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Semaphorin 3F Gene from Human 3p21.3 Suppresses Tumor Formation in Nude Mice[1]

RuiHua Xiang, Albert R. Davalos, Charles H. Hensel, Xiao-Jun Zhou, Christin Tse, and Susan L. Naylor[2]

Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, Texas 78229-3900 [R. X., A. R. D., C. H. H., S. L. N.]; Lawrence Berkeley National Laboratory, Berkeley, California 94720 [A. R. D.]; Myriad Genetics, Salt Lake City, Utah 84108 [C. H. H.]; Department of Pathology, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, P.R. China [X-J. Z.]; and Sagres Discovery Davis, California 95616 [C. T.]

ABSTRACT

Loss of heterozygosity on human chromosome 3p21.3 is a frequent occurrence in many tumor types. In a previous study, our laboratory demonstrated that an 80-kb P1 clone from chromosome 3 suppresses the tumorigenicity of the mouse fibrosarcoma cell line A9. Two cDNAs corresponding to genes encoded on this P1 clone, *semaphorin 3F* (*SEMA3F*) and *N23*, were tested for their effects on *in vitro* and *in vivo* growth characteristics after transfection into mouse A9 cells. Transfection of *SEMA3F* cDNA resulted in complete loss of tumorigenicity in nude mice, whereas transfection of *N23* had no effect. Moreover, SEMA3F also functioned to block apoptosis of transfected A9 cells treated with Taxol or Adriamycin. The human ovarian adenocarcinoma cell line HEY showed a similar result as A9 cells, but the small cell lung cancer line GLC45 was unaffected by expression of *SEMA3F*.

INTRODUCTION

Loss of heterozygosity on human chromosome 3p21.3 occurs in a large number of tumor types (1). By analogy to other known tumor suppressor genes, these data imply the existence of a tumor suppressor gene in this region. In a previous study, our laboratory demonstrated functionally that a 2-Mb fragment of DNA from chromosome 3 suppresses the tumorigenicity of the mouse fibrosarcoma cell line A9 (2). Subsequently, we built a P1 genomic clone contig spanning the region most frequently deleted in SCLC[3] and demonstrated that a single P1 clone from this contig possesses the same tumor suppressor activity as the 2-Mb fragment (3). This P1 clone (P1 294) corresponds to a peak region of loss of heterozygosity in ovarian, testicular, lung, uterine and SCLC (1) and is completely contained within three previously published homozygous deletions in SCLC (4, 5).

During studies to develop additional functional assays for tumor suppression activity, we noted that the mouse hybrid containing the chromosome 3 fragment responded differently from mouse A9 fibrosarcoma cells to a number of chemotherapeutic drugs.[4] For example, mouse A9 cells undergo apoptosis in response to Adriamycin and Taxol, exhibiting characteristic internucleosome cleavage. In contrast, under identical conditions, the chromosome 3p-containing cell hybrid does not undergo apoptosis but arrests in G_2. Apoptosis is a complex process involving multiple pathways and multiple genes that are frequently altered during carcinogenesis. These observations raised the possibility that tumor suppression associated with 3p21.3 was also linked to the modulation of apoptosis.

In this study, genes from P1 294 were identified. cDNA corresponding to two genes were transfected into A9 cells, and the resulting clones were tested for their tumorigenicity in nude mice. In addition, the clones were tested for their response to chemotherapeutic drugs. The human *SEMA3F* gene suppressed growth of A9 fibrosarcoma cells in nude mice as well as altered the response of the A9 cells to Adriamycin and Taxol from apoptosis to growth arrest. Moreover, the human ovarian adenocarcinoma line HEY (6) was also suppressed for tumor growth by expression of *SEMA3F*. In contrast, GLC45 (7), a small lung cancer line, was unaffected by expression of *SEMA3F* either *in vitro* or *in vivo*. These data suggest that there is a specific response of certain tumors to the expression of *SEMA3F*, a member of a family of signaling molecules that have primarily been described in neuronal systems.

MATERIALS AND METHODS

cDNA Identification. P1 clone 294 was the genomic starting material for the isolation of candidate cDNAs. Potential coding sequences were indicated by an *Sst*II small fragment (8), and the isolation of *SEMA3F* cDNA is described by Xiang *et al.* (9). Exon trapping was performed by the method of Buckler *et al.* (10) using the vector pSPL3. Exon trapping products were detected for *N23* (21-1, 16-4) and for an alternatively spliced product of *GNAI2* (clone 162-2). Hybridization selection using the method of Morgan *et al.* (11) yielded a cDNA fragment (clone 327) for *GNAT1* and an ~1-kb fragment of *N23* (clone 325). The *N23* clone was extended by 3′ and 5′ rapid amplification of cDNA ends (Life Technologies, Inc.). *N23* is identical in sequence to clone G17 (GenBank Accession Number U49082), deposited by Latif and colleagues.[5]

