[CANCER RESEARCH 64, 5097–5105, August 1, 2004]

Expression of Chicken Ovalbumin Upstream Promoter-Transcription Factor II Enhances Invasiveness of Human Lung Carcinoma Cells

Roya Navab,[1,2] Juana Maria Gonzalez-Santos,[1,2] Michael R. Johnston,[3] Jiang Liu,[3] Pnina Brodt,[6] Ming-Sound Tsao,[4] and Jim Hu[1,2,5]

[1]Lung Biology Research Programme and Canadian Institutes of Health Research Group in Lung Development, The Hospital for Sick Children, [2]Departments of Laboratory Medicine and Pathobiology, [3]Division of Surgical Oncology at Princess Margaret Hospital, [4]Ontario Cancer Institute/Princess Margaret Hospital, and [5]Department of Paediatrics, The University of Toronto, Toronto, Ontario, and [6]Department of Surgery and Department of Oncology, McGill University and The Royal Victoria Hospital, Montreal, Quebec, Canada

ABSTRACT

Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) plays an essential role in angiogenesis and development. It is differentially expressed in tumor cell lines, but its role in carcinogenesis is largely unknown. We demonstrate here that noninvasive human lung cancer cells become invasive when *COUP-TFII* was expressed. The expression of extracellular matrix degrading proteinases, such as matrix metalloproteinase 2 and urokinase-type plasminogen activator, was upregulated in these cells. This finding was confirmed by transduction of different human lung cancer cell lines with COUP-TFII protein and also by using antisense expression. We observed disorganization of actin filaments and focal adhesion kinase phosphorylation in *COUP-TFII*-transfected human lung cancer cells in addition to the increase in extracellular metalloproteinase activity. These results suggest that *COUP-TFII* may be considered as a new target for anticancer therapies.

INTRODUCTION

Degradation of the extracellular matrix (ECM) and components of the basement membrane by proteases facilitates the detachment of tumor cells, their crossing of tissue boundaries, and invasion into adjacent tissue compartments. Several of these phenotypic alterations result from the dysregulation of gene expression, particularly at the level of transcription (1). Analysis of mRNA expression *in vitro* suggested that alterations in expression of nuclear transcription factors such as COUP-TFs might be involved in this dysregulation (2).

COUP-TFs are the best characterized "orphan" (ligands not yet identified) nuclear receptors (2). They belong to the steroid/thyroid hormone receptor superfamily of nuclear receptor proteins and are required for regulation of gene expression (3), development, differentiation, and homeostasis (4). Angiogenesis in *COUP-TFII* mutant mice is largely impaired (5), and defects mimic the phenotypes exhibited by mice lacking angiopoietin-1 or its receptor, TIE2. It has also been suggested that COUP-TFII plays an important role in mesenchymal-endothelial interactions (5). COUP-TFs are expressed in some tumor cell lines (6), including human endometrial cancer cells (7), lung cancer cells (8), and in adrenal tumors (9), but are not expressed in terminally differentiated epithelial cells. The relationship between *COUP-TFII* expression and cancer development is not known.

Several types of proteases contribute to the degradation of the extracellular matrix: serine proteases (*e.g.*, plasmin and urokinase-type plasminogen activator; uPA; Ref. 10), cysteine proteases (*e.g.*, cathepsins B and L; Ref. 11), and matrix metalloproteinases (MMP; Ref. 12). MMPs are implicated in tumor invasion and metastasis (13). Other features of tumor evolution, including survival, growth, and angiogenesis, may also be dependent on MMPs (14). MMP members are classified into subgroups on the basis of their structure and substrate preference (15). Among these, the gelatinases (MMP-2 and MMP-9) are closely associated with tumor invasiveness and metastasis because of their potent ability to degrade type IV collagen present in the basement membrane that surrounds blood vessels. Elevated levels of gelatinases are found in many types of human cancers (16).

In the extracellular milieu, the activity of MMPs is controlled by tissue inhibitors of MMPs (TIMP; Ref. 17). Although these inhibitors have similar inhibitory activities against most MMPs, they differ in many aspects including their interactions with pro-MMPs, solubility, transcriptional regulation, and tissue specificity (17). TIMP-1 forms complexes with pro-MMP-9, and TIMP-2 and TIMP-4 with pro-MMP-2 (18).

MMPs are produced by cells as proenzymes, and they require additional processing to generate the active enzyme. The activation of pro-MMPs is facilitated by active forms of other MMPs. For example, the activation of pro-MMP-2 requires a membrane-type MMP at the cell surface (16). The activation of pro-MMPs can also be mediated by other groups of proteinases (19). For example, a serine proteinase, plasmin, can activate gelatinases without the action of other metallo- or acidic proteinases (20). It has been shown that increased expression of uPA and its membrane-bound receptor (uPAR; CD87) is closely correlated with an increase in disease recurrence and with early death of lung and other cancer patients (21). Cell membrane-associated uPAR is a key molecule for the induction of pericellular proteolysis, because plasminogen is efficiently activated to plasmin by cell surface-associated interactions with uPAR-bound uPA (22).

Interference with the membrane-associated function of uPAR should result in a reduction in plasminogen activation, which would decrease tumor cell proliferation, invasion, and metastasis (23). In this context, it is important to note that the membrane anchoring of uPAR is not a prerequisite for uPA activation but is necessary for plasminogen activation (22). In addition to the proteolytic function of uPA, the uPA/uPAR interaction induces downstream signaling, resulting in the induction of cell proliferation, adherence, migration, and chemotaxis (24).

We examined the relationship between *COUP-TFII* and ECM proteinases. We demonstrate that *COUP-TFII*-transfected A549 cells (a human lung cancer cell line in which *COUP-TFII* is not normally expressed) acquired invasive ability, had increased *in vitro* tumorigenicity and migratory ability, and displayed enhanced expression of collagenase type IV (MMP-2). These results were confirmed by transduction of different human lung cancer cell lines and a human breast carcinoma cell line (MDA-MB231) with purified COUP-TFII protein.

Received 4/29/03; revised 5/4/04; accepted 6/2/04.

Grant support: Operating Grants from the Canadian Institutes of Health Research, from the Canadian Cystic Fibrosis Foundation, and from Grant Miller Cancer Research (J. Hu). R. Navab held a fellowship from the Canadian Lung Association/Canadian Institutes of Health Research and J. Hu is a Canadian Cystic Fibrosis Foundation Scholar and holds a Premier's Research Excellence Award of Ontario Canada.

Note: Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Jim Hu, Lung Biology Research Program, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. Phone: (416) 813-6412; Fax: (416) 813-5771; E-mail: jhu@sickkids.on.ca.

