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Introducing α(1,2)-linked fucose into hepatocarcinoma cells inhibits vasculogenesis and tumor growth

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The glycoantigen sialyl-Lewis x (sLex) and its isomer sialy-Lewis a (sLea) are frequently associated with advanced states of cancer and metastasis. In a previous work, we have shown that hepatocarcinoma cells (HCC) HepG2 interact with the endothelial E-selectin exclusively through sLe^x oligosaccharides, the synthesis of which could be completely prevented by the α(1,2)-fucosyltransferase-I (FUT1), thus resulting in a strong inhibition of adhesion and rolling on activated endothelial cells. The purpose of the present study was to evaluate the impact of inhibiting sLex synthesis and the subsequent E-selectin adhesion, on HCC tumor growth in nude mice. Four weeks after subcutaneous transplantation of cells, no FUT1-derived tumor could be detected, whereas 75% of control animals developed large size tumor nodules. Between the 4th and the 8th week postinoculation, 33% tumors arose from FUT1-transduced cells but showed a slow growth (nodule volumes less than 500 mm^3), while more than 50% of control tumors reached volumes between 1,500 and 3,000 mm^3. Several parameters were examined, including cell division and proliferation, apoptosis, adhesion to extracellular matrix components and angiogenesis/vasculogenesis. We provide evidence that among all, vasculogenesis was the most clearly affected by FUT1 expression, suggesting that tumor angiomorphogenesis may, at least partly, depend on E-selectin-mediated interaction between HCC and endothelial cells, the inhibition of which remarkably retards tumor growth.

Key words: α(1,2)-fucosyltransferase-I; E-selectin; sialyl-Lewis; antitumor; vasculogenesis

The sialyl-Lewis antigens (sLex and sLea) are blood group-related antigens naturally expressed on leukocytes to initiate leukocyte-endothelium interaction mediated by the cytokine-inducible endothelial adhesion molecule E-selectin.[1,2] In addition to this physiological role in inflammation, sialyl-Lewis antigens are also considered as tumor markers expressed on many cancer cells and therefore, they are thought to be involved in metastasis by initiating tumor cell-endothelium adhesion.[3] Indeed, the expression of sialyl-Lewis antigens on carcinoma cells is frequently associated with advanced states of cancer and a statistically relevant relationship has been established between the postoperative prognosis of patients and the degree of expression of sialyl-Lewis antigens on cancer tissues.[3] Besides, several lines of evidence from selectin-deficient mice studies pointed out the contribution of selectins in facilitating tumor growth and progression.[4,5] In particular, E-selectin and its sLex-containing glycoconjugate ligands have been shown to be involved in the essential events of tumor angiogenesis,[6–9] including the fact that coinjection of tumor cells expressing high level of sLe^x together with endothelial cells constitutively expressing E-selectin, has been found to promote tumor angiogenesis and growth.[10]

We have recently succeeded in strongly blocking the expression of sLex on digestive cancer cells by a virally-delivered α(1,2)-fucosyltransferase-I (FUT1).[11] FUT1 catalyses the transfer of an α(1,2)-linked fucose on the galactose residue of *N*-acetyllactosaminic-type oligosaccharides, including the sLex precursor Galβ1,4GlcNAcβ1-R.[12] Hence, the sLex structure [NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1-R] is replaced by the histo-blood group *H/O* (Fucα1,2Galβ1,4GlcNAcβ1-R) or the double-fucosylated Lewis y antigen [Le^y, [Fucα1,2Gal β1,4(Fucα1,3)GlcNAcβ1-R] which do not bind to E-selectin. Consequently, the FUT1-transduced cells lost their ability to adhere or roll on activated endothelial cells. While performing those experiments we had found that the hepatocarcinoma cells (HCC) HepG2, do not bind to P-selectin but interact with E-selectin exclusively through sLex glycoantigens.[11] We also found that HCC interact with L-selectin in a sulfate-dependent manner, but this interaction is not modified by FUT1 transduction. Thus, HepG2 cells represent a good model to study the role played solely by the inducible endothelial E-selectin and its tumoral partner sLex in the development of hepatocarinomas. Using nude mice subcutaneously xenotransplanted with parental or FUT1-transduced HCC, we show here that FUT1 displays a strong antitumorigenicity due a defect in tumor vascularization.

Material and methods

Cell lines, DNAs and lentiviral transduction

HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Cambrex, Verviers Belgium) and occasionally in serum-free OptiMEM (Invitrogen, Cergy Pontoise, France). The human umbilical vein endothelial cells (HUVEC, Clonetics, San Diego, CA) were cultured in complete Endothelial Cell Growth Medium (EGM-2, Clonetics) as per the manufacturer's instructions. All media were supplemented with 10% foetal calf serum (FCS, Eurobio Les Ullis, France) and the antibiotics penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The lentiviral vector pRRLpgk-green fluorescent protein (GFP).sin18 containing the *FUT1* cDNA downstream of the human phosphoglycerine kinase (pgk) promoter and in frame with the GFP marker (denoted as pL1*FUT1*-gfp) was used for gene delivery. The construction of pL1*FUT1*-gfp vector and the preparation of viral particles as well as the transduction of HepG2 cells with this construct were performed as previously described.[11] HepG2 cells infected with particles of pRRLpgk-GFP.sin18 to express EGFP alone are denoted as parental cells throughout the manuscript. The construct for E-selectin conjugated to the human IgM portion[13] (denoted as E-selectin-IgM chimera) was kindly provided by Dr Minoru Fukuda (The Burnham Institute, La Jolla, CA). The E-selectin-IgM chimera was produced by transiently transfecting the human 293-T cells as described.[11]

Abbreviations: Fuc, fucose; FUT1, α(1,2)-fucosyltransferases-I; Gal, galactose; GlcNAc, *N*-acetylglucosamine; NeuAc, *N*-acetytl neuraminic acid (sialic acid); sLea, sialyl-Lewis a; sLex, sialyl-Lewis x.

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Antibodies, lectins and fluorescence analyses

The anti-human sLe^x mAb KM93, the anti-human E-selectin mAb BBIG-E4 and the anti-mouse CD31 (Clone MEC13.3) were purchased from Seikagaku (Tokyo, Japan), R&D system (Abingdon, UK) and PharMingen (San Diego, CA), respectively. The Alexa Fluor 594-labeled and the cyanin-5 (Cy5)-conjugated secondary antibodies were from Molecular Probes (Eugene, OR) and Jackson Immunoresearch (West Grove, PA), respectively. Rhodamine (RITC)-conjugated *Ulex europaeus* agglutinin-I (UEA-I) was from Sigma-Aldrich (St-Quentin Fallavier, France), the biotin-conjugated *Maackia amurensis* lectin (MAL) was from Vector Laboratories (Burlingame, CA) and the RITC-conjugated streptavidin was from CliniSciences (Montrouge, France).

