Tissue factor pathway inhibitor-2 suppresses the production of active matrix metalloproteinase-2 and is down-regulated in cells harboring activated *ras* oncogenes

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Abstract A human placenta cDNA expression library was screened for genes inducing flat reversion when transfected into a *v-K-ras*-transformed NIH3T3 cell line, DT. One such gene was found to encode a Kunitz-type serine protease inhibitor, tissue factor pathway inhibitor-2 (TFPI-2). While the *TFPI-2* mRNA can be detected in normal human fibroblasts (MRC-5), it is down-regulated in MRC-5 cells expressing an activated *H-ras* oncogene and in the human fibrosarcoma cell line, HT1080. Restored expression of the *TFPI-2* gene in HT1080 cells resulted in the suppression of matrix invasion activity in vitro with concomitant decrease in the relative amount of active matrix metalloproteinase-2 secreted from the cells. When DT cells were cultured in the presence of conditioned medium and extracellular matrix prepared from *TFPI-2*-transfected HT1080 cells, increased attachment and flat reversion were observed. These results suggest that TFPI-2 may be required for the maintenance of the integrity of extracellular matrix in normal tissues and its down-regulation as a result of oncogene activation may contribute to the malignant phenotypes of tumor cells.

Key words: *ras* oncogene; Transformation suppressor gene; Tissue factor pathway inhibitor-2; Matrix metalloproteinase-2; Tumor invasion

1. Introduction

Mutations of *ras* proto-oncogenes are found in a large variety of human tumors [1]. Ras proteins are essential components in various intracellular signaling pathways involved in regulating gene expression and several other aspects of cellular behavior [2–5]. Therefore, it is now important to find targets for these signals relevant to the expression of the malignant phenotype in order to understand the mechanism of cell transformation and to help developing novel strategies for cancer intervention.

To this end, we have been isolating and characterizing genes which induce flat, normal fibroblast-like morphology (or 'flat reversion') when expressed in a *v-K-ras*-transformed NIH3T3 cell line, DT [6]. Products of such reversion-inducing genes we characterized so far include a Ras-related small G-protein (Krev-1/Rap1A) [7,8], a truncated form of the MSX-2 homeobox protein [9], and a novel membrane-anchored glycoprotein (RECK) containing multiple Kazal-type serine protease inhibitor-like domains [10]. Interestingly, the expression of the *RECK* gene is strongly suppressed in many cell lines derived from tumors or transformed by various oncogenes [10,11]. Moreover, when the *RECK* gene was artificially expressed in fibrosarcoma cells, secretion of matrix proteinase-9 was found to be reduced and the invasive and metastatic activities of the cells significantly suppressed [10].

Here we report on the characterization of another reversion-inducing cDNA encoding a Kunitz-type serine protease inhibitor, previously termed tissue factor pathway inhibitor-2 (TFPI-2) [12,13] and placental protein 5 (PP5) [14]. TFPI-2 is an extracellular glycoprotein of about 32 kDa (213 amino acid residues) and is structurally related to TFPI [12,13]. TFPI is a plasma protein which inhibits the initial steps of the extrinsic coagulation pathway and regulates the hemostasis [15,16]. It also inhibits the proliferation of vascular smooth muscle cells in culture [17]. In the enzyme assay system in vitro, TFPI-2 was reported to be a strong inhibitor for trypsin, plasmin, plasma kallikrein and factor XIa amidolytic activity [13]. Little is known, however, about the physiological functions of TFPI-2, except for the finding by Shinoda et al. that it stimulates the proliferation of smooth muscle cells in culture [18]. Our observations reported here indicate that the expression of *TFPI-2* gene is low in cells expressing activated *ras* oncogenes and that this may contribute to the morphological changes associated with cell transformation and the invasive/metastatic behavior of cancer cells.