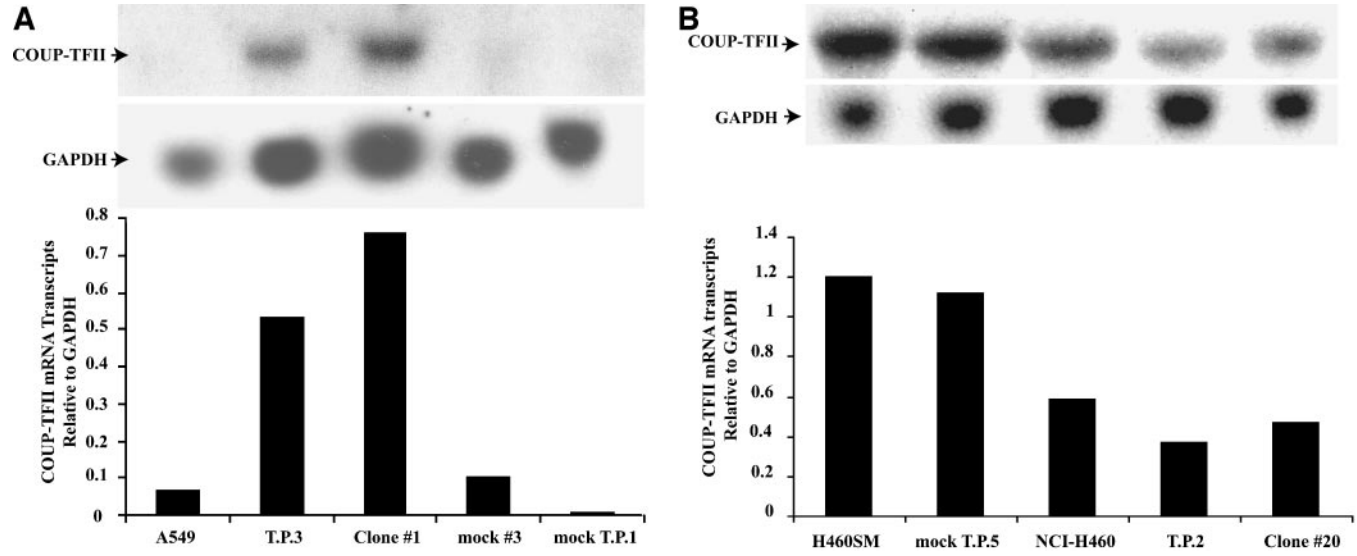


Fig. 1. Northern blot analysis of *COUP-TFII* mRNA in *COUP-TFII*-transfected or *COUPTFII* antisense-transfected cells. *A*, *COUP-TFII* mRNA expression in *COUP-TFII*-transfected (*T.P.3* and *Clone #1*) and mock-transfected (*mock T.P.1* and *mock #3*) or control A549 cells. *B*, expression of *COUP-TFII* mRNA in *COUP-TFII* antisense-transfected (*T.P.2* and *Clone #20*), mock-transfected (*mock T.P.5*) or control H460SM cells as well as in NCI-H460. The intensity of the bands was measured with NIH Image and is expressed as a ratio relative to the intensity of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) bands.

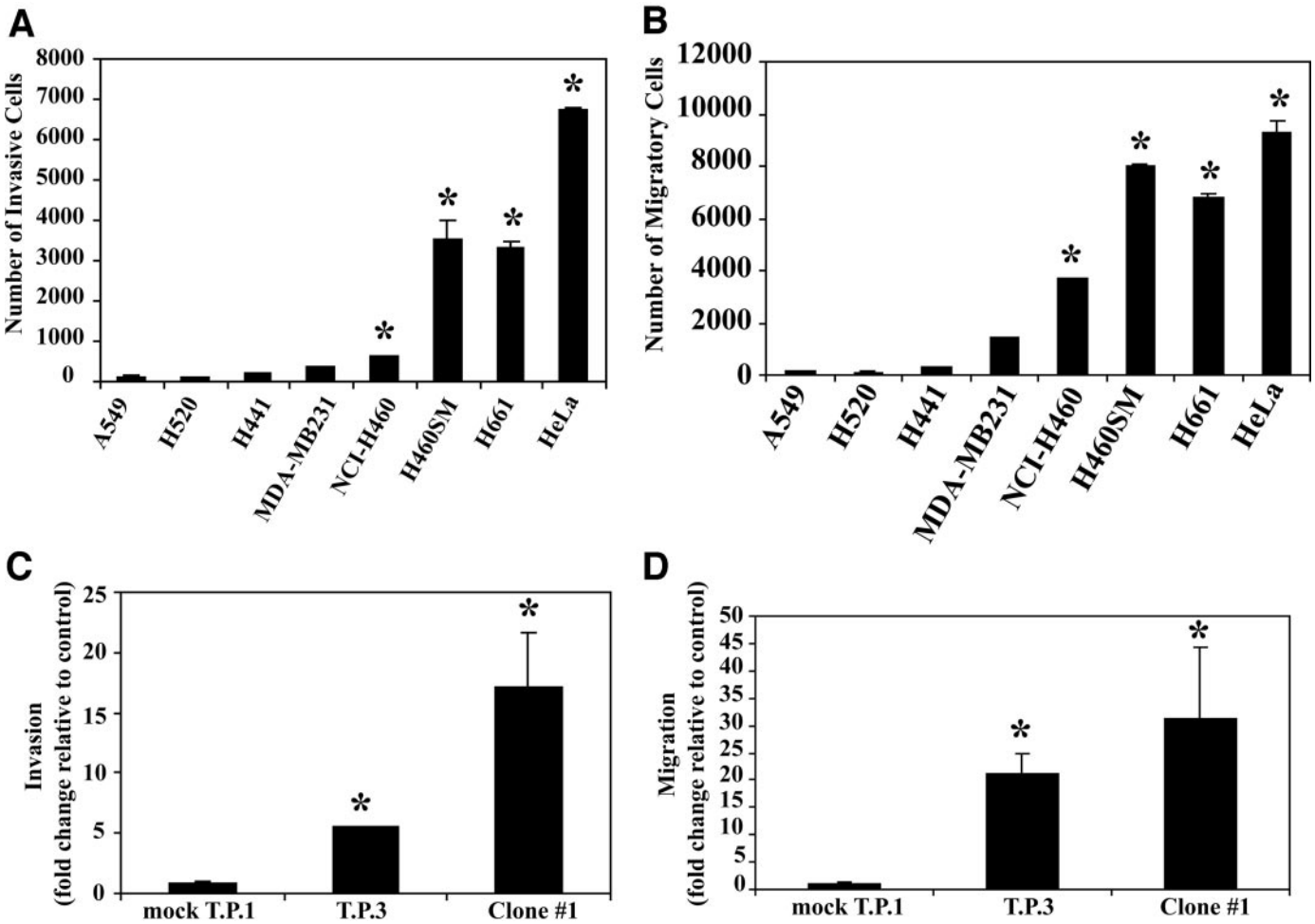


Fig. 2. Correlation of *COUP-TFII* expression with cancer cell invasion and migration. Invasiveness (*A*, *C*, *E*, and *G*) and migratory ability (*B*, *D*, *F*, and *H*) of different tumor cells was assayed on Matrigel- or collagen type IV-coated filters. *Bars*, ±SD from three experiments, each done in duplicate ($n = 6$). Cells analyzed are indicated in each panel. A549 and H460SM cells transfected with a *COUP-TFII* or *COUP-TFII*-antisense plasmid were described in "Results." *, $P < 0.005$ A549, H520, H441, or MDA-MB231 *versus* NCI-H460, H460SM, H661, or HeLa (*A* and *B*). *, $P < 0.005$ controls A549 and mock T.P.1 *versus* T.P.3 and Clone #1 (*C* and *D*). *, $P < 0.005$ controls H460SM, mock #1, and mock T.P.5 *versus* T.P.2, Clone #20, and NCI-H460 (*versus* H460SM; *E* and *F*). *, $P < 0.005$ tumor cells nontransduced or transduced with TAT-HA-Cre *versus* tumor cells transduced with TAT-HA-COUP-TFII (*G* and *H*).

Interestingly, transfection of a very invasive human lung cancer cell line (H460SM) with *COUP-TFII* in the antisense orientation decreased *COUP-TFII* expression level, suppressed their invasive and migratory ability, and profoundly reduced the synthesis of both pro- and active forms of MMP-2. To analyze more specifically the contribution of *COUP-TFII* to the process of invasion, the regulations of human (*h*)*uPA* and/or *huPAR* were studied. Our study suggests a critical role for *COUP-TFII* in human lung cancer invasion.

MATERIALS AND METHODS

Cell Lines. A549, HeLa, and NCI-H460 (American Type Culture Collection, Rockville, MD) were maintained as monolayer culture in DMEM (A549 and NCI-H460) and Eagle's MEM (HeLa) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Burlington, Ontario, Canada). H661, H520, H441, (American Type Culture Collection), and MDA-MB231 (kindly provided by Dr. Wolfgang Vogel from University of Toronto) were maintained as a monolayer culture in RPMI 1640 supplemented with 10% fetal bovine serum. H460SM (25) was grown in RPMI 1640 containing 10% fetal bovine serum.