For fluorescence microscopy, cells were plated in eight-chamber slides (Falcon, Becton Dickinson, USA) and cultured 1 or 2 days before use. Cell monolayers were then rinsed in PBS, fixed in 1% paraformaldehyde for 5 min at room temperature and incubated 5 min at 4°C in PBS containing 1 mg/ml BSA (PBS/BSA). Cells were then incubated with either the anti-sLe^x mAb KM93, the RITC-conjugated UEA-I or the biotin-conjugated MAL, for 30 min at 4°C in PBS/BSA. The KM93 immunoreactivity was then revealed by Alexa Fluor 594-labeled anti-mouse IgM and MAL binding sites were stained by RITC-conjugated streptavidin in PBS/BSA for 30 min at 4°C. The E-selectin binding was performed by incubating cell monolayers at 4°C for 30 min with the conditioned OptiMEM medium recovered from E-selectin-transfected 293T cells as described.[11] Bound E-selectin-IgM molecules were then stained with an Alexa Fluor 594-labeled anti-human IgM secondary antibody in OptiMEM/BSA. Cells were then visualised on a Zeiss Axiovert 200 fluorescence microscope with a 40× objective (Carl Zeiss, Göttingen, Germany). In some experiments the binding of E-selectin-IgM was measured by fluorescence-activated cell sorting (FACS) according to Prorok-Hamon *et al.*[14]

Cell adhesion, in vitro *cell growth and capillary-like tube formation*

Adhesion on purified extracellular matrix (ECM) proteins, including vitronectin, fibronectin, collagen and laminin was performed as described previously.[15] To study the cell growth, cells were seeded at a density of 10^4 cells/well in 96-multiwell culture plates (Falcon) and cultured in complete DMEM medium. Every day, media were removed and cells were fixed in 1% glutaraldehyde, stained by 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured with an ELISA microplate-reader at 600 nm. The *in vitro* Matrigel-based capillary-like tube assay was carried out according to Donovan *et al.*[16] Briefly, parental and FUT1-transduced cells were cultured 24 hr in serum-free OptiMEM and media (denoted as HCC supernatant) were recovered, cleared from detached cells and debris by centrifugation and added onto HUVEC monolayers for 4 hr at 37°C. Fresh OptiMEM medium was used as negative control. HUVEC were then viewed in phase contrast with Zeiss Axiovert 200 microscope as above and images were taken at 0, 8 and 24 hr after the addition of supernatants.

The Effects of HCC-secreted factors were also examined by coculturing parental and FUT1-transduced HCC with HUVEC. To this end, HCC and HUVEC were mixed at the ratio of 4:1 in complete EGM-2 medium and seeded in 6-well plates (10^5 cells/well). After 5 days of culture, cells were harvested, rinsed 3 times with PBS and E-selectin expression was analysed by FACS as follows. Cells were harvested, rinsed in PBS and monodispersed cells were incubated with the anti-E-selectin mAb BBIG-E4 (1/200 dilution) for 1 hr at 4°C in PBS/BSA. The Cy5-conjugated goat anti-mouse IgG (1/200 dilution) was used for counterstaining. Cells were then fixed in 1% paraformaldehyde and samples were analysed by measuring the fluorescence of 10,000 cells and displayed on a 4-decade log scale. Cells incubated without the first antibody and those. incubated with 10 ng/ml TNF-α (4 hr at 37°C), were used as negative and positive control, respectively.

Cell death assays

Cell death assay was performed essentially as described by Remacle-Bonnet *et al.*[17] Briefly, cells at 80% confluency were incubated with or without 40 ng/ml interferon-γ (IFN-γ) for 15 min at 37°C in serum-free DMEM, washed twice and treated for 48 hr at 37°C with TNF-α (50 ng/ml), TNF-related apoptosis-inducing ligand (TRAIL, 100 ng/ml), or anti-Fas mAb (100 ng/ml) in DMEM containing 0.5% foetal calf serum. Cells were then detached rinsed twice in PBS and prepared for DNA fragmentation assay according to Leist *et al.*[18] or to caspase-3 immunostaining (see later).

Mouse tumor transplants and immunohistochemistry

FUT1-transduced and parental cells were suspended in serum-free DMEM medium at 6×10^7 cells/ml, 150 μl of which were then subcutaneously inoculated to 6–8 weeks old female nude mice (nu/nu, Janvier, Le Genest-St-Isle, France). Each animal was injected with parental cells in the left flank and with FUT1-expressing HepG2 cells in the right flank ($n = 12$ animals per experiment). Tumor measures were taken weekly with graduated callipers and volumes were calculated as $(\text{width})^2 \times (\text{length}) \times (\pi/6)$ according to Osborne *et al.*[19] After 8 weeks postinoculation, animals were sacrificed and tumors surgically harvested and either fixed in 4% formaldehyde, paraffin-embedded (for caspase-3 staining) or frozen in liquid nitrogen, stored at −80°C and sectioned (for CD31 staining). Immunostainings were carried out with the Animal Research Kit (ARK, Dako A/S, Glostrup, Denmark), using the antiactive caspase-3 pAb (Promega, Madison, WI) and colors were developed with the Ventana detection system (Ventana, Tucson, Ariz). For CD31 labeling, frozen sections were incubated for 90 min with the rat anti-mouse CD31 diluted at 1/25 in Dakocytomation antibody diluent (Dako) and counterstained for 45 min with Alexa Fluor 594-labeled anti-rat IgG secondary antibody diluted at 1/250 in the same buffer. Slides were then examined by fluorescence microscopy. For each detection, a control was done by omitting the primary antibody to determine the nonspecific binding.

^{35}S-labeling, lectin chromatography and fluorography

^{35}S-labeling, lectin chromatography and fluorography were carried out as described.[20] Proteins fractions were separated on a 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes, which were soaked in 1 M sodium salicylate (pH 6) for 20 min at room temperature, air-dried and subjected to fluorographic analysis, using BioMax XAR Film (Kodak, Chalon-Sur-Saône, France).

Western immunoblotting

Proteins (50 μg per fraction) were separated on a 7.5% (for sLe^x detection) or 15% SDS-PAGE (to detect cleaved caspase-3) and transferred to nitrocellulose membranes as earlier. Membranes were then blocked for 1 hr at 4°C in 25 mM Tris-HCl pH 7.4 containing 0.137 M NaCl, 0.1% Tween-20 (TBS-T) and 5% (w/v) non-fat dry milk (blocking buffer). To detect sLe^x-carrying proteins, membranes were probed with 3 μg/ml of the anti-sLe^x KM-93 overnight at 4°C in the blocking buffer. The KM-93-immunoreactive proteins were then detected after 1 hr incubation at room temperature with phosphatase alkaline-conjugated goat anti-mouse secondary antibody (1/5,000, Sigma-Aldrich) in the blocking buffer and staining with the NBT-BCIP chromogenic system (Kierkergard and Perry, Gaithersberg, MD). To detect cleaved caspase-3, membranes were incubated overnight at 4°C with rabbit anti-human caspase-3 (1:1,000, Cell Signaling, Danvers, MA) in the blocking buffer, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody (1:5,000, Sigma). Detection by the chemiluminescence