2. Materials and methods

2.1. Cell culture

DT cells [6] were cultured in Dulbecco's modified Eagle medium (DME; Gibco) supplemented with 10% fetal calf serum (FCS; JRH Bioscience), 4 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate. Other cell lines were obtained from ATCC and maintained according to the supplier's instruction. MRC-5/*H-ras*^{12V} was generated by infecting the MRC-5 normal human fibroblast strain [19] with a helper-free stock of LXSN retroviral vector [20] carrying the activated human *H-ras* oncogene (*H-ras*^{12V}) prepared using the amphotropic packaging host GP+envAm12 [21]. Transfection with cloned plasmids was carried out using the mammalian transfection kit (Stratagene). For soft agar assay, cells (3×10^3) were plated in suspension in minimal essential medium containing 0.33% Bacto-Agar (Difco), L-glutamine, antibiotics and 10% filtered FCS, and the colonies were counted under a microscope after 3 weeks incubation.

2.2. Expression cloning of reversion-inducing cDNAs

A cDNA library consisting of 2×10^6 independent clones was generated using human placental mRNAs (Clontech) and a newly developed phagemid-type expression vector carrying blasticidin-S (Bla-S)

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resistance marker BSD (N. Akiyama et al., in preparation). The total of 200 μg purified phage DNA was transfected into 6×10^6 DT cells to obtain 1×10^6 independent Bla-S-resistant transfectant colonies. After three cycles of adhesion selection [7] and repeated single-colony isolation, 13 stable revertants were obtained. The plasmid portions of the transfected DNA in these revertant clones were recovered and amplified in bacteria as described previously [10]. Out of 22 plasmids thus recovered, three clones with insert sizes of about 1.2 kb (20d-3), 1.0 kb (8c-3) and 1.4 kb (6a-3), respectively, were found to give rise to flat revertants when transfected into DT cells.

2.3. RNA blot hybridization

For tissue distribution analysis, the ^{32}P-labeled 20d-3 (*TFPI-2*) cDNA was hybridized to pre-blotted human tissue poly(A)$^+$ mRNA (Multiple Tissue Northern Blot, Clontech). The quality and quantity of RNA samples were assessed by hybridization with ^{32}P-labeled β-actin cDNA. For other RNA blot hybridization experiments, 2 μg of poly(A)$^+$ mRNA purified from cultured cells using the Quick Prep Micro mRNA Purification kit (Promega) was analyzed according to the standard protocol [22]. The same blot was analyzed successively with the *TFPI-2* probe, human *H-ras* probe in some cases, and β-actin probe.

2.4. Immunoblot assay

To prepare conditioned medium (CM), cells (1.2×10^6 per 100 mm dish) were plated, incubated overnight, rinsed with phosphate-buffered saline (PBS; 4 ml×3) and further incubated in 11 ml serum-free DME for 24 h. The culture supernatant was harvested, cleared by low speed centrifugation and concentrated to 50 μl with Centricon 10 (Amicon). To prepare cell lysates, the cells on the dish were rinsed with PBS (4 ml×3) and detached by incubating for 20 min in 1.5 ml of 1 mM EDTA in PBS. After suspension by pipetting, the cells were harvested and lysed with 1 ml of 1% sodium dodecyl sulfate (SDS) in Tris-buffered saline (TBS). To prepare the extracellular matrix (ECM) fraction, 1.5 ml of 1% SDS in TBS was added to the dish from which the cells had been removed as described above. After swirling at room temperature for 2 h, the solution was collected. Each of these samples was diluted with one volume of 2×SDS sample buffer and boiled for 5 min. The proteins were separated by SDS–polyacrylamide gel electrophoresis (15%) and transferred onto PVDF membrane (Micron Separations). After blocking with 4% non-fat dry milk in TBS containing 0.01% Tween 20, the membrane was incubated with rabbit polyclonal anti-TFPI-2 antibodies (provided by Drs. K. Udagawa and Y. Miyagi, Yokohama City University) for 2 h. After TBS-washing, the membrane was incubated with alkaline phosphatase-conjugated monoclonal mouse anti-rabbit IgG (Promega) using nitro blue tetrazolium and 5-bromo-3-indolyl phosphate as substrates.

2.5. Invasion and motility assays

For invasion assay, cells (7×10^5) suspended in DME containing 0.1% bovine serum albumin (BSA) were placed in the upper compartment of BioCoat Matrigel Invasion Chamber (Collaborative). The lower compartment was filled with DME containing 10% FCS. After incubation at 37°C in a CO_2 incubator for 24 h, the cells that had penetrated the Matrigel-coated membrane were counted [23]. For motility assay, uncoated Transwell Chambers (Coster), instead of Matrigel chambers, were used and the cells that had migrated across the membrane were counted after 6 h incubation.

