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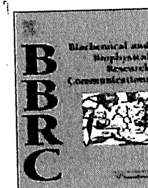

Differential expression of *nanog1* and *nanogp8* in colon cancer cells

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ABSTRACT

Nanog, a homeodomain transcription factor, is an essential regulator for promotion of self-renewal of embryonic stem cells and inhibition of their differentiation. It has been demonstrated that *nanog1* as well as *nanogp8*, a retrogene of *nanog1*, is preferentially expressed in advanced stages of several types of cancer, suggesting their involvement during cancer progression. Here, we investigated the expression of Nanog in well-characterized colon cancer cell lines. Expression of Nanog was detectable in 5 (HCT116, HT29, RKO, SW48, SW620) out of seven cell lines examined. RNA expression analyses of *nanog1* and *nanogp8* indicated that, while *nanog1* was a major form in SW620 as well as in teratoma cells Tera-2, *nanogp8* was preferentially expressed in HT29 and HCT116. In accordance with this, shRNA-mediated knockdown of *nanog1* caused the reduction of Nanog in SW620 but not in HT29. Inhibition of Nanog in SW620 cells negatively affected cell proliferation and tumor formation in mouse xenograft. Biochemical subcellular fractionation and immunostaining analyses revealed predominant localization of Nanog in cytoplasm in SW620 and HT29, while it was mainly localized in nucleus in Tera-2. Our data indicate that *nanog1* and *nanogp8* are differentially expressed in colon cancer cells, and suggest that their expression contributes to proliferation of colon cancer cells.

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1. Introduction

It has been well documented that a set of transcription factors, Oct-3/4, Nanog, and Sox-2, controls self-renewal and pluripotency of embryonic stem (ES) cells [1]. Accumulating reports indicate that these transcription factors, in combination with a set of regulatory microRNAs and transcriptional co-regulators that modify chromatin structure, form a key regulatory network controlling the identity and differentiation of ES cells [2–4].

Recent progress on cancer research revealed that, among the ES-specific transcription factors, Oct-3/4 and Nanog are expressed in a variety of human cancers [5–8]. Especially, expression of Nanog is associated with advanced stage and poor prognosis in some types of cancer [6,9–11].

Nanog is a homeodomain transcription factor that plays a crucial role in maintaining integrity of undifferentiated ES cells, and regulated by a variety of extrinsic and intrinsic signals [12]. Whereas external signal factors including HIF [13], Hedgehog (HH) [14,15], LIF [16], and TGF-β/activin/nodal [17,18] as well as the intrinsic transcription regulators such as Oct-3/4 and Sox-2 [19,20], SMAD [18], Klf4 [21,22], GLI1 [14,15], SATB [23], mSin3A–HDAC [24], β-catenin [25], and Sall4 [26] function to transcriptionally up-regulate Nanog expression, the induction of p53 [27,28] or epigenetic modification of its promoter [29,30] inhibits its expression. Nanog expression is also regulated by ES-specific microRNAs [31,32]. Activated Nanog, in a complex of associated transcription co-factors [33–35], in turn regulates gene transcription of its targets to promote self-renewal of embryonic stem cells and affects differentiation processes [2,34,36]. Presumably through the regulation of the expression of the target genes, Nanog functions to transit to a ground state for pluripotency of ES cells [37], and blocks their differentiation [38].

In addition to *nanog1*, an authentic gene that encodes Nanog, there are 10 pseudogenes for Nanog in the human genome [39,40]. Among them, *nanogp8* is the most recent pseudogene generated during evolution [39], and is regarded as a retrogene because it retains the capacity to code for a 305 amino acid polypeptide that is structurally very similar to the *nanog1* gene product [40], and is expressed in human cells [41]. In fact, ectopic expression of *nanogp8* generates the functional protein [41,42]. Because of high degree of homology between these genes, the protein products of *nanog1* and *nanogp8* are basically indistinguishable on western blot analyses or immunostaining [41,43]. Therefore, protein products of these genes are collectively referred to as Nanog [42,43].

Examination of Nanog expression in clinical studies revealed that Nanog is overexpressed in a variety of cancer [5–11,44,45].

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Indeed, expression of *nanog1* gene was demonstrated at least in some of cancer cell lines [46], and ectopic expression of *nanog1* contributes to stem cell-like properties to cells [5] and promotes cell proliferation [47]. Of note, *nanogp8* gene is rather a major form expressed in many types of cancer [15,41,42], promotes stem cell-like characteristics [15,43], and facilitates progression of cancer [15,41,42]. Thus, it is likely that both *nanog1* and *nanogp8* contribute to cancer development mediated by Nanog.

In human colon cancer, high levels of Nanog expression are associated with advanced stages of cancer and poor prognosis [10]. It is likely that expression of Nanog in colon cancer is functionally important because *nanog1* over-expression in colon cancer cells promotes its proliferation [10]. However, it remains unclear whether *nanog1* or *nanogp8* is a major form that is expressed in colon cancer cells, and the expression and subcellular localization of Nanog in those cells have not been well documented. These issues should be clarified to understand the molecular mechanisms of Nanog in colon cancer development.

In this paper, we investigated expression of Nanog in a set of well-characterized human colon cancer cells. Our data indicate that, while both *nanog1* and *nanogp8* are expressed in most colon cancer cells, they are expressed at varied ratios dependent on cell lines. Examination of subcellular localization indicates Nanog is mainly localized in cytoplasm in at least two colon cancer cells. In addition, Nanog inhibition by shRNAs in colon cancer cells caused growth inhibition in *in vitro* culture and in mouse xenograft, indicating the positive role of Nanog in proliferation of colon cancer cells.

2. Materials and methods

2.1. Cell culture

All colon cancer cells and Tera-2 were cultivated in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). FHC cells were cultivated in a 1:1 mixture of Ham F-12 medium and Dulbecco's modified Eagle medium (Invitrogen) supplemented with 25 mM Hepes (Invitrogen), 10 ng/ml cholera toxin (Calbiochem), 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 100 ng/ml hydrocortisone (Sigma), and 10% fetal bovine serum (Invitrogen).

2.2. Western blot analyses

Cells were lysed in lysis buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM EDTA) supplemented with protease inhibitors, and used for Western blot analyses as previously described [48], with anti-Nanog (ReproCELL, Tokyo), anti-Oct-3/4 (C-10, Santa cruz Biotechnology), anti-Sox-2 (H-65, Santa Cruz Biotechnology), anti-Actin (Sigma, A5316), anti-TopoI (C-21, Santa Cruz Biotechnology), or anti-beta-Tubulin (D-10, Santa cruz Biotechnology).

2.3. Lentivirus preparation and Infection

Lentiviral plasmid vectors expressing *Nanog1* shRNAs (#1, #2) or control shRNAs (#1, #2) were purchased from Sigma (the Mission shRNA clones). Note that *nanog1* shRNA (#2) corresponds to the older version of *nanog1* sequence (NM_024865.1) but not to the current version (NM_024865.2). Lentiviruses that express GFP or RFP (pCDH-CMV-MCS-EF1) were purchased from System Biosciences (CA, USA). The lentiviral plasmids were co-transfected with pLP1, pLP2 and pLP/VSVG (Invitrogen) into 293FT cells (Invitrogen), and virus-containing supernatants were prepared according to the manufacturer's instructions. For infection of the lentiviruses, SW620 cells were incubated with virus-containing supernatants in the presence of 6 μg/ml polybrene. Cells infected with the shRNA-expressing viruses or the control viruses were selected in the presence of 1 μg/ml puromycin. For cells infected with GFP or RFP-expressing viruses, flow cytometry analyses (FacsCalibur, Becton Dickinson) were performed to confirm that >90% of cells were infected.

2.4. RT-PCR analyses

Complementary DNA (cDNA) was synthesized from total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara). For amplification of *nanog* cDNA, the PCR reaction with the cDNA was performed with ExTaq (Takara) for 35 cycles with the following conditions: 94 °C (1 min), 68 °C (1 min), 72 °C (2 min). For amplification of *gapdh* cDNA, the PCR reaction was performed for 30 cycles with the following conditions: 94 °C (1 min), 55 °C (1 min), 72 °C (2 min). The following primer sets were used for PCR reactions: Nanog forward primer (5′-AACATGAGTGTGGATC-CAG-3′), Nanog reverse primer (5′-TCACTCATCTTCACACGTCTT-CAGGTTG-3′), GAPDH forward primer (5′-ACCACAGTCCAGTCCA TCAC-3′), GAPDH reverse primer (5′-TCCACCACCCTGTTGCTGTA-3′).

2.5. Subcellular fractionation

Cells were rinsed in 1× phosphate-buffered saline (PBS), and cytoplasmic and nuclear fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Thermo Scientific). The prepared lysates were used for western blot analyses.

2.6. Immunostaining

Cells were fixed in 4% paraformaldehyde in PBS for 15 min, washed with 1× PBS, permeabilized in PBS/0.1% Triton X-100 for 5 min at 4 °C, and blocked with PBS containing 3% bovine serum albumin (BSA). The fixed cells were then used for immunostaining with anti-Nanog (ReproCELL) and DAPI.

2.7. GFP/RFP competition assays

The GFP-expressing SW620 cells were infected with the lentiviruses that express Nanog shRNA or the control viruses, selected in the presence of 1 μg/ml puromycin, and mixed with RFP-expressing SW620 at a 1:1 ratio, and used for an *in vitro* culture. For mouse xenograft experiments, the infected cells (1×10^6 cells) were suspended in medium containing 50% Matrigel (Becton Dickinson), and subjected for subcutaneous injection into NOG (NOD/Shi-*scid* $IL\text{-}2rg^{null}$) mice. The inhibitory effects of Nanog shRNAs were evaluated by measuring the number of GFP/RFP-positive cells by flow cytometry, and calculating the reduction of GFP/RFP ratios after these experimental procedures.

3. Results and discussion

3.1. Nanog is expressed in a majority of colon cancer cell lines

In order to examine whether ES-specific transcription factors, Nanog, Oct3/4, and Sox2 are expressed in colon cancer cells, we performed western blot analyses of seven colon cancer cells (DLD-1, HCT116, HT29, RKO, SW48, SW480, SW620) as well as normal colon epithelial cells (FHC) and teratoma cells (Tera-2). We observed detectable levels of Nanog and Sox-2 expression in most cell lines, whereas Oct3/4 was not detected in any colon cancer cells

