Suppression of the Malignant Phenotype of Melanoma Cells by Anti-Oncogene Ribozymes

Yukinori Ohta,*[1] Hiroshi Kijima,* Mohammed Kashani-Sabet,† and Kevin J. Scanlon*

*Biochemical Pharmacology, Department of Medical Oncology, City of Hope Medical Center, Duarte; and †Department of Dermatology, University of California at San Francisco, San Francisco, California, U.S.A.

The activation of signal transduction pathways by mutation or overexpression of cellular oncogenes has been associated with neoplastic transformation. In this study, we addressed the therapeutic potential of ribozymes targeted against the activated H-*ras* oncogene as well as against the nuclear proto-oncogenes c-*fos* and c-*myc* in the FEM human melanoma cell line containing a H-*ras* mutation. FEM cells transfected with the anti-*ras* ribozyme were shown to have the longest doubling time, the least DNA synthesis, and the fewest colonies in soft agar when compared with transfectants with ribozymes against c-*fos* or c-*myc* mRNA. Furthermore, anti-*ras* ribozyme clones showed a dendritic appearance in monolayer culture that was associated with enhanced melanin synthesis. These results suggest that the anti-*ras* ribozyme could affect not only the proliferation but also the differentiation process of human melanoma cells *in vitro*. They also reinforce the role of anti-oncogene ribozymes as suppressors of the neoplastic phenotype of melanoma cells. ***Key word:* H-ras.** ***J Invest Dermatol 106:275–280, 1996***

Recent advances in cancer biology have demonstrated that tumorigenesis occurs as a result of genetic alterations in many types of cancers [1,2]. In cutaneous malignancies, various investigators have suggested a linkage between ultraviolet irradiation–induced mutations in oncogenes (e.g., *ras*) and tumor progression [3,4]. Initial reports indicated that ras mutations were detected in 24% of cultured melanoma cell lines [5]. More recent studies have shown mutations in 36% of tumor material from melanoma patients, with an increase to 45% in melanomas beyond Clark's level II [6]. In addition, *in vitro* transformation studies using oncogenes induced a melanoma-like phenotype in cultured melanocytes [7]. Specifically, melanocytes transformed with the activated H-*ras* gene exhibit the malignant phenotype and possess the cytogenetic aberrations observed in primary melanomas [8]. The biological importance of these genes has made them attractive targets for manipulation of gene expression. Recently, many trials have been undertaken to study this concept by using gene modulators such as anti-sense oligodeoxynucleotides or catalytic RNAs (ribozymes) [9,10]. With the proven therapeutic potential of gene-modulating agents in experimental models, trials are being initiated to test the clinical application of this approach.

Hammerhead ribozymes, as used in this study, can promote the site-specific cleavage reaction of the target RNA in a truly catalytic manner [11,12]. For instance, a hammerhead ribozyme designed to target activated H-*ras* mRNA was shown to cleave only the mutated transcripts and leave its normal counterpart unaffected *in vitro* [13]. This anti-*ras* ribozyme was shown to have greater efficacy in inhibiting H-*ras* gene expression and reversing the transformed phenotype than a mutant ribozyme mainly capable of anti-sense activity [14,15]. Ribozymes have been previously examined as anti-oncogene, anti-HIV, and anti–drug resistance agents [14–20].

Recent progress in cell biology has defined *ras*-regulated signal transduction from the plasma membrane to the nucleus. Mutated *ras* oncogene–encoded proteins are presumed to activate the nuclear proto-oncogenes that lay downstream from them, resulting in a transformed phenotypic alteration [21]. In this study, we have attempted to compare the *in vitro* therapeutic potential of ribozymes designed to cleave activated H-*ras* and the nuclear proto-oncogenes c-*fos* and c-*myc* in human melanoma cells possessing a H-*ras* mutation. We used the glucocorticoid-inducible murine mammary tumor virus promoter to demonstrate the reversible effect of ribozymes against oncogenes on the suppression of the melanoma phenotype.

