

**UNIVERSITAT DE BARCELONA**  
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**Expressió diferencial determinant del fenotip  
metastàtic en un model d'adenocarcinoma de pulmó  
humà**

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## CAPÍTOL II

## **La sobreexpressió de Fucosiltransferasa VII és suficient per a l'adquisició del fenotip colonitzador de pulmó a les cèl·lules d'adenocarcinoma de pulmó humà HAL-24Luc**

Les cèl·lules metastàtiques d'adenocarcinoma de pulmó humà HAL-8Luc presenten una expressió incrementada dels gens de les Fucosiltransferases del tipus  $\alpha(1,3)$  [ $\alpha(1,3)$ -Fuc-Ts] quan es comparen amb les cèl·lules HAL-24Luc, les seves equivalents no metastàtiques. Això es correlaciona amb una expressió incrementada de molècules del tipus Lewis<sup>x</sup> (Le<sup>x</sup>) i Lewis<sup>a</sup> (Le<sup>a</sup>) a la superfície cel·lular i amb una capacitat també major d'adhesió a cèl·lules endotelials que expressen E-selectina (Martin-Satue *et al.*, 1998). En el treball aquí presentat hem transfectat les cèl·lules HAL-24Luc de forma estable amb els cDNAs dels enzims  $\alpha(1,3)$ -Fuc-TIV i VII i hem analitzat mitjançant citometria de flux l'expressió de Le<sup>x</sup>, sialil-Le<sup>x</sup>, sialil-Le<sup>x</sup> dimèric, Le<sup>a</sup> i sialil-Le<sup>a</sup>. Les cèl·lules transfectades amb el cDNA de Fuc-TVII únicament sobreexpressen sialil-Le<sup>x</sup> mentre que les cèl·lules transfectades amb el cDNA de Fuc-TIV només presenten sobreexpressió de l'oligosacàrid Le<sup>x</sup>. Mostrem que només les cèl·lules transfectades amb el cDNA per Fuc-TVII són capaces d'adherir-se a les monocapes de HUVECs estimulades amb interleuquina-1 $\beta$ . Així mateix demostrarem que la sobreexpressió de Fuc-TVII a les cèl·lules HAL-24Luc és suficient per a què aquestes cèl·lules adquireixin el fenotip colonitzador de pulmó. Aquesta és la primera demostració directa de la contribució d'una Fucosiltransferasa del tipus  $\alpha(1,3)$  al fenotip metastàtic de cèl·lules humanes d'adenocarcinoma de pulmó.

## Overexpression of $\alpha(1,3)$ -fucosyltransferase VII is sufficient for the acquisition of lung colonization phenotype in human lung adenocarcinoma HAL-24Luc cells

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### SUMMARY

Metastatic human lung adenocarcinoma HAL-8Luc cells display an enhanced expression of  $\alpha(1,3)$ -fucosyltransferases ( $\alpha(1,3)$ -Fuc-Ts) compared with their nonmetastatic counterpart HAL-24Luc cells. This correlates with an increased surface expression of Lewis<sup>x</sup> (Le<sup>x</sup>)- and Lewis<sup>a</sup> (Le<sup>a</sup>)-related molecules and an *in vitro* enhanced adhesive capacity to E-selectin-expressing endothelial cells (Martin-Satué *et al.*, 1998). In the present work we have stably transfected HAL-24Luc cells with the cDNAs for the  $\alpha(1,3)$ -Fuc-TIV and VII enzymes and analysed by flow cytometry the expression of Le<sup>x</sup>, sialyl-Le<sup>x</sup>, sialyl-Le<sup>x</sup> dimeric, Le<sup>a</sup> and sialyl-Le<sup>a</sup>. Fuc-TVII transfectants exclusively overexpress sialyl-Le<sup>x</sup> while Fuc-TIV transfected cells only overexpress the Le<sup>x</sup> oligosaccharide. We show that solely Fuc-TVII transfectants are able to adhere to interleukin-1 $\beta$ -stimulated HUVEC monolayers. We also demonstrate that Fuc-TVII overexpression in HAL-24Luc cells is sufficient for the acquisition of the lung colonization phenotype. This is the first report directly showing the contribution of an  $\alpha(1,3)$ -Fuc-T to the metastatic behaviour of human lung adenocarcinoma cells.

### INTRODUCTION

Lung cancer is the most prevalent cause of cancer death in most countries. In several areas the annual incidence rates among males exceed 100 per 100.000, some of the highest reported for any malignancy (Blot & Fraumeni, 1996). Adenocarcinoma, which arises from glandular epithelia, is a major form of this pathology. Metastatic spread of primary tumour cells is the main cause of death in oncological patients. Aberrant cell surface glycosylation plays an important role on tumour cell adhesion to endothelium, a necessary event leading to extravasation into target organs (Hakomori, 1989).