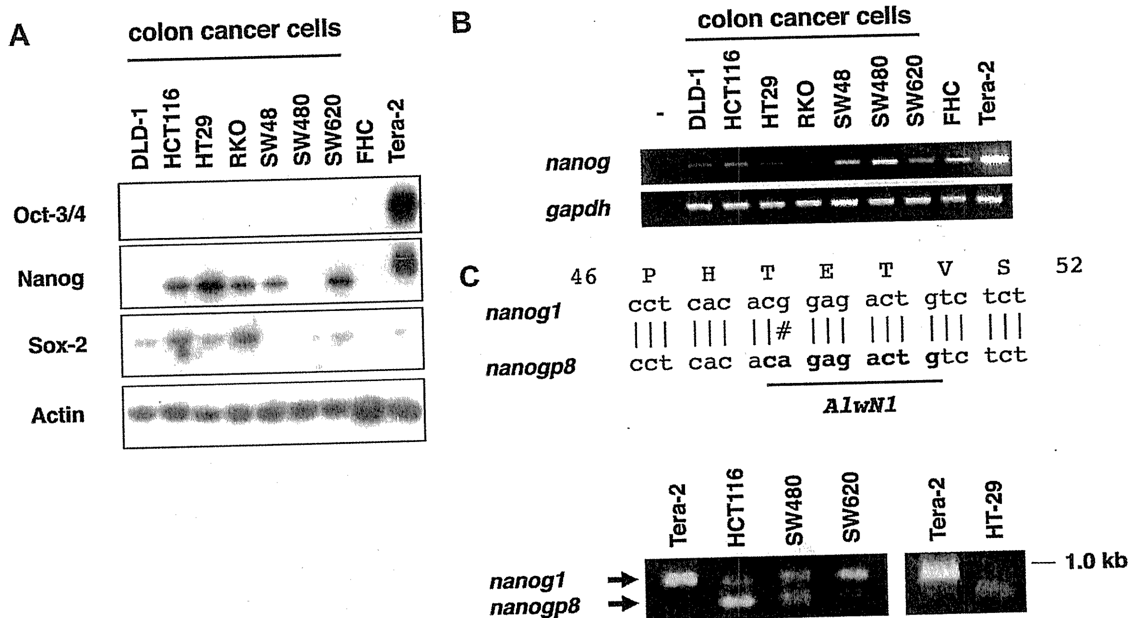

Fig. 1. *nanog1* and *nanogp8* were differentially expressed in colon cancer cells. (A) Western blot analyses of colon cancer cells. Designated colon cancer cells, colon epithelial cells (FHC), and teratoma cells (Tera-2) were used for Western blot analyses with antibodies against Oct-3/4, Nanog, Sox-2, or Actin. (B) RT-PCR analyses of *nanog* and *gapdh* genes for cells shown in A. For PCR amplification of *nanog*, primers that correspond to the N-terminal and the C-terminal sequences of both *nanog1* and *nanogp8* were used. cDNA fragments amplified by PCR were run on a 1% agarose gel and stained with ethidium bromide. (C) Digestion patterns of *nanog* cDNAs with the restriction enzyme *AlwN1* (upper panel). Unique sequence for the restriction enzyme *AlwN1* in *nanogp8* cDNA. Corresponding amino acid sequence is also shown (lower panel). Approximately equal amounts of *nanog* cDNAs from colon cancer cells (HCT116, SW480, SW620, HT-29) and Tera-2, which are shown in B, were digested with *AlwN1* and run on a 2% agarose gel and stained with ethidium bromide. The arrows indicate the position of the predicted size for *nanog1* and *nanogp8* cDNAs after the treatment with *AlwN1*. Note that the digested *nanogp8* cDNA is 148 nt shorter than *nanog1* cDNA.

(Fig. 1A). Nanog in colon cancer cells migrated faster than that in Tera-2 on western blot (Fig. 1A), probably due to its cell type-specific modification [46]. Because of the reported prognostic role of Nanog in progression of colon cancer [10], we focused on investigating the expression of Nanog in the following studies.

3.2. *nanog1 and nanogp8 were differentially expressed in colon cancer cells*

nanog1 and *nanogp8*, genes responsible for Nanog, encode similar polypeptides that differ from *nanog1* by only six nucleotides and two amino acids [40], and their products are indistinguishable on western blot analyses. Therefore, first we examined the total levels of RNA expression of the *nanog* genes by performing RT-PCR analyses with primers that match with the N-terminal and the C-terminal ends of both genes. The RT-PCR analyses of colon cancer cells indicated that the *nanog* genes were expressed in all colon cancer cells examined, although the expression levels were much lower than that in Tera-2 cells (Fig. 1B).

Next, we performed sequencing of the full-length cDNAs that were amplified by RT-PCR from teratoma cells (Tera-2) and from colon cancer cells (HT-29). 4 out of 4 cDNAs derived from Tera-2 corresponded to *nanog1* (data not shown). In contrast, 6 out of 6 cDNAs derived from HT-29 corresponded to *nanogp8* (data not shown), suggesting that *nanog1* and *nanogp8* are major forms of the *nanog* gene that is expressed in Tera-2 and HT-29, respectively.

In order to determine whether *nanogp8* is a dominant form in other colon cancer cells, we took advantage of the difference in their primary nucleotide sequences of the two genes. One of the nucleotide alterations in *nanogp8* should cause its cDNA to be a substrate for a restriction enzyme *AlwN1* (Fig. 1C, upper panel). Digestion of the full length PCR products with *AlwN1* revealed that each colon cancer cell lines expresses *nanog1* and *nanogp8* at varied ratios; *nanog1* is dominantly expressed in SW620 as well as in Tera-2, whereas *nanogp8* was expressed at a relatively higher level in HT-29 and HCT116 (Fig 1C, lower panel). Thus, two *nanog* genes are expressed at different ratios among colon cancer cell lines.

Of note, RT-PCR analyses in Fig. 1B indicated that *nanog1/nonogp8* was expressed in FHC, SW480, and DLD-1 at a level that was comparable to those in other colon cancer cells, although expression of Nanog protein was very low or undetectable in these cells (Fig. 1A), suggesting that Nanog expression is regulated at post-transcriptional levels.

3.3. *nanog1 and nanogp8 are dominant forms of Nanog in SW620 and HT-29 cells, respectively*

Next, we attempted to confirm differential expression of *nanog1* and/or *nanogp8* in colon cancer cells by knocking down these genes with shRNAs. Two shRNAs were used; one sh-RNA (sh-Nanog (#1), TRCN0000004887, sigma) is capable of knocking down both *nanog1* and *nanogp8*, whereas the other (sh-Nanog (#2), TRCN0000004884, sigma) can target only *nanog1*. The lentiviruses expressing these shRNAs were infected into SW620 and HT-29 cells to inhibit Nanog. Indeed, as expected from the distinct expression profiles (Fig. 1C), Nanog was inhibited by both shRNAs in SW620 (Fig. 2A), while it was inhibited by sh-Nanog (#1) but not by sh-Nanog (#2) in HT-29 (Fig. 2B). Combined with data shown in Fig. 1C, these data indicate that *nanog1* and *nanogp8* are mainly responsible for Nanog expression in SW620 and HT-29 cells, respectively.

3.4. *Nanog expression mediates cell proliferation and tumor formation of colon cancer cells*

In order to examine whether Nanog expression in colon cancer cells promotes their proliferation, we determined the growth rate

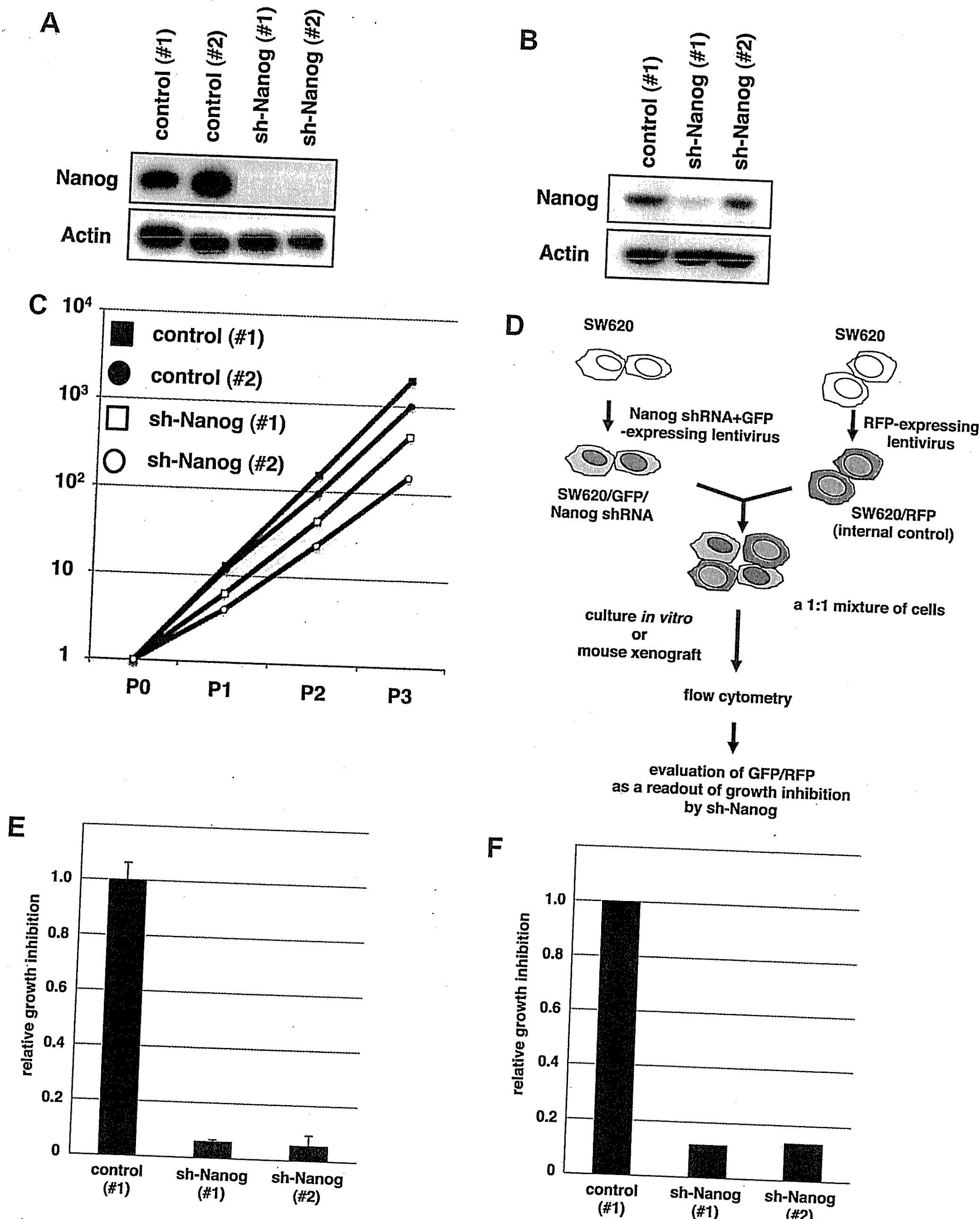

Fig. 2. Nanog expression mediates cell proliferation and tumor formation of colon cancer cells. (A) Western blot analyses of SW620 cells transfected with *nanog* shRNA-expressing lentiviruses or the control viruses. (B) Western blot analyses of HT-29 cells transfected with nanog shRNA-expressing lentiviruses or the control viruses. (C) Growth curves of SW620 cells introduced with lentivirus shown in A *in vitro*. (D) An experimental scheme for the GFP/RFP competition assays (see Section 2). (E) The GFP/RFP competition assays of SW620 cells infected with *nanog* shRNAs under normal culture condition. The shRNA-infected and the control cells were grown *in vitro* under normal culture conditions for seven passages (21 days), and the GFP/RFP ratios before and after the passage was calculated to determine the effects of each shRNA on cell growth. (F) The GFP/RFP competition assays of *nanog* shRNA-infected SW620 cells with in mouse xenograft. The effect of each shRNA on tumor formation was evaluated as described in E.

of SW620 cells after infection of the lentiviruses that express the Nanog shRNA or the control viruses. The inhibition of Nanog negatively affected the proliferation of SW620 (Fig. 2C).

We next questioned whether Nanog inhibition suppresses cell proliferation in a cell autonomous or non-autonomous manner by performing GFP/RFP competition assays under normal cell culture conditions (Fig. 2D). The inhibition of Nanog by shRNAs inhibited cell proliferation of SW620 cells in this assay (Fig. 2E), suggesting that the suppression of cell growth by Nanog inhibition is cell autonomous. Combined with data presented in Fig. 2D, these data indicate that Nanog expression promotes cell proliferation of SW620 cells.