MATERIALS AND METHODS

Cells Human melanoma FEM cells were obtained from Dr. Oystein Fodstad (Oslo, Norway) and their characteristics have been previously described [22,23]. The FEM cell line has a H-*ras* codon 12 mutation, in which the normally glycine-encoding GGC sequence is converted to GUC, encoding valine [17]. Cells were grown in monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum. Three days after seeding, they were treated with 0.5 μM dexamethasone (Dex; Sigma Chemical Co., St. Louis, MO) for 48 h when desired. Thymidine uptake was used to determine the rate of [^{3}H]dThd incorporation into trichloroacetic acid–precipitable material [16]. Colony formation in soft agar was performed as previously described [24]. Briefly, the top layer consisted of 5×10^3 viable cells suspended in 0.3% agarose, RPMI 1640, and serum at concentrations of 1, 10, or 20% with or without 0.5 μM Dex, overlaid on a 1% agarose layer in a 35-mm culture plate. To assess cloning efficiencies in the presence of Dex, cells were first grown for 24 h in culture medium containing 0.5 μM Dex and were then plated under the conditions described above. Colonies were counted under a light microscope 10–14 d after seeding.

Relative Melanin Content of Cells Cells were seeded to achieve a density of approximately 5×10^5 cells/100mm plate after 4 d in culture.

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Reprint requests to: Dr. Kevin J. Scanlon, Montana Building—City of Hope Medical Center, 1500 East Duarte Road, Duarte, CA 91010.

[1] Present address: Department of Dermatology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan.

Abbreviations: Rz, ribozyme; AP-1, activating protein 1.

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After attachment, cells were treated with 0.5 μM Dex when desired. From each plate, 5×10^6 cells were collected by trypsinization and subjected to a 2000*g* centrifugation. Cell pellets were dissolved in 1 ml of 1 N KOH at 80°C for 1 h. This hydrolysate was centrifuged at 12000*g* for 10 min. The relative melanin concentrations of the supernatants were determined by measuring the O.D. at 492 nm [25]. A standard curve was prepared by dissolving known amounts of synthetic melanin (Sigma) in KOH. The results are expressed as: Melanin synthesis (%) = amount (μg) of experimental cells/amount (μg) of control cells × 100.

RNA Sequences of Substrates and Ribozymes The sequence of RNA transcript of activated H-*ras*, c-*fos*, and c-*myc* targeted by the ribozymes is encoded by the following nucleotides (nt): Target activated H-*ras* mRNA (exon 1, 1697–1712nt): 5′-GGC GGC **GUC*** GGU GUG G-3′ (Bold letter and asterisk denote the recognition sequence and site of cleavage reaction, respectively). The *ras*Rz: 5′-CCAC ACC CUG AUG AGU CCG UGA GGA CGA AAC GGC GCC-3′. Target c-*fos* mRNA (exon 1, 914–938nt): 5′-A CUA CGA GGC **GUC*** AUC CUC CCG CUG-3′. The fosRz: 5′-CAG CGG GAG GAU CUG AUG AGU CCG UGA GGA CGA AAC GCC UCG UAG U-3′. Target c-*myc* RNA (exon 2, 1224–1246nt): 5′-C AUC CUG UCC **GUC*** CAA GCA GAG G-3′. The *myc*Rz: 5′-C CUC UGC UUG CUG AUG AGU CCG UGA GGA CGA AAC GGA CAG GAU G-3′. Each hammerhead ribozyme was constructed with the same stem loop region and distinct flanking sequences complementary to the substrate RNA essentially as described by Haseloff and Gerlach [26]. The ribozymes were tested for *in vitro* cleavage of their target RNAs for H-*ras* [16]; for c-*fos* [19]; unpublished results for c-*myc*.

Plasmid Construction The fosRz used in this study was cloned into the plasmid pMAMneo (Clontech) as previously described [19]. Ribozymes against activated H-*ras* or c-*myc* were subcloned into pMAMneo using two synthetic single-stranded oligonucleotides designed to contain sequences for creating two restriction sites (Nhe I and Sal I) following a previously reported method [19]. The plasmid pMAMneo contains the murine mammary tumor virus dexamethasone-inducible promoter to regulate expression of ribozyme RNA transcripts. The sequence and orientation of the inserts were confirmed by dideoxynucleotide sequencing of the construct ribozyme using the Sequenase kit (U.S. Biochemical Corp.).

Transfection by Electroporation Subconfluently growing cells were transfected by electroporation according to a previously published method [16]. The cells were then selected in growth medium containing 500 μg/ml geneticin (G418; Gibco, Gaithersburg, MD) for 3–6 wk. Individual G418-resistant colonies were picked, grown, and screened for expression of the ribozyme by reverse transcriptase–polymerase chain reaction (RT-PCR).

Polymerase Chain Reaction RT-PCR, following that of a commercially available protocol (GeneAmp, Perkin-Elmer-Cetus), was used to detect ribozyme, basic fibroblast growth factor (bFGF), and phosphoglycerate kinase (PGK) expression, and was performed as previously described [19,27].