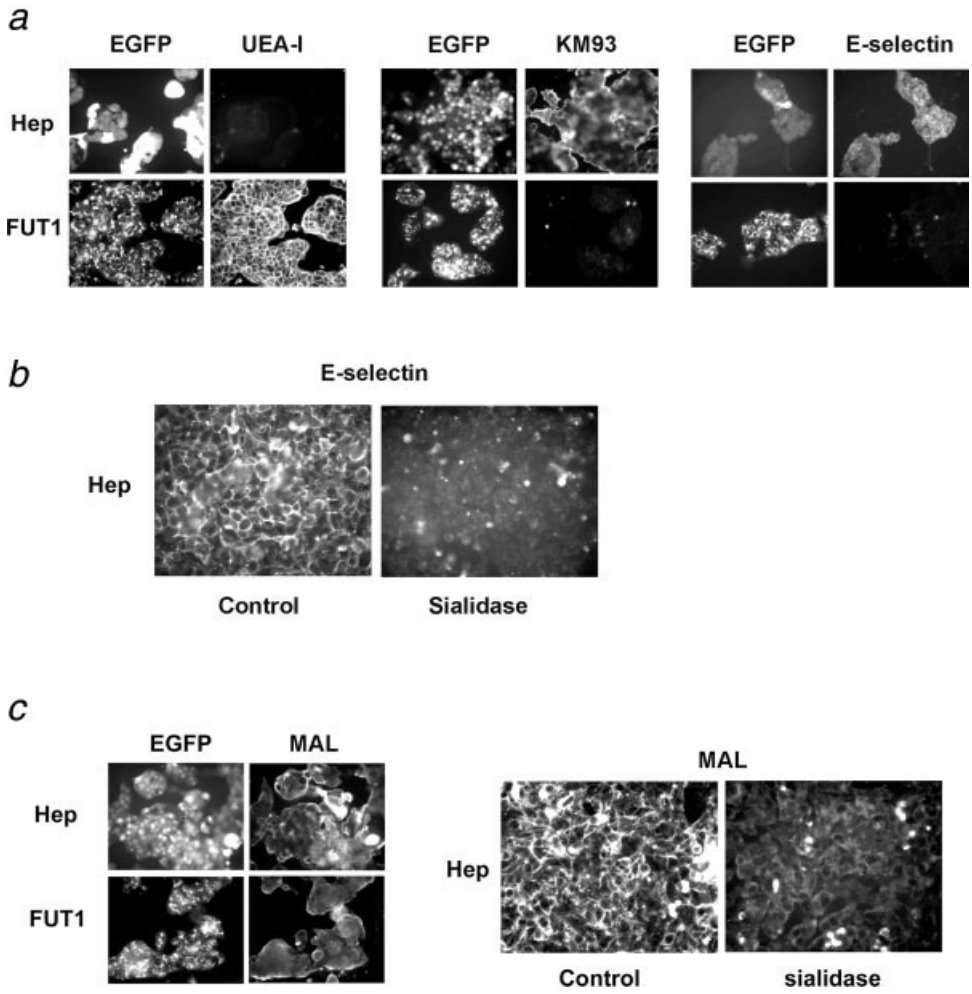

FIGURE 1 – Cell surface expression of α(1,2)-linked fucose, α(2,3)-linked sialic acid, sLe^x epitope and E-selectin binding of parental (Hep) and FUT1-transduced HepG2 cells (FUT1). (*a*) Cells were stained either with RITC-labeled UEA-I lectin (left panel) or with the anti-sLe^x mAb KM93 followed by Alexa Fluor-conjugated anti-mouse IgM antibodies (middle panel). For E-selectin binding (right panel), cells were incubated with the E-selectin-IgM chimera and counterstained with Alexa Fluor-conjugated anti-human IgM. (*b*) Sialic acid dependence of E-selectin binding as demonstrated by treatment of HepG2 cells with the Clostridium perfringens sialidase. The E-selectin binding was performed as in (*a*). (*c*) Comparison of sialic acid-specific staining with Maakia ameurensis lectin (MAL) between parental and FUT1-transduced HCC (left panel) and after sialidase treatment (right panel). MAL staining was performed by incubating cells with biotin-labeled MAL followed by RITC-labeled streptavidin. Cells were visualised by fluorescence microscopy. Original magnification ×40.

reaction was carried out for 1 min, using the ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Sugar analysis

Cells were removed from the flasks, pelleted by centrifugation, resuspended in water (10^7 cells/ml) and sonicated three times for 20 sec at maximum intensity. The samples were then lyophilised and submitted to acid-catalysed methanolysis and gas chromatography-mass spectrometry (GC-MS) analysis as described.[21] The integration of the peaks was performed on the total ion current, using Xcalibur software (Thermo Electron, Courtaboeuf, France). The integration of monosaccharides was performed on the major anomers by applying the relative molar response factor reported elsewhere.[22]

Results

FUT1 efficiently competes with sLe^x-specific sialyltransferases without significantly altering the overall sialylation

To create α(1,2)-fucosylated glycoconjugates, FUT1 has to compete with other terminal glycosyltransferases acting on the same acceptors,[23] among which a number of α(2,3)sialyltransferases involved in the synthesis of sialyl-Lewis a and x (sLe^a and sLe^x), the major E-selectin ligands. The α(1,2)-linked fucose transferred by FUT1, can be detected by the lectin UEA-I.[24] As shown in Figure 1, HepG2 cells are not stained with UEA-I (Fig. 1*a*, left panel), indicating that these cells do not express α(1,2)-fucosylated glycoconjugates and suggesting that they are deficient in FUT1 activity. However, upon the introduction of *FUT1* gene, cells exhibit a strong reactivity to the lectin. Concomitantly with the appearance of UEA-1 reactivity, there is a strong diminution of sLe^x expression, as defined by the KM93 mAb (Fig. 1*a*, middle panel) and the ability of FUT1-transduced cells to bind the E-selectin-IgM chimera (Fig. 1*a*, right panel). As shown in Figure 1*b*, E-selectin binding to HepG2 cells is dependent on cell surface sialic acid, since removing this sugar by the *Clostridium perfringens* sialidase abolishes the HCC/E-selectin interaction. Taken together, these results suggest that the cell surface sialic acid residues, including those supporting the E-selectin binding, have been swapped by α(1,2)-linked fucose residues.

To further evaluate the amplitude of changes in sialic acid content, parental and FUT1-transduced cells were stained with MAL,

TABLE I – GC-MS ANALYSIS OF MONOSACCHARIDES LIBERATED BY ACID-CATALYZED METHANOLYSIS FROM CELL EXTRACTS OF PARENTAL (HEP) AND FUT1-TRANSDUCED CELLS

Monosaccharides	Hep	Hep-FUT1
Fucose	**0.60**	**1.02**
Galactose	1.00	1.11
Mannose	1.95	1.76
GlcNAc	1.00	1.00
Sialic acid	**0.26**	**0.20**

Data are expressed as molar ratios relatively to GlcNAc residues. Values of fucose and sialic acid contents are shown in bold. This is one representative of two independent experiments.

specific for α(2,3)-linked sialic acids.[25] As shown in Figure 1*c*, expressing FUT1 in HepG2 cells does not dramatically alter the overall cell surface sialylation, as judged by MAL staining (Fig. 1*c*, left panel). The lectin staining is indeed dependent on sialic acid content as verified by sialidase treatment, which strongly decreases the lectin reactivity (Fig. 1*c*, right panel). This result was further confirmed by GC-MS. Except for fucose, GC-MS analyses revealed comparable sugar chromatographic profiles between parental and FUT1-expressing cells (Table I). As expected, the fucose content increases from 0.6 to 1.0, as a consequence of FUT1 introduction in HepG2 cells. However, the increase in fucose content is not accompanied by a significant decrease in sialic acid content since the latter slightly shifted from 0.26 to 0.20 in FUT1-transduced cells (Table I). Taken as a whole, the earlier data indicate that while the sLe^x biosynthesis and E-selectin binding are strongly affected by FUT1, only few sugar chains are actually modified by α(1,2)-linked fucose.