We also performed the GFP/RFP competition assays after the subcutaneous injection of the mixture of GFP/RFP cells in immunocompromised NOG mice. Again, the suppression of Nanog inhibited cell proliferation of the transplanted cells. Thus, these data indicate that the suppression of Nanog in SW620 inhibit cell proliferation in mouse xenograft as well as under normal culture conditions.

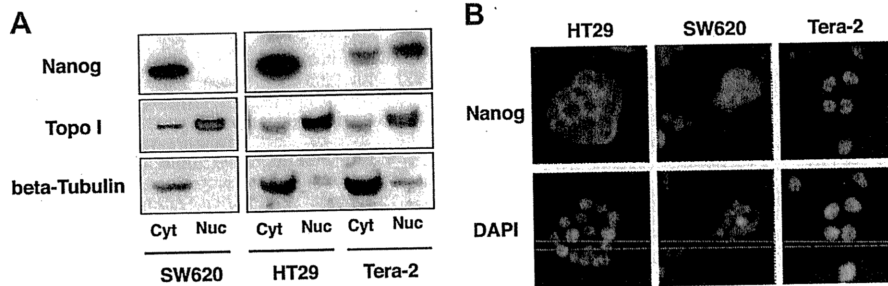

Fig. 3. Subcellular distribution of Nanog in colon cancer cells and teratoma cells. (A) SW620, HT-29, and Tera-2 cells were used for subcellular fractionation, and subcellular localization of Nanog was determined by performing Western blot analyses of the fractionated lysates with anti-Nanog antibody. Anti-TopoI antibody and anti-beta-Tubulin antibodies were also used to evaluate the fractionation. (B) Immunostaining of SW620, HT-29, and Tera-2 cells with anti-Nanog antibody.

3.5. Distinct pattern of subcellular distribution of Nanog in colon cancer cells and teratoma cells

Finally, we determined the subcellular localization of Nanog in colon cancer cells. In agreement with a previous report on immunohistochemical analyses of colorectal cancer [10], both western blot analyses after subcellular fractionation and immunostaining with anti-Nanog antibody indicated that Nanog was mainly localized in cytoplasm in SW620 and HT-29, which was in contrast to its nuclear localization in Tera-2 (Fig. 3A and B). Thus, Nanog is localized mainly in cytoplasm in at least two colon cancer cells.

Taken together, our data indicate that, while Nanog is expressed in a majority of colon cancer cells, *nanog1* and *nanogp8* are expressed with varied ratios among them, and it is likely that the differential expression of both genes contributes to Nanog expression, which promotes proliferation and tumor formation of colon cancer cells. Our data also suggest the importance of post-transcriptional regulation of Nanog expression in colon cancer cells. It will be interesting to determine its molecular mechanism in the future.

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Review Article

Systematic exploration of cancer-associated microRNA through functional screening assays

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MicroRNA (miRNA), non-coding RNA of approximately 22 nucleotides, post-transcriptionally represses expression of its target genes. miRNA regulates a variety of biological processes such as cell proliferation, cell death, development, stemness and genomic stability, not only in physiological conditions but also in various pathological conditions such as cancers. More than 1000 mature miRNA have been experimentally identified in humans and mice, yet the functions of a vast majority of miRNA remain to be elucidated. Identification of novel cancer-associated miRNA seems promising considering their possible application in the development of novel cancer therapies and biomarkers. Currently, there are two major approaches to identify miRNA that are associated with cancer: expression profiling study and functional screening assay. The former approach is widely used, and a large number of studies have shown aberrant miRNA expression profiles in cancer tissues compared with their non-cancer counterparts. Although aberrantly expressed miRNA are potentially good biomarkers, in most cases a majority of them do not play causal roles in cancers when functional assays are performed. In contrast, the latter approach allows screening of 'driver' miRNA with cancer-associated phenotypes, such as cell proliferation and cell invasion. Thus, this approach might be suitable in finding crucial targets of novel cancer therapy. The combination of both types of approaches will contribute to further elucidation of the cancer pathophysiology and to the development of a novel class of cancer therapies and biomarkers. (*Cancer Sci* 2011; 102: 1615–1621)

MicroRNA (miRNA) belongs to a class of non-coding RNA of approximately 22 nucleotides, and post-transcriptionally represses expression of its target genes.[1] miRNA are sequentially processed from precursors, either primary transcripts transcribed from the genome or intronic sequences of protein coding genes ('miRtrons'), by RNase III nuclease Drosha and Dicer. After their processing, miRNA are incorporated into the RNA-induced silencing complex (RISC), and the formed complex in turn represses the expression of the target genes, which have partially complimentary sequences with the miRNA in their 3′ untranslated regions (UTR), either by translational repression or cleavage of the target mRNA.[2] Although 3′ UTR is the main target of miRNA, 5′ UTR and open reading frames (ORF) were also reported as target sites of miRNA.[3] More than 60% of all protein coding genes have conserved miRNA binding sites in their 3′ UTR and are implicated to be the targets of miRNA.[4,5]

While miRNA was initially identified in *Caenorhabditis elegans*, it has been demonstrated that miRNA are evolutionarily conserved in many species, suggesting their universal roles in the regulation of gene expression.[6] The number of miRNA whose expression has been experimentally verified has grown rapidly over the last decade. This is partly because the use of sequencing-by-synthesis technology enabled the identification of novel miRNA with low-level expression.[7,8] Currently, 19 724 mature miRNA from 153 species are registered at the miRNA database (miRBase release 17, http://www.mirbase.org), including 1733 (1424 miRNA genes) in the human and 1111 (720 miRNA genes) in the mouse.[9] However, the functions of the vast majority of these miRNA remain to be elucidated. Because the roles of a miRNA depend on its target mRNA and the consequence of repressing multiple target mRNA under a specific cellular condition is difficult to predict, it is necessary to explore miRNA functions under experimental conditions of interest.

miRNA and cancer

It is estimated that more than 60% of all protein coding genes are the potential targets of miRNA.[4,5] Naturally the reported roles of miRNA are implicated in almost all aspects of cellular functions, including cell differentiation, cell death, cell cycle, developmental timing, inflammation, metabolism and stemness.[10–15] As expected from their involvement in normal physiological functions, dysregulation of miRNA expression has been shown to be involved in the pathogenesis of a wide variety of pathological conditions, such as heart disease, neurodegenerative disease and cancer. The dysregulation of miRNA is implicated in almost all aspects of cancer characteristics, including cell cycle, apoptosis, invasion/metastasis, angiogenesis and hypoxia-resistance.

The first evidence that miRNA is involved in the pathogenesis of cancer was obtained from the study of chronic lymphocytic leukemia (CLL), in which *miR-15a* and *miR-16-1* were identified on a region of the genome that was frequently lost in CLL patients.[16] These miRNA target anti-apoptotic protein BCL2, and their downregulation promotes cancers. Hence, it was proposed that these miRNA have a 'tumor-suppressive' role in the pathogenesis of CLL.[17] Since then, a number of reports have demonstrated the involvement of miRNA in cancers (Table 1).

Whereas miR-15a and miR-16-1 were identified through the study of aberrant chromosomes, most cancer-associated miRNA have been identified through expression analyses of miRNA in cancer tissues or by a 'candidate approach' in which potential cancer-associated miRNA that target known oncogenes or tumor-suppressor genes are evaluated as to whether they have cancer-related phenotypes. Interestingly, some miRNA in turn are directly regulated by cancer-associated transcriptional factors, including Myc, HIF, Stat3, p53 and Twist.[18] Thus, the emerging

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Table 1. Cancer-associated miRNA

Phenotype	miRNA	Function	References
Cell cycle	miR-16	Suppress CDK6, CARD10, CDC27; induce G0/G1 accumulation	41
	miR-17-92	Suppress E2Fs	42
	miR-27a	Suppress Myt-1	43
	miR-34a	Suppress Cdk4/6, cyclin E2, E2F3	20
	miR-122a	Suppress cyclinG1	44
	miR-124a	Suppress CDK6	45
	miR-221/-222	Suppress p27(Kip)	46
	let-7	Suppress Cdk6, Cdc25a, Cyclin D2; induce G0/G1 accumulation	41
Apoptosis	miR-15a/16-1	Suppress Bcl-2; induce apoptosis	17
	miR-22	Suppress p21; induce apoptosis in p53 wild cells	40
	miR-29b	Suppress Mcl-1; suppress apoptosis	47
	miR-34a/b/c	Suppress Bcl-2; induce apoptosis	20
Invasion/ metastasis	miR-10b	Suppress HOXD10; promote invasion/ metastasis	48
	miR-21	Suppress PTEN, Pdcd4; promote motility/ invasion	49
	miR-125a/b	Suppress ERBB2/3; suppress motility/ invasion	50
Angiogenesis	miR-27a	Suppress Zbdb10, promote angiogenesis	43
	miR-17-92	Suppress Tsp1, CTGF; promote angiogenesis	42
	miR-296	Suppress HGS: promote angiogenesis	51
	miR-378	Suppress SuFu, Fus-1; promote angiogenesis	52
Oncogene-associated miRNA	Myc	miR-9, miR-17-92	42
	E2F	miR-17-92	42
	Stat3	miR-21	53
	Twist	miR-10b	48
Tumor-suppressor-associated miRNA	p53	miR-26a, -34, -30c, -103, -107, -182, etc.	18

picture indicates that miRNA and transcriptional factors form an intertwined network during the development of cancers.

Oncogenic miRNA and tumor-suppressive miRNA. miRNA associated with the pathogenesis of cancers can either be classified as oncogenic miRNA (oncomiR) or tumor-suppressive miRNA, although their roles are sometimes dependent on cellular context. As suggested by their name, oncogenic miRNA promote phenotypes associated with cancers, including cell proliferation, invasion and resistance to apoptosis. OncomiR are in many cases upregulated in cancers, and their elevated expression is indispensable for sustained growth of cancer cells.[19] Therefore, inhibition of these miRNA by anti-miRNA can be a new class of molecular-targeted therapy.

In contrast, tumor-suppressive miRNA are miRNA that have anti-tumor functions. Among them is the miR-34 family that represses E2F3, Cdk4, Bcl2 and MET in response to genotoxic stress.[20–22] Of note, miR-34 is a downstream effector of p53, and introduction of miR-34 in cancer cells induces either apoptosis or premature senescence. MiR-34a upregulates p53 by translational repression of SIRT1, thus miR-34a and p53 constitute a positive feedback loop.[23]

Interestingly, miRNA are globally downregulated in many cancer tissues compared with their non-cancerous counterparts.[24] Knockdown of miRNA processing components promote cellular transformation and tumor growth *in vitro* and *in vivo*.[25] Furthermore, reduced expression of Dicer in a subset of lung cancer has been shown to be associated with poor prognosis.[26] These observations suggest that there exists a subset of unidentified miRNA whose downregulation promotes cancer development. Identification of such tumor-suppressive miRNA will be a promising area of future cancer research.

Identification of cancer-associated miRNA for clinical application

Because oncogenic and tumor-suppressive miRNA confer cancer-promoting or cancer-suppressing characteristics to cancers, these miRNA are regarded as potential targets for novel cancer therapies. In addition, aberrantly expressed miRNA can be used for the diagnosis of cancers. Considering that the functions of a substantial proportion of miRNA are not known, systematic exploration of cancer-associated miRNA might be beneficial to detect such clinically relevant miRNA. There are currently two major approaches to explore cancer-associated miRNA: expression analysis and functional assay.

