and absence of RA. Right lower panel: the level of Hes1 mRNA in undifferentiated NT2 cells that had been treated with synthetic miR-23 or synthetic mutant miR-23.

and their various combinations along with the original miR-23 (Figs. 4b,5), which happened to match another cDNA also called *HES1* (human homolog of *Escherichia coli* and zebrafish; GenBank accession number Y07572; Scott et al., 1997; Kawasaki and Taira, 2003; unpublished data). We were informed of this ambiguity by Debbie Marks (Columbia University). Hairy/enhancer of split protein, also called *HES1* gene (accession number NM_005524; Feder et al., 1994), which actually encodes the transcriptional repressor HES1, was the subject of our study and is referred to as Hairy *HES1* (Kawasaki and Taira, 2003). Of note, in our study, the HES1 protein was detected by anti-HES1 antibody and the miR-23 was seen to suppress HES1 protein levels (Kawasaki and Taira, 2003). Thus, although the database sequence homologies raise doubts on the validity of the data demonstrating hairy HES1 as a target for miR-23, protein analysis stands by it.

To further clarify this issue, we investigated the effect of two additional independent miRNA target motifs (Fig. 4b) that were found in the 3′ UTR of Hairy *HES1* mRNA and did not match to homolog *HES1* (Fig. 5). Analysis of the activity of the luciferase reporter that was linked to the 5′ end of the miR-23 target site or the sequence of the other two unique potential target motifs in Hairy *HES1* mRNA revealed that the three independent target motifs (Fig. 4b) act as targets of miR-23 in Hairy *HES1* mRNA; each of these sites have only about 72–77% homology to the target. Thus, although detoured by the long route of confirmation and validation, the consequence of the study remains unchanged in describing that HES1 is regulated by miRNA such as miR-23.

A model for the function of miR-23 in regulation of RA-induced neuronal differentiation of NT2 cells is proposed (Fig. 5). When cells were treated with RA, pre-miR-23 is expressed. The pre-miR-23 is then processed to mature miR-23 by the ribonuclease Dicer. The mature miR-23 negatively regulates expression of Hairy *HES1* gene strongly and homolog *HES1* gene weakly. These events then promote neuronal differentiation of NT2 cells. However, the mechanism of expression of pre-miR-23 and inhibition of translation of both *HES1* mRNAs by miR-23 remains to be addressed in future studies.

In an independent study, we were compelled to recall what we referred to as pseudo-positives in our screens to

search for novel genes using a ribozyme-based discovery system. Many of the target sequences were ignored and forgotten because they did not belong to the coding genome. Those of us who could not ignore the pseudo-positives won this time. T. Kuwabara et al. (2004) used a randomized ribozyme library to isolate genes involved in neuronal development and isolated small RNAs that determine the fate of cells. It is becoming clear that nature has structured sequence information to embed in it not only structural and functional aspects of proteins but also non-coding regulatory elements such as siRNA and miRNA. An era of an unexplored functional RNA world has begun, and is predicted to go a long way in providing information for the understanding of the regulation of biological phenotypes.

Note added in proof: Kawasaki, H. and Taira, K. (2003) Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. Nature 423, 838-842. In this Article, the mRNA that is identified to be a target of microRNA-23 (miR-23) is from the gene termed human 'homolog of ES1' (HES1), accessionnumber Y07572, and not from the gene encoding the transcriptional repressor Hairy enhancer of split HES1 (accession number NM_00524) as stated in this paper. We incorrectly identified the gene because of the confusing nomenclature. Our experiments in NT2 cells had revealed that the protein levels of the repressor Hes1 were diminished by miR-23. Although we have unpublished data that suggest that miR-23 also interacts with Hes1 repressor mRNA specially in NT2 cells, given the incorrect use of the probe for the detection of the target mRNA resulting from our error, we respectfully retracted this paper [Kawasaki, H. and Taira, K. (2003). retraction: Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. Nature *426*, 100].

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