Plasmid Preparation and Transfection. A549 cells were transfected with a pCR 3.1 expression cassette containing a 1.5-Kb mouse *COUP-TFII* cDNA fragment (kindly provided by Dr. Ming-Jer Tsai, Baylor College of Medicine, Houston, TX) or with the empty vector (mock). To suppress the expression of *COUP-TFII*, H460SM cells were transfected with a plasmid containing the *COUP-TFII* coding region in the antisense orientation. This plasmid was made by ligating an *Eco*RI-*Xho*I fragment corresponding to the full-length *COUP-TFII* cDNA into the pEGFP.C1 expression vector (Clontech, Palo Alto, CA), in the antisense orientation.

Transfections were carried out using coprecipitation with calcium phosphate (26), and stable G418-resistant clones were isolated.

COUP-TFII Expression and Purification. To generate a transducible TAT fusion COUP-TFII protein, we used a bacterial expression vector, pTAT-HA (27), which contains an NH_2-terminal 6-histidine leader followed by the 11-amino acid TAT protein transduction domain and a hemagglutinin (HA) tag. The murine *COUP-TFII* coding region, which has >90% homology to its human analog, was inserted into the polylinker region of pTAT-HA. To facilitate the cloning, restriction sites *Nco*I and *Eco*RI were included in the 5′(GCACCATGGCAATGGTAGTCAGCACGTGGC) and 3′(GGAATTCTTATTGAATTGCCATATATGGCCAG) primers, respectively. To express a control protein, we cloned a 1.1-kb *Cre* cDNA sequence into *Xho*I-*Eco*R1 sites of pTAT-HA.

The pTAT-HA-COUP-TFII and pTAT-HA-Cre plasmids were used to transform *Escheria coli* DH5α and, after sequencing confirmation, put into *E. coli* BL21(DE3). The transformed *E. coli* BL21(DE3) were cultured as described (28) and the fusion proteins purified under denaturing conditions (27). The proteins were refolded by gradually decreasing the concentration of guanidine hydrochloride (Gu-HCl) from 6 M to 0 M, by dialysis against a buffer containing 100 mM potassium phosphate (pH 6.0), 500 mM KCl, 10% glycerol, EDTA-free protease inhibitor mixture tablets (Roche), 8 mM β-mercaptoethanol, and 0.1% Tween 20, and then dialysis against PBS containing 10% glycerol, 2 mM DTT, and 50 μM 4-(2-aminoethyl) benzenesulfonylfluoride.

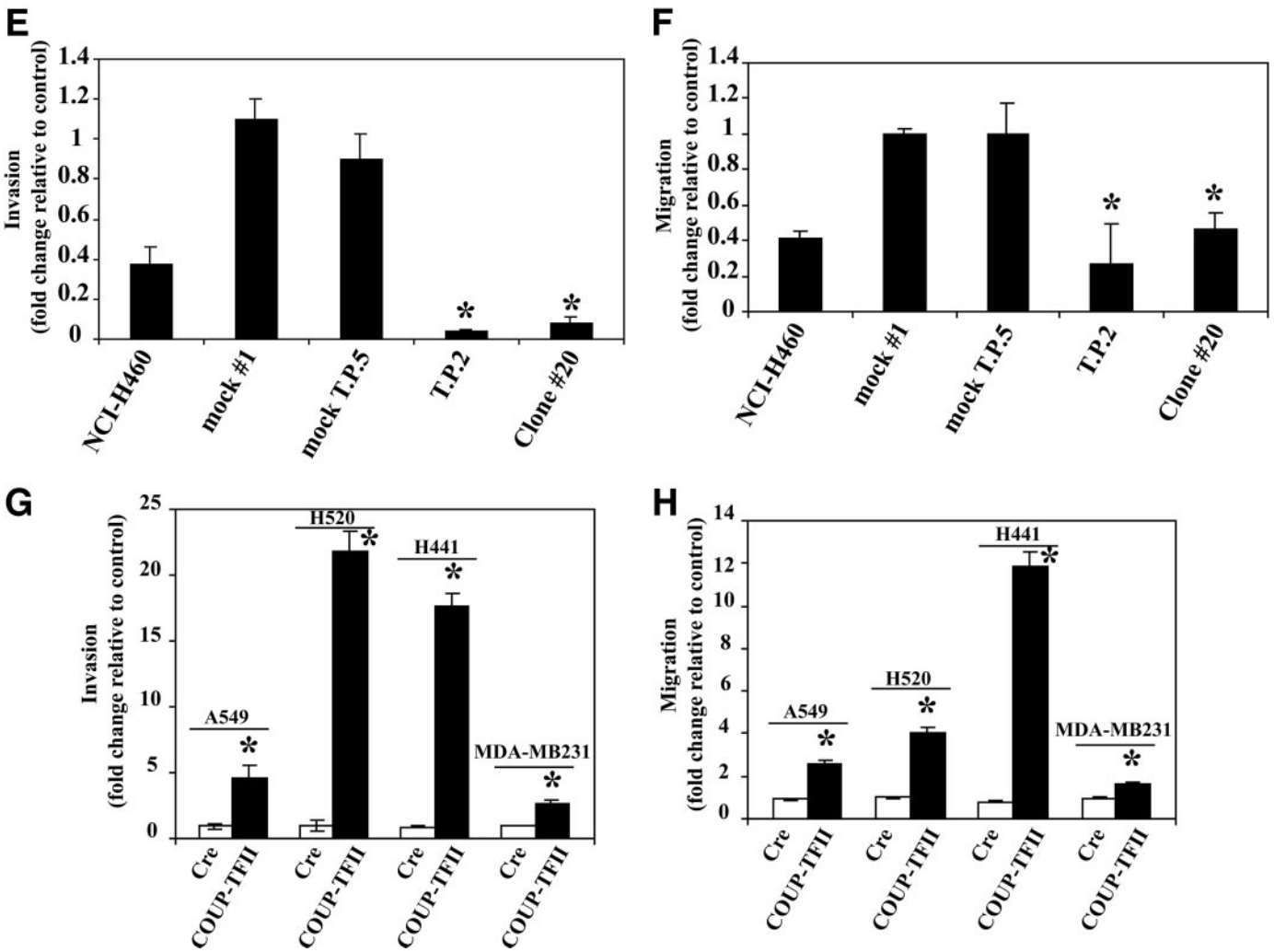


Fig. 2. *Continued.*

Transduction of Tumor Cells with Purified Proteins and Immunostaining. To transduce the tumor cells with TAT-HA-COUP-TFII and TAT-HA-Cre, we followed the method described by Vives *et al.* (29). Cells were seeded at a concentration of 2×10^5 per 10-cm dish (for Matrigel invasion and migration assays) or 3×10^4 cells/well on coverslips (for immunostaining).

The purified proteins were dissolved in serum-free medium at a concentration of 1 nM, incubated at 37°C for 15 min, then incubated with the cells at 37°C for 2–4 h. The cells were then rinsed three times with PBS (pH 7.3) at room temperature and prepared for Matrigel invasion and migration assays or immunostaining. Immunostaining was performed using monoclonal anti-HA antibody (BAbCO, Richmond CA) as described (28).

Tumor Cell Invasion, Migration, and Clonogenic Growth Assays. Invasive and migratory ability of tumor cells was measured using the Matrigel invasion assay (30). The soft agar cloning assay was performed as described (31). Briefly, tumor cells (10^4) were suspended in 0.8% low melting point agarose (Difco Laboratories Inc., Detroit, MI) at room temperature, mixed with an equal volume of 2× concentrated culture medium, and plated onto an agarose bed consisting of 2% low melting point agarose and the same medium. After 12 days, colonies were stained with neutral red and those exceeding 250 μm in diameter were enumerated using an inverted microscope (Leica; DMIRB).