2.6. Gelatin-zymography

Culture supernatants were prepared by incubating cells in DME containing 0.1% BSA (volume: 300 μl per 5×10^5 cells) at 37°C for 6 h. Proteins in the conditioned media were separated, without prior boiling, by electrophoresis through SDS–polyacrylamide (10%) gel containing 1 mg/ml gelatin (Difco) under non-reducing conditions. The proteins in the gel were renatured and stained as described previously [24].

2.7. Cell adhesion and reversion assays using CM and ECM

The protocol described previously [25] was adapted with some modifications. In brief, cells (5×10^5) were seeded onto a 60 mm dish and incubated at 37°C for 2 days. The culture supernatant (CM) was then recovered and filtered through 0.45 μm membrane filter (Millipore). The cells were removed from the dish by treatment with 1 mM EDTA in PBS for 20 min. After pipetting, the suspended cells were removed, and each dish was checked for the complete detachment of the cells under a phase-contrast microscope. To fix the remaining ECM, dishes were dried for 30 min under a laminar flow hood. For cell adhesion assay, DT cells (2×10^5) suspended in the filtered CM were seeded onto the ECM-coated dish and incubated at 37°C. After 90 min incubation, floating cells were thoroughly removed, and the dish was

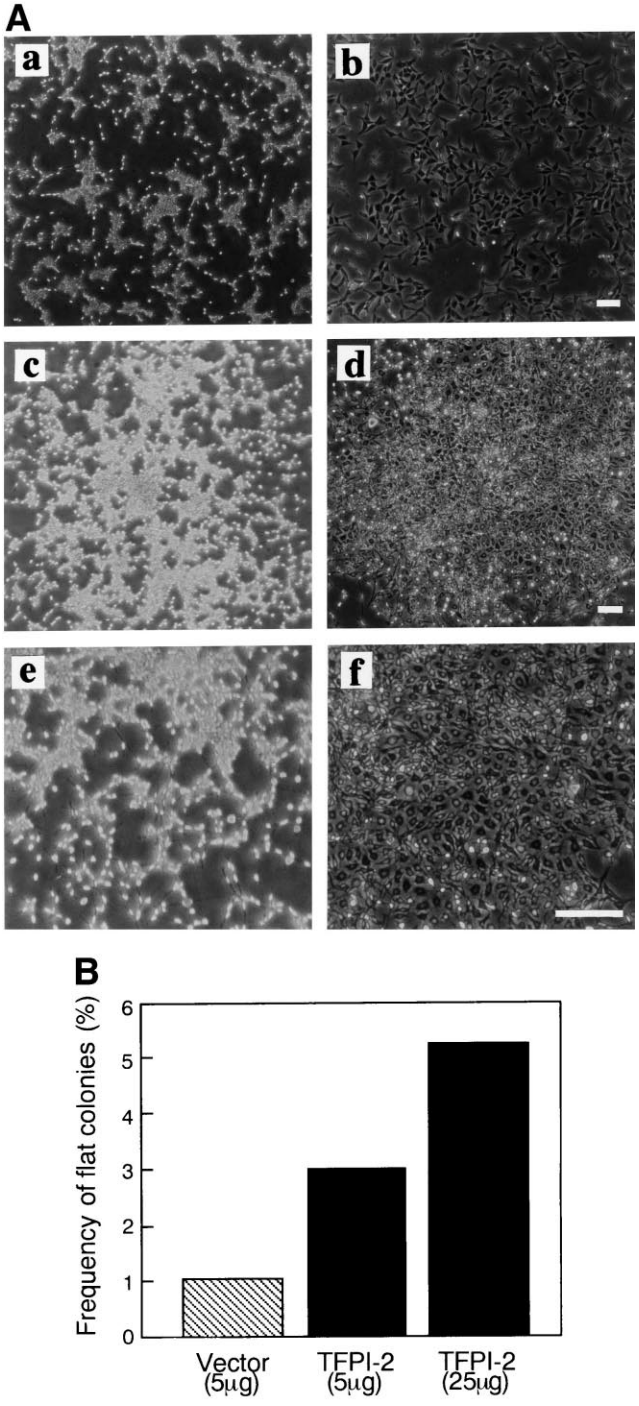

Fig. 1. Reversion-inducing activity of 20d-3 (TFPI-2) cDNA. A: Morphology of the parental DT cells (a), the primary revertant 20d-3 (b), and the colonies obtained after transfection of control vector (c, e) or one of the cDNA clones recovered from the 20d-3 cells (d, f) into DT cells followed by selection with Bla-S for 7 days. Bars represent 100 μm. B: Frequency of flat colonies among total colonies observed after transfection of control plasmid (vector) or 20d-3-containing plasmid into DT cells. The data represent the summary of three separate experiments.

washed with PBS. The remaining cells were then detached using trypsin/EDTA and counted. For reversion assay, DT cells (1×10^3) suspended in the filtered CM supplemented with 10% FCS were seeded onto the ECM-coated dish and incubated at 37°C in a CO_2 incubator. After 7 days incubation, the numbers of all colonies and flat colonies were counted.

3. Results

3.1. Isolation of reversion-inducing cDNAs