Northern Analysis Total RNA and poly(A) mRNA were prepared by the guanidium isothiocyanate method [19]. RNA was size-fractionated and transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, IL). Hybridizations were carried out with probes radiolabeled by the random primer method as described [28].

RESULTS

FEM Cells in Culture FEM human melanoma cells were transfected with pMAMneo containing ribozymes against activated c-H-*ras* (*ras*Rz), c-*fos* (*fos*Rz), and c-*myc* (*myc*Rz) oncogenes by electroporation. These transfectants were treated with G418 for 3–6 wk, and 8–16 G418-resistant colonies were selected. For screening, RT-PCR was performed to demonstrate the presence of ribozyme transcripts in the transfectants. The RNA transcripts of *ras*Rz, *fos*Rz, and *myc*Rz were actively expressed in the different clones (**Figs 1, 2**, *lanes 3–6*). In contrast, no ribozyme expression was detected in FEM control cells transfected with pMAMneo lacking the ribozyme sequences (**Figs 1, 2**, *lanes 1,2*). Ribozyme expression was induced approximately 1.5- to 5-fold by Dex administration (**Figs 1, 2**, *lanes 4,6*) compared with that of untreated cells (*lanes 3,5*). The FEM ribozyme transfectants without Dex treatment also exhibited a detectable level of ribozyme expression. This baseline expression might be linked to the leakage induced by 10% serum in the culture medium [29]. To evaluate the efficacy of accelerated ribozyme expression, transfectants were

