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RESEARCH ARTICLE

The cross-talk between the urokinase receptor and fMLP receptors regulates the activity of the CXCR4 chemokine receptor

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Abstract The receptor (CXCR4) for the stromal-derived factor-1 (SDF1) and the urokinase-receptor (uPAR) are up-regulated in various tumors. We show that CXCR4-transfected cells migrate toward SDF1 on collagen (CG) and do not on vitronectin (VN). Co-expression of cell-surface uPAR, which is a VN receptor, impairs SDF1-induced migration on CG and allows migration on VN. Blocking fMLP receptors (fMLP-R), alpha-v integrins or the uPAR region capable to interact with fMLP-Rs, impairs migration of uPAR/CXCR4-transfected cells on VN and restores their migration on CG. uPAR co-expression also reduces the adherence of CXCR4-expressing cells to various components of the extracellular matrix (ECM) and influences the partitioning of beta1 and alpha-v integrins to membrane lipid-rafts, affecting ECM-dependent signaling. uPAR interference in CXCR4 activity has been confirmed in cells from prostate carcinoma. Our results demonstrate that uPAR expression regulates the adhesive and migratory ability of CXCR4-expressing cells through a mechanism involving fMLP receptors and alpha-v integrins.

Keywords uPAR · Urokinase-receptor · fMLP receptors · CXCR4 · Prostate carcinoma cells

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Introduction

The CXCR4 chemokine receptor is implicated in several physiologic and pathologic processes; in fact, it is a co-receptor for T-trophic HIV, plays a fundamental role in fetal development and in the trafficking of naive lymphocytes, and is a key molecule in the regulation of hematopoietic stem cell (HSC) trafficking from and to the bone marrow (BM). In fact, CXCR4 is expressed in HSCs, and its ligand, the stromal derived factor 1 (SDF1), is largely produced by BM endothelium [1].

CXCR4 and SDF1 are also considered key molecules in invasion and metastasis of several cancers. CXCR4 is highly expressed by several tumors such as leukemias, breast, prostate, lung and brain tumors, and is strongly involved in directing tumor cells to organs that highly express SDF1 (lymph nodes, lungs, liver, bones), which contributes to a suitable microenvironment for metastatic cells [2–5].

Urokinase (uPA) is a serine protease that activates plasminogen to plasmin and binds a specific high-affinity cell-surface receptor, uPAR [6]. uPAR is formed by three homologous domains (D1, D2, D3) anchored to the cell surface by a glycosyl-phosphatidylinositol (GPI) tail. The GPI-anchor confers extreme mobility to uPAR along the

cell membrane and allows its association to lipid rafts, cholesterol-rich microdomains of the plasma membrane, which seem implicated in signal transduction events initiated by cell adhesion to the extracellular matrix (ECM) [7, 8].

uPAR is strongly up-regulated in several cancers and represents a negative prognostic factor [9]. uPAR traditional role was considered the focusing of proteolytic uPA activity on the cell membrane. However, proteolysis-independent uPAR activities have been demonstrated in the last years [9]. uPAR binds vitronectin, a component abundant in tumor-associated ECM [10, 11], interacts with various integrins regulating their activity, interferes with the activity of specific growth-factor receptors, mediates uPA-dependent cell migration and is required for chemotaxis induced by fMet-Leu-Phe (fMLP), a potent leukocyte chemoattractant [9, 12]. Indeed, cell-surface uPAR functionally interacts with fMLP-receptors (fMLP-Rs) through a specific site corresponding to amino acids 88–92 (SRSRY) located in the region linking uPAR domain 1 (D1) to uPAR domain 2 (D2) [13, 14]. The fMLP receptor family comprises three highly homologous receptors: the high and low affinity fMLP-Rs, FPR and FPRL1, and the FPRL2, which does not bind fMLP [15].

uPAR can be cleaved, thus generating cell-surface truncated forms lacking the N-terminal D1 domain. uPAR cleavage negatively regulates most of full-length uPAR activities, since cleaved uPAR does not bind uPA and VN, and does not coimmunoprecipitate with integrins. Nevertheless, cleaved uPAR, still containing the SRSRY sequence at its N-terminus, retains its ability to functionally interact with fMLP-Rs [9, 12].

