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RNA-Mediated Gene Silencing of FUT1 and FUT2 Influences Expression and Activities of Bovine and Human Fucosylated Nucleolin and Inhibits Cell Adhesion and Proliferation

Domenico Palumberi,[1] Silvia Aldi,[1] Leonardo Ermini,[1] Marina Ziche,[2] Federica Finetti,[2] Sandra Donnini,[2] and Floriana Rosati[1*]

[1]*Laboratory of Cell Biology and Glycobiology, Department of Evolutionary Biology, University of Siena, Siena, Italy*
[2]*Laboratory of Farmacology, Department of Molecular Biology, University of Siena, Siena, Italy*

ABSTRACT

In a previous article, we demonstrated the existence of fucosyl-containing *O*-glycans forms of nucleolin in bovine post-capillary venular endothelial cells (CVEC) and malignant cultured human A431 cells. The tool for this discovery was an antibody found to interact strongly and exclusively with nucleolin in total protein extracts. The antibody was originally raised against a mollusc glycoprotein and was demonstrated to be directed against its *O*-glycans, recently found to belong prevalently to the blood group H-antigen type with fucose linked in alpha1, 2 to galactose. Here, we show that si-RNA induced down-regulation of the expression of FUT1 and FUT2, the fucosyltransferases required for the biosynthesis of the terminal glycan motif Fucα-2-Galβ-R, reduced expression of the fucosylated nucleolin glycoforms and their exposure at the cell surface in CVEC. Treatment of the cells with FUT1/2 siRNA also reduced their ability to bind and internalize endostatin and their adhesion efficiency and inhibited cell growth. Expression of FUT1, FUT2, and FUT6 was also analyzed in serum-stimulated versus serum-starved cells and in cells treated with FUT1 and FUT2 siRNA. A reduced expression of fucosylated nucleolin and inhibition of cell growth by suppressing FUT1/2 expression was also tested and shown to be exhibited in human A431 cells. J. Cell. Biochem. 111: 229–238, 2010.

KEY WORDS: NUCLEOLIN; FUCOSYLGLYCOCONJUGATES; FUCOSYLTRANSFERASES; siRNA

Nucleolin, first described by Orrick et al. [1973], is a single copy gene encoded protein [Bourbon et al., 1988] present in the nucleolus where it is primarily related to ribosome biogenesis. A role of nucleolin in DNA replication, recombination, and repair has also been suggested as a result of its ability to bind directly both DNA and proteins involved in these processes. Several observations indicate that nucleolin is a major actor in promoting proliferation. Firstly, nucleolin levels are higher in tumors and actively dividing cells [Derenzini et al., 1995; Sirri et al., 1997]; secondly, over-expression of nucleolin co-operates with oncogenic mutant Ras in a rat embryonic fibroblast transformation assay [Takagi et al., 2005] and thirdly, the anti-proliferative activity of G-rich oligonucleotids (GROs) is perfectly correlated with their ability to bind and perhaps to sequester nucleolin [Bates et al., 1999; Dapić et al., 2003]. However, there is no indication on which of the nucleolin activities are responsible for proliferation.

Nuclear functions are only part of the nucleolin activities [Srivastava and Pollard, 1999]. It has been demonstrated that inactivation of nucleolin also leads to cell cycle arrest and defects in centrosome duplication [Ugrinova et al., 2007]. Moreover, it has recently been indicated that nucleolin is also capable of translocation to the cell surface membrane and an association between cell surface expression and proliferation has been described [Hovanessian et al., 2000]. At cell surface, nucleolin is considered a marker of endothelial cells in angiogenic blood vessels [Christian et al., 2003]. On the plasma membrane nucleolin serves as binding protein for various ligands such as ApoB and apoE-containing lipoproteins on HepG2 cells [Semenkovich et al., 1990], anti-HIV cytokine midkine [Said et al., 2002], lactoferrin [Legrand et al., 2004], L-selectin [Harms et al., 2001], and also the tumor-homing peptide F3 [Christian et al., 2003]. For HIV, cytokine midkine, lactoferrin, and tumor-homing peptide F3, endocytosis of nucleolin with relative

Palumberi and Aldi as coauthors.
*Correspondence to: Prof. Floriana Rosati, Department Evolutionary Biology, University of Siena, Via A. Moro 2, 53100 Siena, Italy. E-mail: rosatif@unisi.it
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ligands and nuclear targeting have been demonstrated thus suggesting for nucleolin a shuttle function between surface and nucleus [Hovanessian et al., 2000; Said et al., 2002]. Nucleolin also undergoes *N*- and *O*-glycosylation. Carpentier et al. [2005] demonstrated that part of the nucleolin present in the extranuclear compartment of Jurkat cells bears complex *N*- and *O*-glycans and more recently that glycosylation is an essential requirement for surface nucleolin expression [Losfeld et al., 2009]. As for other cytosolic and nuclear glycoproteins the biosynthetic pathway of the glycosylated forms of nucleolin is still largely unknown. The lack of a signal sequence for entering the endoplasmic reticulum but at the same time the ability to localize to the plasma membrane support the hypothesis that nucleolin follows no classical glycosylation and surface localization pathways [Carpentier et al., 2005].

In a very recent article, we demonstrated in cultured bovine endothelial cells (CVEC) and malignant cultured human A431 cells that fucosylated glycoforms of nucleolin exist both in nuclear and extranuclear regions [Aldi et al., 2009]. The tool for this discovery was an antibody raised against gp273, a glycoprotein ligand for the sperm–egg interaction in the mollusc bivalve *Unio elongatulus*. The function and immunological properties of gp273 were first indicated to depend on clustered Lewis-like, fucose-containing *O*-glycans [Focarelli and Rosati, 1995; Focarelli et al., 2003]. Structural analysis of the *O*-glycans of gp273 has recently confirmed that most of these structures are indeed rich in fucose but has also demonstrated that fucose residues are mostly found in the context of the blood group H-antigen type with fucose residues linked in alpha1, 2 to galactose [Tissot et al., 2009]. We then postulated that alpha1, 2 fucosyl-containing glycoepitopes were also present in nucleolin and for this reason we decided to investigate the role of alpha1, 2 fucosyltransferases in nucleolin activities. Two different, closely linked loci encode the fucosyltransferases that give rise to the H antigens; both loci encode closely homologous alpha1, 2 fucosyltransferases (FUT1 and FUT2) that transfer fucose in an alpha1–2 linkage to galactose and result in products whose structure is nearly identical.

In this article, we report on how FUT1 and/or FUT2 are involved in nucleolin fucosylation and how this fucosylation influences nucleolin dynamics and activities. Initially, we investigated the relationships between expression levels of mRNA of FUT1, FUT2, and nucleolin in serum-stimulated cell cultures. We also analyzed as a control the alpha(1, 3) fucosyltransferase FUT6. We then investigated nucleolin expression and location in addition to cell proliferation and adhesion in cells after transiently knocking-down of FUT1, FUT2, or both of them.