Carcinoma cells are often enriched with sialylated fucosylated lactosaminoglycans such as sialyl-Lewis<sup>x</sup> (sialyl-Le<sup>x</sup>) and sialyl-Lewis<sup>a</sup> (sialyl-Le<sup>a</sup>) which are recognized by the

endothelial cell adhesion molecule E-selectin (Sawada *et al.*, 1994; Takada *et al.*, 1993; Yamada, N. *et al.*, 1997). It has been proposed that E-selectin promotes attachment of tumour cells to endothelium by molecular interactions similar to those used in leukocyte recruitment to inflammation sites (Bevilacqua & Nelson, 1993; Lowe, 1997; Varki, 1994). The final step in the synthesis of these side-chain oligosaccharides is dependent on the activity of one or more specific  $\alpha$ -(1,3)-fucosyltransferases ( $\alpha$ -(1,3)-Fuc-Ts), and to date, five  $\alpha$ -(1,3)-Fuc-T genes have been cloned: *Fuc-TIII* (Kukowska-Latallo *et al.*, 1990), *Fuc-TIV* (Goelz *et al.*, 1990; Lowe *et al.*, 1991), *Fuc-TV* (Weston *et al.*, 1992a), *Fuc-TVI* (Weston *et al.*, 1992b) and *Fuc-TVII* (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). These enzymes catalyse the transfer of fucose to type 2 chain-based structures (Gal $\beta$ 1-4GlcNAc-R) to generate Le<sup>x</sup>-related molecules and Fuc-TIII (the Lewis type enzyme) can also efficiently use type 1 carbohydrates (Gal $\beta$ 1-3GlcNAc-R) to yield Le<sup>a</sup> and sialyl-Le<sup>a</sup> determinants. Transfection studies have shown that the five known  $\alpha$ -(1,3)-Fuc-T enzymes are able to synthesize the sialyl-Le<sup>x</sup> determinant although the role of Fuc-TIV, the myeloid type enzyme, in this synthesis has only been reported for few cell types such as a CHO-derived cell line (Goelz *et al.*, 1994). This enzyme has, however, high affinity for non-sialylated type 2 acceptor and thus participates on the synthesis of Lewis<sup>x</sup> antigen. Fuc-TV and Fuc-TVI (the plasma type enzyme) have the ability to synthesize Le<sup>x</sup> and sialyl-Le<sup>x</sup> (Weston *et al.*, 1992a; Weston *et al.*, 1992b). Fuc-TVII, a leukocyte-expressed enzyme, is unique in that this enzyme can synthesize only sialyl-Le<sup>x</sup> (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). Several reports have already confirmed the key role of this enzyme in the control of leukocyte trafficking in health and disease (Hiraiwa *et al.*, 1996; Maly *et al.*, 1996). Expression of Fuc-TIV and Fuc-TVII genes had initially been related to poor prognosis in lung cancers (Ogawa *et al.*, 1996) but, more recently, Fuc-TVII has been defined as a more important indicator of a poor prognosis by its participation in sialyl-Le<sup>x</sup> synthesis (Ogawa *et al.*, 1997).

We have previously demonstrated that the metastatic human lung adenocarcinoma cell line HAL-8Luc overexpressed a series of sialylated fucosylated lactosaminoglycans compared to the nonmetastatic HAL-24Luc cells (Martin-Satue *et al.*, 1998). Both cell lines were derived from the same common parental cell line KUM-LK-2 using repeated cloning with limiting dilution technique and i.v. injection in *nu/nu* mice (Inufusa *et al.*, 1991). Our previous characterization of these cells indicated that the lungs are the only organs colonized when HAL-8Luc cells were inoculated either i.v. or i.m. in athymic mice while HAL-24Luc cells did not metastasize. Overexpression of these oligosaccharides correlated with an increased expression of the five  $\alpha$ -(1,3)-Fuc-T genes and enhanced adhesion to activated endothelial cells. Results from those *in vitro* adhesion assays pointed to the relevance of the sialyl-Le<sup>x</sup> oligosaccharide in the binding reaction and indicated a high affinity of this epitope for E-selectin expressed on endothelial cells. These observations

reinforced previous findings in which sialyl-Le<sup>x</sup> had been identified as predicting factor of recurrence in lung adenocarcinoma patients (Ogawa *et al.*, 1994). The aim of the present work was to analyse if  $\alpha(1,3)$ -Fuc-TIV and  $\alpha(1,3)$ -Fuc-TVII overexpression modifies the adhesive properties and lung colonization potential of nonmetastatic human lung adenocarcinoma HAL-24Luc cells.