Both full-length and cleaved uPAR forms can be shed from the cell surface, generating soluble full-length (suPAR) and cleaved (c-suPAR) forms of uPAR. The soluble form of cleaved uPAR, lacking the N-terminal domain and exposing residues 88–92 (SRSRY-c-suPAR), or soluble uPAR peptides, containing the SRSRY sequence, are ligands for all three fMLP-Rs and induce migration of various cell types, including HSCs [13, 16, 17]. The SRSRY-dependent signaling is supported by a cross-talk between the high-affinity fMLP receptor (FPR) and the alpha-v chain of integrinic VN receptors [18]. SRSRY-c-suPAR also regulates the activity of inflammatory chemokine receptors, such as MCP-1 and RANTES receptors, through fMLP-R activation [19].

Independently on uPAR expression, fMLP-Rs have been reported to regulate the activity of other chemokine receptors, including CXCR4 [20–22] and integrins [23]. Recently, increased expression of fMLP-Rs in some tumors has been demonstrated [24].

We recently demonstrated that the suPAR derived peptide, $uPAR_{84–95}$, (residues 84–95), containing the SRSRY sequence, is able to mobilize murine HSCs from BM into the circulation, likely by stimulating HSC migration and/or by impairing migration of human and mouse leukemia cells toward the CXCR4 ligand, SDF1 [17, 25].

These observations prompted us to investigate whether the cell-surface uPAR can interfere with CXCR4 activity, by examining the effect of uPAR expression on CXCR4 functions.

Materials and methods

Reagents

The rabbit anti-uPAR polyclonal antibody and the amino-terminal fragment of uPA (ATF) were from American Diagnostica (Greenwich, CT); the monoclonal antibody R4 was kindly provided by Dr. G. Hoyer-Hansen (Finsen Laboratory, Copenhagen, Denmark). Rabbit polyclonal antibodies against alpha-v and beta1 integrins, the polyclonal antibody directed to phosphorylated ERK 1/2 and the monoclonal antibody against total ERK 2 used in Western-blot analysis, were from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-alpha-v integrin monoclonal antibody (P3G8) used in migration assays was from Chemicon (Temecula, CA); the rabbit poyclonal antibody directed to the N-terminus of CXCR4 was from Upstate (Temecula, CA). FITC-anti-mouse IgG were purchased from Santa Cruz Biotechnology and Alexa Fluor 594 anti-rabbit IgG from Invitrogen (Carlsbad, CA, USA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Bio-Rad (Hercules, CA); fluorescein isothiocyanate-labeled goat anti-rabbit IgG were from Jackson Lab (West Grove, PA). ECL detection kit was from Amersham Biosciences. Polyvinylidene fluoride (PVDF) filters were from Millipore (Windsor, MA). Collagen, laminin, fibronectin were from Collaborative Research (Bedford, MA) and vitronectin from Promega (Madison, WI). The chemotaxis polyvinylpyrrolidone-free (PVPF) filters were from Whatman Int. (Kent, UK) and 96-well flat-bottom microtiter plates were from NUNC (Roskilde, Denmark). uPAR-specific siRNA and the control siRNA were from Qiagen (Valencia, CA, USA); oligofectamine and lipofectamine from Invitrogen. The WKYMVm peptide was synthesized by Innovagen (Lund, Sweden). The rabbit antibody recognizing the SRSRY sequence of uPAR has been developed by PRIMM (Milan, Italy) by using the $uPAR_{84–95}$ peptide (corresponding to uPAR residues 84–95, which include the SRSRY sequence) assembled onto a branching lysine core. The antibody specificity and sensitivity was tested by ELISA; 10 μg/ml of antibody detected up to 2 ng of $uPAR_{84–95}$ peptide.