MATERIALS AND METHODS

CELL LINES

Bovine post-capillary venular endothelial cells (CVEC) were isolated and characterized as previously described [Schelling et al., 1988]. Human epidermoid carcinoma A431 cells were obtained by ATCC (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4,500 mg/L (A431) or 1,000 mg/L (CVEC) glucose, antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin), 2 mM L-glutamine (complete medium), 10% fetal calf serum (FCS; A431) or 10% bovine calf serum (BCS, CVEC; Hyclone, Logan, UT). CVEC were maintained in culture in 1% gelatine-coated dishes. All media and antibiotics for cell culture were purchased from Sigma–Aldrich (St. Louis, MO). Cells were split 1:3 (CVEC) or 1:8 (A431) twice a week.

siRNA TRANSFECTION

Cells were plated at 2.5×10^5 in 60-mm-diameter dishes and transiently transduced with siRNA directed against human FUT1 and FUT2 in the presence of Hiperfect transfection reagent (Qiagen, Milan, Italy) according to the manufacturer protocols. Twelve, 24, 48, and 72 h post-transfection cells were trypsinized, counted, and used for subsequent analyses. siRNA duplex oligoribonucleotides targeting human FUT2 siRNA has been designed by Ambion (Austin, TX) using an all-new algorithm that was developed utilizing the latest in machine-learning methods starting by FUT2 mRNA sequence (GenBank: AK290277.1). Off-target activity was determinated by microarray analysis and cell-based assays. FUT1 siRNA were purchased pre-designed from Invitrogen (Milan, Italy). Control siRNA is a random siRNA provided by Ambion.

ADHESION STUDY

CVEC were evaluated for their ability to adhere after silenced for FUT1 and FUT2. A 96-well microtiter plate was coated with 1% gelatine and washed with sterile PBS. A cell suspension containing 5×10^4 silenced CVECs was plated in DMEM supplemented with 1% BCS. After 1 h of incubation the plate was harvested and cells were fixed with methanol and stained with Diff Quick. The total number of adherent cells was counted with the aid of an ocular grid (0.21 mm^2). Each experiment was run in triplicate.

WESTERN BLOT ANALYSIS

Total protein from whole cell lysate was prepared and applied for Western blotting. In brief, cells prepared as indicated were washed, with phosphate-buffered saline (PBS, 150 mM NaCl, 50 mM KH_2PO_4, pH 7.4), scraped in lysis buffer [(10 mM Tris–HCl, pH 8.0, 1% Triton-X 100, 400 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmetylsulphonylfluoride (PMSF), protease inhibitor cocktail (Sigma–Aldrich)] and incubated for 30 min on ice. Cell lysates were clarified by centrifugation at 13,000*g* for 15 min at 4°C, and the supernatant was collected. Protein concentration was determined with Bicinchoninic Acid (BCA) protein assay reagent kit (Sigma–Aldrich) using bovine serum albumin as a standard. Total proteins (10–40 μg) from the whole cell lysate was separated by 8% SDS–PAGE mini-gel, transferred to a nitrocellulose membrane [Towbin et al., 1979] and probed with a specified primary antibody. Antibodies used are mouse monoclonal anti-nucleolin MS-3 (Santa Cruz Biotechnology, Inc., CA), anti-gp273 [Focarelli et al., 2003] serum and corresponding IgG purified as previously described [Aldi et al., 2009], polyclonal rabbit IgG anti-actin (Sigma–Aldrich) and goat polyclonal IgG anti-FUT2 (Santa Cruz Biotechnology) diluted 1:200, 1:1,000, 1:5,000, and 1: 200, respectively. Bound antibodies were visualized with horseradish peroxidase(HRP)-conjugated goat anti-mouse IgG (Fab specific; Sigma–Aldrich), goat anti-rabbit IgG (BioRad Microscience, Cambridge, MA), rabbit anti-goat IgG (Zymed Laboratories, CA) diluted 1:3,000, 1:20,000, and 1:5,000,

respectively. After extensive rinsing in TTBS, labeled proteins were developed using Immun-Star HRP Chemiluminescent Kit (BioRad Microscience) according to the manufacturer's instructions. Images were digitalized with CHEMI DOC Quantity One programme.

IMMUNOPRECIPITATION

CVEC silenced with FUT1/FUT2 and with scrambled siRNA for 24 h was treated with 50 ng/ml of endostatin (Sigma–Aldrich) for 3 h. After treatment, cells were rinsed with Dulbecco's PBS (Sigma–Aldrich) and lysated into 60 μl of lysis buffer [10 mM Tris–HCl, pH 8.0, 1% Triton-X 100, 400 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmetylsulphonylfluoride (PMSF), protease inhibitor cocktail (Sigma–Aldrich)]. Five hundred microgram of cell lysate were subjected to overnight immunoprecipitation at 4°C with an anti-endostatin rabbit polyclonal antibody (Santa Cruz Biotechnology). Immune complexes were then captured by adding Dynabeads Protein G (Invitrogen, Norway) to the sample and incubating for 1 h at 4°C, followed by magnetic separation. After incubation, beads were washed three times with PBS (150 mM NaCl, 50 mM KH_2PO_4, pH 7.4). The samples were boiled for 5 min and analyzed by SDS–PAGE and Western blotting using the monoclonal anti-nucleolin antibody MS-3.

IMMUNOFLUORESCENCE

Cells (2.2×10^4) were seeded on cover slips in a 24-multiwell plate and cultured for 24 h in appropriate medium. Cells were silenced with siRNA directed against FUT1 and FUT2 and cover slips harvested after at different time intervals. Cells were washed three times with cold PBS and fixed with 4% paraformaldehyde (PFA) for 1 h at 4°C and extensively washed in PBS. After fixing, to monitor intracellular nucleolin, cells were permeabilized in 0.5% Triton X-100 in PBS for 5 min at 4°C. After blocking no specific binding in 3% goat serum for 30 min, cells were incubated with anti-nucleolin (1:50) and anti-gp273 (1:100) diluted in 1% goat serum for 1 h at RT. Cells were then washed in PBS and bound antibodies were detected with secondary antibodies coupled to Alexa Fluor dyes A555 (goat anti-mouse IgG) and A488 (goat anti-rabbit IgG; Invitrogen, OR) both diluted 1:100 and incubated for 30 min at RT. Nuclei were identified with Hoechst 3342 1 μg/ml (Sigma–Aldrich). Finally, the cover slides were mounted in Mowiol 4–88 (Calbiochem, La Jolla, CA) and visualized by fluorescence microscopy Leitz Diaplan (Leica, Heidelberg, Germany), and confocal microscope LSM-510 META (ZEISS, Jena, Germany). The images were processed in Photoshop 5.5 (Adobe Systems, Mountain View, CA).