FUT1 strongly inhibits tumor formation in nude mice

It has been reported that the expression of sLe^x by tumor cells correlates with the development and progression of cancers[26,27] including hepatocellular carcinomas.[28,29] Accordingly and having shown that FUT1 selectively and quantitatively inhibits sLe^x synthesis in HepG2 cells, we investigated the capacity of FUT1-expressing cells to develop tumors. To this end, cells were subcutaneously inoculated to *nu/nu* nude mice and results from two independent experiments, using 12 animals per each experiment, are shown in Figure 2. There is a strong reduction in tumor size in mice inoculated with FUT1-expressing cells, compared to parental counterparts (Fig. 2*a*). To confirm the persistence of the transgenes, the tumor tissue was enzymatically dispersed into single cells and cultured under standard conditions. As shown in Figure 2*a*, almost all parental cells still express the transgene EGFP (cytoplasmic and nuclear staining, left panel). The expression of FUT1-EGFP fusion protein was also sustained in tumors originating from FUT1-transduced cells (presumably Golgi staining, right panel). Together with tumor size reduction, there is a marked difference in tumor occurrence as we noticed that after 4 weeks postinjection, 9 out of the 12 animals (75%) inoculated with HepG2 cells, developed macroscopic tumors while no tumor arose from FUT1-transduced cells (data not shown). After 8 weeks postinjection, only 4 small tumors had appeared on the flanks inoculated with FUT1-infected cells, indicating that FUT1 expression not only reduced the size of nodules, but also caused a significant delay in tumor formation. After 8 week postinoculation, more than 50% of HepG2-derived tumors achieved volumes greater than 1,500 mm^3, whereas FUT1-derived tumors did not exceed 500 mm^3 (Fig. 2*b*).

FUT1 does not affect cell proliferation or adhesion to extracellular matrix proteins and does not sensitize cells to apoptosis

Prompted by these observations, we sought to know whether this phenomenon is due to a defect in cell growth and/or to an increased apoptosis. As shown in Figure 3*a*, there is no obvious

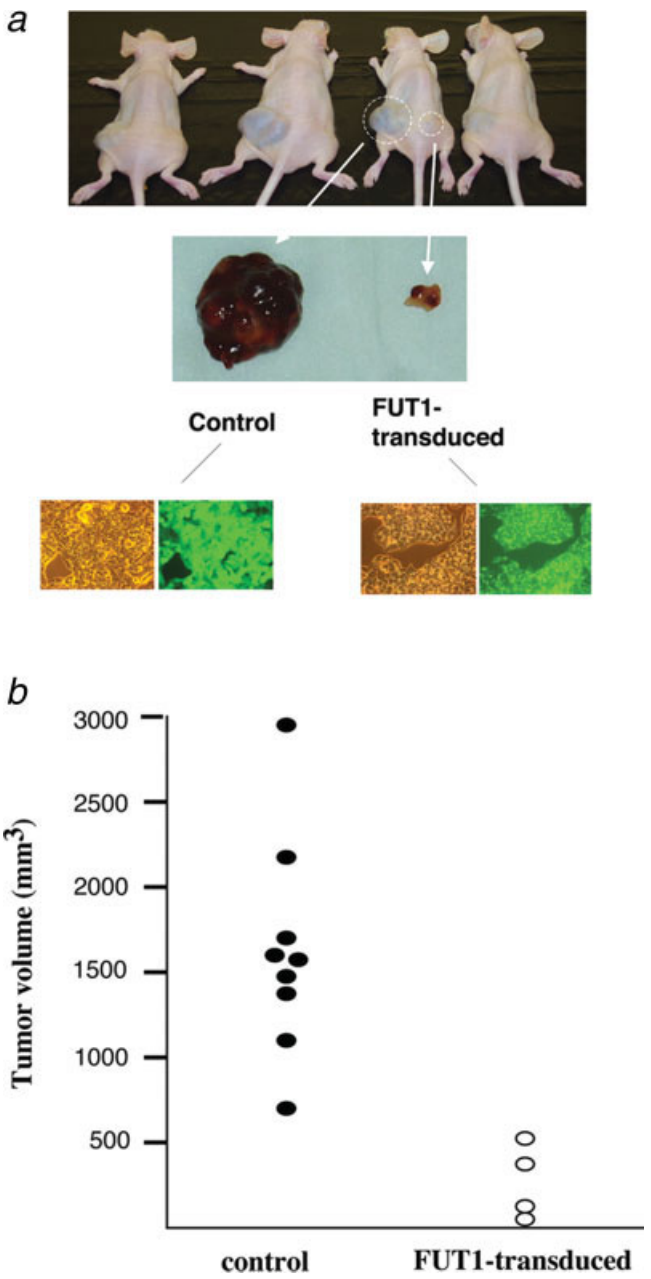

FIGURE 2 – Effect of FUT1 expression on hepatocarcinoma tumor growth. (*a*) *Top*, Photograph of 4 repesentative nude mice, 8 weeks after s.c. inoculation of HCC. Mice (12 animals in each experiment) were injected with 10^7 cells of either parental (in the left flank) or FUT1-expressing HepG2 cells (in the right flank). Tumors were allowed to develop for 8 weeks and the animals were sacrificed thereafter. *Middle,* one of the tumors grown from FUT1-transduced cells (*right*) is presented for size comparison with its parental counterpart (*left*). *Bottom*, persistence of the transgene expression in the tumors. The tumor nodules were enzymatically dispersed into single cells and cultured under standard conditions. Presented are parental cells expressing EGFP (*left*) and those expressing the FUT1-EGFP fusion protein (*right*). (*b*) A representative plot showing the strong FUT1-dependent inhibition of HCC tumor growth. Note that 9 out of 12 animals that have been inoculated with HepG2 cells, developed tumors with sizes ranked from 500 to 3,000 mm^3 while in the same period, only 4 small size tumors appeared on animals injected with FUT1-transduced cells. The statistical significance was determined by the Student's test $p < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