Cell culture

Human embrional kidney 293 (HEK-293) cells were grown in DMEM and PC3 prostate carcinoma cells in RPMI, supplemented with 10% fetal bovine serum (FBS). Transfected cells were grown in DMEM supplemented with 10% FBS and selective antibiotics.

Transfection

uPAR cDNA was cloned in a pcDNA3 vector with resistance to Geneticin, and the resulting plasmid was named uPAR-pcDNA3 [14]. The cDNA coding for CXCR4, cloned in a pcDNA3 vector with resistance to Hygromycin, named CXCR4-pcDNA3, was kindly provided by Dr. R.M. Melillo ("Federico II" University, Naples, Italy). A total of 5×10^6 cells, cultured overnight in 100 mm tissue culture dishes, were transfected with 10 μg of uPAR-pcDNA3 or with the empty vector pcDNA3 by 60 μl of Lipofectamine for 5 h at 37°C (5% CO_2). Transfected cells were selected by Geneticin at 1.5 mg/ml for 15 days, pooled and cultured in the presence of 0.5 mg/ml Geneticin. uPAR- and vector-transfected cells were then transfected again with CXCR4-pcDNA3 or the empty vector pcDNA3 resistant to Hygromicin. Double transfected cells (uPAR/CXCR4-293, CXCR4-293, V-293) were selected by Hygromicin at 0.4 mg/ml for 15 days and then cultured in the presence of 0.5 mg/ml Geneticin and 0.2 mg/ml Hygromicin.

Flow cytometry analysis

The cells were harvested by 2 mM EDTA-PBS and washed in Ca^{2+}/Mg^{2+}-containing PBS. 5×10^5 cells were incubated with 10 μg/ml of anti-uPAR or anti-CXCR4 polyclonal antibodies for 1 h at 4°C. Purified rabbit immunoglobulins were used as a negative control. The cells were then washed and incubated with a fluorescein isothiocyanate-labeled goat anti-rabbit IgG for 30 min at 4°C. Finally, the cells were washed and analyzed by flow cytometry using a FACScan (Becton–Dickinson, Mountain View, CA).

Fluorescence microscopy

Transfected cells were grown on glass slides and stimulated with 100 ng/ml SDF1 or DMEM for 1 h at 37°C. Then, the cells were incubated for 2 h at 20°C with the R4 mouse uPAR-specific antibody (2 μg/ml) and a rabbit CXCR4-specific antibody (10 μg/ml) or nonimmune Ig, washed and further incubated for 1 h at 20°C with FITC anti-mouse IgG (1:100) and Alexa Fluor 594 anti-rabbit IgG (1:200). Cells were visualized using a Zeiss 510META LSM microscope in multitrack analysis.

In internalization experiments with fluorescent *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Ly, the cells, grown on glass slides to semi-confluence, were incubated with buffer, 100 nM fMLP, 100 nM $uPAR_{84–95}$ peptide or 100 nM scrambled peptide for 30 min at 37°C. Then, the cells were washed with PBS, exposed to 10 nM fluorescent *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (Molecular Probes) for an additional 30 min at 37°C. Cells were visualized using a Zeiss 510META LSM microscope. Z-series images represent focal planes corresponding to 0.5 μm vertical interval.

Cell migration assay

Cell migration assays were performed in Boyden chambers using 8-μm-pore-size PVPF polycarbonate filters coated with 5 μg/ml of vitronectin, collagen, fibronectin, or laminin. Transfected cells (2×10^5) were plated in the upper chamber in serum-free medium. 100 nM SDF1 or serum free medium was added in the lower chamber. The cells were allowed to migrate for 4 h at 37°C, 5% CO_2. The cells on the lower surface of the filter were then fixed in ethanol, stained with hematoxylin, and counted at 200× magnification (ten random fields/filter). In a separate set of experiments, transfected cells were preincubated for 1 h at room temperature with 5 μg/ml of polyclonal antibodies directed to uPAR or the $uPAR_{84–95}$ region or 5 μg/ml of a monoclonal antibody directed to alpha-v integrins, or for 1 h at 37°C with 100 nM fMLP, 100 nM $uPAR_{84–95}$ peptide or 5 nM WKYMVm peptide.