A human placental cDNA expression library was screened for clones inducing flat reversion when overexpressed in the *v-K-ras*-transformed NIH3T3 cell line, DT, as described previously [10]. Three biologically active cDNA clones were obtained. One of these, named 20d-3, was found to show the highest reversion-inducing activity and therefore subjected to further characterization.

A dose-dependent increase in the number of flat colonies was observed when the 20d-3 plasmid DNA was transfected into DT cells (Fig. 1). The flat transfectants, when isolated, were found to show reduced proliferative potential both in soft agar and in liquid medium as compared to the parental DT cells, and their growth properties were closer to those of untransformed NIH3T3 cells (data not shown). The nucleotide sequence analysis revealed that the 20d-3 cDNA contains the full coding sequence of TFPI-2 [12,14].

3.2. TFPI-2 gene expression is influenced by oncogenic ras genes

Next we examined the expression of *TFPI-2* mRNA in cell lines and normal human tissues by RNA blot hybridization. Endogenous *TFPI-2* mRNA was undetectable in DT cells, while two species of mRNA could be detected in DT cells transfected with 20d-3 plasmid (Fig. 2A). Strong expression of the transcripts of about 1.2 and 1.4 kb was detected in human placenta, as expected from its alternative name, PP5 [14]. In addition, low level expression could be detected in a wide variety of organs (Fig. 2B), which is consistent with the earlier observation by Miyagi et al. using a limited number of organs [26].

TFPI-2 transcript could also be detected in cultured normal human fibroblasts (MRC-5), whereas it was undetectable in a human fibrosarcoma cell line HT1080 [27] which is known to harbor a mutationally activated *N-ras* gene [28] (Fig. 2C). Interestingly, when we overexpressed an activated form of human *H-ras* gene (*H-ras*^{12V}) in MRC-5 cells, *TFPI-2* mRNA was down-regulated (Fig. 2C). Thus, the low expression of *TFPI-2* gene in HT1080 cells may be attributed, at least in part, to the mutated *N-ras* oncogene.

TFPI-2 expression was also undetectable in the colon carcinoma-derived cell line SW480 [29] (Fig. 2C) which contained mutationally activated *K-ras* oncogene [30]. It is, however, hard to judge whether *TFPI-2* gene was down-regulated in these cells, since its expression in their normal counterpart, colon mucosa, was originally low and barely detectable under these conditions (Fig. 2B).