## MATERIALS AND METHODS

### Cell Lines

Human lung adenocarcinoma cell lines HAL-8Luc (metastatic) and HAL-24Luc (nonmetastatic) (Martin-Satué *et al.*, 1998) were maintained in RPMI 1640 (Bio-Whitaker, Verviers, Belgium) supplemented with 10% (v/v) heat-inactivated FBS (Bio-Whitaker), 2mM L-glutamine (Bio-Whitaker), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml) and 300  $\mu$ g/ml geneticin (G418; Life Technologies, Inc., Gran Island, NY) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

HUVECs were isolated by collagenase digestion from multiple segments of normal-term umbilical cords and cultured as previously described (Lopez *et al.*, 1993). Adhesion assays were performed using endothelial cells within three passages.

### Plasmids and Transfection

Plasmids containing full-length cDNAs for Fuc-TIV (pcDNAI- $\alpha(1,3)$ FT<sub>Mlu</sub>) and Fuc-TVII (pCDM8-FucTVII) were kindly donated by Dr. John B. Lowe from the Howard Hughes Medical Institute (The University of Michigan Medical Centre, Ann Arbor, MI). Plasmid pCEP4 encoding hygromycin resistance as well as the pcDNAI and pCDM8 plasmids were obtained from Invitrogen (San Diego, CA).

HAL-24Luc cells, which were already neomycin resistant, were cotransfected by lipofection with each Fuc-T cDNA-containing plasmid together with the pCEP4 plasmid, using the method supplied by the manufacturer (Life Technologies, Inc., Gran Island, NY). HAL-24Luc cells mock transfected with pCEP4 and the vector devoid of Fuc-T cDNA were used as control clones. Transfected cells were selected by supplementing culture medium with 100  $\mu$ g/ml hygromycin (Boehringer Mannheim, Mannheim, Germany). After limiting dilution culture resistant clones were analysed for surface antigens expression.

### Flow Cytometry

Up to 25 HAL-24Luc clones from each transfection were analysed for cell surface antigen expression by flow cytometric analysis (Epics XL-MCL, Hialeah, FL) as previously described (Martín-Satué *et al.*, 1998) using the following primary mAbs: FH6, an IgM anti-sialyl-Le<sup>x</sup> dimeric, kindly provided by Otsuka Pharmaceutical (Osaka, Japan); CSLEX-1,

an IgM anti-sialyl-Le<sup>x</sup> and Leu-M1, an IgM anti-Le<sup>x</sup> both purchased from Becton Dickinson (San Jose, CA); LE-1, an IgM anti-Le<sup>a</sup> (Ortho Diagnostic, Neckargemünd, Germany); CA19.9, an IgG<sub>1</sub> anti-sialyl-Le<sup>a</sup> (Novocastra Laboratory, Ltd., Newcastle, UK). These specific antibodies were used at saturating concentrations determined by titration.

Cells labeled with irrelevant isotypic murine mAbs (mouse anti-human IgG<sub>1</sub> and mouse anti-human IgM from Becton Dickinson, San Jose, CA) or secondary antibody alone (fluorescein-conjugated goat anti-mouse IgG; DAKO, A/S, Copenhagen, Denmark) were used as controls.

Mean channels of fluorescence were recorded for every analysis, and the ratios of mean channels of fluorescence for each mAb and control pair were also calculated.

### Northern Blot

4µg Poly (A)<sup>+</sup> RNA from transfected and untransfected HAL-24Luc cells were fractionated by formaldehyde/agarose gel electrophoresis and transferred to nylon membranes (Hybond-N<sup>+</sup>; Amersham International, Buckinghamshire, United Kingdom). The membranes were hybridized with <sup>32</sup>P-labelled cDNA probes in a solution containing 50% (v/v) formamide, 5x SSC, 50mM sodium phosphate buffer pH 6.5, 250 µg/ml sheared salmon sperm DNA, 10x Denhart's solution and 10% dextran sulphate overnight at 42°C. Following hybridization the membranes were washed twice for 10 min at RT in 2x SSC, 0.1% SDS and once in 0.2x SSC, 0.1% SDS for one hour at 68°C. Finally the membranes were autoradiographed by exposure to Hyperfilm MP (Amersham International, Buckinghamshire, United Kingdom). Blots were stripped and reprobed with human β-actin cDNA as a control for RNA integrity and loading consistency. Band intensity was analysed with the Molecular Analyst/PC 1.4 software (BioRad, Hercules, CA) and calibrated by comparison with the β-actin bands.

Fuc-Ts and β-actin human cDNA probes were obtained in our laboratory (Martin-Satue *et al.*, 1998).