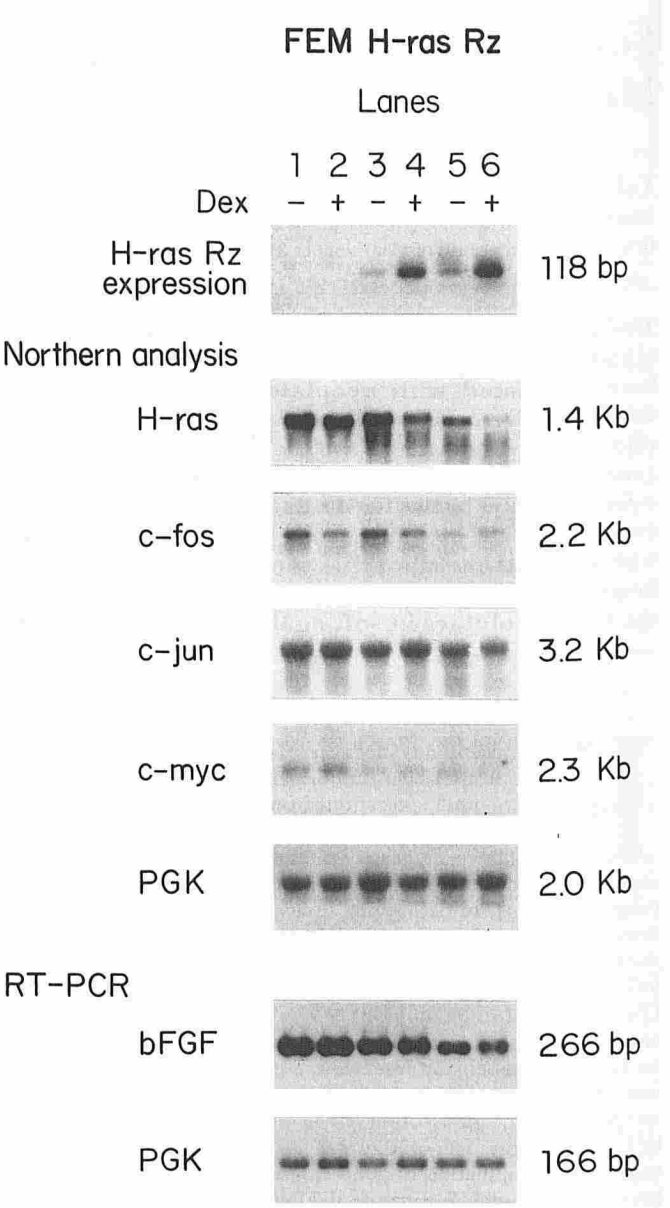

Figure 1. Gene expression of FEM*ras*Rz transfectants. *Lanes 1* and *2*, FEMpMAMneo vector only clone; *lanes 3* and *4*, FEM*ras*Rz clone 1 (Ras-1), *lanes 5* and *6*, FEM*ras*Rz clone 6 (Ras-6). Total RNA was prepared from log-phase cells grown in either the absence (−) or presence (+) of 0.5 μM dexamethasone (*Dex*) for 48 h. *PGK*, phosphoglycerate kinase; *Kb*, kilobases; *bp*, base pairs. Gene expression has been normalized (18) to FEM cells (vector only). H-*ras* (1.0, 0.8, 0.95, 0.3, 0.25, and 0.05); c-*fos* (1.0, 0.35, 1.2, 0.3, 0.1, and 0.15); c-*jun* (1.0, 1.0, 0.85, 1.0, 0.6, and 0.65); c-*myc* (1.0, 1.2, 0.05, 0.05, 0.04, and 0.02); PGK (1.0, 1.0, 1.25, 1.1, 1.2, and 1.2); bFGF (1.0, 0.95, 0.8, 0.8, 0.35, and 0.3); PGK (1.0, 1.2, 0.8, 0.95, 0.95, and 0.85).

transiently exposed to 0.5 μM Dex for 48 h. At this concentration of Dex, no significant alterations were observed in parent FEM cells (data not shown) and control FEM cells transfected with pMAMneo only **(Table I, Fig 3)** on cell growth, morphology, and melanin synthesis.

Gene Expression in FEM Cells To determine whether these ribozymes could affect expression of the substrate RNA, we examined the mRNA levels of each target gene by Northern analysis. In *ras*Rz clones Ras-1 and Ras-6, the expression of H-*ras* mRNA was decreased compared with control cells. Moreover, clone Ras-6, with a relatively larger amount of ribozyme expres-