3.3. Effects of TFPI-2 expression on the growth properties of HT1080 cells

To examine the role of *TFPI-2* gene in the regulation of cell growth and malignant behavior of the cells, we established HT1080 cells transfected with an expression vector containing the *TFPI-2* cDNA. *TFPI-2* mRNA could be detected in these transfectants but not in the vector-transfected control cells (Fig. 3A). Considerable fractions of TFPI-2 protein were found to be secreted into the ECM and CM of the cDNA-transfected cells (Fig. 3B). Cellular morphology, however, was

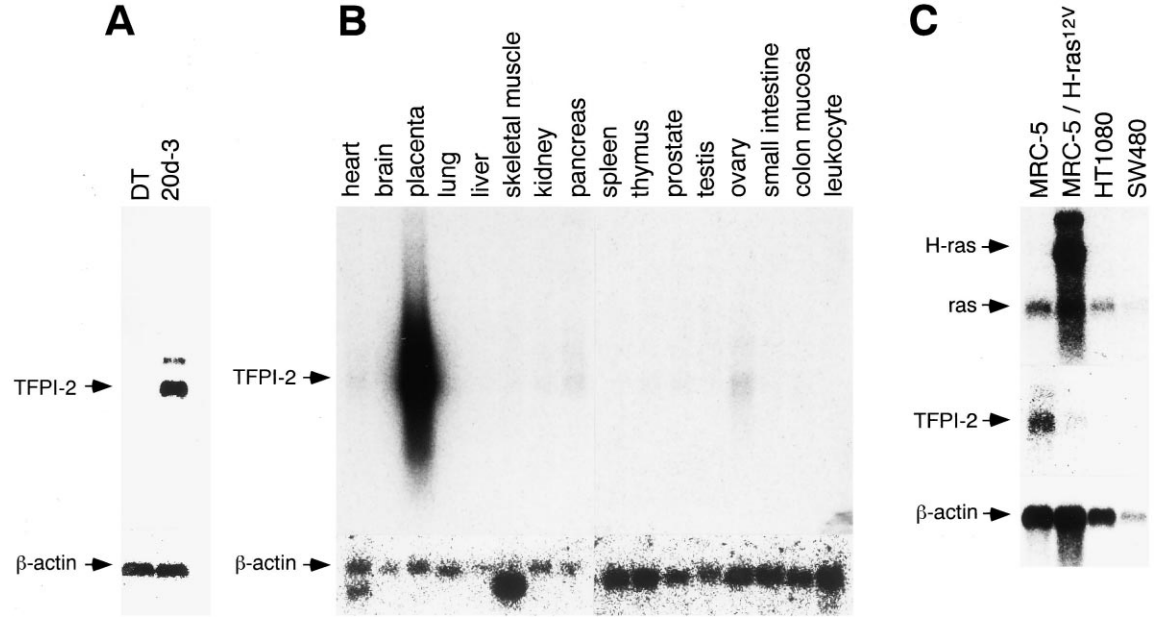

Fig. 2. Detection of *TFPI-2* mRNA in cell lines and normal human tissues by RNA blot hybridization. A: Mouse cell lines. Poly(A)$^+$ RNA from parental DT cells (left lane) or the 20d-3-transfected DT cells (right lane) was probed with 20d-3 cDNA (top panel) and then re-probed with β-actin cDNA (bottom panel). B: Normal human tissues. Human multiple tissue blots were probed with *TFPI-2* cDNA (top panel) and then with β-actin cDNA (bottom panel). C: Cultured human cells. Poly(A)$^+$ RNA form MRC-5 (normal fibroblast), MRC-5 infected with a retroviral vector carrying the *H-ras*^{12V} oncogene, HT1080 (fibrosarcoma) or SW480 (colon adenocarcinoma) were probed successively with *H-ras*, *TFPI-2* and β-actin cDNAs.