In migration assays with PC3 cells, 1×10^5cells/filter were loaded and the cells were allowed to migrate for 2 h at 37°C, 5% CO_2. In migration assays with uPAR-silenced PC3 cells, cells have been transfected with 50 nM uPAR-specific siRNA or control siRNA oligonucleotides (Qiagen) and 4 μl of Oligofectamine, incubated for 48 h at 37°C, 5% CO_2, and then harvested and loaded in Boyden chambers. Transfection efficiency (nearly 90%) and functional specificity were monitored with the RNAi starter kit by using Alexa Fluor 488 labeled nonsilencing siRNA (Qiagen). uPAR silencing was assessed by Western-blot analysis with uPAR-specific antibodies and RT-PCR.

Reverse-polymerase chain reaction

Total cellular RNA was isolated by lysing cells in TRIzol Solution according to the supplier's protocol. RNA was precipitated and quantitated by spectroscopy. Total RNA (5 μg) was reversely transcribed with random hexamer primers and 200 U of M-MLV reverse transcriptase. Then, 1 μl of reversely transcribed DNA was amplified using FPRL2 specific 5′ sense (AGTTGCTCCACAGGAATC CA) and 3′ antisense (GCCAATAATGAAGTGGAGGA TGAGA) primers. PCR was performed in a thermocycler

for 40 cycles at 61°C. The reaction products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination.

Lipid rafts analysis

Cells were harvested by 2 mM EDTA-PBS and resuspended in Ca^{2+}/Mg^{2+}-containing PBS. Washed cells (1×10^6) were plated in 35-mm plates coated with 10 μg/ml of CG or VN and incubated for 1 h at 37°C. Cells were then lysed in lysis buffer (20 mM MOPS, 0.15 M NaCl, 1% Triton-X 100 and protease inhibitors) for 30 min at 4°C. The protein content was measured by a colorimetric assay (Bio-Rad) and 2 mg of protein was diluted to 1 ml, mixed with 1 ml of 80% sucrose in MOPS and placed at the bottom of a centrifuge tube. The samples were overlaid with 4 ml of 30%, 2 ml of 5%, 4 ml of 0% sucrose in MOPS and centrifuged at 39,000 rpm for 17 h using a SW41Ti rotor in a Beckman L7-55 ultracentrifuge at 4°C. After centrifugation, the top 4 ml was removed and the remaining 8 ml was harvested as 1 ml fractions. Equal volumes (60 μl) of each fraction were analyzed by Western blot.

Western-blot analysis

Samples were electrophoresed in 10% SDS-PAGE and transferred onto a PVDF filter. The membrane was blocked with 5% nonfat dry milk and probed with 1 μg/ml of anti-uPAR, anti-integrin, anti-caveolin or anti-CXCR4 polyclonal antibodies, or with 2 μg/ml of the anti-$uPAR_{84–95}$ polyclonal antibody. Finally, washed filters were incubated with horseradish peroxidase-conjugated secondary antibodies and detected by ECL.

Analysis of ERK 1/2 activation

Transfected cells were serum starved for 20 h, harvested by 2 mM EDTA-PBS and plated in wells pre-coated with 10 μg/ml of CG or VN (10^6 cells/35 mm well) at 37°C, 5% CO_2. At different time points, cells were lysed in 1% Triton X-100/PBS in the presence of protease and phosphatase inhibitors and the protein content was measured by a colorimetric assay. 30 μg of protein was electrophoresed in 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% nonfat dry milk and probed with 1 μg/ml rabbit anti-phospho-ERK 1/2 and, then, with mouse anti-ERK 2 (as a loading control) antibodies. Finally, washed filters were incubated with horseradish peroxidase–conjugated anti-rabbit or anti-mouse antibodies; specific bands were detected by ECL.