Elucidation of cancer-associated miRNA through expression profiling. A body of evidence indicated there is a number of miRNA aberrantly expressed in cancer tissues in comparison with their non-cancerous counterparts (Table 1). Currently there are several techniques available for miRNA expression analysis including cloning, northern blotting, serial analysis of gene expression (SAGE), microarray, quantitative RT-PCR, *in situ* hybridization and sequencing-by-synthesis technology. Analyses of aberrant chromosomes and methylation status have also been performed to elucidate the underlying mechanisms of aberrant miRNA expression.[16,27] Among them, microarray is an experimental technique that is most widely used for genome-wide miRNA expression profiling. miRNA appear to be relatively stable in various storage conditions of cancer tissues, including fresh frozen tissues and formalin-fixed paraffin-embedded tissues. The relative stability of miRNA makes them good candidates for biomarkers, and expression profiling of miRNA by microarray has become an intense focus of current cancer research.[28] miRNA can also be detected in body fluids, especially blood, and they exhibit altered expression profiles in cancer patients, making them a new class of biomarker.[29–32]

Microarrays have been successfully used for genome-wide expression profiling of miRNA as well as protein-coding genes. However, there have been some technical drawbacks for microarray analysis of miRNA. Because mature miRNA are as short as ~22 nucleotides and members of a miRNA family are highly similar, discrimination of mature miRNA and their precursors is necessary for precise expression profiling of mature miRNA. To overcome the problem, several novel technologies are beginning to emerge for miRNA profiling, including the use of hairpin-structured or locked-nucleic acid (LNA) probes, length-adjusted probes and microfluidics platforms.[33–35] Another drawback of microarray analyses of miRNA is its lack of a proper normalization method. Because of such drawback of the microarray, quantitative RT-PCR (qRT-PCR) is widely used for the quantification of individual miRNA as well as genome-wide miRNA profiling.

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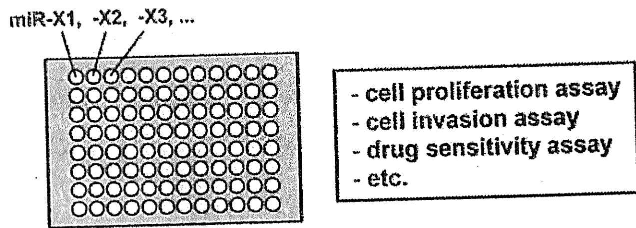

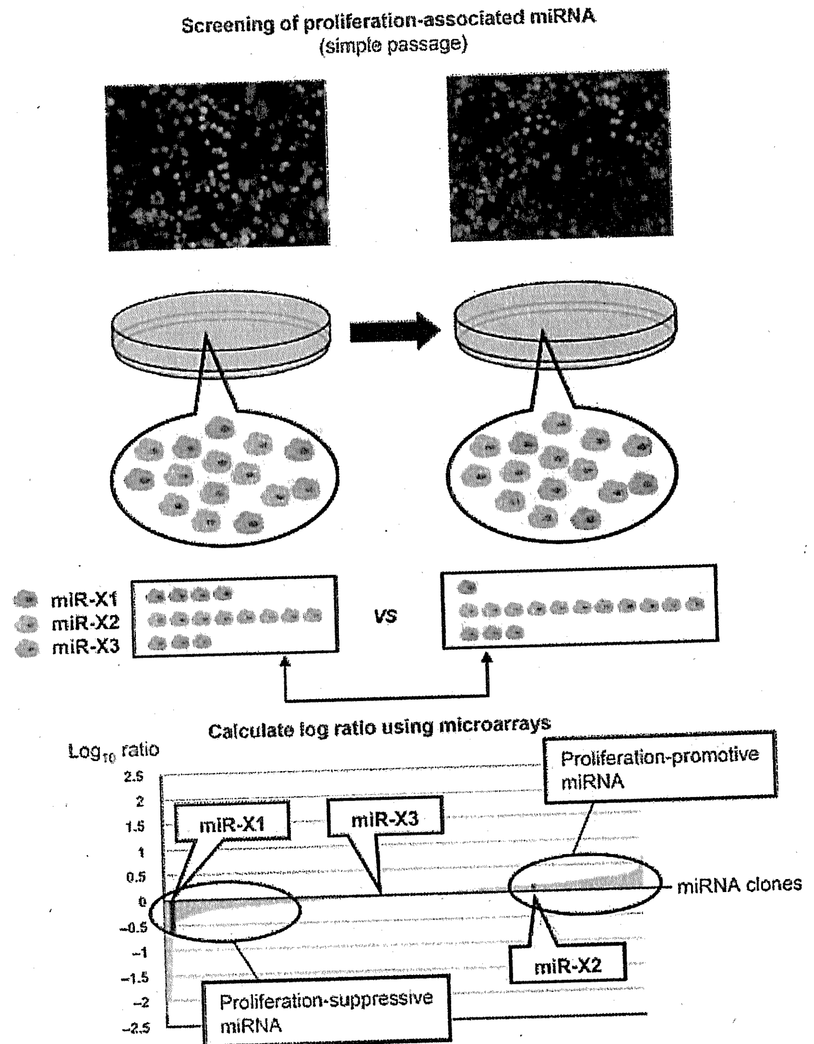

Fig. 1. Schematic view of the functional screening assays. In a single-plex format (A), miRNA undergo functional assays (e.g. cell proliferation assay, cell invasion assay, drug sensitivity assay, etc.) individually in separate wells of a multi-well plate. In a multi-plex format with viral-based expression vectors (B), a large number of cells are infected with a virus library expressing miRNA and then undergo phenotypic screening (e.g. cell proliferation). In the screening of proliferation-associated miRNA, cells transduced with each miRNA clone change their proportions in the whole cell population according to their effects on cell proliferation, which can be quantified using microarrays. For example, cells transduced with a proliferation-suppressive miRNA (miR-X1) or a proliferation-promotive miRNA (miR-X2) increase or decrease their proportion, respectively.

Alternative approach to detect cancer-associated miRNA: functional screening assay of miRNA

Another approach to elucidating miRNA that are associated with cancers is a functional screening assay (Fig. 1).[36–38] This is an assay that enables identification of miRNA that are causally linked to phenotypes of interest, irrespective of their levels of expression. Although there are several methodological variations among these assays, they are basically composed of the following two steps: (i) systematic introduction of miRNA into cells; and (ii) detection of exogenously introduced miRNA that confer cancer-associated phenotypes. Either the arrayed single-plex assay or the pooled multi-plex format with a virus-based expression vector is available for the functional screening assay of miRNA (Table 2).

Single-plex format using multi-well plate. In a single-plex assay, each miRNA is individually introduced to cells and their effects on cells are separately examined in a functional assay in multi-well plates (i.e. 96-well or 384-well format). Synthetic miRNA-mimics or vector-based miRNA can be used under this setting (Table 3). Using 319 synthetic miRNA in a 96-well plate format and chronometric cell viability assay, Nakano *et al.*[37]

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Table 2. Representative cancer-associated phenotypes assayed in the functional screening assays of miRNA and genome-wide RNAi screening

	miRNA	RNAi
Array based (single-plex)	Cell proliferation/survival[54]	Partners of *KRAS*[55]
Pool based (multi-plex)	Cell proliferation/survival[36]	Cell proliferation/ survival[56–58]
	Cellular transformation[38]	Resistance to nutlin-3[59]
	Cell migration and invasion[60]	p53-dependent proliferation arrest[61]
		Resistance to chemotherapy[62]
		Suppressor of epithelial cell transformation[63,64]
		Tumor suppressor in a mouse lymphoma model[65]

conducted single-plex gain-of-function analysis of miRNA associated with cell proliferation and identified a number of miRNA that increase or decrease cell viability in DLD-1 colon cancer cells. Among them are miR-362, -491 and -132, which do not exhibit aberrant expression in clinical colorectal cancer (CRC) samples.

Multi-plex format with viral-based expression vectors. A pooled multi-plex format with a virus-based expression vector is more complex but can be used for a wide range of applications.[39] Vector-based miRNA, especially those expressing miRNA precursors by retrovirus- or lentivirus-based vectors, are generally used in multi-plex assays, and stable expression of introduced miRNA allows functional screening under various experimental settings (Table 3). In general, a pooled library of miRNA is transduced to a single large cell population and biological selection of the transduced cells is performed. After selecting a cell sub-population with a phenotype of interest, miRNA that cause phenotypic changes are identified by sequencing. Although the viral-based approach has been successfully used to detect oncogenes or OncomiR, in general detection of these factors relies on the growth advantage they confer, and it is difficult to detect anti-miR via such an approach.

An alternative approach to identify cancer-related miRNA from library-transduced cells was initially demonstrated by Voorhoeve *et al.*[38] Genetic screening of miRNA was performed using a retrovirus library of miRNA precursors (~500 bp) and DNA barcode arrays. They successfully identified miR-372 and miR-373 as oncogenic miRNA that cooperate with oncogenic K-ras mutation in immortalized primary fibroblasts (BJ/ET cells). This is an example of the positive screening assay in which a cell population with phenotypes of interest (e.g. cell proliferation) increases during the selection process and the responsible clones are identified either by sequencing or microarrays. The combination of the miRNA-expressing virus library and the custom-made microarray can be used in the negative screening assay ('drop-out' screening) in which a cell population decreases in the selection process.

We have successfully identified miRNA that negatively regulate cell proliferation in pancreatic cancer cells using a lentivirus library of ~450 miRNA precursors and custom-made microarray.[36] Changes in the relative abundance of a miRNA clone (e.g. miR-X1) in the whole cell population were quantified by the comparison of differently labeled miRNA clones recovered

Table 3. Representative commercially available miRNA library

	Species	Structure
Synthetic miRNA-like molecules		
miRNA mimic (gain-of-function analysis)		
Pre-miR miRNA precursor molecule (Ambion, Austin, TX, USA)	H, M	Double stranded
miRIDIAN microRNA Mimic (Thermo Fisher Scientific, Lafayette, CO, USA)	H, M, R	Double stranded
miScript miRNA Mimics (Qiagen, Hilden, Germany)	H, M, R	Double stranded
MISSION (Sigma-Aldrich, St. Louis, MO, USA)	H	Double stranded
miRNA inhibitor (loss-of-function analysis)		
miRCURY LNA microRNA inhibitor (Exiqon, Vedbaek, Denmark)	H, M	Single stranded
Anti-miR miRNA inhibitors (Ambion)	H, M	Single stranded
miRIDIAN microRNA hairpin inhibitor (Thermo Fisher Scientific)	H, M, R	Single stranded
miScript miRNA inhibitors (Qiagen)	H, M, R	Single stranded
miArrest (GeneCopoeia, Rockville, MD, USA)	H, M, R	Single stranded
Virus vector-based miRNA		
miRNA (gain-of-function analysis)		
Virus vector (non-barcoded)		
Lenti-miR microRNA (System Biosciences, Mountain View, CA, USA)	H	HIV based, expressing miRNA precursors
miRNA library (miR-Lib) (NKI, Amsterdam, The Netherlands)	H	MSCV based, expressing miRNA precursors
miExpress (GeneCopoeia)	H, M, R	FIV based, expressing miRNA stem-loop
miRIDIAN shMIMIC microRNA (Thermo Fisher Scientific)	H	HIV based, expressing mature miRNA incorporated into a universal scaffold
Non-viral vector		
miExpress (GeneCopoeia)	H, M, R	Non-viral vector expressing miRNA precursor
miRNA inhibitor (loss-of-function analysis)		
miRZIP (System Biosciences)	H	HIV based
miArrest (GeneCopoeia)	H, M, R	HIV based

FIV, feline immunodeficiency virus; H, human; HIV, human immunodeficiency virus; M, mouse; MSCV, murine stem cell virus; R, rat.