RNA Isolation and Northern Blot Analysis. Total RNA was prepared using Trizol reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. RNA blots on Hybond nylon (Amersham, Oakville, Ontario, Canada) were probed with ^{32}P-labeled 1.5 kb mouse *COUP-TFII* cDNA, 905-bp human *GAPDH* cDNA (Ambion Inc., Austin, TX), or full-length cDNA of human *uPA* or *uPAR* (provided by Dr. Shafaat A. Rabbani, The Royal Victoria Hospital, Montreal, Quebec, Canada). DNA probes were prepared by random primer extension (30). Relative amounts of the mRNA transcripts visualized by autoradiography were analyzed using NIH Image software and normalized to the internal *GAPDH* control.

Actin Filament Staining. *COUP-TFII*-transfected and mock-transfected or control A549 cells were seeded on coverslips, washed twice with PBS, and fixed with 3% paraformaldehyde.

Cells were permeabilized with 0.25% Triton X-100. Actin was stained with rhodaminephalloidin for 30 min.

Focal Adhesion Kinase (FAK) Phosphorylation Analysis. *COUP-TFII*-transfected and mock (vector) -transfected or control A549 cells were serum-starved for 24 h, washed twice with PBS, and collected as a single cell suspension in DMEM with or without 400 μg/ml G418 plus 0.25 mg/ml BSA. Cells were kept in suspension for 60 min at 37°C and then seeded on fibronectin-coated or noncoated culture plates. Cells were harvested as described (32), and the lysate was then centrifuged at 100,000 × *g* for 30 min at 4°C. The resulting supernatant represented the cytoplasmic fraction and was subjected to immunoprecipitation.

Immunoprecipitations. Immunoprecipitation was performed as described (31). Ten μg of polyclonal rabbit anti-FAK antibodies (kindly provided by Dr. Jun-Lin Guan, Cornell University, Ithaca, NY) was added to the cytoplasmic fraction for 2 h at 4°C. Immune complexes were precipitated by incubation with protein A Sepharose beads for 60 min at 4°C. The beads were washed four times with the hypotonic buffer (31), resuspended in Laemmli SDS sample buffer (33), and boiled for 10 min. The eluted proteins were separated by electrophoresis on 8% SDS- polyacrylamide gels under reducing conditions.

Western Blot Analysis. Proteins (30 μg) were separated by electrophoresis on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose filters (Bio-Rad, Hercules, CA). The blots were incubated in TNT buffer [0.15 M NaCl (pH 7.5) containing 0.05% Tween 20 and 10 mM Tris] containing 5% skimmed milk and 2% BSA, or 3% skimmed milk (for FAK phosphorylation detection), and probed with MMP-2 antiserum or an anti-TIMP-2 antibody (Oncogene Research Products, Boston, MA). The blots were also probed with anti-FAK or anti-FAK phosphotyrosine (Tyr 397; Upstate, Lake Placid, NY) antibodies at a 1:1000 dilution. To visualize the bands, blots were incubated with horseradish peroxidase-conjugated antimouse or rabbit IgG antibodies, and detected by enhanced chemiluminescence (Amersham, Baie d'Urfé, Quebec, Canada).

Gelatin and Casein Zymography. *COUP-TFII*-transfected A549 cells and *COUP-TFII*-antisense-transfected H460SM cells, together with the control and mock-transfected cells, were incubated in serum-free medium at 37°C for 48 h. The conditioned medium was then collected, dialyzed, and concentrated by freeze-drying at −50°C. Proteins in concentrated conditioned medium (30 μg/sample) were diluted in nonreduced SDS sample buffer and separated by electrophoresis in 10% SDS-polyacrylamide gels copolymerized with 1 mg/ml of gelatin or casein (for MMP-2 or plasmin activity detection, respectively). Gels were washed with 2.5% Triton X-100 for 1 h and then twice in Tris-HCl (pH 8.0) for 15 min at room temperature. The gels were incubated with substrate buffer [50 mM Tris-HCl (pH 8.0) and 10 mM $CaCl_2$] for 18 h at 37°C. The gels were then stained with Coomassie brilliant blue and destained until the clear bands of lysis appeared. To confirm the lytic bands, gels were treated with 20 mM EDTA (a metalloproteinase inhibitor) or 500 μg/ml amino-n-caproic acid (a plasmin inhibitor) in the substrate buffer for 18 h at 37°C.

Immunocytofluorometry. A549 and H460SM cells were cultured in DMEM and RPMI 1640, respectively, without serum. The *COUP-TFII*-transfected A549 and *COUP-TFII* antisense-transfected H460SM cells were cultured in serum-free medium containing 400 μg/ml G418 for 24 h, dispersed, and seeded in 96-well plates (Falcon, Lincoln Park, NJ) at a density of 10^5 cells/well. The cells were washed three times with serum-free medium, incubated at 37°C for 30 min, and then incubated for 1 h on ice with 5 μg/ml of monoclonal antibody against huPAR (CD87; American Diagnostica Inc., Greenwich, CT). After extensive washing with cold medium, the cells were incubated with FITC-labeled goat antimouse IgG antibody (1:200) for 1 h on ice, washed, then fixed in PBS containing 1% paraformaldehyde. The labeled cells were analyzed by flow cytofluorometry using a FACScan System (BD Bioscience).

Statistics. The two-tailed *t* test was used to analyze differences in the invasiveness and migration of tumor cells.

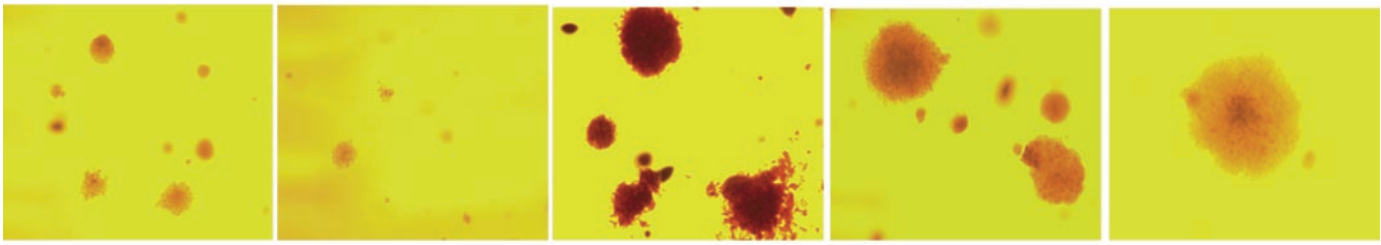
RESULTS

COUP-TFII Expression Is Correlated with Cell Invasion. It is reported that *COUP-TFs* are expressed in some human lung cancer cell lines, such as H460 and H661, but not expressed in others, such as A549, H520, and H441(34). To test the role of *COUP-TFII* in tumor progression, we stably transfected A549 cells with a plasmid vector (pCR3.1) expressing *COUPTFII* cDNA or with pCR3.1 as a mock control. As shown by Northern blot analysis (Fig. 1*A*), an isolated stable clone (clone 1) and a total population of 20–30 clones (T.P.3) expressed higher levels of *COUP-TFII* mRNA than their parental cell line A549 or the mock controls (mock 3 from a single clone and mock T.P.1 from a mixture of ~20 clones). *COUP-TFII* expression was higher in clone 1 than in T.P.3. (Fig. 1*A*).

As a complementary approach, we generated stable cell lines of H460SM cells expressing *COUP-TFII* antisense RNA. According to Northern blot analysis, cells stably transfected with antisense *COUP-TFII* (T.P.2 from total population of ~20 colonies and clone 20 from a single clone) showed 59–68% reduction in *COUP-TFII* mRNA expression compared with H460SM or mock T.P.5 (a mixture of ~20 clones; Fig. 1*B*). NCI-H460, the parental cell line of H460SM, also exhibited a lower level (48%) of *COUP-TFII* expression compared with H460SM.