Transfection of Cells. A9 cells (an L cell derivative; Ref. 12) were first transfected with pUHD172-1neo (13) to yield clones making the tet repressor protein (A9 TetR). Those clones were screened for expression of a functional transactivator by transient transfection with pUHC13-3, which contains a luciferase reporter gene. The positive clone A9TetR (2–8)[4] was chosen for stable cotransfection with cDNA and ptkHyg with a ratio of 10:1 cDNA: ptkHyg (13) as linear plasmids. A *SEMA3F* cDNA (9) clone including nucleotides 9–2373 (GenBank NM_004186) encoding all but the 20 COOH-terminal amino acids was cloned into the tetracycline-inducible vector pUHC10-3 (Ref. 13; clone N10). A cDNA encoding the entire N23 protein was also cloned into the pUHC10-3 vector (bases 14–2264; GenBank U49082).[5] Cells were cotransfected with cDNA and ptkHyg using Lipofectamine (Life Technologies, Inc.). The cells were then selected for growth in DMEM/F12 medium supplemented with 10% FBS and 800 μg/ml hygromycin. Individual hygromycin-resistant colonies were cloned and cultured in hygromycin-containing medium. Clones containing the respective cDNA were identified using DNA primers. The DNA-positive clones were then assayed by RT-PCR for mRNA expression.

After our initial experiments, other constructs for *SEMA3F* were tested (Table 1). A cDNA containing the entire coding sequence (bases 9–2416, GenBank NM_004186) was placed in the tet vector (N15 clones). Other clones were constructed in the pTracer-SV40 vector (Invitrogen; SV3FL clones). N10, N15, and SV3FL constructs all contained the longer of the two splice forms of *SEMA3F* (5, 9, 14). The shorter spliced form (the product is 31 amino acids shorter) was also introduced using the pTracer vector (SV3FS).

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[2] To whom requests for reprints should be addressed, at Department of Cellular and Structural Biology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900. Phone: (210) 567-3842; Fax: (210) 567-6781; E-mail: Naylor@uthscsa.edu.

[3] The abbreviations used are: SCLC, small cell lung cancer; SEMA3F, semaphorin 3F; RT-PCR, reverse transcription-PCR; VEGF, vascular endothelial growth factor.

[4] A. Davalos, R. H. Xiang, and S. L. Naylor. Human SEMA3F encoded by genomic DNA from chromosome 3p21.3 blocks non-neuronal cell internucleosomal cleavage in response to apoptotic stimuli, submitted for publication.

[5] Database deposition: All sequences have been reported previously. Human *SEMA3F* (U38276 and Ref seq NM_004186). N23 cDNA is the same sequence as G17 (U49082).

Table 1 *SEMA3F constructs injected into nude mice*

Construct	Vector	Promoter	Sema3F bases (NM_004186)	Alternative splice	Total clones	RT-PCR + clones	Tumors of >200 mm^3/total independent clones injected[a]
A9 constructs							
N10	pUCH10-3	hCMV	9-2373	+	46	5	0/4
N15	pUCH10-3	hCMV	9-2436	+		1	0/1
SV-3FS	pTracer-SV40	SV40	9-2436 (Δ535–624)	−	7	4	0/4
SV-3FL	pTracer-SV40	SV40	9-2436	+	20	6	0/6
HEY constructs							
SV-3F	pTracer-SV40	SV40	9-2436	+	30	9	2/9[b]
N10	pUCH10-3	hCMV	9-2436	+	20	6	2/6[b]
GLC45 constructs							
SV-3F	pTracer-SV40	SV40	9-2436	+	16	6	4/4[c]

[a] For each clone tested, five nude mice received injections. After 3 weeks, the control clones averaged 1800 mm^3.
[b] Explants from the clones that grew tumors no longer expressed *SEMA3F* as determined by RT-PCR.
[c] All of the explants from the clones that grew tumors expressed *SEMA3F* as determined by RT-PCR.

HEY cells (6) were transfected using the tet system as well as with pTracer-SV40 containing *SEMA3F* constructs. GLC45 was transfected with the pTracer-SV40 *SEMA3F* cDNA construct. Both HEY and GLC45 did not express endogenous *SEMA3F*.