FACS ANALYSIS

Cells (2×10^5), harvested by subconfluent culture dish or after silencing with siRNA targeting FUT1 and FUT2, were used for flow cytometric analysis. Single-cell suspensions were stained with anti-nucleolin MS-3 or anti-gp273, followed by Alexa Fluor A488-conjugated goat anti-rabbit or anti-mouse IgG (Invitrogen) and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

TABLE I. Primers Used for RT-PCR in this Study

Gene	Forward	T_m (°C)
FUT 1 forward	CCGGTTTGGTAATCAGATGG	60.18
FUT 1 reverse	CTCAAGTCCGCGTACTCCTC	60.01
FUT 2 forward	ATCATGACCATTGGGACGTT	60.06
FUT 2 reverse	GTGCTTGAGTAAGGGGGACA	60.11
FUT 6 forward	CCTTCGAGAACTCCTTGCAC	59.99
FUT 6 reverse	CCAGCGAAAGTAGCTCAGGT	59.64
Nucleolin forward	CGTTCGGGCAAGGATAGTTA	60.05
Nucleolin reverse	AGCCACCTTCACCCTTAGGT	59.99

RT-PCR

Total RNA from cells was extracted with the RNeasy Mini reagent kit (Qiagen), according to the manufacturer's recommendations. Two microgram of total RNA, was reverse transcribed using 1× RT-PCR buffer with 25 mM Mg^{2+}, 500 μM dNTPs, 6 U Masterscript RT enzyme, 0.2 U prime RNase inhibitor solution (Eppendorf, 5 PRIME, Hamburg, Germany), 100 μM Oligo(dt) primer (Quiagen, Hilden, Germany) in a final reaction volume of 20 μl. Reactions were carried out at 50°C for 30 min in a My Cycler™ thermal cycler (BioRad Microscience), followed by a 10 min step at 4°C. Primers for the measurement of FUT1, FUT2, FUT6, and Nucleolin mRNA expression were selected using the software Primer 3 [Rozen and Skaletsky, 2000] available on-line at http://www-genome.wi.mit.edu., and were synthesized by Invitrogen (for sequence see Table I). Primers were always chosen according to the following parameters: length between 18 and 25 bases, optimal 20–22 bases; T_m comprised between 57 and 65°C, optimal T_m 60–62°C; length of amplification product between 200 and 300 bp. We evaluated the specificity of all sequences using the program Blast (www.ncbi.nlm.nih.gov). The T_m was calculated according to the formula reported by Sambrook et al. [1989].

For each sample, 500 ng of cDNA products were added to 25 μl of PCR mix containing 2 unit of Masterscript PCR Enzyme Mix (Eppendorf), 25 nm of Mg^{2+}, 200 μM of dNTPs in presence of the specific primers for FUT1, FUT2, FUT6, and Nucleolin (Table I), together with the β-actin (Invitrogen) used as an internal standard. The samples and the standard were then subjected to 40 cycles of amplification at 95°C for 2 min, followed by 94°C for 15 s, the appropriate annealing temperature for 20 s (Table I) and 68°C for 45 s, in the My Cycler™ thermal cycler (BioRad Microscience). The conditions were chosen so that none of the RNAs analyzed reached a plateau at the end of the amplification protocol, that is, they were in the exponential phase of amplification. Each set of reactions always included a no-sample negative control and a negative control containing RNA instead of cDNA to rule out genomic DNA contamination. The PCR products were then loaded onto ethidium bromide-stained 1.2% agarose gels in TBE [Sambrook et al., 1989]. A wide range DNA ladder molecular weight marker (Sigma–Aldrich), was run on every gel to confirm expected molecular weight of the amplification product. Acquisition of gel was performed by Chemi Doc XRS (BioRad Microscience).

REAL-TIME RT-PCR

Total RNA from CVEC cells was extracted with the RNeasy Mini reagent kit (Qiagen), according to the manufacturer's recommendations.

Total RNA (1.5 μg) was reverse transcribed using the iSCRIPT cDNA Synthesis kit (BioRad Microscience), in a final reaction volume of 40 μl. Reactions were carried out at 42°C for 30 min in an iCycler™ thermal cycler (BioRad Microscience). One hundred nanogram (5 μl) sample of the cDNA was added to 25 μl of PCR mix containing 300 nM of each primer and 2× IQ SYBR Green Supermix (BioRad Microscience). After the PCR reaction, melting curve analysis was performed. Samples were run in triplicate and experiments were repeated twice. In each sample the presence of β-actin mRNA expression was also tested. β-actin mRNA was expressed in all samples at constant and reproducible levels as shown by the values of threshold cycle (15–14 ΔC_t). In terms of absolute expression of β-actin mRNA this difference was not statistically significant. The samples and the standards were then subjected to 40 cycles of amplification at 95°C for 15 s and 60°C for 60 s in the iCycler™ thermal cycler (BioRad Microscience). Quantification data are presented as cycle threshold (C_t) and the results normalized to the (-actin gene. Quantification of mRNA levels used ΔC_t values calculated from the formula $\Delta C_t = C_t$ (target gene)$-C_t$ (β-actin gene). Expression of the target gene in ratio to β-actin expression was calculated with the formula: target gene expression/β-actin expression $= 2^{(-\Delta C_t)}$.

RESULTS

FUT 2 IS OVER-EXPRESSED IN SERUM-ACTIVATED CELLS

We have recently reported that fucosylated glycoforms of nucleolin exist [Aldi et al., 2009] with the possibility of fucose being linked in alpha1, 2 [Aldi et al., 2009; Tissot et al., 2009]. In order to investigate whether and which of the two alpha1, 2 fucosyltransferases FUT1 and FUT2 was involved in nucleolin fucosylation we analyzed their expression in serum-activated cells. This was done because nucleolin is known to be expressed at low levels in serum deprived non-dividing cells [Sirri et al., 1997] and at high levels in serum-activated cells. The level of mRNA of FUT6, known to catalyze the transfer of an L-fucosyl group to an acceptor molecule to form an alpha(1, 3) linkage, was evaluated as a control. Changes in the expression of nucleolin and its fucosylated forms recognized by the anti-gp273 antibody as a consequence of serum activation were also monitored. The mRNA levels were analyzed by quantitative real-time RT-PCR (C_t-values in relation to an internal standard) and the nucleolin and fucosylated nucleolin forms were quantified by immunoblot using the anti-nucleolin antibody MS-3 and the anti-gp273 IgG in starved (0.1% serum) and serum stimulated (10% serum) cells for different time intervals. The results are shown in Figure 1. It was found that mRNA levels of both nucleolin and FUT2 increased an average of seven times in serum-stimulated cells compared to non-serum-stimulated ones but whereas level of nucleolin mRNA increased during the first 12 h and reached a maximum 24 h after serum stimulation, that of FUT2 increased approximately 12 h later (Fig. 1A). mRNA of FUT1 also resulted in an increase as a consequence of cell stimulation but not at a rate comparable to that of FUT2. No relationship between levels of the mRNA of FUT6 and stimulation was observed. The results of immunoblot analysis (Fig. 1B,C) confirmed the results of Sirri et al. [1997] since nucleolin was detected at very low levels in cells cultured at 0.1% serum and at progressively higher levels in cells activated with 10% serum for increasing time intervals. However, a different behavior was observed for nucleolin peptide and fucosylated nucleolin forms. The nucleolin peptide recognized by the MS-3 antibody was clearly detectable, albeit at low levels, whereas anti-gp273 IgG did not detect any component in the lysate cells cultured in 0.1%. In line with the mRNA results, the nucleolin recognized by the MS-3 antibody reached a maximum 24 h after serum stimulation (Fig. 1B), whereas fucosylated nucleolin glycoforms progressively increased up to 36 h after serum stimulation (Fig. 1C). Taken together these first results indicated FUT2 as a