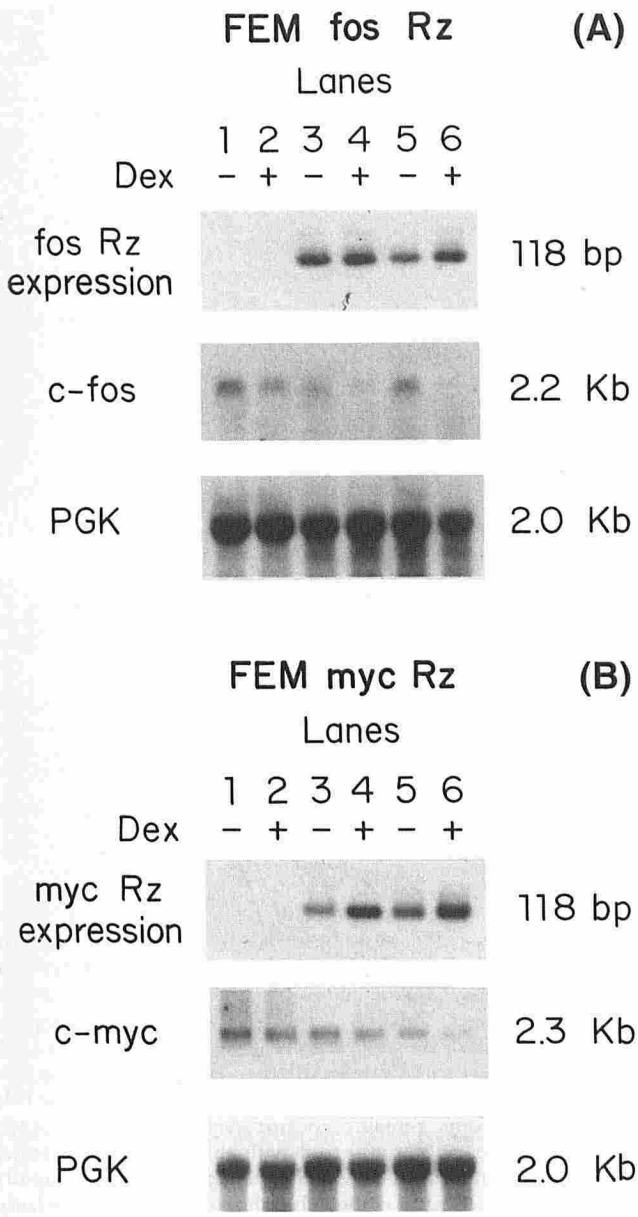

Figure 2. Expression of ribozymes and target transcripts in FEM-*fos*Rz and *myc*Rz clones. The *fos* ribozyme was expressed in FEM cells (*A*) and *fos* gene expression was characterized and compared to phosphoglycerate kinase (*PGK*) gene expression. *Lanes 1* and *2*, FEMpMAMneo vector only clone; *lanes 3* and *4*, FEM*fos*Rz clone 3 (Fos-3); *lanes 5* and *6*, clone 4 (Fos-4). Gene expression has been normalized (18) to FEM cells (vector only). c-*fos* (1.0, 0.35, 0.2, 0.01, 0.4, and 0.01); PGK (1.0, 1.0, 1.0, 1.1, 1.2, and 0.9). The c-*myc* ribozyme was expressed in FEM cells (*B*) and the c-*myc* gene expression was characterized and compared with PGK gene expression. *Lanes 3* and *4*, FEM*myc*Rz clone 3 (Myc-3); *lanes 5* and *6*; clone 10 (Myc-10). c-*myc* (1.0, 0.85, 0.8, 0.7, 0.6, and 0.2); PGK (1.0, 1.2, 1.3, 1.15, 1.2, and 1.3). Each transfectant was treated with Dex (0.5 μM) for 48 h, when desired.

sion than clone Ras-1, exhibited correspondingly less H-*ras* mRNA **(Fig 1)**. Thus, there appeared to be an inverse correlation between the level of ribozyme and target mRNA expression. Transfectants containing the *fos*Rz (clones Fos-3 and -4) and the *myc*Rz (clones Myc-3 and -10) also demonstrated a similar inverse correlation **(Fig 2)**.

The effects of ribozyme action on cell growth and DNA synthesis were investigated **(Table I)**. The doubling time was 2.3 to 2.9 times longer in Dex-treated *ras*Rz-expressing FEM cells than in the control Dex-treated clone, ranging from 47.7 to 58.6 h (Ras-1 and Ras-6, respectively). Clone Ras-6, with a greater amount of ribozyme expression and lesser amount of target *ras* mRNA, had a longer doubling time than clone Ras-1. Transfectants expressing *fos*Rz and *myc*Rz also had increased doubling times, though to a lesser extent than *ras*Rz clones. In the *fos*Rz clones, 1.9- and 1.7-fold longer doubling times were shown in Fos-3 and Fos-4 cells, respectively. Myc-3 and Myc-10 cells demonstrated 1.5- and 1.7-fold longer doubling times compared to control cells. The increased doubling time in ribozyme-expressing transfectants was associated with diminished DNA synthesis as exhibited by [^{3}H]thymidine incorporation into DNA **(Table I)**. In two selected clones with each ribozyme, the ability of the ribozyme to reduce target mRNA expression correlated with prolonged doubling time and decreased DNA synthesis. Colony formation in soft agar, an indication of anchorage-independent growth, was decreased in ribozyme-treated cells, with the greatest growth inhibition evident in Ras-6 cells **(Table I)**.

In order to guard against clonal variation following the transfection and selection process, the ribozyme-containing transformants from four clones were pooled and analyzed for *in vitro* growth characteristics. The pooled *ras*Rz expressing cells continued to exhibit more sluggish *in vitro* cell growth followed by the pooled *fos*Rz and *myc*Rz clones **(Table I)**.

FEM Cellular Morphology Morphologically, control FEM cells transfected with pMAMneo were spindle or triangle shaped **(Fig 3*A*)** similar to parent FEM cells (data not shown). This control clone was unaffected by Dex treatment. In contrast, ribozyme transfectants showed an altered morphology **(Fig 3*B*)**, even in the absence of Dex (data not shown). Dex-treated *ras*Rz clones revealed a more dendritic shape compared with the control cells **(Fig 3*B*)**. The *fos*Rz induced a significant bipolarity in shape **(Fig 3*C*)**. MycRz clones had a more flattened, somewhat reticulated appearance **(Fig 3*D*)**. These clones morphologically reverted to control FEM cells without Dex treatment within 2–3 wk (data not shown). Relative melanin content was also increased in the transfectants **(Table I)**: 2.62 and 3.53 times (Ras-1 and -6, respectively); 1.82 and 2.15 times (Fos-3 and -4, respectively); and 2.30 and 2.61 times (Myc-3 and -10, respectively). The same pattern of increased melanin expression was observed when the pooled clones were analyzed **(Table I)**. Thus, among these anti-oncogene ribozymes, *ras*Rz clones demonstrated the longest doubling time, the least DNA synthesis, and the lowest efficiency of soft agar colony formation. Moreover, the *ras*Rz stimulated melanin synthesis most effectively.

In terms of gene expression, decreases in activated H-*ras* expression were associated with downregulation of c-*fos* expression **(Fig 1)**. However, the expression of c-*jun*, encoding the other component of the AP-1 complex, was not altered in transfectants. The expression of c-*myc* was decreased in ribozyme clones of FEM cells. Using RT-PCR, the expression of bFGF was depressed in the FEM *ras*Rz clone, Ras-6, whereas control PGK expression was not altered.