***In Vitro* Apoptosis Assays.** The apoptosis assays used are those described by Davalos *et al.*[4] Cells were plated at 2×10^6 cells/T75 flask in DMEM/F12 + 10% FBS with or without 1 μg/ml of doxycycline with replacement of medium plus drug 48 h after seeding. The following concentrations were used: Adriamycin at 1 μM and Taxol at 0.5 μM. Fragmentation of cellular DNA was measured after drug treatment. Adherent and nonadherent cells were pooled, washed in PBS, and resuspended in ice-cold buffer containing 0.15 M NaCl, 10 mM Tris (pH 7.4), 2 mM $MgCl_2$, and 1 mM DTT (15). The samples were placed on ice for 40 min. The nuclei were isolated by centrifugation, resuspended in ice-cold buffer containing 0.35 M NaCl, 10 mM Tris (pH 7.4), 2 mM $MgCl_2$, and 1 mM DTT with a 20-min incubation on ice. The samples were centrifuged, and the supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The remaining low molecular weight DNA was precipitated with 2.5 volumes of ethanol and 10 mM $MgCl_2$. The samples were resuspended in 20 μl of Tris-EDTA (pH 8.0) and treated with RNase A (0.5 mg/ml) for 2 h at 37°C and then for 1 h with proteinase K (1 mg/ml) at 50°C (15). Electrophoresis was performed in a 1.5% agarose gel run in TAE buffer [40 mM Tris acetate, 1 mM EDTA (pH 8.0)] for 5 h at 2 V/cm.

Visual examination of apoptotic cells was after treatment with 1 μM Adriamycin for 48 h. The cells were then isolated, and 5×10^3 cells were placed on a glass slide, fixed, and stained with eosin (Sigma). Two hundred cells were examined per slide for apoptotic characteristics, and at least three slides were examined for each condition.

***In Vivo* Assay for Tumorigenesis.** A9 cells (5×10^5) were injected into the shoulders of 5 BALB/c *nu/nu* mice. The average tumor volume is plotted. Tumor volume was assessed at 2-day intervals by the formula (1/2 × width × width × length). Tumors were explanted at 4 weeks and placed in culture for 3 days before harvesting for DNA and RNA. The same procedure was used for GLC45 and HEY constructs except that 2×10^6 cells were injected.

Western Blots. SEMA3F and neuropilin-2 were detected using Western blots.[4] The antibody to SEMA3F was generated using peptide corresponding to the human SEMA3F amino acids 155–178. Neuropilin-2 antibody was purchased from Santa Cruz Biotechnology. The SEMA3F control was the *in vitro* transcription/translation product (Promega) from the pTracer construct.

Soft Agar Assay. One thousand and 10,000 cells were plated in 60-mm soft agar plates consisting of α-MEM containing 10% FBS in a 0.4% agar medium. Agar plates containing cells were carefully placed into the incubator and were supplemented with medium once per week. After 3 weeks, the number of cell colonies formed is determined by staining the agar plates with *p*-iodonitrotetrazolium violet (Sigma). Colonies are quantitated using a NucleoTech Imaging Workstation equipped with a colony counting program.

RESULTS

Gene Identification. To isolate the genes encoded in P1 294, we have used a combination of techniques including exon trapping (10), cDNA hybridization selection (11), and CpG island identification (8). Four independent genes were isolated by these techniques (Fig. 1). cDNA fragments of α transducin (*GNAT1*; Ref. 16), an inhibitory G protein (*GNAI2*; Ref. 17), and a gene with transmembrane and mitochondrial targeting motifs (*N23*) were isolated by hybridization selection. Complete cDNA for *N23* and several clones corresponding to the human *SEMA3F* gene (9) were isolated from commercially available cDNA libraries using either exon trapping clones (*N23*) or a CpG island-containing fragment from P1 294 (*SEMA3F*) as probes. Additional analysis of publicly available sequence from the region corresponding to P1 294 (Ref. 18; GenBank accession numbers AC004693, AC002077, and AC000063) using GRAIL did not yield further predicted coding regions.

Transfection of Candidate Clones. We hypothesized that the tumor suppressor gene encoded in P1 294 should contain mutations in SCLC tumor samples. However, single-strand conformational polymorphism analysis and direct sequencing did not reveal mutations in any of the candidate genes in the 26 tumor samples examined. Although no mutations were found, previous results from our laboratory and others (5, 9, 19) demonstrated that *SEMA3F* is expressed at low or undetectable levels in most SCLC cell lines, although it is expressed in normal lung. Thus, we chose to use a functional approach to identify the gene in P1 294 that suppresses tumor formation in nude mice. *N23* was found to be ubiquitously expressed by Northern

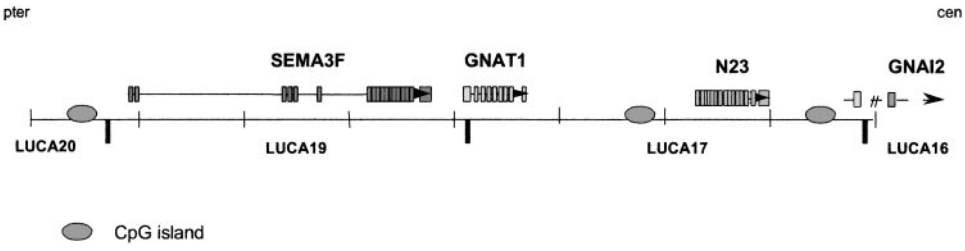

Fig. 1. Map of genes from P1 294. Locations of the genes are given as compared with the sequence generated by Washington University. P1 294 corresponds to the cosmid clones Luca16, Luca17, Luca19, and Luca20 described by Wei *et al.* (18). The shaded exon of *GNAI2* is an alternative splice detected in one hybridization clone (162-2). *Ovals*, the location of CpG islands. *Hash marks*, 10 kb. *Solid bars*, the limits of each of the Luca cosmids.