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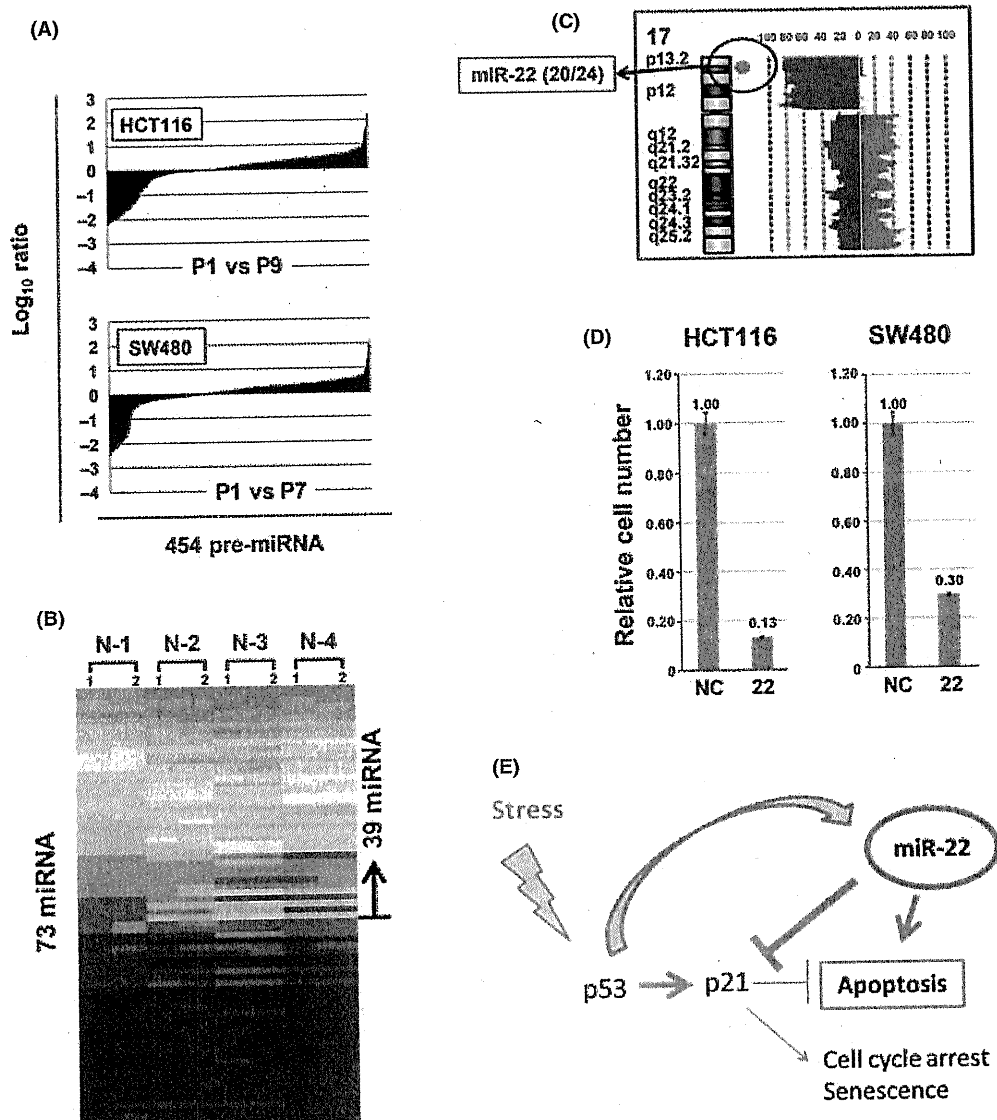

Fig. 2. (A) Results of dropout screening. HCT116 and SW480 cells were transduced with a lentivirus pooled miRNA expression library at multiplicity of infection (MOI) of 3. Exogenous transduced miRNA precursor genes were PCR amplified from genomic DNA recovered from cells immediately after library infection (passage 1, P1) and after several passages (P9 in HCT 116 cells and P7 in SW480 cells). Amplified DNA were labeled with Cy3 (P1) and Cy5 (P9 and P7), and competitively hybridized onto a custom-made microarray. Graphs are a scattered plot of the $\log_{10}$ ratio of each array set. (B) Expression profile of miRNA in non-cancerous parts of four colon cancer specimens using microarrays. A heat map was made using expression levels of 73 dropout miRNA. (C) Copy number aberration of chromosome 17 in 24 human colon cancer patients. Green and red indicate loss and gain, respectively. The position of the miR-22 gene is shown by the red dot. (D) Cell proliferation assay. HCT 116 and SW480 cells were transfected with 5 nM of either miR-negative control (NC) or miR-22 (22) and incubated for 5 days. Cell viability was measured by MST (Promega, Fitchburg, WI, USA) assay. Error bars indicate standard deviation in triplicate cultures. (E) Possible roles of miR-22 as an intrinsic molecular switch. In the exposure to oncogenic stresses, activated p53 transcriptionally activates both p21 and miR-22. MiR-22 represses p21 directly through the inhibition of translation and enhancement of mRNA degradation. Repression of p21 might cause the changes of cellular state from cell cycle arrest to apoptosis.

immediately after infection or after several passages (Fig. 1B). Five miRNA exhibited remarkable reduction in their abundance ($\log_{10}$ ratio <-1), indicating the proliferation-suppressive effect of these miRNA. Interestingly, one of these five miRNA was miR-34a, a representative tumor-suppressive miRNA that is transactivated by p53.[20,21] MiR-34a does not exhibit aberrant expression in pancreatic cancers or it exhibits mild upregulation in colorectal cancers. We have also reported that miR-222, which is upregulated in pancreatic cancers and has been shown to be tumor promotive by targeting p27, PUMA and PPP2R2A, has a tumor-suppressive effect in pancreatic cancer cells. These results suggest a methodological advantage of this functional screening assay to detect hidden cancer-related genes, and illustrates the need to explore miRNA functions in various tissues as the functions of miRNA are sometimes context-dependent, further showing the importance of experimental validation of cancer-associated miRNA using a functional screening assay. The flexibility of a multi-plex format assay also warrants exploration in more complicated settings, including *in vivo* settings. Moreover, use of lentivirus vectors broadens the possible application of the assay to primary or non-dividing cells, including neural and stem cells.

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miR-22, a novel tumor-suppressor gene, identified by functional genetic and comprehensive genomic analyses. The usefulness of a functional screening assay in the exploration of tumor suppressive miRNA can further be strengthened by combining the results of the assay with other data, especially those of clinical samples. We integrated the functional screening assay, expression profiling and chromosome analysis to identify miRNA species that function as tumor-suppressor genes in colon cancer[40]. Tumor-suppressor miRNA were defined as miRNA that: (i) repress cell proliferation; (ii) is expressed in normal colon tissues; (iii) is located at a frequently lost region on the chromosome; and (iv) is downregulated in colon cancers. As indicated in Figure 2A, considerable numbers of miRNA clones dropped out during the culture of HCT 116 or SW480 cell lines. Expression analysis of proliferation-suppressive ('drop-out') clones in non-cancerous colon tissues (Fig. 2B) and copy number analysis of colon cancer tissues (Fig. 2C) led to a novel tumor-suppressor miRNA miR-22 that satisfies the aforementioned four criteria.

The activity of repression for cell proliferation was again assessed by the introduction of miR-22 in both HCT 116 and SW480 cells (Fig. 2D). Interestingly, miR-22 induced apoptosis only in p53 wild-type cells, but it caused cell cycle arrest in p53 mutant cells.[40] Furthermore, we found that miR-22 is a transcriptional target of p53 and directly represses p21. Our findings define an intrinsic molecular switch that controls apoptosis by direct repression of p21 in response to strong stresses, in which cells should eliminate severely damaged cells to prevent malignant transformation (Fig. 2E).

In summary, functional screening of miRNA using either a single-plex or multi-plex format is a powerful genetic approach for the systematic elucidation of miRNA that cause cancer-associated phenotypes independent of their expression. Moreover, by combining the functional screening assay with expression profiling and genomic analysis, it should further facilitate the identification of novel cancer-associated miRNA that have a vital role in cancer pathophysiology, such as the miR-34 family and miR-22.

Perspectives

There is growing interest in the clinical application of miRNA. Aberrantly expressed miRNA in cancer tissues are good candidate biomarkers for the diagnosis of cancers and the prognosis of cancer patients, as has been shown by a large number of studies.[18] In addition, they are suitable as a good biomarker because of their ease of detection, high stability in clinical specimens and availability from the blood of cancer patients. Furthermore, miRNA themselves can be a novel class of molecular targets in cancer therapy. In theory, suppression of upregulated oncogenic miRNA by anti-miRNA or introduction of downregulated tumor-suppressive miRNA by synthetic or viral-vector based miRNA might be effective to cure cancer, although the development of effective drug delivery systems is another challenge. Identification of regulators of the mechanisms of cancer-associated miRNA will provide novel therapeutic targets. Despite many obstacles, exploration of cancer-associated miRNA will contribute to the elucidation of the pathogenesis of cancers and the development of novel cancer therapies and biomarkers.

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Disclosure Statement

The authors have no conflict of interest.

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Cancer Research

Tumor Suppressor *miR-22* Determines p53-Dependent Cellular Fate through Post-transcriptional Regulation of p21

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Abstract

Selective activation of p53 target genes in response to various cellular stresses is a critical step in determining the ability to induce cell-cycle arrest or apoptosis. Here we report the identification of the microRNA *miR-22* as a p53 target gene that selectively determines the induction of p53-dependent apoptosis by repressing p21. Combinatorial analyses of the AGO2 immunocomplex and gene expression profiles identified *p21* as a direct target of *miR-22*. Induction of p21 was inhibited by *miR-22* after exposure to the genotoxic agent Adriamycin (doxorubicin; Bedford Laboratories), sensitizing cells to p53-dependent apoptosis. Interestingly, the activation of *miR-22* depended on the intensity of the stresses that induced cells to undergo apoptosis in the presence of *p21* suppression. Our findings define an intrinsic molecular switch that determines p53-dependent cellular fate through post-transcriptional regulation of p21. *Cancer Res; 71(13); 4628–39. ©2011 AACR.*

Introduction

The p53 tumor suppressor network plays a crucial role in the prevention of malignant transformation in normal cells by maintaining the integrity of signaling pathways in response to various oncogenic stresses, including DNA damage, acute activation of oncogenes, and hypoxic conditions (1). The outcome of p53 activation in response to cellular stresses ranges from the induction of cell-cycle arrest for DNA repair to apoptosis for the complete elimination of damaged cells (2–4). The commitment to one of these alternative cellular fates depends on the set of p53 target genes induced by different stresses. Induction of cell-cycle arrest is mediated by the activation of the cyclin-dependent kinase inhibitor *CDKN1A* (hereafter referred to as p21), whereas apoptosis is induced by the activation of pro-apoptotic genes, including *NOXA* (5), *PUMA* (6), and *BAX* (7) that encode the regulators of intrinsic apoptosis pathways.

Post-translational modifications of p53 are involved in the selective activation of its various target genes leading to apoptosis (8, 9). Phosphorylation of p53 at serine 46 (Ser46), mediated by HIPK2 (10), regulates apoptotic pathways through the activation of *p53AIP1* (11). Furthermore, acetylation of p53 at lysine 120 (K120) by Tip60 is essential for the expression of *PUMA* (12). Ongoing work focuses on the elucidation of p53 function and its regulation as a transcriptional factor.