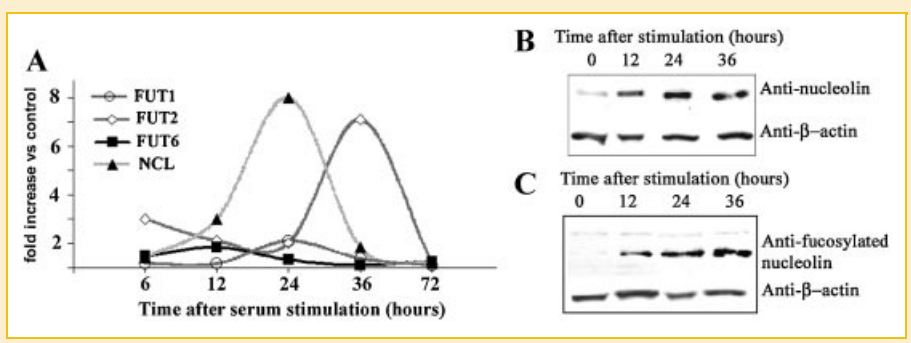

Fig. 1. Expression profiles of FUT1, 2, 6, and nucleolin genes, and immunoblot analysis of nucleolin and fucosylated nucleolin in serum-activated CVEC. A: 1.5 μg of total RNA extracted from serum-starved and serum-activated cells for different time intervals. CVEC were reverse transcribed as described in Materials and Methods Section and 100 ng of the cDNA was added to 25 μl of PCR mix containing 300 nM of each primer and 2× IQ SYBR Green Supermix. Each sample was also tested for mRNA expression of β-actin. The samples and the standards were then subjected to 40 cycles of amplification at 95°C for 15 s and 60°C for 60 s in the iCycler™ thermal cycler (BioRad Microscience). Quantification data are presented as cycle threshold (C_t) and the results normalized to the β-actin gene. Quantification of mRNA levels used ΔC_t values calculated from the formula $\Delta C_t = C_t$(target gene)$-C_t$(β-actin gene). Expression of the target gene in ratio to β-actin expression was calculated with the formula: target gene expression/β-actin expression $= 2^{(-\Delta C_t)}$. The graphic shown in this figure is representative of three experiments obtained with similar results. B, C: Western blot of nucleolin (B) and fucosylated nucleolin (C) in serum-starved and serum-activated cells for different time intervals. At the indicated times cells were harvested and protein extracts were analyzed with the monoclonal anti-nucleolin antibody MS-3 and the anti-fucosylated nucleolin antibody (anti-gp273). Equal loading was analyzed using an anti-β-actin antibody. The blots shown in this figure are representative of three experiments obtained with similar results.

plausible candidate for the enzyme responsible for nucleolin fucosylation.

siRNA-MEDIATED DOWN-REGULATION OF FUT2 AND FUT1 DECREASES EXPRESSION OF FUCOSYLATED NUCLEOLIN IN CVEC

To detect whether FUT2 was effectively involved in nucleolin fucosylation we down-regulated FUT2 expression with a siRNA designed to target FUT2 (FUT2 siRNA). A siRNA control was also used. FUT2 depletion was checked by analyzing the level of the corresponding mRNA by semi-quantitative RT-PCR and by immunoblot analysis of the protein with an anti-FUT2 antibody. Results indicated that the mRNA expression of FUT2 was greatly reduced in cells transfected with siRNA of FUT2 for 12 h and the protein expression was found to significantly decrease in cells 24 h after incubation in siRNA of FUT2 (Fig. 2A). No depletion at all was observed with the control siRNA. The effect of down-regulating FUT2 expression on the expression of fucosylated nucleolin was then investigated by immunoblot analysis using anti-gp273 IgG (hereafter termed anti-fucosylated nucleolin antibody). The results indicated that FUT2 could not be the sole alpha1,2 fucosyltransferase playing a role in nucleolin fucosylation since only 48 h depletion of FUT2 resulted in a slight decrease of the amount of the fucosylated nucleolin glycoforms recognized by the antibody (Fig. 2B) whereas depletion over longer interval times resulted in no difference in the expression of the fucosylated nucleolin glycoforms between cells transfected with FUT2 siRNA and those transfected with scrambled siRNA (data not shown).

We then investigated whether depletion of FUT2 influenced expression of FUT1. This was done by analyzing the mRNA levels of this enzyme at different time intervals after FUT2 depletion. The results shown in Figure 3A revealed that 24 h depletion of FUT2 resulted in a threefold increase of FUT1 expression. We then investigated whether there was also a regulatory role of FUT1 on FUT2 expression. This was done by analyzing the mRNA levels of FUT2 after different intervals of FUT1 depletion. As shown in Figure 3B, depletion of FUT1 resulted in a fourfold increase in FUT2 expression but this occurred after 48 h depletion. Taken together these results suggest that alpha1, 2 fucosylation of nucleolin occurs through a balanced action between both enzymes.

We thus analyzed how the contemporary introduction in the cells of the siRNA for FUT2 and a siRNA designed to target FUT1 (FUT1 siRNA) influenced the expression of fucosylated nucleolin. Down-regulation of both the fucosyltransferases was checked and proved by analyzing the corresponding mRNA levels by RT-PCR (Fig. 3C) and fucosylation of nucleolin by immunoreactivity analysis of cell lysates with the anti-fucosylated nucleolin antibody at different time intervals after depletion (Fig. 3D). The results confirmed that FUT1 contributes to nucleolin fucosylation since the level of the fucosylated forms of the protein progressively declined at increasing time intervals after FUT1 and FUT2 depletion (Fig. 3D).