### Endothelial Cell Adhesion Assays

HUVECs were cultured in fibronectin-coated 96-well microtiter plates. Confluent endothelial cells were induced for E-selectin expression by incubating with 10 U/ml human recombinant IL-1β (Boehringer Mannheim, Mannheim, Germany) for 4 hours at 37°C previous to the experiment.

HAL-8Luc, HAL-24Luc and Fuc-T-transfected HAL-24Luc cells were detached by trypsinization, labeled with 200 µCi of sodium [<sup>51</sup>Cr] chromate (Amersham International, Amersham, UK) and added in RPMI 1640 with 2% FBS to both stimulated and nonstimulated HUVEC monolayers at a nominal density of 1x10<sup>5</sup>/well. After a 30 min incubation period at 4°C nonadherent cells were removed by aspiration and following two

washes with 2% FBS medium bound cells were lysed with 25 mM NaOH, 0.1% SDS and radioactivity was determined using a gamma counter (1261 Multigamma; WALLAC Oy, Turku, Finland). Adhesion percentages were calculated as the ratio of bound cell radioactivity to total radioactivity from  $1 \times 10^5$  cells.

For E-selectin blockade, IL-1 $\beta$ -stimulated HUVEC monolayers were incubated with 10  $\mu$ g/ml anti-human E-selectin mAb, P2H3 (Chemicon International Inc., Temecula, CA) for 30 min at 4°C previous to the binding assay.

For inhibition assays, labeled tumour cells were first incubated with the above described mAbs: Leu-M1, CSLEX-1, FH6, LE-1 or CA19.9 at a saturating concentration of 50  $\mu$ g/ml for 30 min at room temperature and then added to HUVEC monolayers. Isotypic irrelevant murine mAbs (mouse anti-human IgG<sub>1</sub> and mouse anti-human IgM from Becton Dickinson, San Jose, CA) were used as controls.

### **Experimental Metastasis Assay**

Metastasis assays were performed on 7-week-old female BALB/c homozygous nude (*nu/nu*) mice (CRIFFA S.A., Barcelona, Spain) maintained in a specific pathogen-free environment.

The above described HAL-24Luc Fuc-T-transfectants ( $5 \times 10^5$  cells) suspended in 0.1 ml serum-free RPMI 1640 were injected in the lateral tail vein to assess for lung colonization potential. Untransfected HAL-8Luc and HAL-24Luc cells as well as mock transfectants were also inoculated as controls in these experiments. Assays were done in duplicate and five animals were used for each replica. Animals were sacrificed 10 weeks after injections. No animals died before the time of sacrifice. Following postmortem examination, lungs were collected for histopathological analysis.

Samples were fixed in 4% (w/v) paraformaldehyde in 0.1M PBS for 2 h at room temperature, dehydrated and paraffin-embedded. Nominal 6  $\mu$ m-thick sections were mounted on glass slides and routinely stained with haematoxylin and eosin. Analysis of these samples was performed following "systematic random sampling" methodology (Pakkenberg & Gundersen, 1988). Specimens were observed and photographed with a Reichert-Jung Polyvar2 light microscope.

## **RESULTS**

### **Flow cytometry**

Fuc-T transfected HAL-24Luc cell clones were examined by flow cytometry for surface expression of the following fucosylated lactosaminoglycans: Le<sup>x</sup>, sialyl-Le<sup>x</sup>, sialyl-Le<sup>x</sup> dimeric, Le<sup>a</sup> and sialyl-Le<sup>a</sup>.



Fuc-TIV transfectants only overexpressed Le<sup>x</sup> while Fuc-TVII transfected cells only showed enhanced expression of sialyl-Le<sup>x</sup> oligosaccharide. The highest antigen expressing clone from Fuc-TIV and Fuc-TVII transfections were named 24FT4 and 24FT7 respectively and selected for experiments. The ratios of mean fluorescence intensity were 172 for Le<sup>x</sup> in 24FT4 cells and 48 for sialyl-Le<sup>x</sup> in 24FT7 cells. No significant increases in Le<sup>a</sup>, sialyl-Le<sup>a</sup> or sialyl-Le<sup>x</sup> dimeric expression were detected in any of the clones analysed. Mock transfectants displayed the same antigenic pattern as untransfected cells indicating that the transfection process did not modify the constitutive expression of these oligosaccharides. Cytofluorometric results are illustrated in Fig. 1.

### Northern Blot Analysis

All the Fuc-T transfectants expressed higher mRNA levels for the analysed Fuc-Ts than the parental HAL-24Luc cells or the mock transfectants (Fig.2). Mock transfectants expressed amounts of Fuc-TIV mRNA equivalent to those of HAL-24Luc cells, showing that cotransfections did not affect the constitutive Fuc-TIV expression on these cells. Mock transfectants as well as HAL-24Luc cells showed undetectable levels of Fuc-TVII mRNA.