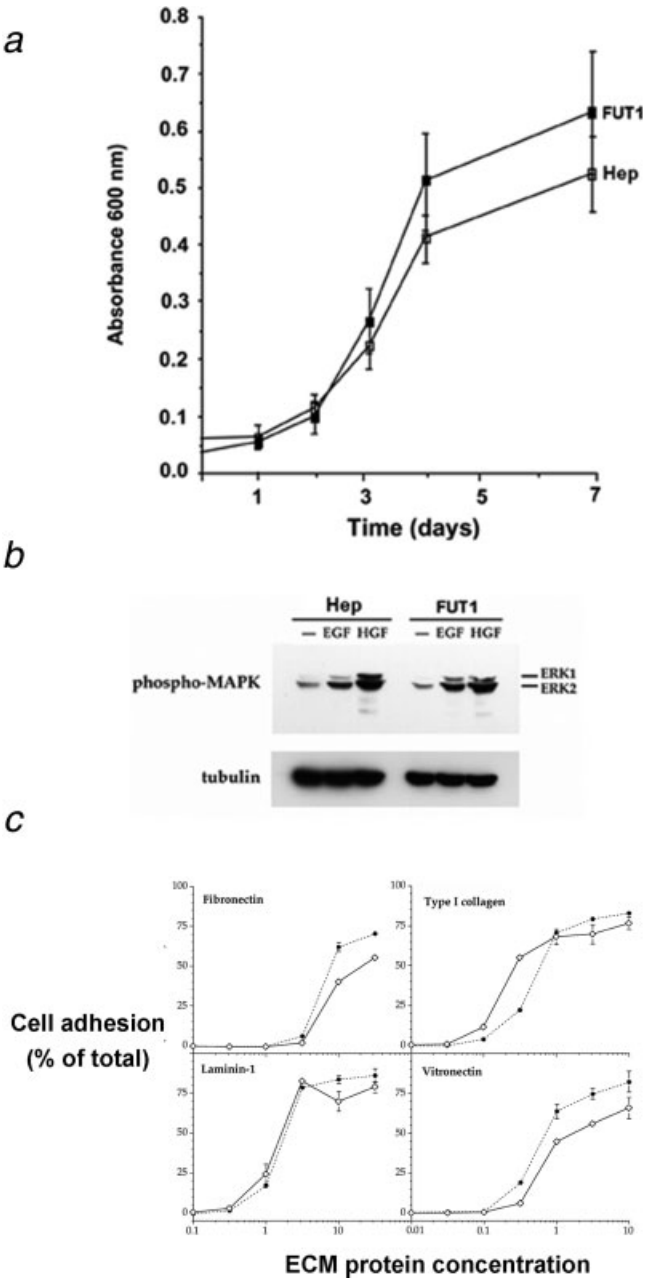

FIGURE 3 – Effect of FUT1 expression on the growth rate, and adhesion to ECM proteins. (*a*) Comparison of growth rates of parental and FUT1-transduced cells. Cells were seeded at a density of 10^4 cells/well in 96-multiwell culture plates. At the indicated days, the media were removed and cells were fixed in 1% glutaraldehyde, stained by 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured with an ELISA microplate-reader at 600 nm. (*b*) MEK/MAPK assay following incubation of parental (Hep) and FUT1-transduced cells (FUT1) without (−) or with EGF or HGF. (*c*) Adhesion of parental (squares) and FUT1-expressing cells (dots) to purified extracellular matrix (ECM) proteins. Adherent cells were counted by the crystal violet technique as described in (*a*) and plotted *versus* protein concentration.

difference in growth rates *in vitro* between the two cell lines. Rather, FUT1-transduced cells grow even slightly faster than their parental counterparts, especially between the 4th and the 7th day of culturing. This result was further confirmed by MEK/MAPK assay, the activation of which has been shown to correlate with cell proliferation.[30] Here also, there is no difference in the extent of ERK1/2 phosphorylation upon stimulation of both cells with either the epidermal growth factor (EGF) or the hepatic growth factor (HGF) (Fig. 3*b*). Other stimulating reagents, including FCS, Insulin-like Growth Factor (IGF) or the phorbol ester PMA, were used without significantly differentiating between the two cell lines (data not shown).

Cell adhesion molecules and extracellular matrix components have pivotal roles in tumor growth and metastasis and are glycosylated molecules. Therefore and having previously shown that adhesion of cancer cells to ECM proteins was influenced by the glycosylation state of integrins,[31] we investigated whether the effect of FUT1 could be related to an altered interaction with ECM components. Parental and FUT1-expressing cells were compared with regard to adhesion to fibronectin, laminin-1, type-1 collagen and vitronectin and results are presented in Figure 3*c*. Again, with the exception of adhesion on vitronectin and fibronectin, which is slightly increased for FUT1-expressing cells, no significant difference could be noticed between the two cell lines. Altogether, these data suggest that neither cell growth nor apoptosis or ECM-mediated adhesion may account for the anti-tumorigenic effect of FUT1.

The FUT1-induced tumor growth inhibition and/or retardation could be because of an increased susceptibility to apoptosis. To address this issue, we incubated cells with three well-known death-inducers that are TNF-α, TRAIL and anti-Fas antibody.[32] We thus evaluated the susceptibility to the inducers of parental and FUT1-expressing cells by detection of cleaved caspase-3 and by DNA fragmentation. In preliminary experiments, we found that none of the molecules was efficient unless cells were first sensitised by IFN-γ, consistent with data reported by others and pointing to the remarkable resistance of HepG2 cells to death-inducers.[33] Yet, among the three inducers, only TRAIL was found to trigger a detectable effect either on caspase-3 activation or DNA fragmentation. In fact, as shown in Figure 4, the expected 17 and 19-KDa cleaved forms of caspase-3 are obtained after incubation with TRAIL for 48 hr (Fig. 4*a*). No such an effect could be seen with either TNF-α or anti-Fas antibody (data not shown). However, no significant difference in caspase-3 activation could be seen between parental and FUT1-expressing cells, indicating that the introduction of FUT1 gene in HepG2 cells does not increase their susceptibility to the apoptotic agents. Interestingly, when the DNA fragmentation was examined, the parental cells appeared even more sensitive to TRAIL than their FUT1-infected counterparts (Fig. 4*b*), consistent with previously reported data on FUT1-mediated resistance to apoptosis of the rat colon cancer cells.[34] Lastly, the immunohistogical staining with the anti-caspase-3 pAb of paraffin-embedded sections does not show more apoptotic bodies in one than in the other tumor (Fig. 4*c*). Taken as a whole, these data do not allow linking the anti-tumorigenic effect of FUT1 to an increased susceptibility to apoptosis.

Vasculogenesis is inhibited in FUT1-derived tumors

We next sought to explore the possibility of a defect in tumor angiogenesis as it has been reported that E-selectin is associated with essential events of angiogenesis, including endothelial cell proliferation and migration.[6–9] To assess vascularization, frozen sections of the tumors were stained for the mouse CD31, an endothelial cell determinant and examined by fluorescence microscopy. As shown in Figure 5*a*, there is a striking difference between the tumors with respect to the vascular architecture and microvessel density. In fact, while many mouse CD31-positive cells are present in both tumors, they clearly achieved mature phenotype in control tumors but not in FUT1-derived ones. These data suggest that either i) new vessels are first established in FUT1-tumors and further eliminated or ii) the microenvironment of FUT1-expressing tumors does not allow endothelial cells to undergo differentiation towards vasculogenesis.