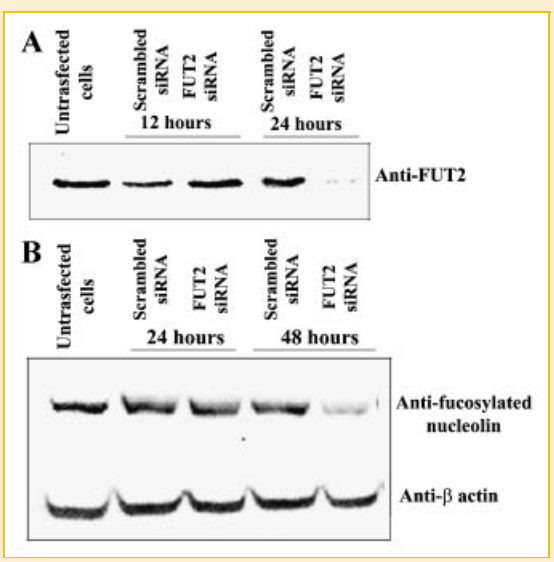

Fig. 2. siRNA targeting FUT2 lowers expression of fucosylated nucleolin in CVEC. Western blot of FUT2 (A) and fucosylated nucleolin (B) in cells transfected with scrambled siRNA and FUT2 siRNA. At the indicated times cells were harvested and protein extracts were analyzed by Western blotting with anti-FUT2 (A) and anti-gp273 (B). Equal loading was verified using anti-β-actin. Data are from three independent experiments.

INACTIVATION OF FUT1/2 EXPRESSION INHIBITS SURFACE NUCLEOLIN EXPRESSION, ALTERS ITS SHUTTLE ACTIVITIES AND AFFECTS CELL ADHESION AND CELL PROLIFERATION IN CVEC

To study the impact of down-regulating FUT1 and FUT2 expression on nucleolin dynamics and localization at the cell surface, we analyzed the FUT1/2 depleted cells by FACS and double immunofluorescence using the anti-fucosylated nucleolin antibody and the anti-nucleolin antibody MS-3. We first evaluated our antibody for its capacity to reveal the cell surface nucleolin in FACS analysis in comparison with the commercial antibody MS-3 and we found that, consistently with our previous results [Aldi et al., 2009], the nucleolin glycoforms recognized by our antibody are expressed at high levels at the cell surface and that the anti-nucleolin antibody MS-3 is unable to reveal them (data not shown). Successive FACS analyses were then performed at different time intervals after FUT2 and FUT1/2 depletion using our antibody. The results showed a decline in fucosylated nucleolin expression from a percentage of about 50–60% of positive cells in cells transfected with scrambled siRNA to about 35% in FUT2 and about 20% in FUT1/2 transfected cells after 20 h transfection and a decline from about 40% to about 20 and about 10% respectively in FUT2 and FUT1/2 after 40 h transfection (Fig. 4A). In Figure 4B, the results of an experiment of 20 h depletion are shown where the shift of the FACS peak of FUT1/ 2 (Fig. 4C) depleted cells is greater than that observed in cells only treated with siRNA directed against FUT2 (Fig. 4B). Immunofluorescence analysis of cells not permeabilized with anti-fucosylated nucleolin antibody confirmed a progressively reduced reactivity at cell surface (data not shown). Analysis of the permeabilized cells with the anti-fucosylated nucleolin and anti-nucleolin MS-3 antibodies showed that the nucleolin forms recognized by the anti-fucosylated nucleolin antibody surrounding the nucleus gradually disappeared in 24 h and were almost completely abolished in 48 h (Fig. 5a). At nuclear level, a gradual decrease of immunoreactivity to anti-fucosylated nucleolin antibody was also

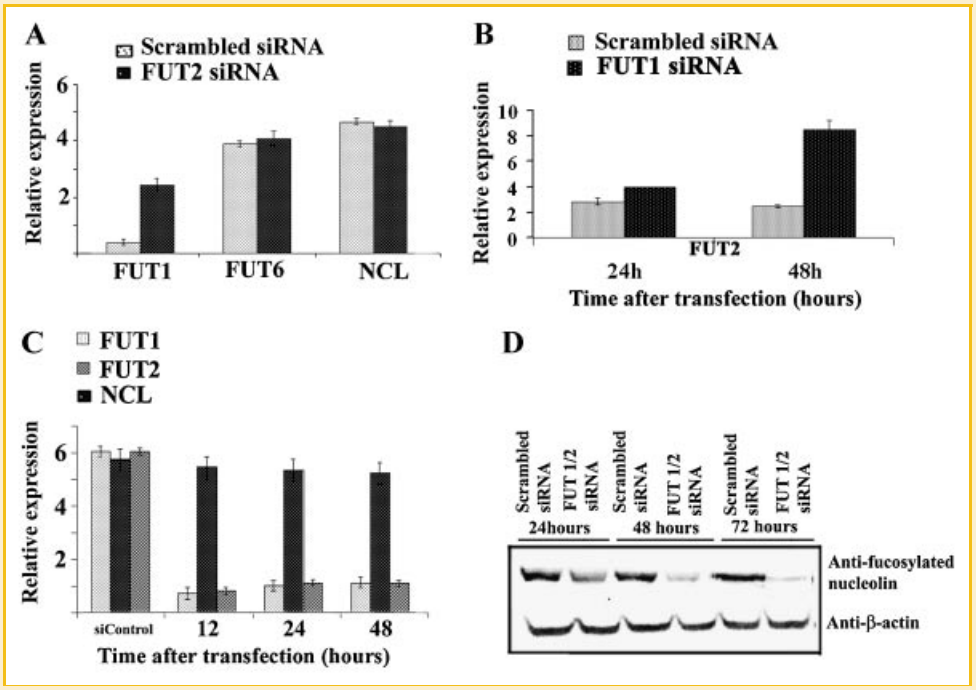

Fig. 3. siRNA targeting both FUT1 and FUT2 inhibits expression of fucosylated nucleolin in CVEC. A: Quantitative RT-PCR of FUT1, FUT6, and nucleolin in cells transfected with scrambled siRNA and with siRNA against FUT2 for 24 h. Total RNA was extracted 24 h after transfection and used for cDNA synthesis and quantitative PCR performed with FUT1, FUT6, nucleolin, and β-actin specific primers. Data were normalized to the amount of β-actin mRNA. Data are from three independent experiments. B: Quantitative RT-PCR of FUT2 in cells transfected with scrambled siRNA and siRNA against FUT1 for 24 and 48 h. Data are from three independent experiments. C: Quantitative RT-PCR of FUT1, FUT2, and nucleolin in cells transfected with scrambled siRNA for 48 h (siControl) and in cells transfected with siRNA against FUT1 and FUT2 for 12, 24, and 48 h. Data are from three independent experiments. D: Western blot of fucosylated nucleolin in transfected cells with scrambled siRNA and a mix siRNA against FUT1 and FUT2. At the indicated times cells were harvested and protein extracts were analyzed with the anti-gp273 antibody. Equal loading was analyzed using an anti-β-actin antibody. The blot shown in this figure is representative of three experiments obtained with similar results.

observed (Fig. 5a). This analysis also revealed a 20% and 40% increase of multinuclear cells in FUT1/2 cells respectively depleted for 24 and 48 h with respect to the transfected cells with control siRNA (Fig. 5a,b). Nucleolin has been described to bind different ligands including endostatin at cell surface and to transport them to the nucleus [Shi et al., 2007]. In order to investigate if a lowered fucosylation of nucleolin altered this shuttle activity we analyzed nucleolin-mediated endocytosis of endostatin in FUT1/2 depleted cells. Cells were FUT1/2 depleted for 20 h and then the corresponding lysate and that of cells treated with control siRNA were immunoprecipitated with an anti-endostatin antibody. The immunoprecipitates were then analyzed for the presence of nucleolin with the anti-nucleolin antibody MS-3. As shown in Figure 6A, the amount of nucleolin immunoprecipitated with endostatin in the FUT1/2 siRNA treated cells was greatly lower than that present in cells transfected with scrambled siRNA. The decrease might reflect the decrease in cell-surface expression of nucleolin thus confirming the role of fucosylation in nucleolin localization at surface or, alternatively, might indicate a direct role of fucosylation in the shuttle activity of nucleolin.