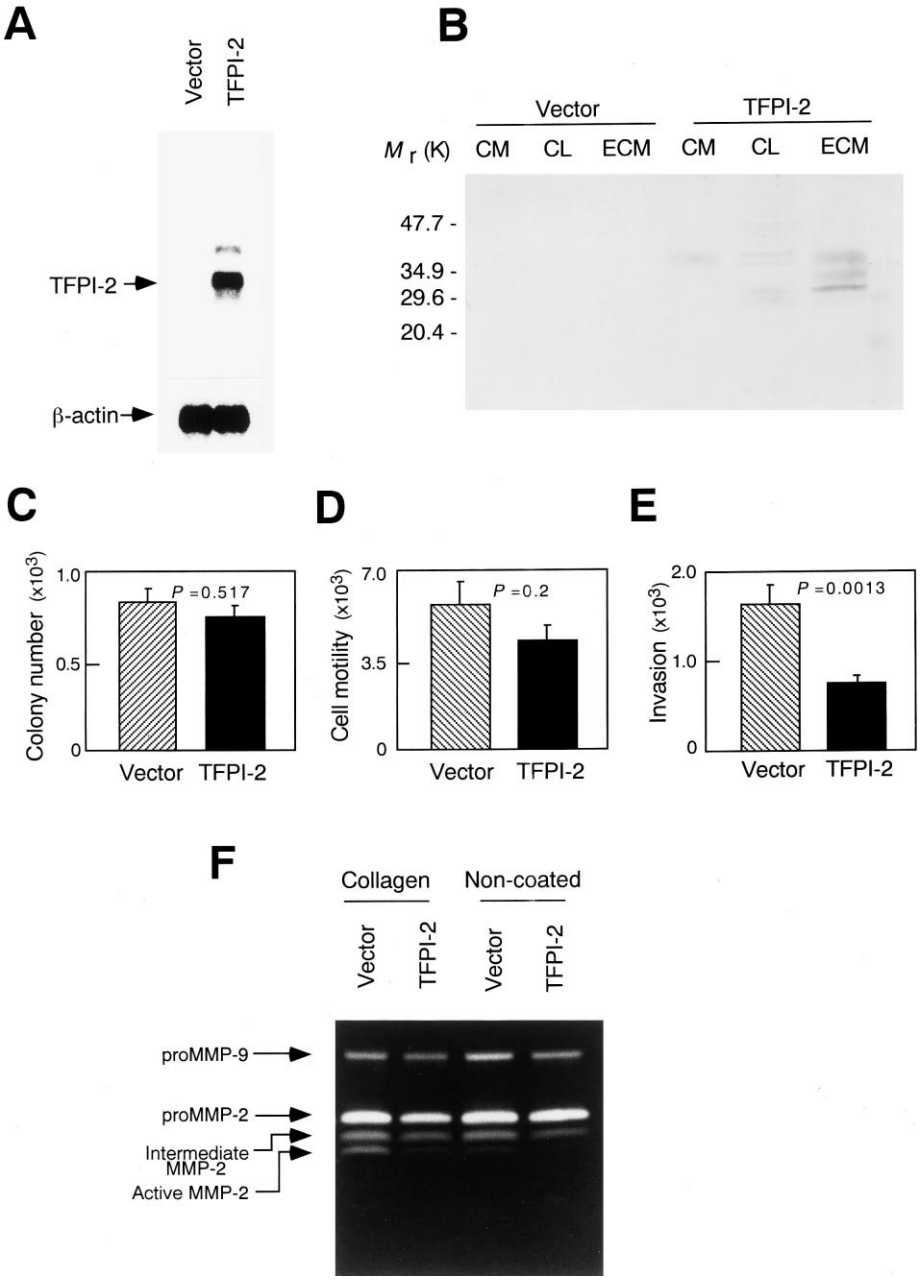

Fig. 3. Effects of TFPI-2 expression in HT1080 cells. A: Detection of *TFPI-2* mRNA in HT1080 cells stably transfected with control vector (left lane) or the vector expressing *TFPI-2* (right lane). The experimental conditions were similar to those in Fig. 2. B: TFPI-2 proteins in the CM, cell lysate (CL) or ECM prepared from these transfectants were analyzed by immunoblot assay using anti-TFPI-2 polyclonal antibody. C: Effects on anchorage-independent growth. The transfected cells plated in soft agar medium were incubated for 3 weeks, and the colonies larger than 0.1 mm in diameter were scored (two independent experiments). D: Effects on cell motility ($n=6$). E: Effects on invasive activity ($n=12$). Error bars in C, D and E represent S.E.M., and P values from Student's t-test are also presented. F: Effects on secreted gelatinase activities. The transfected cells were plated on a regular tissue culture dish (non-coated) or the dish coated with collagen type I (collagen), and the culture supernatants were analyzed by gelatin-zymography. Positions of respective gelatinase species are indicated.

not appreciably affected in these transfectants (not shown); the parental line itself was heterogeneous in morphology to some extent.