DISCUSSION

The activation or mutation of cellular oncogenes plays a crucial role in tumor biology [21]. Mutations in ras genes have been found in approximately 15% of solid tumors [30]. A recent report has suggested that *ras* mutations were detected in 36% of primary melanomas [6]. Interestingly, while *ras* mutations were not found in level I melanomas, most of the mutations occured in melanomas beyond level II, indicating that *ras* mutations may play an important role in melanoma progression.

This is supported by our results demonstrating that Dex-treated *ras*Rz transfectants (Ras-6) represented longer doubling times, lower thymidine incorporation rate, and fewer number of colonies in soft agar, compared with those observed in *fos*Rz or *myc*Rz clones. The levels of disrupted target mRNA were not exactly the same in all ribozyme clones; however, our results indicate that

Table I. Growth Characteristics and Melanin Synthesis of Ribozyme-Transfected FEM Human Melanoma Cells

Cell Lines[a]	Dex[b]	Doubling Time (h)	[^{3}H]Thymidine Uptake[c] (%)	Colonies in Soft Agar Serum: 1%/20%[d]	Melanin Contents[e] (%)
FEMpMAMneo					
	−	20.3	100	198/576	100
	+	20.4	96.6	162/500	118
FEM*ras*Rz					
Clone 1	−	35.2	30.4	0/56	156
	+	47.7	21.3	0/31	262
Clone 6	−	32.2	31.9	0/48	153
	+	58.6	18.2	0/15	353
Pooled clones	+	60.1	23.5	1/20	311
FEM*fos*Rz					
Clone 3	−	31.4	47.0	18/112	185
	+	39.2	37.0	1/28	215
Clone 4	−	30.9	54.0	27/134	160
	+	35.5	51.0	3/40	182
Pooled clones	+	38.5	39.0	3/35	245
FEM*myc*Rz					
Clone 3	−	26.8	77.6	31/188	139
	+	30.8	33.1	11/52	230
Clone 10	−	27.5	57.0	22/155	242
	+	35.2	32.4	4/48	261
Pooled clones	+	33.1	38.0	8/51	222

[a] FEMpMAMneo, FEM cells transfected with pMAMneo vector only; FEM*ras*Rz, FEM*fos*Rz, and FEM*myc*Rz, FEM cells transfected with a ribozyme targeted against activated H-*ras*, c-*fos*, and c-*myc*, respectively.

[b] Dexamethasone (Dex) was used at a concentration of 0.5 μM.

[c] To determine the rate of [^{3}H]thymidine uptake in acid-soluble material, cells were grown for 48 h in medium with or without Dex, and were pulsed for 1 h with [^{3}H]thymidine, washed, acid-precipitated, and counted. The FEMpMAMneo (−Dex) (100%) represented 3.29 fmol/mgDNA/h. These results represent the mean of three separate experiments. There was less than 10% variation in the data points.

[d] Five thousand viable cells were grown in 0.3% agar with 1 or 20% fetal bovine serum. Thirteen days after seeding, colonies equal to or greater than 250 μm in diameter were counted. The data are presented as the mean of triplicate plates. There was less than 10% variation in the data points.

[e] An equal number of cells was harvested and lysed in 1 N KOH. The hydrolysate was centrifuged at 12000*g*, and the O.D. at 492 nm was determined. Melanin contents (100%) in control FEMpMAMneo (−Dex) represented 12.1 $\mu g/10^6$ cells. There was less than 10% variation in the data points.

*ras*Rz had the strongest impact on the proliferation of FEM human melanoma cells. The FEM cells were shown to express a more differentiated phenotype as determined by morphological changes, altered melanin content, and response to phorbol esters [22,23]. Morphologically, *ras*Rz clones had elongated dendritic processes and expanded more sparsely compared with control cells. Furthermore, of all the ribozyme clones tested, the largest amount of melanin was demonstrated in Ras-6 cells. Giuffrè *et al* suggested that induction of differentiation in melanoma cells by phorbol esters may lead to increased dendricity, diminished proliferation capacity, and enhanced melanin synthesis [31]. Other reports demonstrated the loss of pigmentation in v-H-*ras* transformed melanocytes [7,32]. Although the precise differentiation pathway in melanoma remains unclear, our findings indicate that the *ras*Rz can induce a differentiated phenotypic change in FEM melanoma cells. Our results indicate that suppression of activated ras genes leads to profound effects on cell growth and differentiation of melanoma cells *in vitro*.