Recently, the regulation of gene expression by small noncoding RNAs, including microRNAs (miRNA), has been reported to play crucial roles in the maintenance of homeostasis in a wide range of cellular processes, including differentiation, control of cell proliferation, and stress responses (13–15). The important feature of miRNAs is the targeting of multiple cellular mRNAs, resulting in the efficient activation or repression of intracellular or intercellular signaling networks at specific times during animal development. miRNA dysfunction therefore causes defects in the integration of signaling networks essential for the maintenance of cellular homeostasis.

miRNA dysfunction has been suggested as a dominant cause of the onset of human disorders, especially cancers. Indeed, aberrant expression of miRNA genes was observed in almost all types of human cancers (16, 17). As a consequence of miRNA dysfunction, cancer cells acquire properties that favor the activation of oncogenic pathways or the repression of tumor-suppressive networks, contributing to cancer progression and metastasis (18–22). *MiR-21* was shown to repress *PTEN,* activating the phosphoinositide 3-kinase (PI3K)–AKT pathway and reflecting its oncogenic role (23). By contrast, *miR-34a* was identified as a p53-regulated tumor-suppressive miRNA in human colon cancer and shown to induce p53-dependent apoptosis or premature senescence, forming a positive feedback loop with p53 (24–28). The function of miRNAs as oncogenes or tumor suppressor genes is therefore well known, and

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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it implies that the incorporation of miRNA species as critical components of intracellular signaling pathways is crucial for the reconstitution of integrated cancer-related networks necessary to fully clarify the molecular basis of carcinogenesis.

To analyze the connection between miRNAs and signaling networks, a functional genetic screening method named "dropout assay" was recently established using a lentivirus miRNA expression library and a home-made microarray to quickly and efficiently isolate tumor-suppressive miRNAs (29). In the present study, an *in vitro* functional genetic screen and comprehensive genomic screens of clinical samples were used to identify tumor suppressor miRNAs in colon carcinogenesis, with the resulting identification of *miR-22* as a tumor suppressor gene. A p53–*miR-22*–p21 axis was identified as a crucial regulatory component involved in the determination of p53-dependent apoptosis. Our results suggest that *miR-22* is an intrinsic molecular switch or sensor for the determination of p53-dependent cellular fate in response to distinct stresses, and *miR-22* dysfunction could affect the anticancer barrier against various oncogenic insults.

Materials and Methods

Cell culture

HCT 116 (HCT 116 $p53^{+/+}$) and HCT 116 $p53^{-/-}$ (30) were kindly provided by Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD). These cell lines were authenticated by morphologic inspection, and mycoplasma testing using PCR. The activation of p53 pathways was confirmed by checking the induction of p53 target genes after exposure to DNA damage before starting the experiments. The SW480 colon cancer cell line was obtained from the American Type Culture Collection and authenticated as described above. Mutation of *TP53* was confirmed by sequencing. These cell lines were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated FBS in humidified air with 5% CO_2.

Clinical samples

Paired surgical specimens of primary human colon cancers and surrounding noncancerous colon tissue counterparts were obtained from patients treated at the Teikyo University Hospital (Mizonokuchi, Kanagawa, Japan) with documented informed consent in each case. Institutional review board approval for the analysis of clinical samples was obtained at each institute.

Functional miRNA dropout screening

Functional dropout screening to identify tumor suppressor miRNAs was carried out according to our recent publication (29). HCT 116 cells were transduced with a pooled lentivirus miRNA expression library (SBI) at a multiplicity of infections (MOI) of 3. Cells were incubated in complete medium for 3 days (P1) and subjected to sequential passages every 3 days. After 9 passages, genomic DNA was prepared from P1, P5, and P9 cells and subjected to array CGH analysis using a home-made microarray.

Quantitative real-time PCR

For quantitative expression analysis of miRNAs, total RNAs from colon cancer patients were reverse-transcribed by Multiscribe RT and miRNA-specific miRNA primers (ABI), and quantitative real-time PCR (qRT-PCR) was carried out by using a TaqMan microRNA assay kit (ABI). The comparative cycle threshold (C_t) method was applied to quantify the expression levels of miRNAs. Relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. *U48* small nuclear RNA was used as an internal standard.

Chromatin immunoprecipitation sequencing

HCT 116 cells were treated with 5-fluorouracil (5-FU; 0.375 mmol/L) for 9 hours, and chromatin immunoprecipitation (ChIP) was conducted by using anti-p53, antimonomethylated or antitrimethylated histone H3 K4, or antitrimethylated histone H3 K36 antibodies. ChIP-isolated DNA was subjected to the sequencing using an Illumina platform.

AGO2-IP on ChIP analysis

The AGO2-IP on ChIP assay was carried out according to a previous report with minor modifications (31). In brief, HCT 116 cells stably expressing HA-AGO2 were transfected with either *miR-22* (Pre-miR precursor molecule, Ambion) or miR-NC (Pre-miR miRNA Precursor Molecules Negative Control #2, Ambion) for 24 hours, and immunoprecipitated using anti-HA agarose beads. AGO2-bound RNA was eluted in boiling water, and the Trizol-LS reagent was added to extract total RNAs. AGO2-bound total RNAs were cleaned further using an RNeasy column and subjected to microarray analysis.

Reporter plasmid construction and luciferase assay

Amplification of the 3′ UTR of *p21* mRNA was carried out by PCR from HCT 116 genomic DNA using a primer set (Supplementary Table S1). The DNA fragment was fused to the 3′ end of a *firefly* luciferase reporter gene in a pmirGLO dual luciferase vector (Promega). Site-directed mutagenesis of a *miR-22* target site of *p21* mRNA was carried out by using a PrimeSTAR Max high fidelity DNA polymerase using the pmirGLO-*p21* 3′UTR plasmid as a template. HCT 116 cells, seeded at 5×10^4 cells/mL, were cotransfected with 200 ng of reporter plasmid and 10 nmol/L of either *miR-22* or miR-NC using Lipofectamine 2000. After incubation for 24 hours, luciferase activities were determined by using a dual luciferase assay kit (Promega). Luciferase activity was normalized by *Renilla* luciferase activity as an internal standard.

Immunoblot analysis

Cells were lysed in lysis buffer consisting of 25 mmol/L Tris-HCl (pH7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.1% SDS and 1× proteinase inhibitor cocktail, and equal concentrations of protein samples were loaded on a 10% to 20% polyacrylamide gradient gel (ATTO). After electrophoresis, proteins were transferred to a PVDF membrane, and immunoblot analysis was conducted by the standard method.

Supplementary information

Supplementary information includes extended Materials and Methods, 8 figures, and 4 tables.

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Results

Identification of *miR-22* as a candidate tumor suppressor miRNA by functional genetic and comprehensive genomic screens

A screening method for the efficient identification of tumor suppressor miRNAs in colon cancer was established and is depicted in Supplementary Fig. S1A. Tumor suppressor miRNAs were defined as follows; (i) repressor of cell proliferation, (ii) expression in normal colon tissue, (iii) high-frequency loss of their chromosomal positions, and (iv) downregulation in human colon cancers. Following these criteria, a functional genetic screening, namely a "dropout assay," was conducted using a lentivirus miRNA expression library (29) to isolate repressors of cell proliferation in a colon cancer cell line (Supplementary Fig. S1B). HCT 116 cells were transduced with a pooled lentivirus library containing 454 miRNA species and propagated for 3 weeks with sequential passages. Genomic DNA from the first passage (P1), fifth passage (P5), and ninth passage (P9) cell populations was prepared, and copy numbers of each miRNA clone in these cells were compared by array CGH analysis using a home-made microarray (Supplementary Fig. S1B). A total 55 miRNA clones were reproducibly dropped out in a culture time-dependent manner (Supplementary Fig. S1C and Table S2). Among these dropout clones, 24 miRNAs were confirmed for their expression in normal tissue (Supplementary Fig. S1D). Furthermore, we carried out array CGH analysis (aCGH) to examine autosomal copy number aberrations using 24 colon cancer patients and finally identified 6 miRNA clones whose genes show hemizygous deletions in cancers with a high frequency (>30%), as candidates for tumor suppressor gene in colon

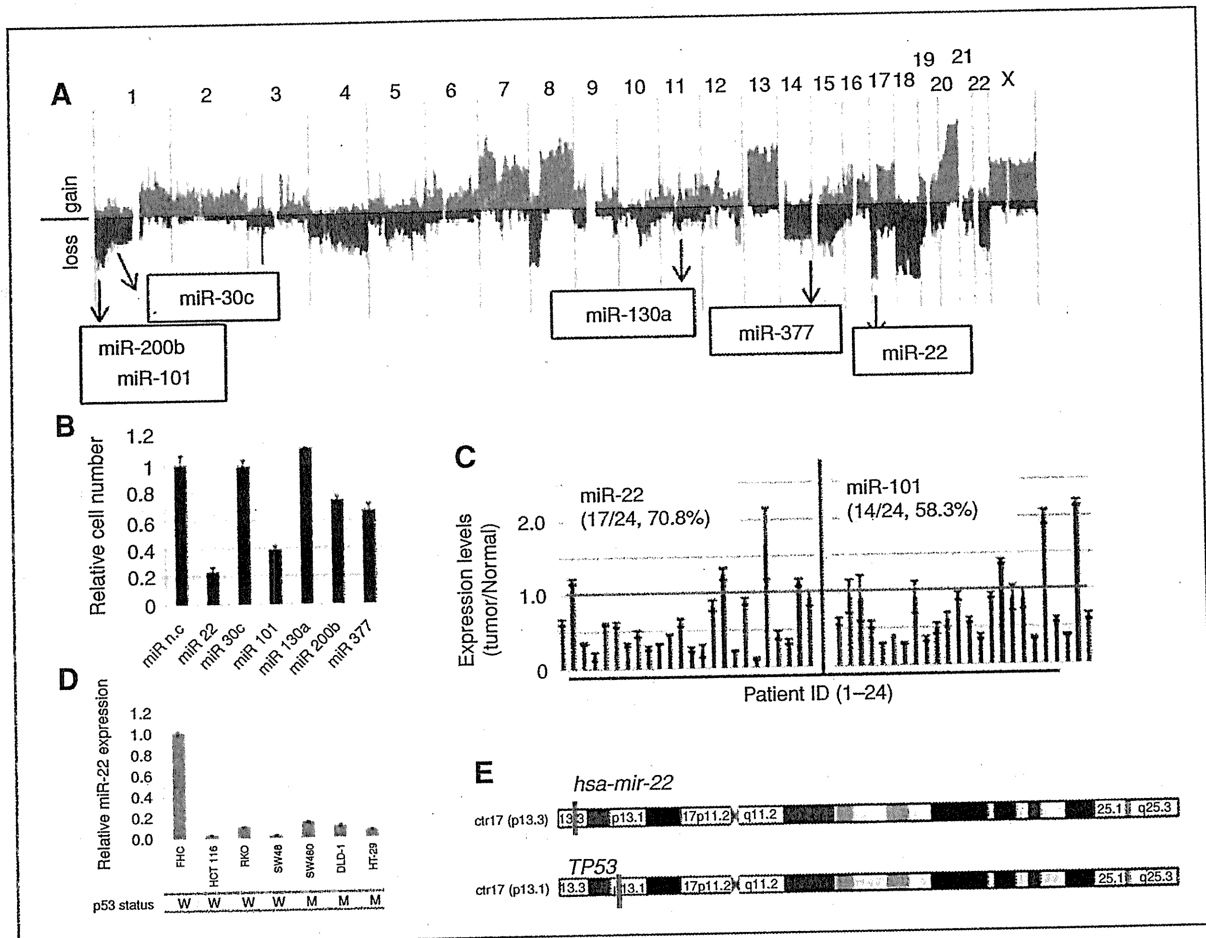

Figure 1. A, result of copy number aberrations in 24 human colon cancer samples. Red and green indicate chromosomal gain and loss, respectively. Chromosomal positions of 6 identified miRNA genes are shown in the CGH result. B, cell proliferation assay. HCT 116 cells were transfected with each synthetic miRNA and incubated for 5 days. Cell viability was measured by MST assay. Error bars indicate SD in triplicate cultures. C, expression of *miR-22* and miR-101 in human colon cancer patients. Expression levels of *miR-22* and *miR-101* were quantified by TaqMan microRNA qRT-PCR. The graphs show the relative expression levels of *miR-22* and *miR-101*, calculated by adjusting their expression levels to matched normal counterparts in each cancer sample. The red line indicates the relative expression level of 1.0. D, expression of *miR-22* in human colon cancer cell lines and normal colon-derived FHC cells. The genomic status of *TP53* in cancer cell lines is indicated. E, chromosomal positions of *miR-22* and *TP53* genes on chromosome 17.