Next we compared the growth properties of the vector-transfected cells and 20d-3-transfected cells. First, we could not detect any significant effects on the ability of these pooled transfectants to form colonies in soft agar (Fig. 3C). Second, we could detect no significant difference in their cell motility (Fig. 3D). Interestingly, however, a significant suppression of matrix invasion was observed in *TFPI-2*-expressing cells (Fig. 3E). To explore the reason for this effect, we examined the levels of gelatinase activities in the CM by gelatin-zymography (Fig. 3F). Interestingly, the relative intensity of the 56 kDa band corresponding to the mature, active form of matrix metalloproteinase-2 (MMP-2) was significantly reduced in *TFPI-2*-expressing cells, irrespective of the substrate used for cultivation (i.e. collagen-coated or uncoated) (Fig. 3F).

3.4. TFPI-2 protein and flat reversion

To confirm the role of TFPI-2 protein in the flat reversion

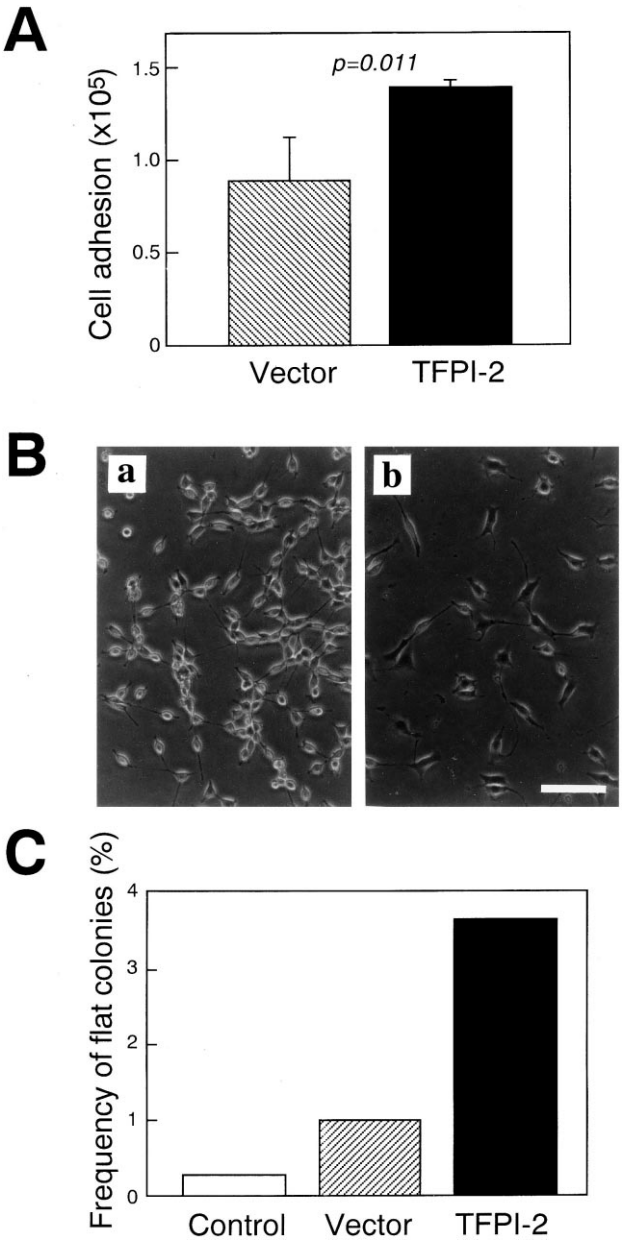

Fig. 4. Effects of TFPI-2 expression on cell adhesion. A: Effects of CM and ECM from transfected HT1080 cells on substrate attachment of DT cells. DT cells were plated in the presence of CM and ECM prepared from the HT1080 cells transfected with control vector or the vector expressing *TFPI-2* gene, and after 1.5 h, cells attached to the dish were counted ($n=4$). B: Morphology of the DT cells incubated for 7 days in the presence of CM and ECM prepared from the vector-transfected HT1080 cells (a) or the *TFPI-2*-expressing cells (b). C: DT cells were plated onto regular tissue culture dishes with fresh medium (Control) or in the presence of CM and ECM from the vector-transfected HT1080 cells (Vector) or the *TFPI-2*-expressing cells (TFPI-2). After incubation for 7 days, the ratio of flat colonies and total colonies was determined. The data represent the summary of two independent experiments.