To examine whether *COUP-TFII* expression correlates with the invasiveness of the cancer cell lines, we determined *in vitro* the cell invasiveness using reconstituted basement membrane (Matrigel) invasion assays. As shown in Fig. 2, *A* and *B*, human lung cancer cell lines A549, H520, and H441 and a human breast carcinoma cell line (MDA-MB231), which do not express *COUP-TFII* (34), had poor invasive and migratory abilities. In contrast, human lung cancer cell lines NCI-H460, its highly invasive variant H460SM, H661, and HeLa cells exhibited higher levels of invasiveness (~2-, 14-, 13-, and 27-fold increases in invasiveness compared with A549, H520, H441, and MDA-MB231, respectively) and migratory ability (~7-, 16-, 14-, and 19-fold increases in migration compared with A549, H520, H441, and MDA-MB231, respectively; Fig. 2, *A* and *B*) in agreement with their levels of *COUP-TFII* expression (34).

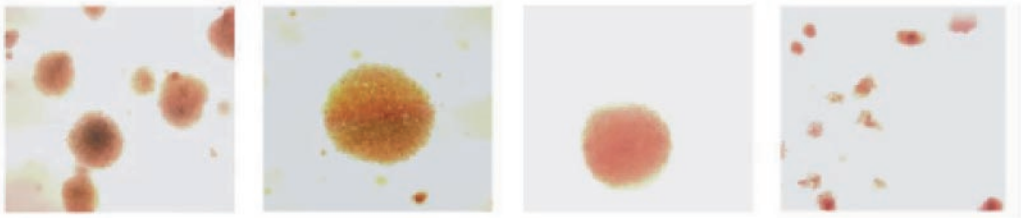
A



B

Cell Lines	No. of Colonies	Size of Colonies (μm)
A549	41± 3	18±0
mock T.P.1	39± 8	17±3
Clone #1	82± 10	37± 8
T.P.3	64± 4	50± 7
HeLa	57± 5	160± 29

C



D

Cell Lines	No. of Colonies	Size of Colonies (μm)
NCI-H460	1188±18	129±40
H460SM	1755±31	329±30
mock T.P.5	1618±122	583±221
T.P.2	1210±127	29±9

Fig. 3. Anchorage-independent growth (or tumorigenicity) of *COUP-TFII*- and antisense *COUP-TFII*-transfected tumor cells. *A* and *C*, light microscope view of the colonies in agar. *B* and *D*, summary of the size and number of colonies. Results represent total number of colonies/plate and are expressed as means of three experiments, ±SD. The definition of the cells are described in "Results."

Using *COUP-TFII*-transfected cells, we observed that in both T.P.3 and clone 1, invasion was significantly ($P < 0.05$) increased (~5- and 17-fold) relative to nontransfected and mock-transfected A549 cells (Fig. 2*C*). In addition, we discovered a significant increase ($P < 0.05$; approximately 20–30-fold) in the migratory ability of the *COUP-TFII*-transfected A549 cells compared with controls (Fig. 2*D*), measured on uncoated or type IV collagen-coated filters. Accordingly, we found that the level of invasion and migration were significantly ($P < 0.05$) decreased (approximately 14–27-fold in invasion and approximately 2–4-fold in migration) in H460SM cells expressing *COUP-TFII*-antisense mRNA (Fig. 2, *E* and *F*).

To additionally confirm the role of *COUP-TFII* in lung cancer invasiveness, we transduced A549, H520, and H441 with purified TAT-HA-COUP-TFII and TAT-HA-Cre (as a control) fusion proteins. We also used a human breast carcinoma cell line (MDA-MB231) to represent another type of tumor cell to determine whether the effect of COUP-TFII was restricted to lung cancer. The HIV-1-derived TAT protein is used in this system to mediate cell entry of the fusion protein (35). Immunocytochemistry analysis was performed with an anti-HA antibody, and both fusion proteins were found in the nucleus (data not shown; Supplementary Fig. 1). We found a significant ($P < 0.05$) increase in the invasive and migratory ability of all of the tumor cells, A549, H520, H441, and MDA-MB231 (~5-, 22-, 18-, and 3-fold in invasion; ~3-, 4-, 12-, and 2-fold in migration; Fig. 2, *G* and *H*) transduced with the purified COUP-TFII protein, compared with controls (nontransduced or transduced with TAT-HA-Cre).

Anchorage-Independent Growth of Tumor Cells Correlates with COUP-TFII Expression. To investigate the role of *COUP-TFII* in regulation of human lung cancer tumorigenicity, the clonogenicity of the cells was measured in semi-solid agarose plates. We found an increase of >100% in the size of the colonies formed by *COUP-TFII*-transfected A549 cells (Fig. 3, *A* and *B*). In agreement with this finding, we also detected an 89% reduction in the size of the colonies

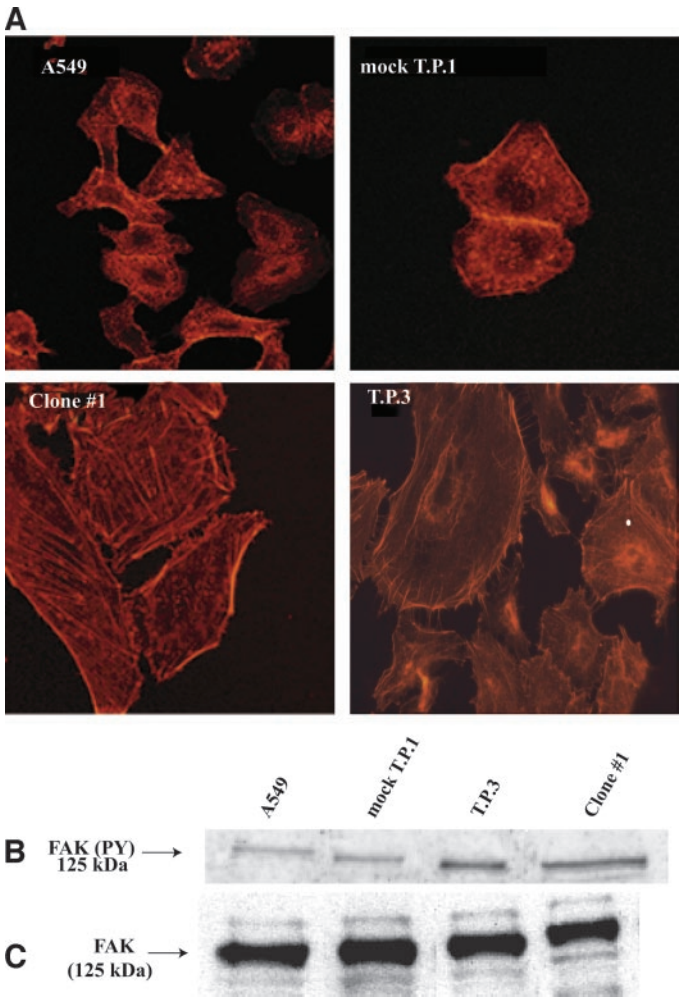


Fig. 4. *COUP-TFII* involvement in actin filament disorganization and focal adhesion kinase (*FAK*) activation. *A,* involvement of *COUP-TFII* in actin filament distribution. Shown is the confocal laser scanning microscope images of actin filaments in *COUP-TFII*-transfected and control or mock-transfected A549 cells. *B,* FAK phosphorylation. FAK in lysates of *COUP-TFII*-transfected and nontransfected or control A549 cells was immunoprecipitated with anti-FAK antibodies and immunoblotted with anti-FAK-phosphotyrosine antibodies. *C,* total FAK protein levels detected by immunoblotting with anti-FAK antibodies.

formed by *COUP-TFII* antisense-transfected H460SM cells (Fig. 3, *C* and *D*). For both cell types the number of colonies relative to control cells changed slightly (Fig. 3, *B* and *D*).