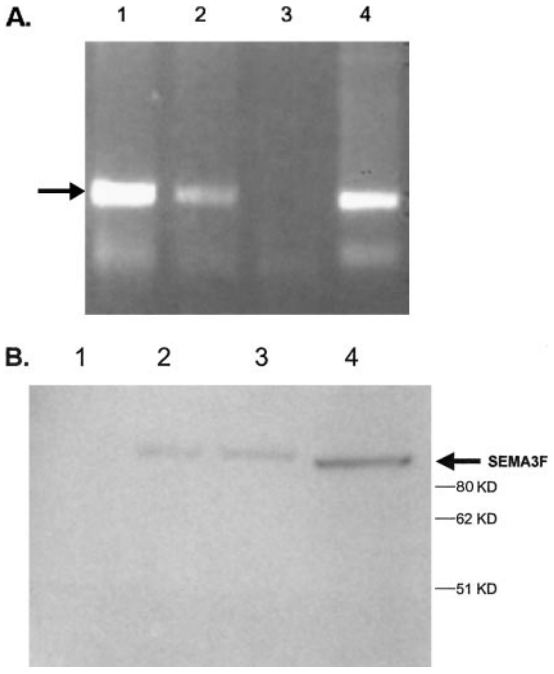

Fig. 2. Expression of SEMA3F. *A*, RT-PCR was performed on RNA extracted from transfectants expressing SEMA3F (*Lanes 1* and *2*) as well as control A9 (*Lanes 3*) and plasmid control (*Lane 4*). cDNA was produced by reverse transcriptase (*Lanes 1*, *2*, and *3*). *B*, Western blot of 30 μg of protein from A9 (*Lane 1*), transfectants (*Lanes 2* and *3*), and *in vitro* transcription/translation product from the pTracer construct (*Lane 4*).

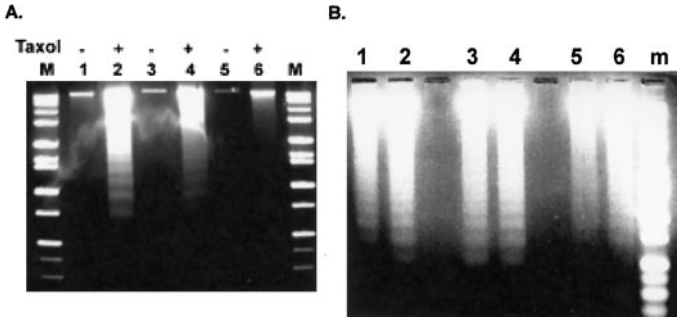

Fig. 3. *A*, DNA fragmentation assay of control A9 cells (*Lanes 1* and *2*), A9 TetR cells (*Lanes 3* and *4*), and a *SEMA3F* transfectant N10 1-1 (*Lanes 5* and *6*) either untreated (*Lanes 1*, *3*, and *5*) or treated for 24 h with 0.5 μM Taxol to induce apoptosis. DNA fragmentation is noted in the controls but not the semaphorin transfectant. A9 TetR clones transfected with ptkHyg vector alone undergo DNA fragmentation comparable with A9 TetR cells. All SEMA3F-expressing constructs have this same response. *B*, DNA fragmentation assay of N23 clones. All clones expressing *N23* underwent apoptosis in response to treatment with Adriamycin. *N23* clones N23 T-6 (*Lanes 1* and *2*), N23 17-6 (*Lanes 3* and *4*), and N23 18-4 (*Lanes 5* and *6*) were treated with doxycyclin (1.0 μg/ml for 24 h; *Lanes 2*, *4*, and *6*) or unmodified medium (*Lanes 1*, *3*, and *5*), then exposed to Adriamycin (1.0 μM for 48 h). DNA fragmentation assay is as described.[4]

analysis and by RT-PCR. One gene, *GNAI2*, is only partially encoded on P1 294 and therefore cannot be responsible for the tumor suppressor activity. *GNAT1* has been shown by us and by others to be undetectable in normal lung tissue even using RT-PCR (data not shown) and therefore was excluded as a candidate. Consequently, *SEMA3F* and *N23* are the only remaining candidates from this P1 clone. Each of these cDNAs was cloned into the pUHC10-3 (13) tetracycline-inducible vector and cotransfected along with ptkHyg (3, 20) into A9 cells stably expressing the tet repressor protein (A9 TetR). All of the resulting clones examined expressed either *N23* or *SEMA3F* (Fig. 2*A*) constitutively, although some clones induced higher levels of expression after tetracycline exposure. We have developed an antibody to a peptide corresponding to the human SEMA3F amino acids 155–178. This antibody does not detect protein in A9 cells but detects protein in the transfectants in amounts proportional to mRNA expression (Fig. 2*B*).