Adhesion assay

Flat-bottom 96-well microtiter plates were coated with 10 μg/ml of laminin, vitronectin, fibronectin, collagen or 1% heat-denatured BSA-PBS as a negative control, and incubated overnight at 4°C. The plates were then blocked 1 h at room temperature with 1% heat-denatured BSA-PBS. Cells were harvested by 2 mM EDTA-PBS and resuspended in Ca^{2+}/Mg^{2+}-containing PBS. 10^5 cells were plated in each coated well and incubated for 1 h at 37°C. Then, wells were washed and attached cells were fixed with 3% paraformaldehyde in PBS for 10 min and then incubated with 2% methanol for 10 min. The cells were finally stained for 10 min with 0.5% crystal violet in 20% methanol. Stain was eluted by 0.1 M sodium citrate in 50% ethanol, pH 4.2, and the absorbance at 540 nm was measured with a spectrophotometer.

Statistical analysis

Differences between groups were evaluated by Student's *t* test using PRISM software (GraphPad, San Diego, CA). $p \leq 0.05$ was considered statistically significant.

Results

HEK-293 cells transfected with uPAR and CXCR4 cDNAs express both receptors which colocalize on their surface

To investigate whether the cell-surface uPAR can interfere with CXCR4 activity, uPAR- and CXCR4-negative HEK-293 cells were transfected with a cDNA coding for CXCR4, with cDNAs coding for uPAR and CXCR4, or with both the empty vectors, as negative controls; the corresponding transfected cells were named CXCR4-293, CXCR4/uPAR-293, V-293. Flow cytometry analysis with anti-uPAR and anti-CXCR4 antibodies showed a high expression of uPAR in uPAR-transfected cells and a moderate but comparable expression of CXCR4 on the cell surface of both uPAR-negative and uPAR-expressing cells (Fig. 1a). uPAR expressed by CXCR4/uPAR-293 cells was mostly in the full-length form, as shown by Western-blot analysis of CXCR4/uPAR-293 cell lysates with uPAR-specific antibodies (Fig. 1b).

Analysis by confocal microscopy of CXCR4/uPAR-293 cells further confirmed cell-surface expression of both receptors and showed CXCR4 colocalization with uPAR, which increased following SDF1 stimulation (Fig. 2). Accordingly with previous reports [26], uPAR-transfected 293 cells showed formation of extensive lamellipodia. Interestingly, uPAR localized both at the peripheral lamellipodia and at the more internal "lamella proper" of

a Mean fluorescence Intensity

	V-293	CXCR4-293	CXCR4/uPAR-293
Nonimmune Ig	10.8	9.1	7.4
Anti-CXCR4 Ab	12.2	52.0	37.0
Anti-uPAR Ab	12.6	8.1	474.7

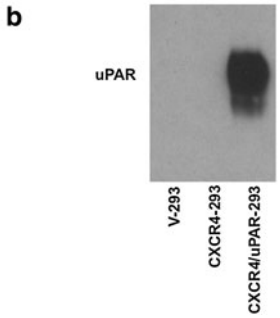

Fig. 1 uPAR and CXCR4 are expressed by transfected HEK-293 cells. **a** HEK-293 cells were transfected with CXCR4 cDNA (CXCR4-293), with uPAR and CXCR4 cDNAs (CXCR4/uPAR-293), or with empty vectors (V-293). Transfected cells were analyzed by flow cytometry with anti-CXCR4 antibodies, anti-uPAR antibodies or nonimmune immunoglobulins (Ig). **b** V-293, CXCR4-293 and CXCR4/uPAR-293 cells were lysed in 1% TRITON X-100 and 5 μg of proteins were analyzed by Western blot with uPAR-specific antibodies. uPAR, expressed only in CXCR4/uPAR-293 cells, was mainly in the full-length form

the leading edge of spreading cells [26], whereas CXCR4 colocalized with uPAR only at the "lamella proper". SDF1 stimulation increased uPAR and CXCR4 focusing in dots at the "lamella proper", likely corresponding to focal complexes [27].