An increase in anchorage-independent growth and tumorigenicity requires changes in the organization of actin filaments (36). Our analysis of actin filaments showed a change in distribution of actin filaments; we observed more stress fibers in the *COUP-TFII*-transfected A549 cells than controls (Fig. 4*A*). Because phosphorylation of p125-FAK is linked to the regulation of cytoskeletal reorganization, cell spreading, and migration, it was of interest to examine changes in FAK phosphorylation. We found that FAK phosphorylation was increased in *COUP-TFII*-transfected A549 cells (T.P.3 and clone 1) when they were cultured on fibronectin-coated plates (Fig. 4*B*), whereas the total FAK levels were comparable with that in nontransfected and *COUP-TFII*-transfected A549 cells (Fig. 4*C*). Interestingly, in H460SM cells expressing *COUP-TFII* antisense RNA, no changes were observed in the distribution of actin filaments. However, the cells acquired a more rounded morphology, which was particularly apparent when they were seeded on Matrigel-coated culture plates (data not shown; Supplementary Fig. 2).

COUP-TFII Expression Changes the Profile of ECM-Degrading Proteinases. Because *COUP-TFII* is known to play an important role in angiogenesis (37), we investigated whether *COUP-TFII* can regulate the expression and activity of ECM-degrading proteinases involved in angiogenesis, such as MMP-2. We analyzed the activity of MMP-2 in *COUP-TFII*-transfected A549 cells (T.P.3 and clone 1) and found a marked increase in the level of MMP-2-mediated gelatinolytic activity, as assessed by zymography (Fig. 5*A*). The increase in MMP-2 activity could be due to an increase in MMP-2 expression. Therefore, we measured MMP-2 levels in conditioned media of *COUP-TFII*-transfected A549 cells. Using Western blot analysis we found an increase in the production of pro-MMP-2 (72 kDa) in *COUP-TFII*-transfected A549 cells (Fig. 5*B*). This was not due to differences in TIMP-2 or MT1-MMP synthesis in these cells, as revealed by Western blotting using the respective antibodies (data not shown). Moreover, in *COUP-TFII*-antisense expressing cells (T.P.2 and clone 20), a noticeable reduction in MMP-2 activity level relative to controls was observed (Fig. 5*C*). In addition, H520 and H441 cells transduced with the recombinant TAT-HA-COUP-TFII protein showed higher pro-MMP-2 activity levels compared with control or nontransduced cells (data not shown; Supplementary Fig. 3).

To investigate the effect of *COUP-TFII* on plasmin production, casein zymography was performed on conditioned media derived from *COUP-TFII*-transfected A549 and *COUP-TFII*-antisense-expressing H460SM cells. Two caseinolytic bands (81–83 kDa) were observed in *COUP-TFII*-transfected A549 cells (clone 1 and very weak bands observed for T.P.3; data not shown; Supplementary Fig. 4*A*). Conversely, in *COUP-TFII* antisense-transfected

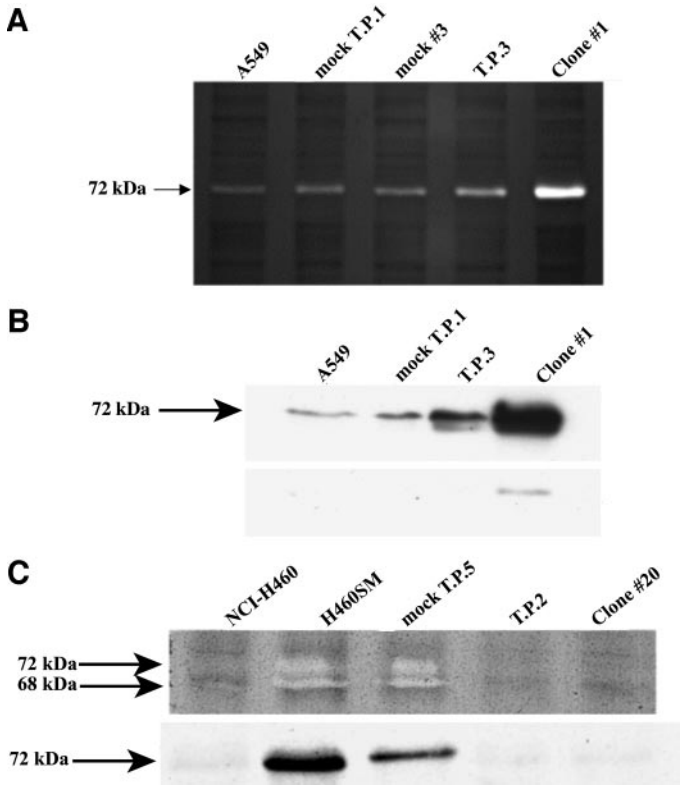


Fig. 5. Correlation of matrix metalloproteinase (MMP)2 expression with *COUP-TFII* expression. *A,* zymographic analysis and *B,* Western blot analysis of MMP-2 activity in conditioned media from *COUPTFII*-transfected, mock-transfected, and control A549 cells. *C,* gelatin zymography of MMP-2 activity (*top panel*) and Western blot analysis of MMP-2 (*bottom panel*) from *COUP-TFII* antisense-transfected, mock-transfected, and control H460SM cells together with NCI-H460 cells.