Apoptotic Response Detected by Internucleosome Cleavage. Four independent A9 TetR transfectants each were found to express *SEMA3F* and *N23*. As a first step in analyzing the effects of these genes on A9 growth properties, we challenged the clones as well as

Table 2 *Soft agar assay for SV3FL clones*[a]

Name of clone	Plate 1	Plate 2
SV3FL (1-1)	10	6
SV3FL (1-3)	5	4
SV3FL (7-1)	8	7
A9pT-SV (vector)	534	545

[a] 10^3 cells were inoculated per plate.

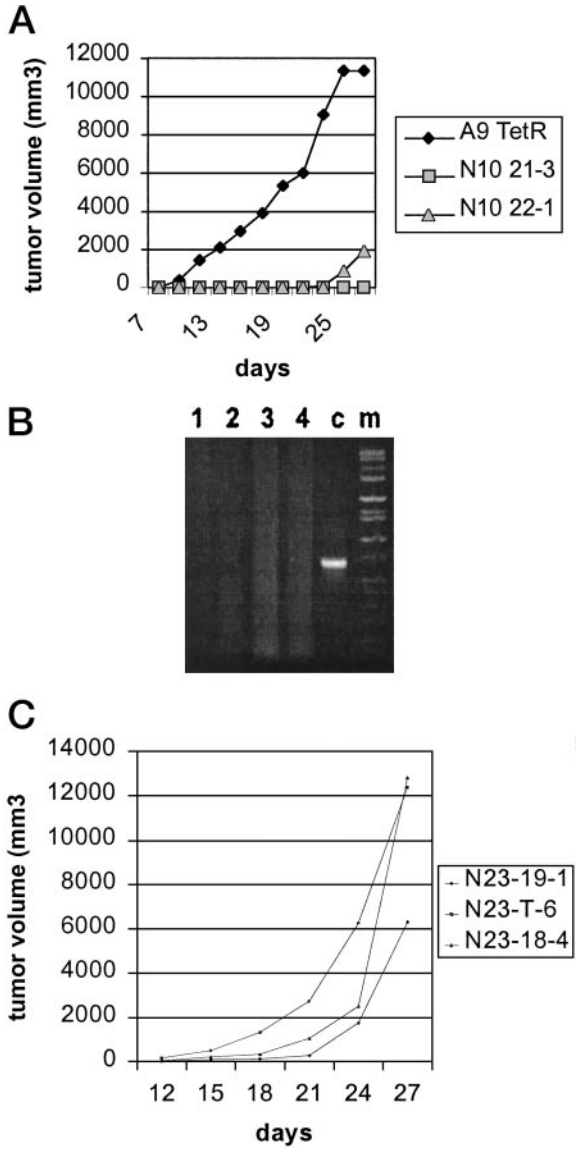

Fig. 4. *A*, tumor growth in nude mice, N10 clones. A9 TetR control cells and two independent *SEMA3F* (N10) transfectants (N10 21-3 and N10 22-1) are shown. *B*, DNA analysis in tumor explants. After sacrifice of the animals shown in *A* at 28 days, the tumor mass was explanted into culture. Human *SEMA3F* DNA was assayed by PCR using species-specific primer. Explants from four N10 tumors (*Lanes 1–4*) show no human semaphorin DNA. Control plasmid and markers are in *Lanes c* and *m*, respectively. *C*, tumor growth in nude mice, *N23* clones. Nude mice were injected as above for three N23-expressing clones (N23 19-1, N23 T-6, and N23 18-4). All clones produce tumors comparable with A9 TetR controls.

parental A9 TetR cells with the apoptosis-inducing agents Taxol or Adriamycin. Upon challenging transfectants with Adriamycin or Taxol, clones transfected with *N23* cDNA as well as parental A9 TetR controls underwent apoptosis as evidenced by internucleosome cleavage of chromatin (Fig. 3*A*). In contrast, clones expressing *SEMA3F* cDNA did not have an apoptotic response but rather arrested growth (Fig. 3*B*). Transfectants were also tested for cloning efficiency in soft agar. *SEMA3F* cDNA transfectants showed a marked reduction in cloning efficiency (<10% of controls; Table 2).