### Endothelial Cell Adhesion Assays

We have studied the relative ability of Fuc-T-transfected HAL-24Luc cell lines, 24FT4 and 24FT7, to adhere to IL-1 $\beta$ -stimulated HUVEC monolayers. Mock transfected HAL-24Luc cells as well as metastatic HAL-8Luc cells were also subjected to adhesion assays and used as controls. Since we have previously shown that HAL-8Luc cells significantly adhere to E-selectin expressing HUVEC monolayers (Martin-Satue *et al.*, 1998) we have taken the amount of HAL-8Luc binding in these adhesion assays as 100%. Normalized values for the mean and SD of these binding results are illustrated in Fig. 3.

The sialyl-Le<sup>x</sup>-overexpressing cells 24FT7 showed extensive adhesion to IL-1 $\beta$ -stimulated HUVEC monolayers but did not detectably bind to nonactivated endothelial cells. On the contrary, 24FT4 cells were unable to bind to HUVEC cells regardless of their activation state (Fig.3A). None of the mock transfectant clones analysed was able to adhere to endothelial cells, indicating that transfection process by itself did not alter the adhesive properties of HAL-24Luc cells.

Blocking of sialyl-Le<sup>x</sup> oligosaccharides with the CSLEX-1 mAb resulted in 90% inhibition of binding in Fuc-TVII-transfected cells (Fig. 3B), demonstrating the key role of this determinant in the adhesion. Moreover, pretreatment of stimulated HUVEC monolayers with anti-E-selectin mAb completely abolished the binding of tumour cells. Irrelevant mAbs used as controls as well as other anti-fucosylated antigen mAbs tested had no effect on adhesion.

### Experimental Metastasis Assays

To analyse if Fuc-T overexpression in HAL-24Luc cells modifies their lung colonization potential, we have i.v. inoculated athymic mice with 3 Le<sup>x</sup>-overexpressing Fuc-TIV transfected HAL-24Luc cells, including the 24FT4 clone and 4 sialyl-Le<sup>x</sup>-overexpressing Fuc-TVII transfected HAL-24Luc cells, including the 24FT7 clone. Untransfected HAL-8Luc, HAL-24Luc cells and HAL-24Luc mock transfectants were also inoculated and used as controls in these experiments.

76% (19 of 25) mice i.v. inoculated with Fuc-TVII-overexpressing HAL-24Luc cells developed lung metastases. Similarly, 90% (9 of 10) mice i.v. inoculated with HAL-8Luc cells and used as positive controls for the inoculation procedure, developed pulmonary colonies. No metastases were detected in mice i.v. inoculated with either untransfected, mock-transfected or Fuc-TIV-overexpressing HAL-24Luc cells. These results are summarized on Table 1.

Metastases derived from mice i.v. inoculated with Fuc-TVII-overexpressing HAL-24Luc cells were associated with the basal lamina of the terminal bronchiole epithelium as was also previously described for the HAL-8Luc-inoculated mice (Martin-Satue *et al.*, 1998). A typical lung metastasis from 24FT7-inoculated mouse is shown in Fig.4.

### DISCUSSION

Side-chain oligosaccharides such as sialyl-Le<sup>x</sup> and sialyl-Le<sup>a</sup> in both glycolipids and glycoproteins on the surface of tumour cells had already been identified as tumour associated antigens (Hakomori, 1989). More recently, these epitopes have also been characterized as E- and P-selectin counterreceptors expressed in several leukocyte subsets and participate in the recruitment of leukocytes during inflammation (Varki, 1994). Similar mechanisms have been proposed to participate in the metastatic spread of primary tumour cells. It has been suggested that P-selectin may mediate the formation of large aggregates of tumour cells and platelets that could be retained in the thin capillaries of the lung promoting growth and organ colonization (Kim *et al.*, 1998). Previous reports have shown that sialyl-Le<sup>x</sup> expression might be a useful indicator of blood vessel invasion and recurrence in lung adenocarcinoma (Ogawa *et al.*, 1994). We have also demonstrated that enhanced surface display of sialyl-Le<sup>x</sup> on human lung adenocarcinoma cells correlated with their metastatic ability and participated in the adhesion of tumour cells to E-selectin-expressing endothelial cells (Martin-Satue *et al.*, 1998). Expression of  $\alpha$ -(1,3)-Fuc-TVII gene has also been related to poor prognosis in lung cancer because of its participation in sialyl-Le<sup>x</sup> synthesis (Ogawa *et al.*, 1997). To determine the role of Fuc-TIV and Fuc-TVII in the metastatic behaviour of lung adenocarcinoma cells we have overexpressed these enzymes in the nonmetastatic HAL-24Luc cells.