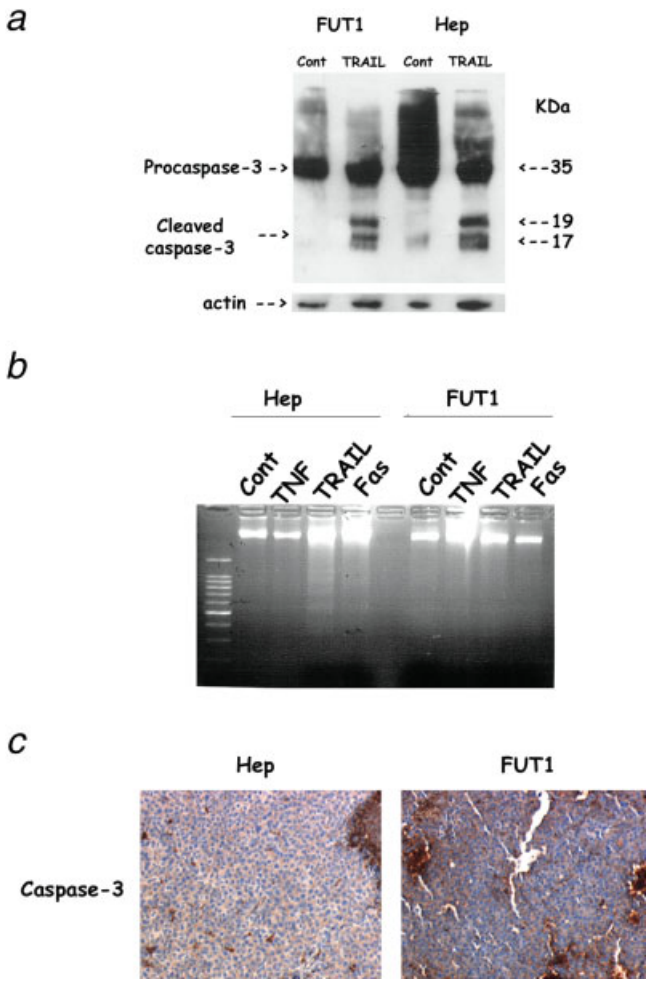

FIGURE 4 – Caspase-3 activation and DNA fragmentation. (*a*) Parental (Hep) and FUT1-transduced HepG2 cells (Hep) were incubated with 40 ng/ml IFN-γ for 15 min at 37°C and treated without (cont) or with the TRAIL (100 ng/ml) for 48 hr. The cleaved caspase-3 was then detected by western blotting as described under Material and Methods section. (*b*) Cells were incubated as earlier without (cont) or with TNF-α (50 ng/ml), TRAIL (100 ng/ml), or anti-Fas mAb (100 ng/ml) for 48 hr and prepared for DNA fragmentation assay. (*c*) Immunohistological studies of apoptosis. Tumors from parental (Hep) and FUT1-expressing cells (FUT1) were paraffin-embedded, sectioned and stained for the active caspase-3 as described under Material and Methods section. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

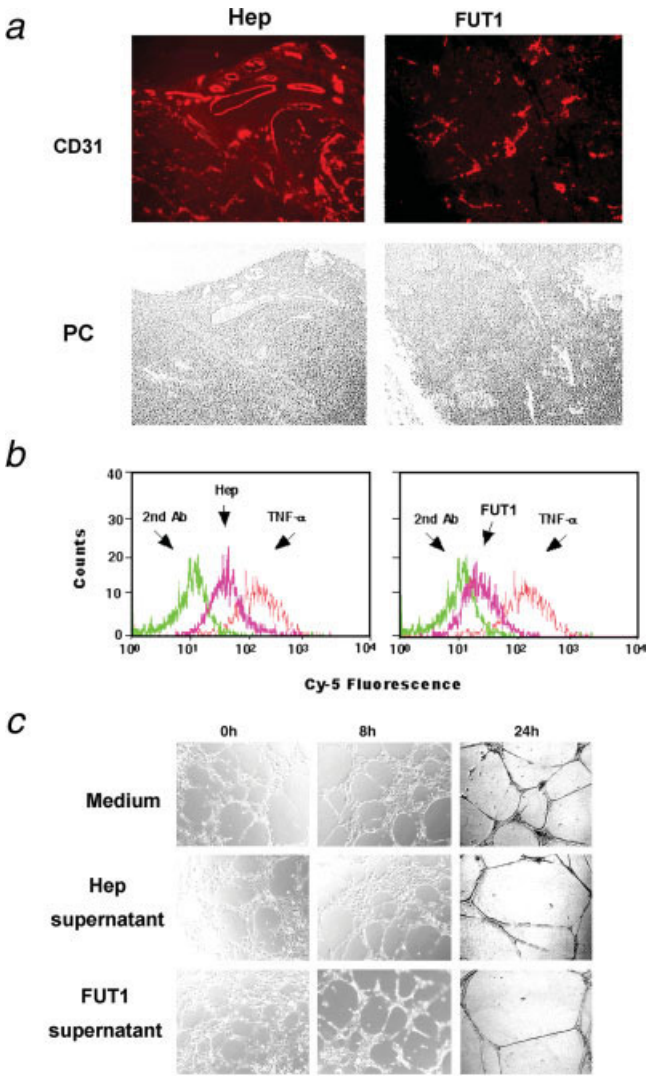

FIGURE 5 – Analysis of vasculogenesis, E-selectin induction and *in vitro* tubulogenesis. (*a*) Immunohistological analysis of tumor vascularization. Cryosections of tumors from parental (Hep) and FUT1-transduced cells (FUT1) were immunostained with the anti-mouse CD31 as described in Material and Methods section and visualised by fluorescence microscopy. PC: phase contrast. (*b*) FACS analysis of E-selectin expression induced on HUVEC following HUVEC/HCC coculture. HCC and HUVEC were mixed at the ratio of 4:1 and cultured for 5 days. Cells were then harvested and stained with the anti-human E-selectin mAb BBIG-E4, followed by cyanin-5 (Cy5)-conjugated goat anti-mouse IgG. Autofluorescence (denoted as 2nd Ab) was determined by incubating cells without the anti-E-selectin mAb. FACS data of either parental (Hep) or FUT1-transduced cells (FUT1) were compared to fluorescence of HUVEC incubated with 10 ng/ml TNF-α for 4 hr at 37°C and examined for E-selectin expression (see TNF-α). (*c*) Effect of culture media on microtubular formation by HUVEC in Matrigel. The *in vitro* Matrigel-based capillary-like tube assay was carried out as described in Material and Methods section. HUVEC were incubated with the fresh medium (medium) or the conditioned media from either parental cells (Hep supernatant) or FUT1-infected cells (FUT1 supernatant) and viewed in phase contrast at 0, 8 and 24 hr later. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The latter possibility could depend on the abolition of E-selectin-mediated HCC/endothelial cell interaction. In fact, it is known that human digestive cancer cells secrete humoral factors that can induce endothelial E-selectin expression,[10] which in turn can support HCC/endothelial cell interaction. Therefore, we cocultured parental and FUT1-transduced cells with HUVEC to allow for direct and continuous cell–cell and cell–microenvironment interactions. The expression of E-selectin on HUVEC was then analysed by FACS. As shown in Figure 5*b* (left panel), coincubating HepG2 cells with HUVEC induces a significant amount of E-selectin on the endothelial cells, albeit to a lesser extent than the level reached with TNF-α. However, in the presence of FUT1-infected cells (Fig. 5*b*, right panel) there is a reduced effect on E-selectin expression compared to control cells. This result suggests that FUT1-expressing cells secrete less E-selectin inducers than their parental counterparts and thus, the proangiogenic activity of FUT1-derived medium might be weaker than the medium from parental cells. We therefore compared the conditioned media with respect to their proangiogenic activities *in vitro*, by incubating them with HUVEC for different periods. As shown in Figure 5*c*, the supernatant from parental cells and FUT1-transduced cells have comparable effects on tubule formation, indicat-

ing that both supernatants have similar proangiogenic potentials, *in vitro*. These results suggest that the *in vitro* capillary-like tube formation i) is not relevant to E-selectin induction on HUVEC and ii) does not reproduce the inhibition of vasculogenesis by FUT1 *in vivo*.

E-selectin ligand characterization

The data presented earlier suggest that whereas mouse endothelial cells are able to infiltrate both tumors, their ability to form vessels (vasculogenesis) is strongly impaired in the presence of FUT1-transduced cells. We then hypothesised that the defect in E-selectin induction (see Fig. 5*b*) and the loss of E-selectin binding (see Fig. 1*a*, right panel) would provide an explanation for the anti-angiogenic effect of FUT1 and hence, the molecule(s) bearing sLex determinants, might play a central role in this phenomenon. So far, a variety of sLe^x-carrier species, including *N*- and *O*-glycoproteins, as well as glycolipids, have been assigned to E-selectin ligands (counter-receptors) in the immune system.[1] However, only few structural data are available on those molecules expressed on cancer cells in general, and on HCC in particular. Therefore, the next set of experiments was aimed to collect structural information on the E-selectin ligand(s) expressed on HepG2 cells, the glycosylation and function of which might have been modified by FUT1-catalysed α(1,2)-fucosylation.