Tumor Suppression in Nude Mice. To test whether the expression of the candidate genes correlated with suppression of tumor growth in nude mice, we injected each of the A9 transfectants into five male BALB/c nude mice. Shown is a plot of the average tumor volume for the five animals (Fig. 4). Tumor growth was monitored for 28 days when the mice were sacrificed, and explants were established from any resulting tumors. As shown in Fig. 4*A*, the clones expressing semaphorin did not form (*e.g.*, clone N10 21-3) tumors or showed a vast reduction in tumorigenicity (*e.g.*, clone N10 22-1). Explants of the tumors from clone N10 22-1 were assayed for human semaphorin DNA. None of the tumors had detectable human semaphorin DNA, indicating that tumor outgrowth resulted from loss of the semaphorin cDNA (Fig. 4*B*). In contrast, all of the *N23*-expressing clones produced tumors in nude mice at a size comparable with the parental A9 TetR cell line (Fig. 4*C*). Explants from tumors of the *N23* transfectants retain *N23* DNA and express the cDNA as determined by RT-PCR.

A total of 16 other *SEMA3F* transfectants were tested for suppression of A9 tumor growth. These included one N15 and six SV3FL clones that contain the full coding region of the longer splice product and four SV3FS clones that expressed the shorter splice form (see "Materials and Methods"). All clones that expressed *SEMA3F* constructs suppressed tumor formation in nude mice (Table 1) and did not undergo apoptosis in response to Adriamycin, Taxol, or tumor necrosis factor α. In addition, two clones that had deleted part of *SEMA3F* were injected. These transfectants produced tumors comparable with the A9 control. These data indicate that expression of *SEMA3F* cDNA in mouse A9 cells results in suppression of tumor growth.

GLC45 Small Cell Lung Cancer Line. We tested the human SCLC line GLC45 for its response to expression of *SEMA3F*. In contrast to A9 cells, transfectants expressing *SEMA3F* grew as well or better than GLC45 cells transfected with vector only in nude mice (Fig. 5). Tumors that were explanted still expressed high levels of *SEMA3F*. In addition, the *SEMA3F* and the vector transfectants were both sensitive to apoptosis-inducing stimuli such as Taxol (data not shown). These results are not surprising, considering that Timmer (21) was unable to demonstrate an effect of a PAC from chromosome 3p21 containing *SEMA3F* on the growth of GLC45. These data also imply that the expression of *SEMA3F* has an effect only in specific cells.

HEY Ovarian Adenocarcinoma Line. Because in our hands two of the SCLC lines that have homozygous deletions in this region (GLC20 and NCI H740) do not readily form tumors in nude mice, we examined other tumor cell lines that show deletions in 3p21.3. Rimessi *et al.* (22) transferred chromosome 3 into human HEY cells. The HEY cell line was described by Buick *et al.* (6) as being derived from a human epithelial adenocarcinoma and having deletions of chromosome 3p. The microcell hybrids carrying a normal chromosome 3 were suppressed for growth in nude mice (22).

We transfected *SEMA3F* cDNA into HEY cells using either the tet vector (13) or the pTracer construct. In both systems, HEY cells expressing *SEMA3F* showed reduced tumorigenicity in nude mice (Fig. 6). In those cases where tumor cells grew out, *SEMA3F* was no longer expressed. HEY cells expressing *SEMA3F* also showed a marked reduction of growth in soft agar (Fig. 6*E*). The *SEMA3F*-transfected HEY cells showed a modest resistance to apoptosis after exposure to Adriamycin (Fig. 6*D*). All three cell lines, A9, HEY, and GLC45, express neuropilin-2, a receptor for SEMA3F. Consequently, the difference in the response to *SEMA3F* expression is most likely specific for the cell lines tested.

DISCUSSION

In this study, we have shown that the expression of the *SEMA3F* gene suppresses tumor formation in nude mice as well as alters the cellular response to drugs inducing apoptosis. A9 cells expressing human *SEMA3F* were completely blocked for tumorigenesis in nude mice. This result is specific for *SEMA3F*, because transfection of a gene located on the same P1 clone, N23, had no effect either on tumorigenicity or on apoptosis. In addition, the observed tumor suppression/apoptosis inhibition is not attributable simply to overexpression of the transfected *SEMA3F* gene, because identical results were obtained after transfer of an intact human chromosome 3, possessing a single copy of *SEMA3F* with its natural promoter, into A9 cells (2). These results strongly suggest that human SEMA3F has specific tumor suppressor activity. Combined with the observed homozygous