Finally, to determine if FUT1/2 depletion affected cell adhesion and proliferation the adhesion and growth rate of CVEC were measured and compared to those of cells transfected with scrambled siRNA. Cell adhesion was evaluated by counting the total number of adherent cells after plating cells transfected with FUT1/2 and scrambled siRNA for 24 h in DMEM supplemented with 1% BCS (Fig. 6B). Depletion of FUT1/2 resulted in a decreased efficiency of adhesion in cells treated with FUT1/2 siRNA with respect to those treated with scrambled siRNA. Growth rate was evaluated by counting cells at different time intervals after transfection (Fig. 6C). Twenty-four hours after transfection, when the level of FUT1/2 decreases and immunoreactivity of surface nucleolin to the anti-fucosylated nucleolin antibody lowers, the number of cells was found not to increase as it occurred in cells transfected with scrambled siRNA. The number of cells increased but by only half that in the control cells, 48 h after transfection. The number of cells did not further increase 72 h after transfection. At this time also the transfected cells with scrambled siRNA reduced their growth rate probably because they reached confluence.

INACTIVATION OF FUT1/2 EXPRESSION INHIBITS EXPRESSION OF FUCOSYLATED NUCLEOLIN AND AFFECTS CELL PROLIFERATION IN HUMAN A431 CELLS

We therefore tested whether depletion of FUT1 and FUT2 also inhibited expression and activities in human cultured cells. To do so we analyzed the protein extract of human epidermoid carcinoma A431 cells after introducing siRNA in the cells for both FUT1 and FUT2. Down-regulation of both the fucosyltransferases was checked and proved by analyzing the corresponding mRNA levels by RT-PCR (data not shown). Immunoreactivity analysis of cell lysates with the

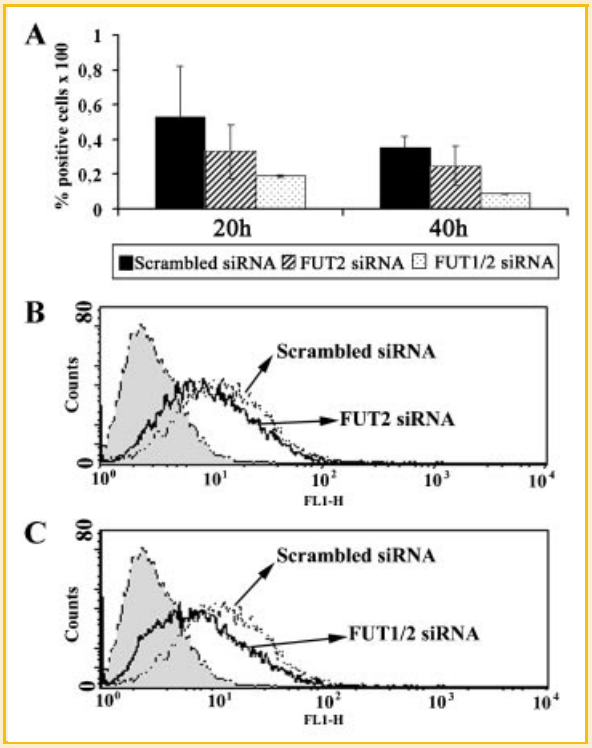

Fig. 4. Knocking down of FUT1/2 gene expression decreases cell surface expression of fucosylated nucleolin in CVEC. A: Flow cytometric analysis of fucosylated nucleolin expression on FUT2 and FUT1/2 transfected CVEC cells after 20 and 40 h transfection. The histogram depicts the results of three independent experiments as mean values. Mean values are calculated from the integrals of the curves of FUT2 or FUT1/2 depleted cells obtained by flow cytometry minus those of negative control isotype immunoglobulin stains. B, C: FACS analysis of anti-fucosylated nucleolin (anti-gp273) antibody binding to CVEC. Propidium negative (living) cells were gated for the analysis. In B, 20 h FUT2-and in C 20 h FUT1/2 transfected cells.

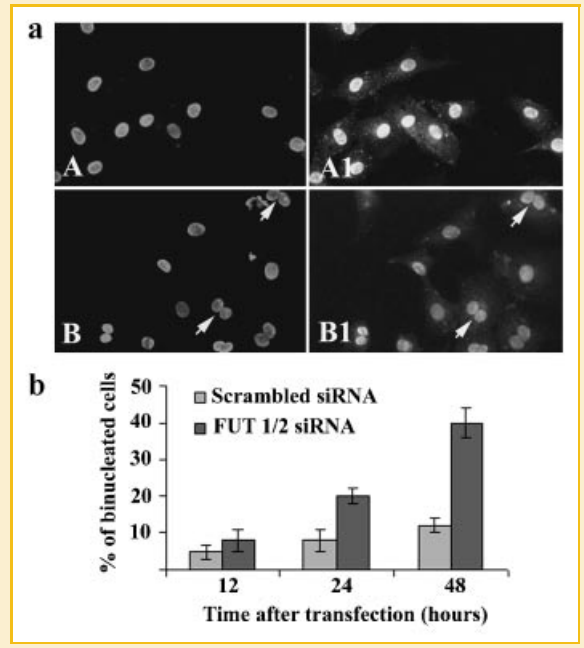

Fig. 5. Decrease of FUT1/2 expression in CVEC increases the amount of polynucleated cells. Cells transfected with scrambled siRNA (A, A1) and siRNA directed against FUT1 and FUT2 (B, B1) were fixed at 48 h after transfection and examined by immunofluorescence using the MS-3 (A, A1) and anti-gp273 (B, B1) antibodies followed by the corresponding secondary antibodies coupled to Alexa Fluor Dyes (red for MS-3. and green for anti-gp273). a: Immunofluorescence of the cells transfected with scrambled siRNA (A, A1) and with FUT1/2 siRNA (B, B1) for 48 h. b: Quantification of polynucleated cells (arrows) in cells transfected with scrambled siRNA and with FUT1/2 siRNA by counting 400 cells in two independent experiments for each time.

anti-fucosylated nucleolin antibody 24 and 48 h after depletion also confirmed a gradual reduction of reactivity in these cells (Fig. 7A).