Since E-selectin binding is abolished as a consequence of FUT1 introduction in HepG2 cells, the E-selectin counter-receptor should have been changed into an α(1,2)-fucosylted glycoconjugate and thus, it is possible to isolate this molecule by the mean of the α(1,2)-fucose specific lectin UEA-1. For this purpose, parental and FUT1-transduced cells were metabolically labeled with ^{35}S-methionine and detergent extracts were prepared and subjected to UEA-1 affinity chromatography. As shown in Figure 6*a*, a significant amount of ^{35}S-labeled material from FUT1-expressing cells is retained on the column and is specifically eluted with 0.1 M fucose, whereas roughly no radioactivity is eluted from the column loaded with HepG2 cell extract, consistent with results from Figure 1*a*, indicating that these cells do not express α(1,2)-fucosylated glycoconjugates. Fluorography of samples from total cell extract, flowthrough and fucose-eluted fractions is shown in Figure 6*b*. Again, no ^{35}S-labeled proteins could be seen in eluted fractions (Elu) from parental cells, while three major proteins of 50, 80 and 150 kDa were retained on UEA-1 column from FUT1-expressing cell extract. These data are in accordance with those from MAL staining and GC-MS analysis (see Fig. 1*c*, and Table I), confirming that indeed, only few molecules are fucosylated in HepG2 cells as a result of FUT1 activity.

To determine which of the 3 proteins carry sLe^x antigens that could serve as E-selectin counter-receptor, total cell extracts were resolved on SDS-PAGE, blotted and membranes were probed with the anti-sLe^x mAb KM-93. As shown in Figure 6*c*, only two protein species of 80–90 and 150 kDa originating from HepG2 cells exhibit an immunoreactivity towards KM93, indicative of the presence of the glycoantigen sLe^x on these molecules. As expected, the KM93 staining disappeared when material from FUT1-infected cells is tested; except for a 200 kDa species that does not correspond to any one of the KM93-positive proteins. Together, the earlier results suggest that the molecule mediating E-selectin interaction with HepG2 cells might i) be one of the two 80–90 kDa and 150 kDa proteins or both and ii) have their sLe^x structures changed by FUT1, so that they can no longer interact with the endothelial E-selectin.

Interestingly, it has been recently reported that CD44, a receptor of the extracellular matrix hyaluronan having multiple angiogenesis-related properties, comprizes two 80–90 and 150 kDa variants that function as high affinity E- and L-selectin ligands on colon carcinoma cells.[35] HepG2 cells, which also interact with both E- and L-selectin and express two major sLe^x-protein carriers of 80–90 and 150 kDa (this study), has been reported to express the CD44 proteins.[36] It is therefore tempting to investigate whether our two KM93-positive proteins are related to the CD44. In a preliminary set of experiments, we tentatively explored this possibility by targeting the CD44 molecule with two widely used anti-human CD44 antibodies defining distinct epitopes of the proteins [*i.e.*, the J.173 mAb (Coulter-Immunotech, Marseille, France) and the F10-44-2 mAb (Southern Biotech, Birmingham, AL)]. Unexpectedly, none of the J.173 mAb or the F10-44-2 mAbs stains HepG2 cell surface while, under the same conditions, the colon carcinoma cells HT29 exhibit a strong immunoreactivity towards the antibodies (data not shown). Other investigations in this direction, including proteomic research are ongoing to yield insights into the structure of the 80–90 and 150 kDa proteins described in this study.

In this regard and to further assess whether the E-selectin binding sites are carried by *O*-linked glycoproeins (mucin-type) or *N*-glycosylated proteins, we used the benzyl-α-*N*-acetylgalactosamine (Benzyl-GalNAc), which inhibits *O*-linked oligosaccharide elongation or the α-mannosidase-I inhibitor deoxymannojirimycin (dMJ), which blocks the maturation of *N*-linked oligosaccharides.[32] Cells were cultured for 72 hr with the drugs and assayed for E-selectin-IgM binding and analysed by FACS as described.[14] As shown in Figure 6*d*, benzyl-GalNAc strongly inhibits E-selectin binding (9.6% remaining activity) whereas dMJ showed no effect. Together with the sialic acid-dependence of HepG2/E-selectin interaction (see Fig. 1*b*), these data suggest that the E-selectin counter-receptors expressed on HepG2 cells are sialylated *O*-glycoproteins, the so-called sialomucins, thus sharing this feature with other previously described selectin ligands.[1,2]

Discussion

The prominent findings of this study are i) FUT1 prevents HepG2 tumor growth in a nude mice model, ii) the anti-tumorigenic effect of FUT1 is due to a defect in vasculogenesis, iii) although strongly inhibiting the sialylation of sLe^x precursors, FUT1 has a minimal effect on the overall sialic acid content and iv) three major glycoproteins were identified as substrates for FUT1, two of which (presumably sialomucins) carry sLe^x structures that may support E-selectin binding and could be candidates to play a critical role in tumor growth of HCC.

The synthesis of sLe^x-decorated *O*-glycans is controlled by an ordered series of sugar transfer reactions, acceptor specificities and synergistic/competition mechanisms between glycosyltransferases.[13,37] It is proposed that glycosyltransferases are organised in the same order in which they act; such that those acting early in glycan biosynthesis are localised to the *cis* and *medial* compartments, whereas enzymes acting later tend to localize to the *trans*-Golgi cisternae and the *trans*-Golgi network (reviewed in[38]). Accordingly, the sLe^x-specific α(2,3)-sialyltransferases (denoted as ST3GalTs) have been proposed to localize to the late Golgi compartments[39,40] and our earlier studies have shown that FUT1 overlaps very well with the α-mannosidase-II and the core2 β(1,6)-*N*-acetyglucosaminyltransferase (C2GnT-I) in the cis/medial Golgi compartments.[41] Hence, our strategy exploits the facts that FUT1 shares the same acceptor specificity with the sLe^x-acting ST3GalTs while being localised up-stream to these enzymes. Unexpectedly and despite this fact, the sialic acid content of FUT1-transduced cells is only slightly decreased compared to parental cells, indicating that a few set of glycoconjugates are actually α(1,2)-fucosylated by the enzyme.