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Table 1. Genomic status of *hsa-miR-22* and *TP53* genes in 24 human colon cancers

		has-miR-22 (17p13.3)		TP53(17p13.1)					
		chrl7:1,563,947-1,564,081		Chrl7:7,520,037-7,531,588					
Sample No	Patient ID	CNA	Expression (T/N)	CNA	Mutation	Exon	Codon	WT (A.A.)	Mut (A.A.)
1	1002	—	0.635	—	—				
2	1004	—	1.184	—	missense	7	237	ATG (M)	ATA (I)
3	1008	Loss	0.587	Loss	missense	6	193	CAT (H)	CGT(R)
4	1010	Loss	0.172	Loss	missense	8	285	GAG (E)	AAG (K)
5	1011	—	0.602	—	—				
6	1013	Loss	0.001	Loss	—				
7	1014	Loss	0.321	Loss	missense	7	230	GAA (E)	AAA (K)
8	1015	Gain	0.470	Gain	—				
9	1016	Loss	0.272	Loss	insertion (4)	7	280		
10	1017	Loss	0.327	—	—				
11	1018	Loss	0.455	—	—				
12	1019	—	0.614	—	—				
13	1022	Loss	0.248	Loss	missense	7	245	GGC (G)	TGC (C)
14	1023	Loss	0.227	Loss	missense	7	245	GGC (G)	TGC (C)
15	1024	Loss	0.835	Loss	missense	7	248	CGG (R)	CAG (Q)
16	1025	Loss	1.285	Loss	missense	5	175	CGC (R)	CAC (H)
17	1027	Loss	0.227	Loss	missense	7	248	CGG (R)	CAG (Q)
18	1028	Loss	0.888	Loss	missense	5	158	CGC (R)	CAC (H)
19	1029	Loss	0.114	Loss	—				
20	1033	Loss	1.668	Loss	deletion (1)	8	267		
21	1035	Loss	0.429	Loss	deletion (18)	5	174		
22	1036	Loss	0.339	Loss	missense	5	152	CCG (P)	CTG (L)
23	1037	Loss	1.139	Loss					
24	1039	Loss	0.922	Loss	deletion (6)	7	235	AAC (N)	ATG (M)

Two patients, sample numbers 10 and 11, showed hemizygous loss of the *miR-22* gene locus with intact *TP53*. Three patients, sample numbers 1, 5, and 12, showed downregulation of *miR-22* with intact *TP53*.

cancer (Fig. 1A and Supplementary Fig. S1E). Two of them, *miR-22* and *miR-101*, showed strong inhibition of cell proliferation in HCT 116-p53$^{+/+}$ cells by MST assay (Fig. 1B). As shown in Fig. 1C, *miR-22* and *miR-101* showed reduced expression in 70.8% and 50.3% of colon cancer cases, respectively, when compared with their normal counterparts. *MiR-22* also showed significant downregulation in 6 colon cancer cell lines in comparison with FHC cells derived from normal colon epithelium (Fig. 1D), which was not observed for *miR-101* (data not shown). Interestingly, CGH analysis showed deletion of the *miR-22* locus without loss or mutation of *TP53* localized to the 6Mb centromeric region of the *miR-22* gene in 2 colon cancer patients, and 3 other cases showed a significant reduction of *miR-22* expression (Fig. 1E and Table 1). Furthermore, in a copy number assay using another set of colon cancer samples, 5 of 36 cases showed hemizygous deletion of *miR-22* locus with intact copy of *TP53* (Supplementary Fig. S2).

Induction of apoptosis by *miR-22* in p53 wild-type colon cancer cells

Cell proliferation assays using the HCT 116-p53$^{+/+}$, HCT 116-p53$^{-/-}$, and p53 mutant SW480 cell lines showed a significant repression of cell proliferation by *miR-22* in 3 cell lines (Supplementary Fig. S3A). Interestingly, *miR-22* induced apoptosis selectively in HCT 116-p53$^{+/+}$ cells (Fig. 2A and B). In contrast, it caused cell-cycle arrest in HCT 116-p53$^{-/-}$ and SW480 cells (Supplementary Fig. S3B and C). These results indicate that *miR-22* acts as a growth repressor in colon cancer cells, and that its ability to induce apoptosis depends on the *TP53* status. Indeed, the expression profile of HCT 116 cells in the presence of *miR-22* showed significant modulation of

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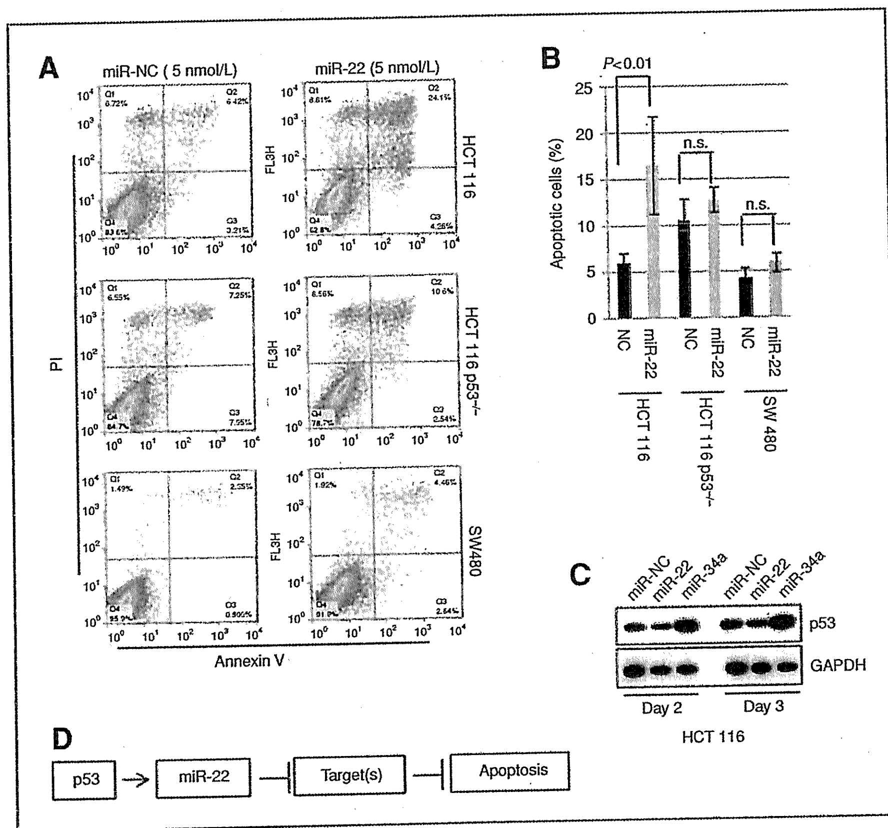

Figure 2. A, fluorescence-activated cell sorting (FACS) analysis. HCT 116, HCT 116-p53$^{-/-}$, and SW480 were transfected with 5 nmol/L of *miR-22* or miR-NC, incubated for 3 days, and subjected to FACS analysis. B, quantification of apoptotic cells. Apoptotic cells were quantified by using 4 independent FACS experiments. Data indicate the mean value with SD. Statistical analysis was carried out by *t* test. C, p53 is not activated by *miR-22*. Cells, transfected with *miR-22* or miR-NC, were incubated for 2 or 3 days, and subjected to immunoblotting. D, hypothesis of *miR-22* function in the p53 network.

cellular p53 network (Supplementary Fig. S3D and Tables S3 and S4). Furthermore, the introduction of *miR-22* into HCT 116 cells did not show upregulation or stabilization of p53, suggesting that *miR-22* may function downstream of the p53-induced apoptotic pathways, and that its role in the induction of apoptosis could be mediated by the repression of p53 target genes (Fig. 2C and D).

Identification of the *miR-22* gene as a direct transcriptional target of p53

As shown in Fig. 3A, *miR-22* is encoded within exon 3 of the *C17orf91* gene, which is located on the minus strand of the 17p13.3 region of the human chromosome, and consensus sequence of p53 binding sites (p53BS) was identified at a 5′ upstream region and within the intron 2 of the *C17orf91* gene (Fig. 3A and Supplementary Fig. S4). The expression of *miR-22* was assessed in HCT 116-p53$^{+/+}$ cells treated with 100 ng/mL of Adriamycin (ADR; doxorubicin, Bedford Laboratories), a genotoxic agent leading to activation of p53, for 24 hours. The result indicated that mature *miR-22* was increased considerably by ADR treatment in HCT 116-p53$^{+/+}$ cells, but not in HCT 116-p53$^{-/-}$ cells (Fig. 3B). The expression of *C17orf91* was induced only in HCT 116-p53$^{+/+}$ cells by ADR (Fig. 3C). Transcriptional activation of *miR-22* was also found in HCT 116 cells after treatment with 5-FU, which was confirmed by qRT-PCR and reporter gene analyses (Supplementary Fig. S5A–C). Furthermore, introduction of a cDNA encoding *C17orf91*, cloned by using a gene-specific primer set (Supplementary Fig. S4), into cells clearly showed an increase of mature *miR-22* in both p53 wild-type and p53$^{-/-}$ HCT 116 cells (Fig. 3D). These results suggest that *miR-22* expression is regulated by p53 at the transcriptional level, not by p53-dependent processing during the maturation of the miRNA (32). Indeed, p53 binding on p53BS located at 5′ upstream and intron 2 of the *miR-22* gene was significantly enhanced after exposure to 5-FU evidenced by p53 ChIP (Supplementary Fig. S5D and E). Furthermore, this was also confirmed by ChIP-sequencing (ChIP-Seq) analysis (Fig. 3E), indicating that *miR-22* is a direct transcriptional target of p53. The concurrent increase in tri-methylation of lysine 4 of histone H3 (33) evidenced transcriptional activation of the *miR-22* gene after exposure to 5-FU (Fig. 3E).