Recent reports using anti-sense RNA to downregulate the nuclear proto-oncogenes c-*fos* and c-*myc* showed that the malignant phenotype was partially reversed in activated *ras*-transformed NIH 3T3 cells [29,33]. These observations suggested that nuclear proto-oncogenes were involved in the maintenance of the activated *ras*-transformed phenotype in murine fibroblasts. EJ human bladder cells commonly used in experimental models have a homozygous mutation pattern of H-*ras* [34]. Assuming that RNA modulators such as the rasRz could completely disrupt the activated *ras* transcripts, cells having no normal *ras* counterpart will be greatly affected with an inability to maintain the steady-state expression level of oncogenes located downstream of the *ras*-related pathway. We previously reported that a ribozyme against activated *ras* transcripts could almost completely reverse the malignant phenotype of EJ *ras*-transformed NIH 3T3 cells and EJ bladder carcinoma itself [16,35]. In this regard, FEM human melanoma cells, having the heterozygous pattern of H-*ras* mutation, provide an intriguing comparison of the efficacy of ribozymes targeting activated *ras* and nuclear proto-oncogenes.

The control of gene expression by c-*fos* is believed to play a critical role in the cellular response to growth or differentiation factors. Recent observations have suggested that alterations in normal c-*fos* expression can lead to oncogenesis [36]. Yamanishi *et al* reported that c-*fos* mRNA levels were increased in rapidly proliferating neonatal melanocytes and in human melanomas [37]. In contrast, other reports indicated that c-*fos* expression was not increased in murine melanoma cell lines [38]. Even though the role of c-*fos* in melanoma remains controversial, the present study indicates that FEM cells transfected with the *fos*Rz had a longer doubling time than control FEM cells, yet still grew more rapidly than *ras*Rz clones. FosRz clones also showed enhanced melanin synthesis, though this enhancement was the least of the three ribozymes. Morphological alterations in *fos*Rz clones revealed a spindle-like shape and tightly packed colony formation. These findings on cell growth and melanin content may indicate that direct down-modulation of c-*fos* expression by the ribozyme may be involved more in regulation of proliferation rather than of differentiation in FEM human melanoma cells.

c-*myc*, another nuclear proto-oncogene examined in this study, has been thought to participate in the response of cultured cells to differentiation agents and mitogenic stimuli. The introduction of high levels of exogenous c-*myc* into cell lines, either by injection of purified protein or introduction of c-*myc* or v-*myc* expression vectors, results in the induction of growth factor independent DNA synthesis and in some cases induces stable cell proliferation [39]. Furthermore, c-*myc* anti-sense oligonuleotides inhibit the proliferation of the promyelocytic cell line HL-60 [40] and mitogen-stimulated peripheral T cells [41]. In melanoma, 50% of melanoma cell lines exhibit c-*myc* overexpression, which may be related to metastatic capacity [42,43]. These alterations have been mostly observed in high-passage cell lines, however, suggesting the possibility of culture artifacts [44]. In our study, the *myc*Rz could decrease the growth rate, but its efficacy was lowest among the three ribozymes. In general, c-*myc* expression is decreased in a number of cell lines following exposure to differentiation stimuli,