To find out how FUT1 affects hepatocarcinoma tumor growth, we examined several parameters, including cell proliferation, apoptosis, adhesion to extracellular matrix proteins and angiogenesis/vasculogenesis. We provide evidence that among all, vasculogenesis was the most clearly affected by FUT1 expression. In fact, using the mouse-specific CD31-immunoreactivity, control tumors appear as hypervascularised with a well organised network of capillaries whereas FUT1-derived tumors exhibit sparse groups of endothelial cells with no visible blood vessel formation (see

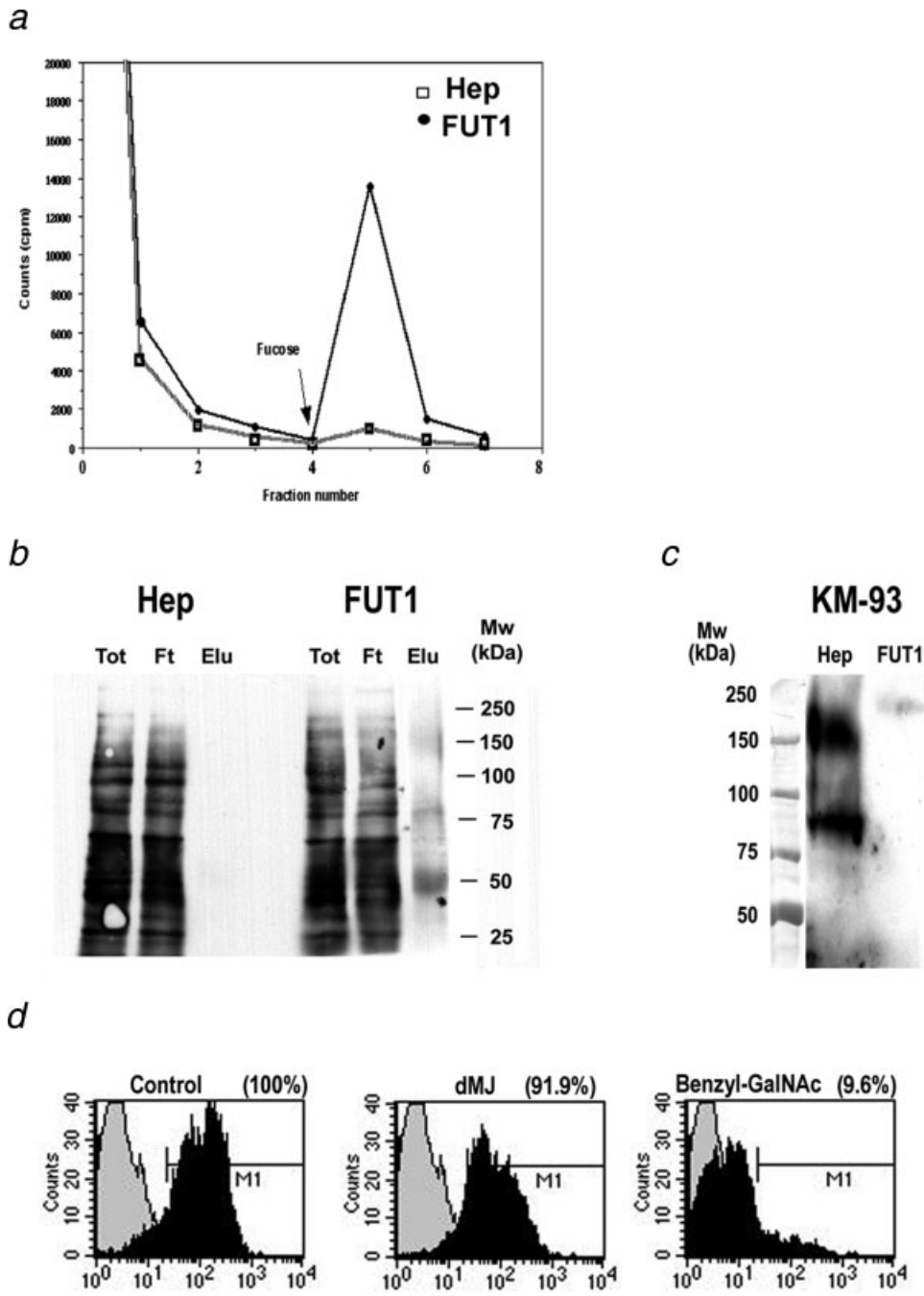

FIGURE 6 – E-selectin ligand characterization: (*a*) Cell extracts were prepared from ^{35}S-methionine-labeled parental (Hep) or FUT1-transduced HCC (FUT1) and incubated batchwise with UEA-I-Agarose beads for 2 hr at 4°C. The rinse fractions (flowthrough) and the eluates (Fucose) were collected and radioactivity was monitored by scintillation counting. (*b*) The flowthrough (FT) and the fucose-eluted (Elu) fractions were collected and compared to a sample from total extract (Tot) by SDS-PAGE and fluorography. (*c*) Sample extracts from parental (Hep) and FUT1-expressing cells (FUT1) were separated by SDS-PAGE and analysed immunoblotting with the anti-sLe^x mAb KM-93. The molecular weight standards are shown on the figure sides of (*b*) and (*c*). (*d*) FACS analysis of E-selectin binding sites: HepG2 cells were cultured with benzyl-GalNAc or deoxymannojirimycin (dMJ) for 72 hr and assayed for E-selectin binding. The numbers between brackets indicate the remaining E-selectin binding activity.

Fig. 4). This suggests that mouse endothelial cells are able to infiltrate FUT1-derived xenografts but they are not able to undergo vasculogenesis, likely because of the scarcity or inefficiency of proangiogenic factors present in the tumor microenvironment. This possibility has been ruled out since both cell-derived conditioned media were found to have comparable effects on tube formation by HUVEC. Yet, factors secreted from FUT1-cells have less potency in inducing E-selectin expression on HUVEC in coculture experiments (see Fig. 4*b*), providing a possible involvement of E-selectin-mediated adhesion in tumor vasculogenesis.

Other groups have previously reported on the effects of FUT1 in cancer cells particularly on sensitivity to apoptosis[34,42] and anticancer drugs.[43] Regarding apoptosis, our data clearly diverge from those of Goupille *et al.*[42] who observed an increased tumorigenicity

in the presence of FUT1 while we herein show that, on the contrary, FUT1 expression induced a dramatic anti-tumorigenic effect. Since this group used colon carcinoma cells, the explanation for this discrepancy may reside in the cell line used. Nevertheless, our and their results are consistent with respect to the fact that FUT1-expressing cells exhibited an increased resistance to apoptotic inducers.[34] Other studies showed that blocking α(1,2)-fucosylation increased cell sensitivity to anticancer drugs.[43] Interestingly, we have previously shown that FUT1 affected E-selectin-mediated adhesion in some cell lines, including HepG2 cells (see also the present study) and the colon carcinoma cells HT29, but had no effect on other cells, such as pancreatic cancer cells BxPC3,[11] suggesting that the effect of FUT1 is cell-dependent.

Accumulating evidence suggests that circulating endothelial progenitor cells (EPC) contribute to vascular healing and remodelling under physiopathological conditions and, E-selectin-mediated adhesion has been clearly implicated in the initial cell arrests and recruitment of EPC.[44] Accordingly, since blood vessels are not formed in FUT1-derived tumors despite of the presence of murine endothelial cells (see Fig. 4*a*), it is possible that the CD31-positive cells are originating from the circulating EPC. However, it is still unclear how these cells could infiltrate FUT1-expressing cells since the latter do not express sLe^x and do not display E-selectin-dependent interaction with endothelial cell.[11] In addition to cell–cell adhesion, the angiogenic processes require numerous levels of regulation through gene expression of growth factors (particularly VEGF) and growth factor receptors. One or several of these features might be modified by FUT1-catalysed α(1,2)-fucosylation. In this regard, both VEGF and VEGF receptor are glycosylated proteins and the role of glycosylation on VEGF structure and function has been addressed. Interestingly, it was found that glycosylation is important for effective secretion of this molecule.[45] Further studies will focus on which of the proangiogenic factors are actually modified by FUT1 and the impact of this modification on their functions *in vivo*.

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