In the present work, we have stably transfected the nonmetastatic human lung adenocarcinoma HAL-24Luc cell line with cDNAs encoding the  $\alpha$ -(1,3)-Fuc-T IV and VII enzymes and analysed the resulting changes in antigenic expression, adhesive ability and metastatic potential of the transfected cell lines. Our cytofluorometric results indicated that 24FT4 and 24FT7 cells exclusively overexpressed Le<sup>x</sup> and sialyl-Le<sup>x</sup> determinants respectively. FH6 mAb did not detect any increase in sialyl-Le<sup>x</sup> dimeric antigen expression. Neither Le<sup>a</sup> nor sialyl-Le<sup>a</sup> had been expressed in any of the clones analysed and both showed the same background expression level as the parental HAL-24Luc cells confirming that neither of these enzymes recognize type 1 chain oligosaccharides. The pattern of Lewis-related antigens displayed by the transfectants studied in this work is in accordance with that from previously reported transfection experiments in which Fuc-Ts were overexpressed in other cell lines such as CHO or leukocyte-derived cells (Natsuka *et al.*, 1994; Sasaki *et al.*, 1994). Synthesis of sialyl-Le<sup>x</sup> involves sialylation of a type 2 lactosamine core (Gal $\beta$ 1-4GlcNAc-R) followed by the addition of one fucosyl residue. Untransfected HAL-24Luc cells did not produce sialyl-Le<sup>x</sup> but Fuc-TVII transfection of these cells resulted in sialyl-Le<sup>x</sup> overexpression, indicating that the sialylated intermediates are not the limiting molecules and pointing to the relevance of Fuc-TVII in this reaction.

Endothelial cell adhesion experiments performed with the selected Fuc-T transfectants showed that only sialyl-Le<sup>x</sup>-overexpressing HAL-24Luc cells (24FT7 clone) had the ability to bind to IL-1-stimulated HUVECs in contrast with the null adhesion capacity displayed by 24FT4, mock transfected or untransfected cells. Binding inhibition achieved either by blocking sialyl-Le<sup>x</sup> on the tumour cells or by E-selectin blockade on HUVECs emphasized the key role of these molecules in the binding reaction.

By inoculation of transfected cells in athymic mice we demonstrated that Fuc-TVII overexpression is sufficient for acquisition of lung colonization phenotype by HAL-24Luc cells. The absence of metastatic foci in 24FT4 inoculated mice is likely related to the null *in vitro* E-selectin adhesion capacity shown by these cells. Our experiments strongly suggest the participation of the endothelial E-selectin in the initial stages of lung colonization by the HAL adenocarcinoma cells. Although in a different cell system, the existence of a mechanism similar to that described by Kim *et al.* (1998) contributing to colonization can not be excluded. Furthermore it may help explain organ selectivity.

The fact that the sole Fuc-TVII transfection rendered the HAL-24Luc cells metastatic emphasizes the convenience in using models consisting of genetically closely related cell lines such as the HAL-8Luc/HAL-24Luc for this work. Our results not only reinforce previous findings in which Fuc-TVII expression has been reported as an indicator of poor prognosis in lung carcinoma (Ogawa *et al.*, 1996; Ogawa *et al.*, 1997) but also directly demonstrate for the first time the role of this enzyme on the acquisition of lung colonization behaviour by human lung adenocarcinoma cells.