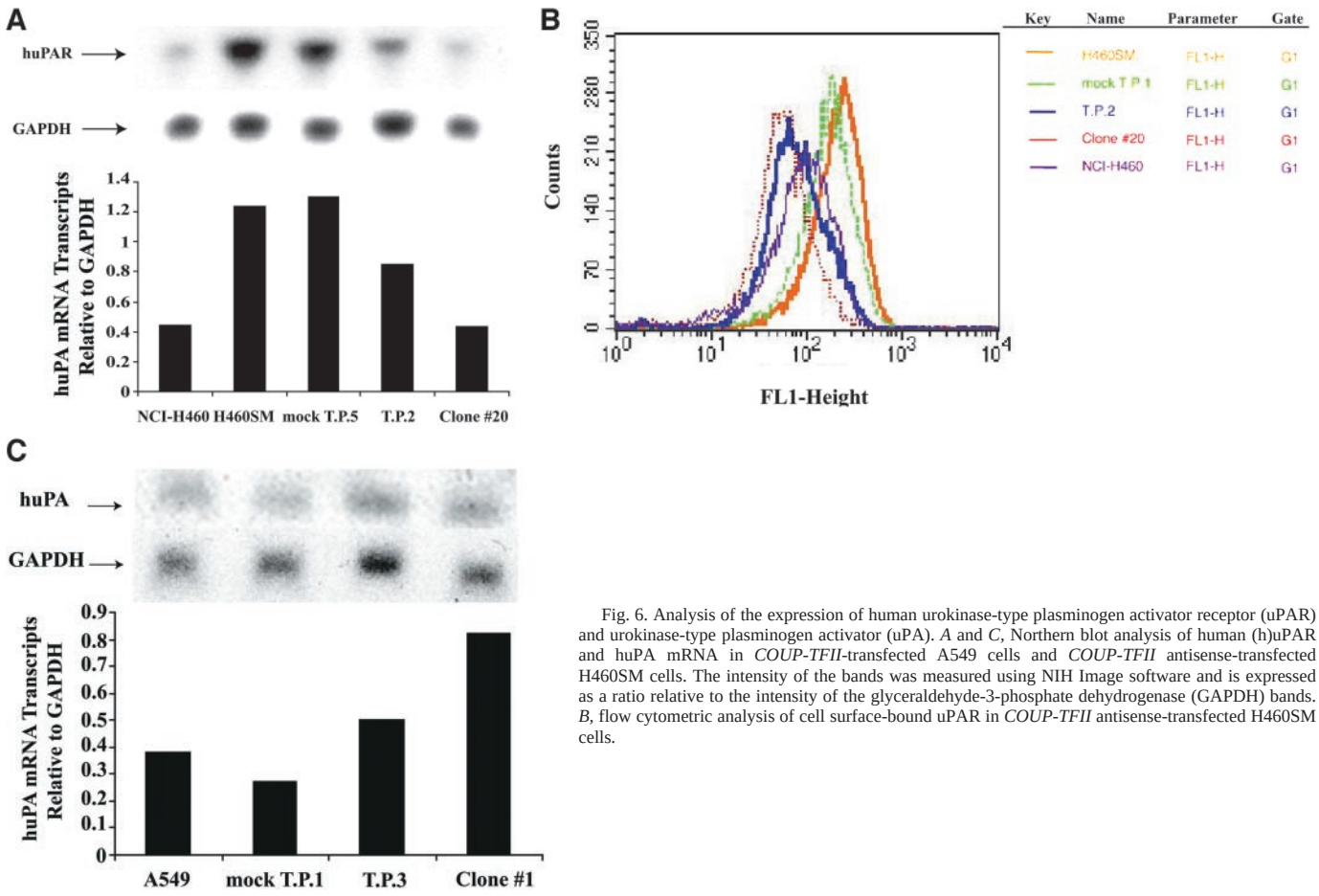


Fig. 6. Analysis of the expression of human urokinase-type plasminogen activator receptor (uPAR) and urokinase-type plasminogen activator (uPA). *A* and *C*, Northern blot analysis of human (h)uPAR and huPA mRNA in *COUP-TFII*-transfected A549 cells and *COUP-TFII* antisense-transfected H460SM cells. The intensity of the bands was measured using NIH Image software and is expressed as a ratio relative to the intensity of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands. *B*, flow cytometric analysis of cell surface-bound uPAR in *COUP-TFII* antisense-transfected H460SM cells.

cells (T.P.2, clone 20), NCI-H460, the two caseinolytic bands could not be observed (data not shown; Supplementary Fig. 4*C*). These caseinolytic activities could be blocked by the plasmin inhibitor amino-n-caproic acid (data not shown; Supplementary Fig. 4, *B* and *D*). The levels of MT1-MMP and TIMP-2 protein were analyzed by Western blotting, and no difference was observed (data not shown).

To determine whether antisense *COUP-TFII* transfection altered uPAR function in H460SM cells, we analyzed cell surface receptor *huPAR* expression and synthesis by Northern blotting and flow cytometry. Northern blot analysis on H460SM cells revealed a 33% or 66% reduction in the steady-state mRNA levels of *huPAR* in T.P.2 and clone 20 cells, respectively (Fig. 6*A*). The reduction in huPAR synthesis was additionally confirmed by immunocytofluorometry using a monoclonal antibody against huPAR (Fig. 6*B*). There was no difference in the number of the human uPAR in the *COUP-TFII*-transfected A549 cells using immunocytofluorometry (data not shown). We found a 1.5-fold increase in the steady-state level of *huPA* mRNA in T.P.3 and a ~2.5-fold increase in clone 1 (Fig. 6*C*).

DISCUSSION

We demonstrated that cancer cell invasiveness correlates with *COUP-TFII* expression in several lung cancer cell lines as well as in a breast carcinoma line. Stable expression of *COUPTFII* in A549 cells increased the motility and invasiveness of the tumor cells. Transfection of a highly invasive human lung cancer cell line (H460SM) with a vector expressing *COUP-TFII* antisense cDNA reduced migration and invasion, providing negative as well as positive evidence for the involvement of *COUP-TFII* in invasion. In accordance with our analyses of stable cell lines, we found noticeable increases in the invasive and migratory ability of A549 cells and other human lung cancer cells transduced with TAT-COUP-TFII fusion protein.

The increase in the anchorage-independent growth of *COUP-TFII*-transfected A549 cells coincided with a change in the distribution of actin filaments (36). Interestingly, no difference was seen in the distribution of actin filaments in *COUP-TFII* antisense-transfected H460SM cells (data not shown). However, their morphology was altered, as they appeared as typical polygonal, rounded epithelial cells and lost the more elongated, mesenchymal appearance of the parental cells. This pattern of morphology has been observed when tumor cells are invasive and they start to invade through junctional margins by extending pseudopodia-like cytoplasmic processes (38).

The changes observed in the actin filaments or the morphology of the cell prompted us to examine the phosphorylation of p125-FAK. Activation of FAK, overexpressed in several human cancers, induces survival, proliferation, and motility of cells in culture (32). In our study, FAK phosphorylation of Tyr^{397} in *COUP-TFII*-transfected A549 cells was increased, mainly in cells grown on fibronectin-coated plates. It is conceivable that, after integrin-mediated cell adhesion (there is similar levels of integrin $\beta 1$ production in both *COUP-TFII*-transfected and nontransfected A549 cells; data not shown), FAK undergoes tyrosine phosphorylation that eventually leads to cytoskeletal disorganization. Whether

distinct effector proteins (such as Rho GTPases) are involved in the reorganization of stress fibers in *COUP-TFII*-transfected cells is our future work.

The functional importance of FAK activation in human tumor growth *in vivo* has been elucidated to be dependent on uPAR-integrin β1 association (39). Also, binding of the uPA to its receptor (uPAR) is involved in the activation of MMP-2 (20). The importance of tumor-associated proteases in invasion and metastasis has been demonstrated for a variety of solid malignant tumors (40). Therefore, we examined changes in tumor-associated proteases in *COUP-TFII*-transfected cells. The increase in activity and production of MMP-2 in *COUP-TFII*-transfected A549 cells encouraged us to investigate whether non-MMP proteinases were involved in the activation of MMP-2. There is compelling evidence to indicate that cell migration and invasion depend on the coordinated enzymatic activities of metallo- and serine proteinases (40). The mechanism of pro-MMP-2 activation has been described in association with the uPA-plasmin cascade, whereby uPA binds uPAR, leading to the conversion of plasminogen to plasmin (41). The appearance of plasmin in the conditioned medium suggests that plasminogen/plasmin conversion can still occur on the surface of the cells, but the enzyme may be rapidly released (42). Alternatively, it is possible that plasminogen activation occurs in the conditioned medium after it is released from the cell surface (43). It should be noted that uPAR and the uPA/uPAR complex have also been identified as receptors for the ECM protein vitronectin (44), whereas plasmin can mediate cell detachment from the ECM (45). Changes in extracellular plasmin levels could be the result of changes in huPAR expression and plasminogen/plasmin conversion. We noticed a decrease in both the expression and synthesis of huPAR after transfection of H460SM cells with *COUP-TFII* antisense. Although the number of huPAR receptors in A549 cells is low, the high level of huPA expression in the *COUP-TFII*-transfected A549 cells may be involved in the regulation of factors downstream of the uPA/uPAR complex, leading to the elevated expression of plasmin (which, in turn, can activate MMP-2).

FAK is associated with integrin β1 regardless of its state of activation, but only in the presence of high levels of uPAR is integrin β1-associated FAK phosphorylated (39). It is conceivable that a high level of uPA/uPAR complex on the cell surface clusters integrins and that ligand-induced conformational change increases the proximity between FAK molecules leading to their *trans*-phosphorylation (46). Other reports also show that uPAR blocking can down-regulate the signaling pathway mediated by integrin and induce cancer dormancy *in vivo* (47). On the basis of the results of our study, we propose that the regulation of *huPA/huPAR* expression by COUP-TFII promotes the association of uPA/uPAR complex with integrin β1, leading to FAK phosphorylation as well as MMP-2 synthesis and activation.

ACKNOWLEDGMENTS

We thank Anan Wang for technical assistance, and Dr. David R. Koehler and Deanna Yaniw for reviewing the manuscript.