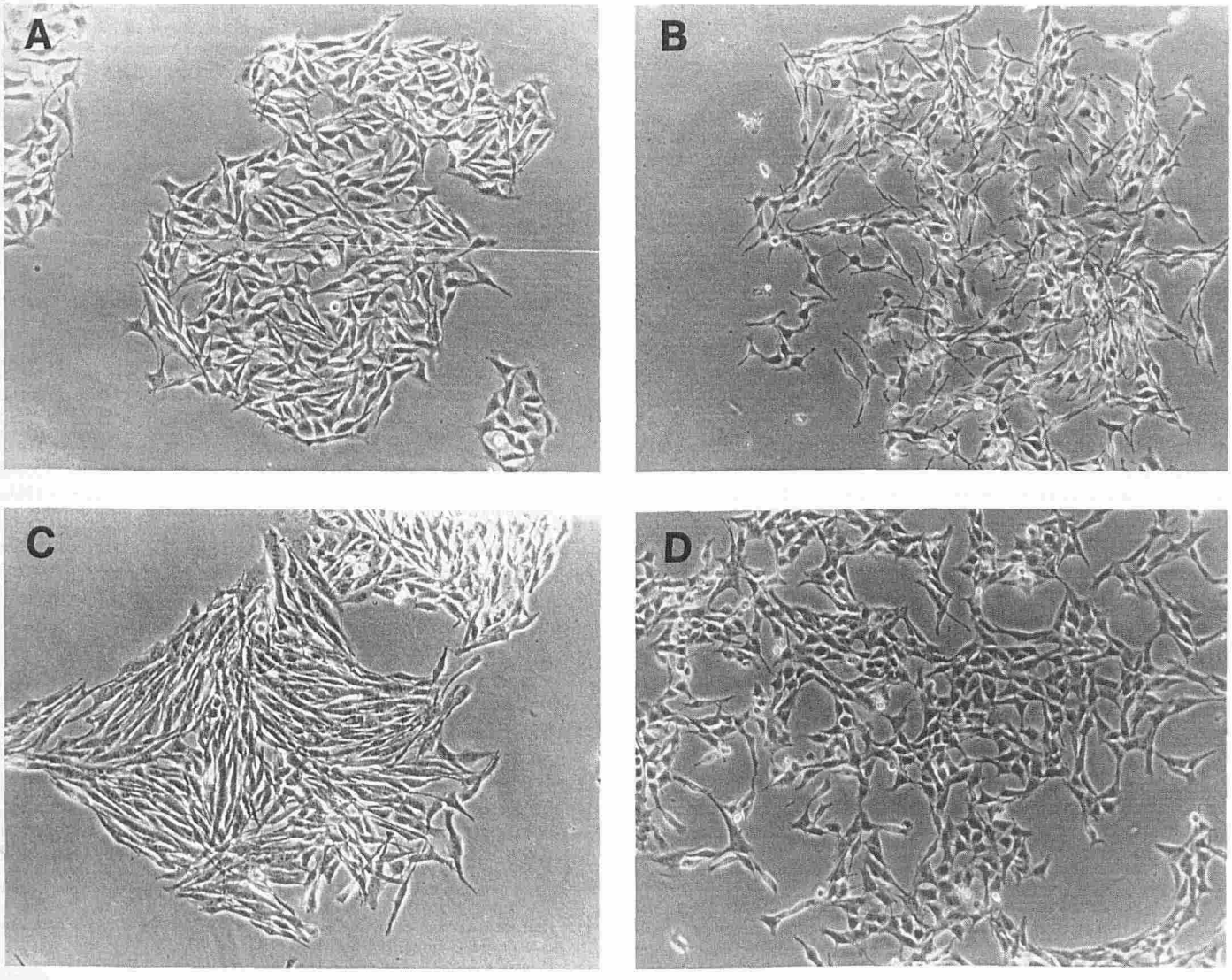

Figure 3. Morphology of FEM ribozyme transfected cells. *A*) FEMpMAM*neo*. *B*) FEM*ras*Rz clone 6. *C*) FEM*fos*Rz clone 3. *D*) FEM *myc*Rz clone 10. These cells were grown for 4 d in the presence of 0.5 μM Dex in monolayer culture. *Magnification*, $\times$100.

suggesting that *myc* downregulation may be important for terminal differentiation [45]. In fact, differentiated melanoma cells treated with bromodeoxyuridine reveal decreased c-*myc* mRNA levels and exhibit a flat morphology [46]. In our studies, the *myc*Rz clones were shown to produce larger amounts of melanin than the *fos*Rz clone. In addition, the *myc*Rz clones exhibited increased surface area, with an expanded, reticular pattern of colony formation. These alterations may be interpreted as a sign of accelerated differentiation.

In *ras*Rz transfectants, decreased H-*ras* gene expression was associated with reduced levels of c-*fos* and c-*myc* mRNA. This is consistent with a previous report suggesting that *ras*-transformed fibroblasts overexpressed c-*fos* and c-*myc* [45,47]. We have also demonstrated the decreased expression of bFGF in the Ras-6 clone, with the longest doubling time. This growth factor may contribute to tumor progression of human melanoma as evidenced by the suppression of melanoma cell growth after inhibition of bFGF activity by antisense oligonucleotides [48]. Moreover, the human bFGF gene contains a potential AP-1 binding site within the core promoter region [49], suggesting its regulation by the Fos/Jun heterodimer.

In conclusion, these observations indicate that targeting a gene upstream in the signal transduction pathway (such as H-*ras*) was most effective in the reversion of the malignant phenotype of human melanoma cells. Moreover, they suggest that the effect of the *ras*Rz may be partly mediated through the downregulation of genes in the *ras*-related pathway, including possibly c-*fos* and c-*myc*. In terms of clinical application, even though ribozyme strategies are still in their incipient stages, our results suggest the possibility that anti-oncogene ribozymes could play a role as suppressors of the neoplastic phenotype.

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