initially observed in DT cells, we prepared both CM and ECM either from control HT1080 cells, vector-transfected cells or *TFPI-2*-transfected cells and observed the morphology of DT cells incubated in the presence of these materials (Fig. 4). First, the CM and ECM prepared from *TFPI-2*-expressing cells support the attachment of DT cells more efficiently than the CM and ECM from vector-transfected cells (Fig. 4A). Second, after incubation for several days, DT cells form flat colonies (Fig. 4B) at higher frequencies in the presence of CM and ECM prepared from *TFPI-2*-expressing cells than in the presence of CM and ECM from control cells (Fig. 4C). Experiments with CM alone or ECM alone gave less clear results (data not shown), suggesting cooperative effects between these components.

4. Discussion

In this study, we isolated the *TFPI-2* cDNA as a transformation suppressor gene against a *v-K-ras*-transformed NIH3T3 cell line. We also found that *TFPI-2* gene is low in the cells harboring activated *ras* oncogenes and the restored expression of *TFPI-2* gene in such tumor cells resulted in suppression of their invasive behavior. These findings are consistent with the model that TFPI-2 may represent the proteins whose expression in normal cells is important for suppression of malignant behavior and whose down-regulation due to oncogenic mutations would contribute to the expression of malignant phenotypes. Another example of such protein is the large membrane glycoprotein RECK which we recently identified through similar expression cloning [10,11].

TFPI-2 contains serine protease inhibitor domains and secreted into CM and ECM. TFPI-2 protein may therefore play a role in maintenance of ECM through direct inhibition of ECM-degrading serine proteinases such as plasmin. Recently, Rao et al. reported that ECM degradation could be inhibited by recombinant TFPI-2 protein [31]. Iino et al. also reported that anti-TFPI-2 antibody caused detachment of *TFPI-2*-expressing endothelial cells from culture flask [32]. These results are consistent with our finding that *TFPI-2* expression enhanced cell adhesion. We also found that the CM and ECM derived from the cells expressing TFPI-2 protein enhanced cell adhesion and induced morphological reversion of DT cells. Likewise, Higgins et al. reported that another serine proteinase inhibitor, plasminogen activator inhibitor-1, induced flat reversion of *v-ras*-transformed rat kidney cells [33]. All these findings point to the roles of TFPI-2 in cell adhesion and morphological changes associated with cell transformation.

It is unclear at the moment how we could reconcile our findings and the finding by Shinoda et al. that TFPI-2 stimulates the proliferation of bovine aortic smooth muscle cells in culture [18]. The question of whether these apparently opposite results reflect cell type-specific action of TFPI-2 or the manifestations of a common molecular mechanism should be explored in future studies.

Several proteinase inhibitors have been implicated as metastasis suppressors [34]. Our data suggest that TFPI-2 suppresses Matrigel invasion by down-regulating the levels of secreted, active MMP-2. Baramova et al. reported that, besides MT1-MMP, plasmin can be involved in the activation of pro-MMP-2 [35]. Therefore, TFPI-2 may inhibit tumor invasion by interfering this process. During the course of this study, Rao et al. reported that the exogenous addition of recombinant TFPI-2 protein inhibited cell invasion in vitro [31], further supporting our interpretation that the secreted product of the transfected 20d-3 cDNA itself was responsible for the observed inhibition of invasion.

TFPI-2 gene has been mapped on human chromosome 7q22 [36]. Loss of this region is observed in many types of

human tumors [36]. Miyagi et al. reported that the *TFPI-2* gene expression was detectable in differentiated ovarian carcinoma cell lines, but undetectable in undifferentiated lines [26]. Also, Udagawa et al. reported that the *TFPI-2* gene expression was undetectable in several choriocarcinoma cell lines [37]. These findings suggest possible involvement of *TFPI-2* gene mutations and/or down-regulation in human carcinogenesis. Thus, it would be of interest to study possible correlation between the levels of *TFPI-2* gene expression and the prognosis of the patients in various types of tumors.

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