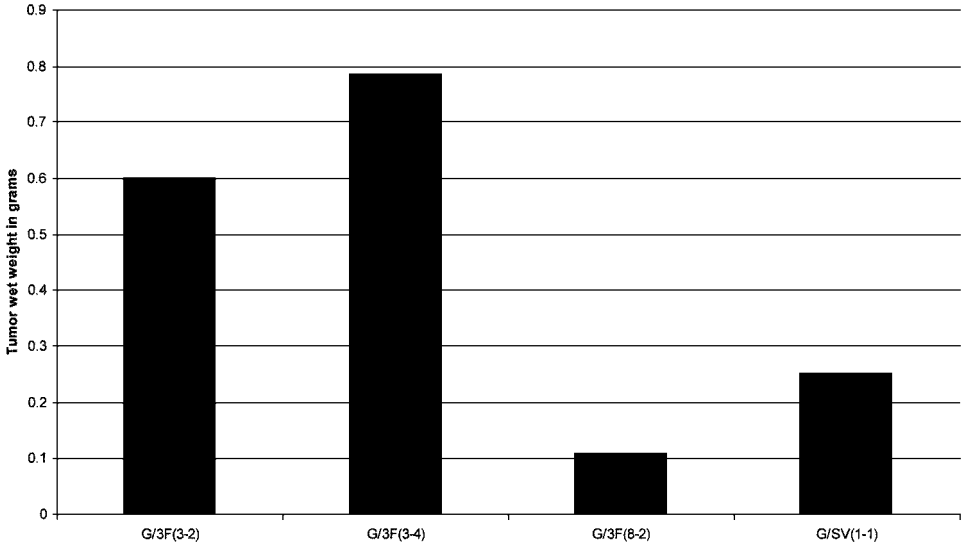

Fig. 5. Transfection of GLC45. pTracer transfectants of *SEMA3F* [*G/3F(3-2), G/3F(3-4),* and *G/3F(8-2)*] were isolated, and five animals of each construct and the control [*G/SV(1-1)*] were injected into nude mice described. The cells were harvested at 28 days, and the tumors were weighed. RNA extracted from the transfectant tumors all express SEMA3F.