REFERENCES

1. Batlle E, Sancho E, Franci C, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells Nat Cell Biol 2000; 2:84–9.
2. Smirnov DA, Hou S, Liu X, Claudio E, Siebenlist UK, Ricciardi RP. COUP-TFII is up-regulated in adenovirus type 12 tumorigenic cells and is a repressor of MHC class I transcription. Virology 2001;284:13–9.
3. Navab R, Wang Y, Chow YH, et al. Regulation of human Clara cell 10 kD protein expression by chicken ovalbumin upstream promoter transcription factors (COUP-TFs). Am J Respir Cell Mol Biol 2002;27:273–85.
4. Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members Annu Rev Biochem 1994;63:451–86.
5. Zhou C, Tsai SY, Tsai M. From apoptosis to angiogenesis: new insights into the roles of nuclear orphan receptors, chicken ovalbumin upstream promoter-transcription factors, during development. Biochim Biophys Acta 2000;1470:M63–8.
6. Kieback DG, Runnebaum IB, Moebus VJ, et al. Chicken ovalbumin upstream promoter transcription factor (COUP-TF): an orphan steroid receptor with a specific pattern of differential expression in human ovarian cancer cell lines Gynecol Oncol 1993;51:167–70.
7. Kieback DG, Levi T, Kohlberger P, et al. Chicken ovalbumin upstream promoter–transcription factor (COUP-TF) expression in human endometrial cancer cell lines. Anticancer Res 1996;16:3371–6.
8. Wu Q, Li Y, Liu R, et al. Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization. EMBO J 1997;16:1656–69.
9. Shibata H, Ando T, Suzuki T, et al. Differential expression of an orphan receptor COUP-TFI and corepressors in adrenal tumors. Endocr Res 1998;24:881–5.
10. Kjoller L, Petersen HH, Andreasen PA. The urokinase-type plasminogen activator system in cancer metastasis: a review. Biochem J 1998;329:55–63.
11. Chapman HA, Riese RJ, Shi GP. Emerging roles for cysteine proteases in human biology Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. Annu Rev Physiol 1997;59:63–88.
12. Yu AE, Hewitt RE, Connor EW, Stetler-Stevenson WG. Matrix metalloproteinases. Novel targets for directed cancer therapy. Drugs Aging 1997;11:229–44.
13. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev. Biol 2001;17:463–516.
14. Chambers AF, Matrisian LM. Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 1997;89:1260–70.
15. Yong VW, Power C, Forsyth P, Edwards DR. Metalloproteinases in biology and pathology of the nervous system. Nat Neurosci 2001;2:502–11.
16. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993;9:541–73.
17. Henriet P, Blavier L, Declerck YA. Tissue inhibitors of metalloproteinases (TIMP) in invasion and proliferation. APMIS 1999;107:111–9.
18. Goldberg GI, Marmer BL, Grant GA, Eisen AZ, Wilhelm S, He CS. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. Proc Mine Med Off Assoc SA 1989;86: 8207–11.
19. Nagase H. Activation mechanisms of matrix metalloproteinases. Biol Chem 1997; 378:151–60.
20. Mazzieri R, Masiero L, Zanetta L, et al. Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. EMBO J 1997;16:2319–32.
21. Montuori N, Mattiello A, Mancini A, et al. Urokinase-mediated posttranscriptional regulation of urokinase-receptor expression in non small cell lung carcinoma. Int J Cancer 2003;105:353–60.
22. Li Y, Sarau HM, Nuthulaganti P, et al. Functional analysis of the cellular receptor for urokinase in plasminogen activation. Receptor binding has no influence on the zymogenic nature of pro-urokinase. J Biol Chem 1996;271:20231–4.
23. Bugge TH, Kombrinck KW, Xiao Q, et al. Growth and dissemination of Lewis lung carcinoma in plasminogen- deficient mice. Blood 1997;90:4522–31.
24. Dumler I, Kopmann A, Weis A, et al. Urokinase activates the Jak/Stat signal transduction pathway in human vascular endothelial cells. Arterioscler Thromb Vasc Biol 1999;19:290–7.
25. Liu J, Blackhall F, Long IS, et al. Modeling of lung cancer by an orthotopically growing H460SM variant cell line reveals novel candidate genes for systemic metastasis. Oncogene;2004; in press.
26. Graham FL, Bacchetti S, McKinnon R, Stanners C, Cordell B, Goodman HM. Transformation of mammalian cells with DNA using the calcium technique, In: Baserga R, Croce C, Rovera G, editors. Introduction of macromolecules into viable mammalian cells. New York: Wiley-Liss; 1980. p. 3–25.
27. Nagahara H, Vocero-Akbani AM, Snyder EL, et al. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Genet 1998;4:1449–52.
28. Gonzalez-Santos JM, Wang A, Jones J, Ushida C, Liu J, Hu J. Central region of the human splicing factor Hprp3p interacts with Hprp4p. J Biol Chem 2002;277:23764–72.
29. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem 1997;272:16010–7.
30. Navab R, Mort JS, Brodt P. Inhibition of carcinoma cell invasion and liver metastases formation by the cysteine proteinase inhibitor E-64. Clin Exp Metastasis 1997;15: 121–9.
31. Navab R, Chevet E, Authier F, Di Guglielmo GM, Bergeron JJ, Brodt P. Inhibition of endosomal insulin-like growth factor-I processing by cysteine proteinase inhibitors blocks receptor-mediated functions. J Biol Chem 2001;276:13644–9.
32. Rigacci S, Rovida E, Sbarba PD, Berti A. Low Mr phosphotyrosine protein phosphatase associates and dephosphorylates p125 focal adhesion kinase, interfering with cell motility and spreading. J Biol Chem 2002;277:41631–6.

33. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nat Toxins 1970;227:680–5.
34. Lin B, Chen GQ, Xiao D, et al. Orphan receptor COUP-TF is required for induction of retinoic acid receptor beta, growth inhibition, and apoptosis by retinoic acid in cancer cells. Mol Cell Biol 2000;20:957–70.
35. Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. Cell 1988;55:1189–93.
36. Pawlak G, Helfman DM. Cytoskeletal changes in cell transformation and tumorigenesis. Curr Opin Genet Dev 2001;11:41–7.
37. Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Dev 1999; 13:1037–49.
38. Sawada M, Shii J, Akedo H, Tanizawa O. An experimental model for ovarian tumor invasion of cultured mesothelial cell monolayer. Lab Anim 1994;70:333–8.
39. Aguirre Ghiso JA. Inhibition of FAK signaling activated by urokinase receptor induces dormancy in human carcinoma cells in vivo. Oncogene 2002;21:2513–24.
40. Vassalli JD, Pepper MS. Tumour biology. Membrane proteases in focus. Nat Toxins 1994;370:14–5.
41. Le DM, Besson A, Fogg DK, et al. Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. J Neuroradiol 2003;23:4034–43.
42. Durko M, Brodt P. Suppression of type I collagenase expression by antisense RNA in melanoma cells results in reduced synthesis of the urokinase-type plasminogen activator receptor. Biochem Biophys Res Commun 1998;247:342–8.
43. Mullins DE, Rohrlich ST. The role of proteinases in cellular invasiveness. Biochim Biophys Acta 1983;695:177–214.
44. Wei Y, Waltz DA, Rao N, Drummond RJ, Rosenberg S, Chapman HA. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J Biol Chem 1994;269:32380–8.
45. Mohanam S, Chintala SK, Go Y, et al. In vitro inhibition of human glioblastoma cell line invasiveness by antisense uPA receptor. Oncogene 1997;14:1351–9.
46. Zhao JH, Reiske H, Guan JL. Regulation of the cell cycle by focal adhesion kinase. J Cell Biol 1998;143:1997–2008.
47. Yu W, Kim J, Ossowski L. Reduction in surface urokinase receptor forces malignant cells into a protracted state of dormancy. J Cell Biol 1997;137:767–77.

Expression of Chicken Ovalbumin Upstream Promoter-Transcription Factor II Enhances Invasiveness of Human Lung Carcinoma Cells

Roya Navab, Juana Maria Gonzalez-Santos, Michael R. Johnston, et al.

Cancer Res 2004;64:5097-5105.