**ACKNOWLEDGEMENTS**

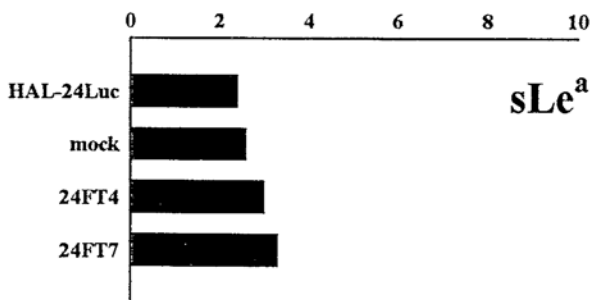
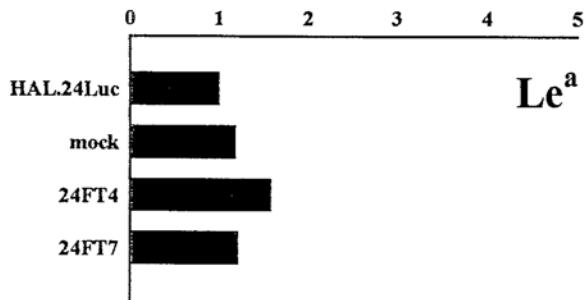
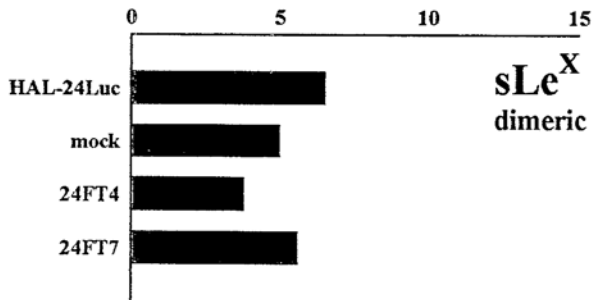
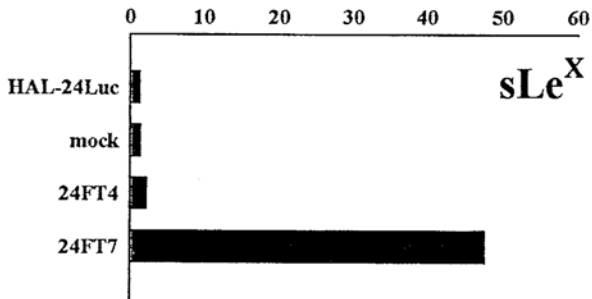
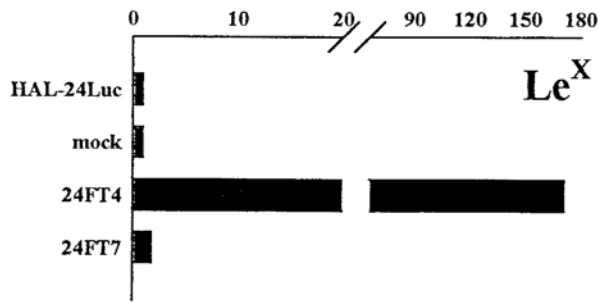
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**FIGURES  
and TABLE**

**Figure 1.** Flow cytometry analysis of HAL-24Luc cells, mock transfectants and 24FT4 and 24FT7 clones. Cells were labeled with mAb against Le<sup>x</sup> (Leu-M1 mAb), sialyl-Le<sup>x</sup> (CSLEX-1 mAb), sialyl-Le<sup>x</sup> dimeric (FH6 mAb), Le<sup>a</sup> (LE-1 mAb) and sialyl-Le<sup>a</sup> (CA19.9 mAb). Bars represent the ratio of mean fluorescence intensities between each mAb and its control. Data correspond to one representative of five similar experiments. Notice difference in the ordinate scales.

Mean Fluorescence Intensity Ratio



**Figure 2.** Northern blot analysis of mRNA from HAL-24Luc (24), mock-transfected (*M*) and Fuc-T-transfected cells (*FT4*, *FT7*). Blots were hybridized with the <sup>32</sup>P-labeled Fuc-TIV and Fuc-TVII cDNA probes as described in "Materials and Methods". After a first autoradiographic exposure, the probes were stripped out and the blots were rehybridized with the β-actin probe (*β-act*). Band sizes are indicated in kilobases at the *left* of each set.