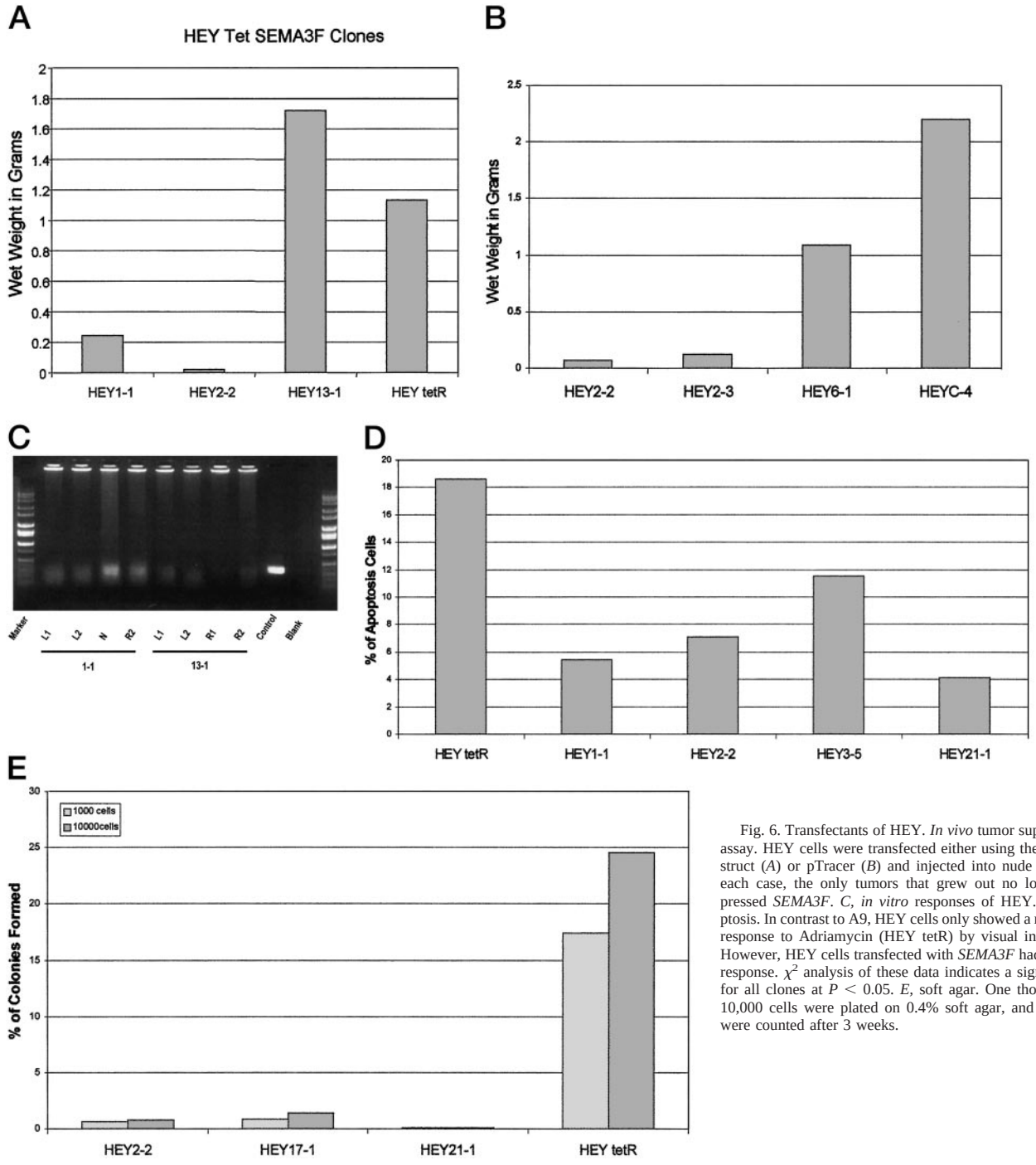

Fig. 6. Transfectants of HEY. *In vivo* tumor suppression assay. HEY cells were transfected either using the tet construct (*A*) or pTracer (*B*) and injected into nude mice. In each case, the only tumors that grew out no longer expressed *SEMA3F*. *C, in vitro* responses of HEY. *D,* apoptosis. In contrast to A9, HEY cells only showed a moderate response to Adriamycin (HEY tetR) by visual inspection. However, HEY cells transfected with *SEMA3F* had a lesser response. χ^2 analysis of these data indicates a significance for all clones at $P < 0.05$. *E,* soft agar. One thousand or 10,000 cells were plated on 0.4% soft agar, and colonies were counted after 3 weeks.

deletions and universal loss of heterozygosity at the semaphorin locus in a variety of tumor types, the data presented here implicate SEMA3F as a tumor suppressor gene located on chromosome 3p21.3.

The A9 cells expressing *SEMA3F* arrest at G_2 in response to drugs such as Taxol or Adriamycin.[4] This response is not unlike that seen with retinoblastoma protein null cells after transfection with a retinoblastoma gene construct. Rb1 knockout mice die *in utero* and exhibit massive apoptosis in several organs, including those of the nervous system (23). If rb −/− myocytes are transfected with an Rb-expressing construct, apoptosis is inhibited (24). Likewise, in SAOS-2, which lacks wild-type Rb, radiation induces apoptosis (25). In these cells also, transfection of wild-type Rb results in arrest at G_2 after radiation exposure. Thus, lack of a tumor suppressor gene product can alter the apoptotic response as well as lead to tumor formation in the animal (26).

The significance of the A9 response to semaphorin expression is heightened by the result with HEY ovarian adenocarcinoma cells. Rimessi *et al.* (22) reported that chromosome 3 suppressed tumor formation in HEY cells, and they had suggestive evidence that 3p21 could be involved. Our data indicate that SEMA3F can suppress tumor formation by HEY cells. In contrast, we did not observe suppression of the SCLC line GLC45. Although it is only a single SCLC cell line, this result raises the question of whether *SEMA3F* plays a role in SCLC. To date, no mutations in *SEMA3F* have been found in SCLC tumors (5, 9, 19, 27). However, Brambilla *et al.* (28) have seen changes in cellular distribution of SEMA3F in lung tumors.

Because we tested for the expression of neuropilin-2, at least part of this pathway is intact. The significant result with HEY cells would indicate that *SEMA3F* is key to some tumors or tumor types.

The semaphorin family of proteins has been identified largely as signaling proteins involved in neuronal development (29–33) having members with chemoattractant and repulsion functions (34). *SEMA3F* has the motifs of a secreted protein of the class 3 semaphorins (35). Another secreted semaphorin (3A or collapsin) has been shown to effect neuronal collapse by the induction of apoptosis (36, 37). Additionally *SEMA3C* has been shown to be overexpressed in tumors (38), and *SEMA3E* expression correlated with metastatic properties (39). Both these observations are in contrast with our results and others (5, 9, 19) for *SEMA3F*. Because members of this family of proteins can have opposing signaling roles in neuronal development (30), it is not surprising that different semaphorins would have different roles in apoptosis and tumorigenesis.

Chen *et al.* (40) have identified a high affinity receptor for this protein as neuropilin-2. Neuropilin-1 also binds this semaphorin (40). All three cell lines express neuropilin-2. Soker *et al.* (41) isolated clones for a receptor for VEGF ($VEGF_{165}$) that are identical to neuropilin-1 (six of seven clones) and neuropilin-2 (one of seven clones). Specific VEGF isoforms bind to the neuropilin receptors (42). These data combined with the functional data presented here foster speculation for the mode of action of *SEMA3F* in tumorigenesis. The results of the soft agar assay would indicate that there is an intrinsic change in the cell that alters its growth properties. Because *SEMA3F* is expressed in most tissue types (5, 9, 19, 27), it is surprising that surrounding cells cannot supply sufficient quantities of SEMA3F protein. This observation would suggest that SEMA3F acts in an autocrine manner, and secretion by the cell itself is necessary to achieve concentrations for inhibition of growth. A decrease in SEMA3F expression, because of haploinsufficiency or methylation of the remaining allele, could tip the balance of suppression and growth. Alternatively, it is the change in cellular distribution that affects this balance (28). Because VEGF is also a factor in tumor progression, the expression of SEMA3F may further inhibit the vascularization of the tumor.

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RuiHua Xiang, Albert R. Davalos, Charles H. Hensel, et al.

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