To determine if inhibition of alpha1, 2 fucosylation also affected cell proliferation in these cells, the growth rate of FUT1/2 depleted A431 cells was measured and compared to that of cells transfected with scrambled siRNA. This was done by counting cells at different time points after transfection (Fig. 7B). As with CVEC the growth rate of FUT1/2 depleted cells was greatly reduced in comparison to the cells transfected with scrambled siRNA.

DISCUSSION

In a previous article [Aldi et al., 2009], we demonstrated the existence of fucosyl-containing *O*-glycans forms of nucleolin in CVEC and malignant cultured human A431 cells. We arrived at this conclusion using the evidence that an antibody directed against fucosyl-containing *O*-glycans of an invertebrate glycoprotein [Focarelli et al., 2003], interacted strongly and exclusively, in total protein extracts of CVEC and A431 cells, with a protein of an MW of 110 kDa, identified by mass spectrometry as nucleolin [Aldi et al., 2009]. The protein against which the antibody was raised has a mass of 273 kDa (gp273) and is the ligand molecule for the sperm–egg interaction in the mollusc bivalve *U. elongatulus* [Focarelli and Rosati, 1995]. The fact that the antibody against gp273 specifically recognized nucleolin was confirmed by comparing the effects of anti-gp273 IgG and the anti-nucleolin monoclonal antibody MS-3, by nucleolin depletion experiments and by immunoblot analysis of the protein after chemically removing the *O*-glycans [Aldi et al., 2009]. Anti-gp273, hereafter termed anti-fucosylated nucleolin, was also demonstrated to strongly detect nucleolin on the plasma membrane and cytoplasm by immunofluorescence, in contrast to commercial anti-nucleolin antibodies [Aldi et al., 2009]. The structural analysis of the gp273 *O*-glycans has confirmed that most of them are indeed fucosylated and has demonstrated that they are of the blood group H-antigen type with fucose linked in alpha1, 2 to galactose [Tissot et al., 2009]. It was thus likely that nucleolin *O*-glycans contain similar terminal motives. To explore this hypothesis and to examine whether the enzymes required for the biosynthesis of the glycan motif Fucα2-Galβ-R effectively play a role in expression and activities of the fucosylated nucleolin glycoforms, we used siRNA to repress the α2FTs, FUT1, and FUT2 in CVEC. FUT1 and FUT2 are known to be active in all mammalian species tested so far [Oriol et al., 2000]. They have GDP-Fuc as a single possible donor substrate, but they can use various acceptor substrates of glycolipids and *O*- or *N*-linked chains of glycoproteins [Abrantes et al., 2009].

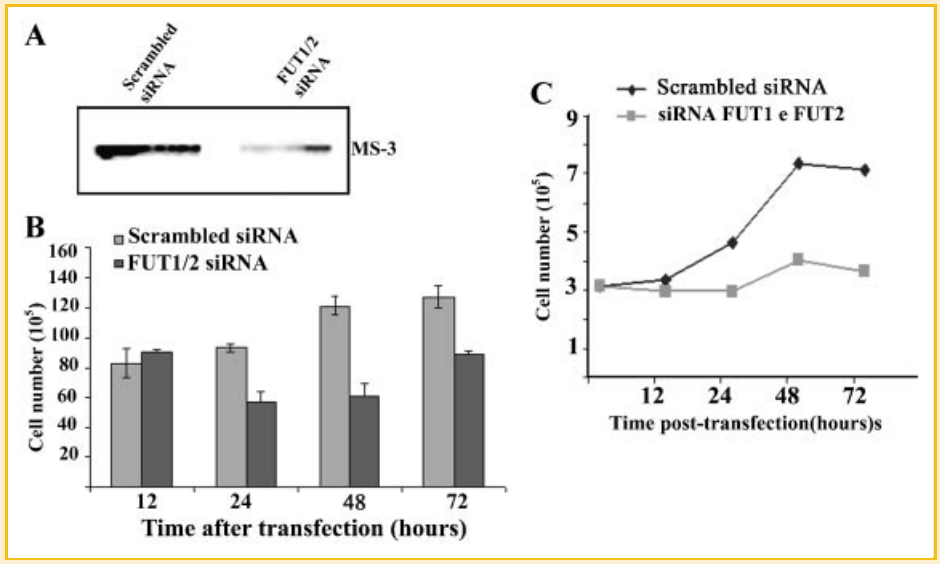

Fig. 6. Knocking down of FUT1/2 gene in CVEC inhibits nucleolin-mediated internalization of endostatin, cell adhesion and proliferation. A: Western blot of nucleolin in immunoprecipitated protein by anti-endostatin antibody. Cells transfected with scrambled siRNA and with FUT1/FUT2 siRNA for 24 h cells were treated with endostatin then lysed and the protein extracts immunoprecipitated with an anti-endostatin antibody. Immunoprecipitates were separated in SDS–PAGE, electroblotted onto nitrocellulose and incubated with anti-nucleolin antibody. B: Cell adhesion test—Cells transfected with scrambled siRNA and siRNA directed against FUT1 and FUT2 were fixed at indicated time intervals after transfection with methanol and stained with Diff Quick. The total number of adherent cells was counted with the aid of an ocular grid (0.21 mm^2). Each experiment was run in triplicate. C: Growth curve of CVEC cells transfected with FUT1/2 siRNA or with scrambled siRNA. Cells were counted at the indicated after transfection. The graph represents the average of six independent experiments.

Our results demonstrated that FUT1 and FUT2 both participate in the fucosylation of *O*-glycans of nucleolin. In fact, expression of the fucosylated forms of the protein was greatly reduced in FUT1/2 depleted cells. In a first series of experiments we have had only suppressed expression of FUT2. This is because only the mRNA level of this transferase increased at the same level as the mRNA level of nucleolin in serum stimulated proliferating cells, in comparison with non-dividing serum deprived cells. FUT2 was effectively found to participate in nucleolin fucosylation but FUT1 was also demonstrated to play a significant role in this process. Suppression of FUT2 gene actually reduced the amount of the fucosylated protein but only during a short period after depletion after which the amount of the fucosylated forms of the protein turned out to be similar to that of non-depleted cells. We suggest that this occurs because FUT1 is able to substitute the function of FUT2 in FUT2 depleted cells. The rationale for this hypothesis is that FUT2 depletion causes an over-expression of FUT1 mRNA and, conversely, FUT1 depletion causes an over-expression of FUT2 mRNA. Suppression of both FUT1 and FUT2 genes expression thus gave rise to a progressive reduction of the fucosylated forms of nucleolin. Suppression of FUT1/2 gene expression was also proved to reduce expression of fucosylated nucleolin on human A431 cancer cells. Suppression of the enzymes expression also produced in CVEC a reduced exposure of the fucosylated nucleolin at cell surface and also in this case reduction in FUT1/2 depleted cells was greater than in those depleted for the sole FUT2. As previously reported in immunofluorescence experiments [Aldi et al., 2009] FACS analyses confirmed that our antibody but not the commercial anti-nucleolin antibody MS-3, strongly detects cell surface nucleolin. Since in the nucleus both the antibodies reveal nucleolin, this finding suggests a high presence and exposure of fucosylated nucleolin glycoforms in this region as well as a masking of the polypeptide backbone.