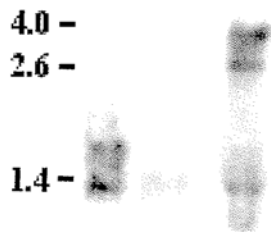


Fuc-TIV

Fuc-TVII

24 M FT4

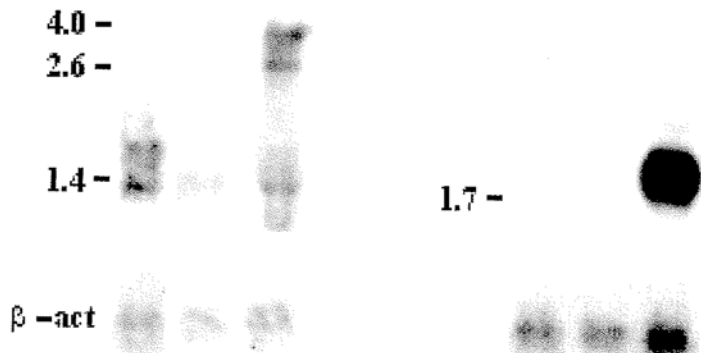
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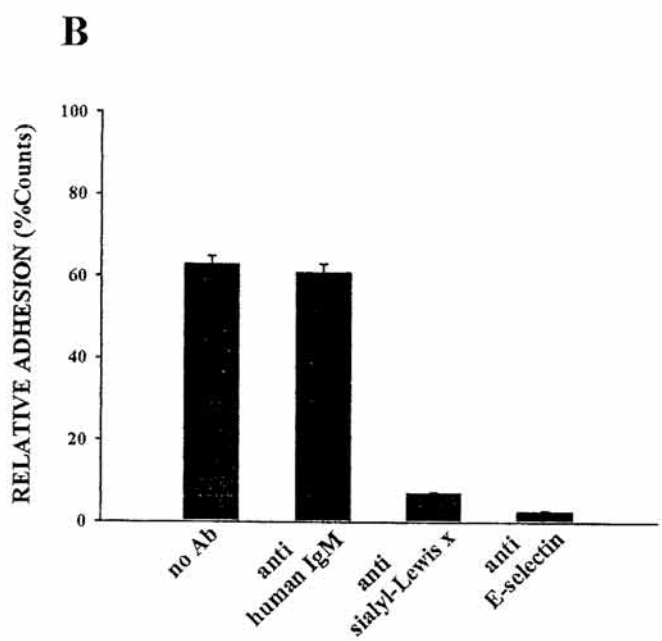
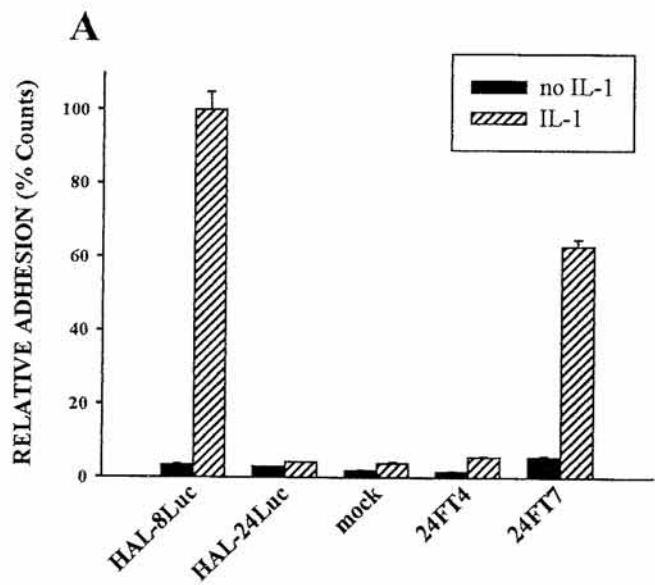
1.7 -

$\beta$ -act

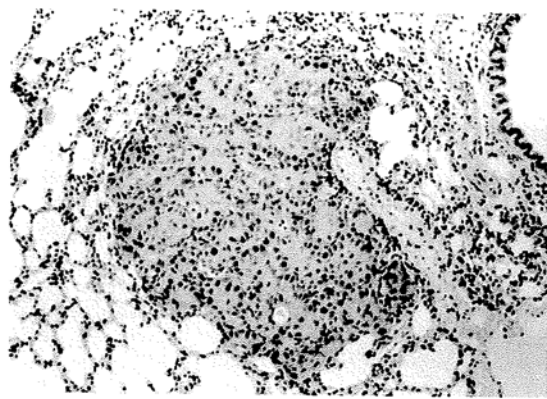
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**Figure 3.** Adhesion of tumour cells to HUVEC monolayers. Data show normalized values corresponding to the fraction of bound cells and represent the means of four different experiments, each performed in six replicates. **A**, adhesion of: HAL-8Luc, HAL-24Luc, mock-transfected HAL-24Luc, 24FT4 and 24FT7 cells to nonstimulated (solid bars) or IL-1 $\beta$ -stimulated (striped bars) HUVECs. **B**, adhesion of 24FT7 cells to IL-1-stimulated HUVECs after no treatment, pretreatment of tumour cells with an irrelevant mAb (anti human IgM), anti-sialyl-Le<sup>x</sup> mAb or pretreatment of HUVECs with anti-E-selectin mAb.



**Figure 4.** Light micrograph of lung metastasis developed by 24FT7-inoculated mouse showing a tightly packed tumour cell mass partially occupying the aerial spaces. *Bar*, 100  $\mu\text{m}$ .



**Table 1.** Production of lung metastasis by tumour cells i.v. injected in athymic mice. Entries in bold face indicate the clones whose analysis is described.

Cell line	Number of mice with lung metastasis / total	Total number of lung colonies / mice
HAL-8Luc	9/10	8-25
HAL-24Luc	0/10	0
Mock-transfected HAL-24Luc	0/10	0
<b>24FT4</b>	<b>0/10</b>	<b>0</b>
24FT4b	0/5	0
24FT4c	0/5	0
<b>24FT7</b>	<b>8/10</b>	<b>4-30</b>
24FT7b	4/5	5-20
24FTc	4/5	10-25
24FTd	3/5	7-20