Identification of *p21* as a direct target of *miR-22*

To identify the *miR-22* target mRNAs involved in p53-dependent apoptosis, AGO2-immunoprecipitation (AGO2-IP) on ChIP analysis (31) was applied to screen mRNA species enriched in the AGO2 complex in a *miR-22* dependent manner; an *in silico* database search was further carried out using candidate mRNAs. This strategy was expected to lead to the efficient identification of responsible miRNA

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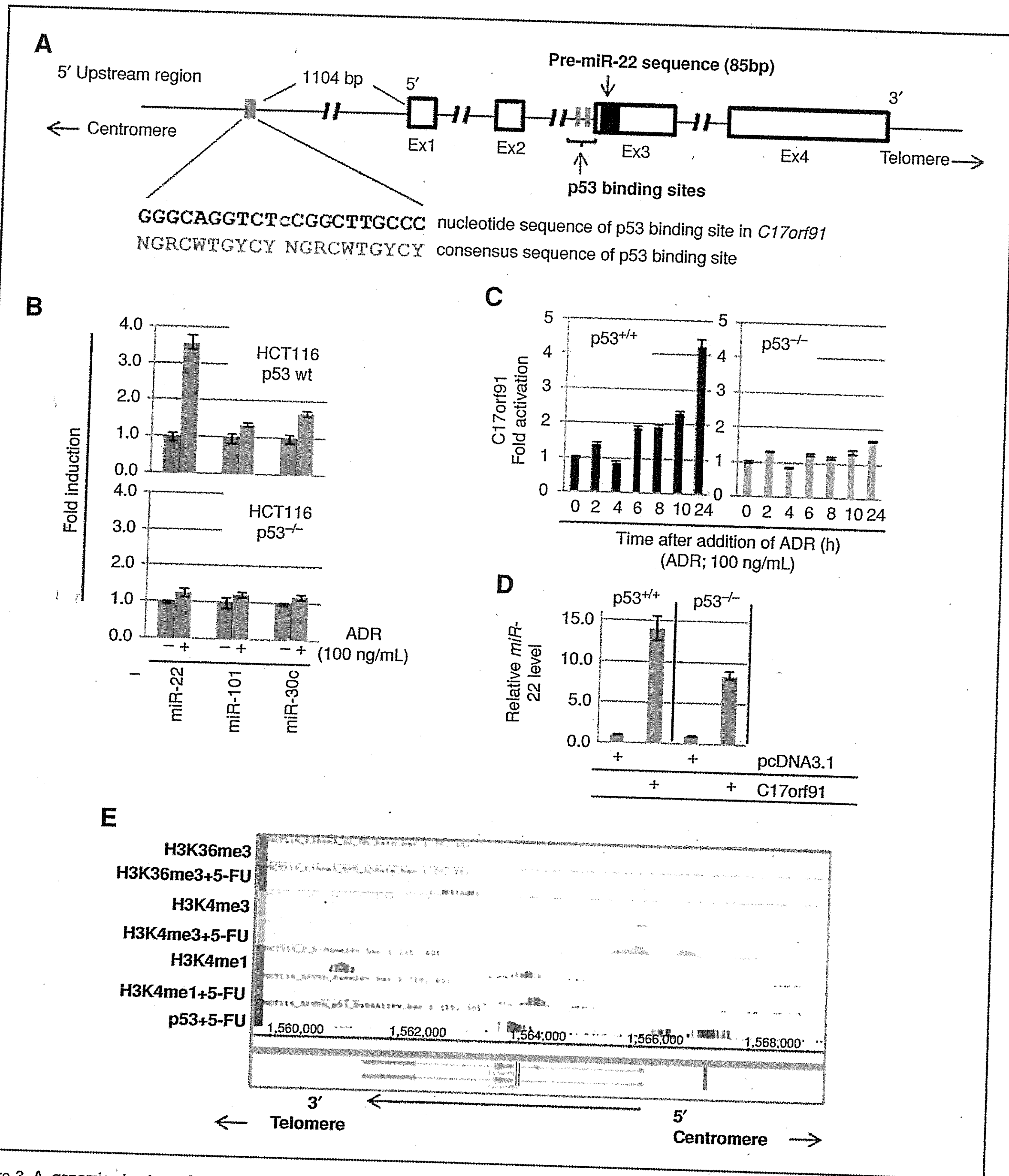

Figure 3. A, genomic structure of *miR-22* and its host gene, *C17orf91*. Genomic structure of C17ORF91 is indicated. Open boxes show exons and the region-encoded pre-*miR-22* is indicated by a closed box. Red boxes are p53 binding site at 5′ upstream region and within intron 2. Consensus sequences of p53 binding sites located 5′ upstream of exon1 of *C17orf91* are shown. B, induction of *miR-22* expression after addition of the genotoxic agent ADR in p53-wild type (wt) and p53−/− HCT 116 cells. Cells were cultured in the presence or absence of ADR (100 ng/mL) for 24 hours. Mature-type miRNAs were measured by qRT-PCR. *miR-101* and *miR-30c*, whose expression was not affected by p53, were used as negative controls. C, upregulation of *C17orf91* by ADR. The cells were treated with ADR (100 ng/mL) for the indicated times, and *C17orf91* was quantified by TaqMan qRT-PCR. D, upregulation of *miR-22* by introduction of *C17orf91* cDNA. Cells were transfected with an expression vector containing *C17orf91* cDNA (Supplementary Fig. S4) for 48 hours. The expression of *miR-22* was analyzed by qRT-PCR. E, ChIP-sequence analysis. Genomic region of C17orf91 indicates opposite direction as shown in (A) because of C17orf91 gene encoded on minus strand in the chromosome 17. HCT 116 cells were treated with a DNA-damaging agent, 5-FU, for 9 hours, and ChIP was carried out by using the indicated antibodies. Red boxes show p53BS located at 5′ upstream region and within intron 2. The direction of *C17orf91* gene is indicated by arrows.

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targets. HCT 116 cells, stably expressing HA-AGO2, were transfected with *miR-22*, and the AGO2 complex was precipitated with anti-HA antibody, followed by the microarray analysis of the precipitated RNAs (Supplementary Fig. S6A). After calculation of the enrichment score (Supplementary Fig. S6B), 10 mRNAs were selected as *miR-22*–dependent AGO2-bound mRNAs, which included regulators of apoptosis and the cell cycle (Supplementary Fig. S6C and D). A search of the TargetScan database (34) using the top10 mRNAs revealed that only *p21* was a potential target for *miR-22*. Indeed, *p21* had a potential *miR-22* target sequence, whose site was conserved among the other mammalian species (Fig. 4A and Supplementary Fig. S6E). The expression of a luciferase reporter gene fused with the 3′ UTR of *p21* mRNA was suppressed by the introduction of *miR-22* (Fig. 4B). This suppression was significantly reduced by the introduction of mutations into the *miR-22* response sequence (Fig. 4B, Mut1 and Mut2), indicating that *miR-22* represses *p21* directly. Furthermore, ectopic expression of *miR-22* in HCT 116-p53$^{+/+}$ cells reduced p21 protein levels (Fig. 4C). Suppression of *p21* mRNA levels was also observed by introduction of *miR-22* (Fig. 4D). These results show that *miR-22* controls *p21* expression by both inhibition of translation and degradation of mRNA.

As shown in Fig. 4E, *miR-22* inhibited the ADR-induced upregulation of p21. Immunocytochemical analysis showed no nuclear accumulation of p21 in *miR-22* introduced cells, even after a 10-hour ADR treatment (Fig. 4F). To show that this repression occurs at a post-transcriptional, but not at a transcriptional level, the ADR-induced increase in *p21* mRNA was quantitatively assessed in the presence or absence of *miR-22*. As expected, transcriptional activation of *p21* was observed with similar kinetics as the p53 response in both miR-NC and *miR-22* introduced cells after ADR treatment (Fig. 4G). These observations suggest that *miR-22* directly represses *p21* expression via a post-transcriptional mechanism.

Sensitization of p53-dependent apoptosis by *miR-22*

p21 is known to be a key regulator of cell-cycle arrest after the activation of p53, and also an inhibitor of apoptosis (35). Thus, we analyzed the effect of *miR-22* levels on the p53-dependent apoptosis. HCT 116-p53$^{+/+}$ cells were transfected with either *miR-22* or miR-NC, and apoptotic cells were quantified by FACS in the presence or absence of ADR. As shown in Fig. 5A, cells transfected with miR-NC showed a slight increase of the Annexin V and PI double-positive fraction after 12-hour exposure to 100 ng/mL of ADR (Fig. 5A, top right and B). The introduction of low amounts (2 nmol/L) of *miR-22* slightly enhanced the induction of apoptosis compared with those with miR-NC in the absence of ADR (Fig. 5A, bottom left, and B). The addition of ADR caused a marked increase of apoptotic cells in *miR-22*–transfected cells (Fig. 5A, bottom right, and B), indicating that *miR-22* sensitizes cells to p53-dependent apoptosis induced by DNA damage. Next, we analyzed the effect of p21 protein levels on *miR-22*-induced apoptosis. *MiR-22* caused significant repression of p21 upregulation by ADR treatment for 24 hours (Fig. 5C, lanes 2 and 5, and Supplementary Fig. S7A). The introduction of p21 ORF showed the reduction of apoptosis, evidenced by the decrease in PARP-1 cleavage (36), in cells transfected with *miR-22* (Fig. 5C, lanes 5 and 11). This was reproducibly detected (Supplementary Fig. S7B).

These results suggest that endogenous levels of *miR-22* are a cellular determinant for the induction of apoptosis through the repression of p21. On the other hand, p21 knockdown induced the cleavage of PARP-1 (Supplementary Fig. S7C). This was consistent with previous reports that p21 deficiency sensitizes cells to apoptosis (37, 38). However, p21 knockdown was not as prominent as is observed by *miR-22* introduction. This strongly suggests that other factors, being also regulated by *miR-22*, could be involved in the sensitization of p53-dependent apoptosis by *miR-22*. Furthermore, inhibition of *miR-22* by expression of an antisense *miR-22* transcript causes the substantial decrease of S-phase cells, suggesting the cell-cycle arrest at G_1 phase (Supplementary Fig. S7D).

Transcriptional activation of *miR-22* depending on the intensity of stresses

To examine whether the expression of *miR-22* and *p21* levels correlate with the induction of apoptosis in a physiologic setting, the kinetics of *miR-22* and *p21* mRNA expression was examined by treating cells with different doses of ADR. As expected, HCT 116 cells treated with 50 ng/mL of ADR showed cell-cycle arrest, but no apoptosis, with rapid increments of *p21* at both mRNA and protein levels; upregulation of *miR-22* was not observed, even after the ADR-mediated activation of p53 (Fig. 6A, left graph, and 6B, left). Under a high-dose exposure to ADR, in contrast, the expression levels of *p21* mRNA and *miR-22* increased from 8 hours after the addition of 200 ng/mL of ADR (Fig. 6A, right). Interestingly, p21 protein levels were not elevated significantly after 36 hours of incubation with ADR, despite the striking increase in *p21* mRNA level (Fig. 6B, top right). The PARP-1 cleavage was observed at a similar kinetics with *miR-22* expression (Fig. 6B). Similarly, a significant activation of *miR-22* accompanying the repression of p21 protein and increase of PARP-1 cleavage was also observed in HCT 116 cells after exposure to high doses of actinomycin D (Act D), an inhibitor of RNA polymerases that activates p53 (ref. 39; Fig. 6C and D). ChIP analysis indicated the enhancement of p53 binding to p53BS in the *miR-22* gene only after addition of high doses of Act D (Supplementary Fig. S8A and B). Interestingly, treatment with deferoxiamine, an inducer of HIF1α that stabilizes and activates p53 (40), did not upregulate *miR-22* or *p21* mRNA and did not induce apoptosis despite the activation of p53 (Fig. 6C and D). These results indicate that the activation of *miR-22* regulated by p53 is dependent on the strength and type of stresses.

Discussion

In the present study, *miR-22* was identified as a strong candidate for tumor suppressor gene in human colon cancers,

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