Although nucleolin is primarily known as being localized in the nucleolus [see Srivastava and Pollard, 1999; Ginisty et al., 1999] its presence in the cytoplasm and at cell surface has been substantiated by many investigators in different cells [Semenkovich et al., 1990; Hovanessian et al., 2000; Legrand et al., 2004; Aldi et al., 2009] and its function as a receptor to internalize ligands and translocate them into the nucleus frequently reported [see Srivastava and Pollard, 1999]. In endothelial cells of angiogenic blood vessels, surface nucleolin specifically binds endostatin and transport it in the nucleus [Shi et al., 2007]. Our data demonstrated that inhibition of the alpha1, 2-fucosylation reduces this shuttle activity of nucleolin and in fact the amount of nucleolin immunoprecipitated by the endostatin antibody was greatly reduced in the FUT1/2 depleted cells in comparison with cells treated with scrambled siRNA. Reduction might merely reflect a lowered expression of nucleolin at surface of FUT1/2 depleted cells thus confirming the importance of fucosylation for the surface localization or might indicate a role of fucosylation for the binding activity of nucleolin.

N- and *O*-glycosylation have been reported to occur in the extranuclear and cytoplasmic nucleolin [Carpentier et al., 2005; Losfeld et al., 2009]. Fucosylated forms of nucleolin are also described as being present in the nucleus [Aldi et al., 2009]. Nucleolin is known to play important roles in nuclear activities ranging from chromatin structure and dynamics [Mongelard and Bouvet, 2007] to mitotic machinery formation [Ugrinova et al., 2007]. Its depletion has been reported to give rise to multiple or very

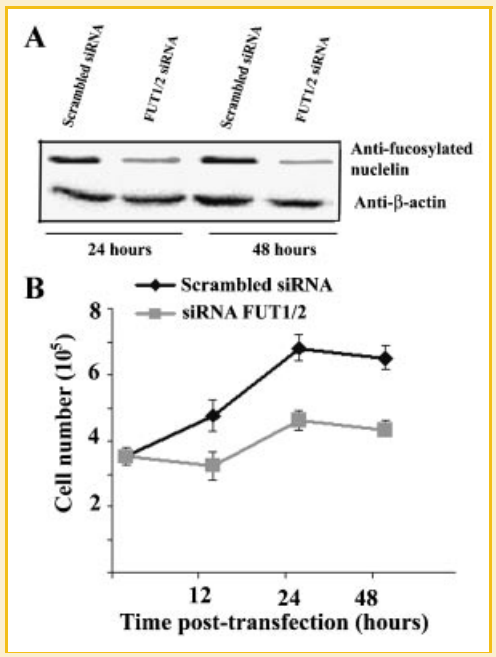

Fig. 7. Suppression of FUT1/2 expression inhibits expression of fucosylated nuleolin and cell proliferation in human A431 cancer cells. A: Western blot of fucosylated nucleolin in transfected A431 cells with scrambled siRNA and a mix siRNA against FUT1 and FUT2. At the indicated times cells were harvested and protein extracts were analyzed with the anti-fucosylated antibody. Equal loading was analyzed using an anti-β-actin antibody. Data are from three independent experiments. B: Growth curve of A431 cells transfected with FUT1/2 siRNA or with scrambled siRNA. Cells were counted at the indicated after transfection. The graph represents the average of five independent experiments.

large nuclei [Ugrinova et al., 2007]. The results of the present study suggest that fucosylation might be important for nucleolin activities related to cell division since FUT1/2 depletion results in an increasing presence of multinuclear cells. However, participation to cell division of other substrates of FUT1 and FUT2 cannot be excluded. Our results also indicate that alpha1, 2 fucosylation influences cell adhesion and cell growth since inactivation of FUT1 and FUT2 resulted in a decreased cell adhesion efficiency and cell growth both in CVEC and in human A431 cells. Current knowledge including the data presented in this work indicates that the inhibition of fucosyltransferases activities by antisense or siRNA may alter cellular properties including adhesion and proliferation. In particular, alpha2-fucosylated glycans have been indicated as responsible of binding activity and signal transduction of the epidermal growth factor receptor (EGF-R) on A431 cells [Defize et al., 1988; Zhang et al., 2008] and the same glycans present in CD44 variants have been described to influence cell motility and resistance to apoptosis [Goupille et al., 1997; Goupille et al., 2000]. In addition, properties such as resistance to drugs [Cordel et al., 2000; Iwamori et al., 2005] or to killer lymphocytes [Marionneau et al., 2000] and ability to form metastasis [Aubert et al., 2000; Mejias-Luque et al., 2007] have been also associated to an altered alpha2-fucosylation. The changes in cell adhesion and proliferation observed by us as a consequence of FUT1/2 depletion might therefore depend upon a diminished fucosylation of one or more of the alpha2 fucosylated glycoproteins expressed by CVEC and A431. Nucleolin could be one of these but the results reported in this study cannot make such a direct correlation. We are currently investigating by means of lectins for the presence and role of all alpha1, 2 fucosylated glycoproteins of the surface of CVEC and other highly proliferating cells.

Where fucosylation takes place in the cell is an open question and, the site also remains to be elucidated where nucleolin is *N*- and *O*-glycosylated [Carpentier et al., 2005]. Nucleolin has a signal to enter the nucleus but it lacks a signal for entering endoplasmic reticulum. It has been subsequently suggested that both glycosylation and translocation on the plasma membrane occur through non-conventional pathways in the cell [Carpentier et al., 2005].

In summary, our previous [Aldi et al., 2009] and present results indicate that nucleolin glycoforms containing α2-fucosylated *O*-glycans exist and that α2-fucosylation influences most of the multifaceted functions of nucleolin probably including those playing a role in cell proliferation. Glycosylation is long known to modulate protein functions and to occur as quickly and dramatically as the process of phosphorylation at various stages of oncogenesis [Hakomori, 1985]. In particular, over expression of fucosylated epitopes is reported as associated with tumor cell phenotypes [Monzavi-Karbassi et al., 2004] and down-regulation of FUT1 described as able to impair in vitro angiogenesis [Moheler et al., 2008] and to inhibit tumor growth [Zhang et al., 2008]. How fucosylation of nucleolin is regulated in the cells and how fucosylation modulates nucleolin activities remain to be explored. However, it is important to note in this context that the anti-fucosylated nucleolin antibody only recognizes the nucleolin form at 100–110 kDa whereas it does not interact at all with its proteolitic fragments recognized instead by the anti-nucleolin antibody [Aldi et al., 2009] thus suggesting that one of the functions of fucosylation is modulation of the turnover of nucleolin by protecting it against proteolysis or autoproteolysis.

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