These results demonstrate that CXCR4 and uPAR are expressed in transfected cells and colocalize on the cell surface, thus supporting the possibility of a functional interaction of these two receptors.

uPAR expression regulates SDF1-induced cell migration on VN and CG

In order to elucidate the potential influence of cell-surface uPAR in CXCR4 activity, we examined the capability of transfected cells to migrate toward SDF1, the CXCR4 ligand, on different ECM components. Firstly, the baseline migration of transfected cells on ECM components was assessed. Transfected cells were plated on filters coated with fibronectin (FN), laminin (LM), collagen (CG) or vitronectin (VN), and allowed to migrate in the absence of chemoattractants. The assay showed that the expression of both receptors does not affect he baseline migration of HEK-293 cells on specific substrates (Fig. 3a).

Then, transfected cells were allowed to migrate toward SDF1 (Fig. 3b). CXCR4-293 and CXCR4/uPAR-293 cells migrated with similar efficiency on FN and LM; thus, both CXCR4- and CXCR4/uPAR-293 cells express a functional CXCR4 on their surface. Interestingly, transfected cells behaved differently on the other two substrates. In fact, CXCR4-293 cells migrated efficiently toward SDF1 on CG, whereas they were unable to migrate on VN; conversely, CXCR4/uPAR-293 cells migrated efficiently on VN and very poorly on CG.

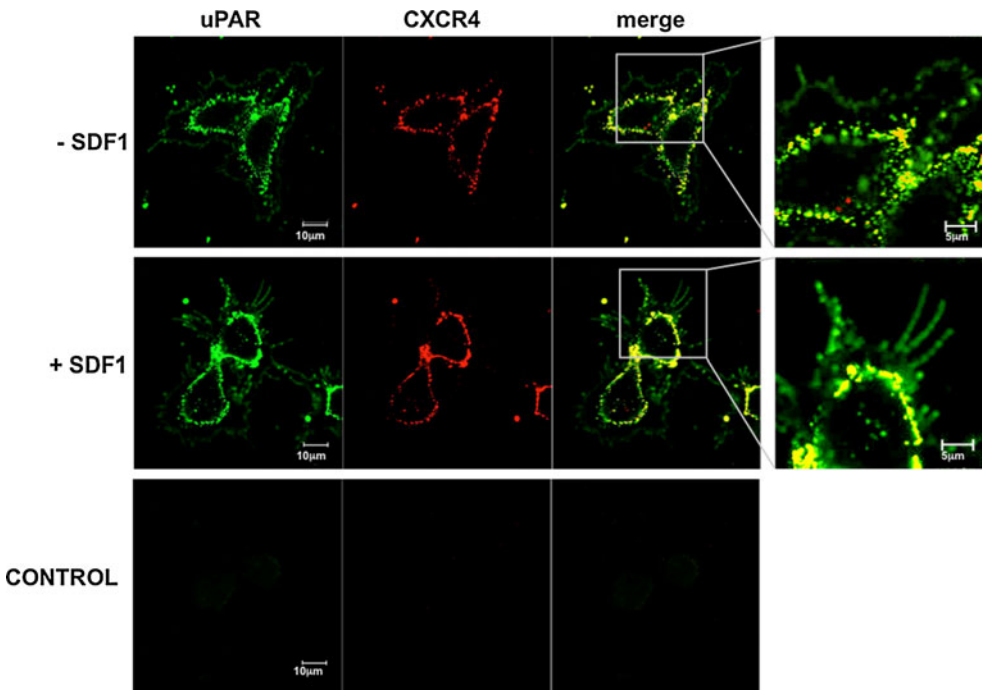

Fig. 2 uPAR and CXCR4 colocalize on the surface of transfected HEK-293 cells. HEK-293 cells transfected with CXCR4 and uPAR cDNAs (CXCR4/uPAR-293) were grown on glass slides and stimulated with SDF1 or buffer for 1 h. Then cells were incubated with the mouse uPAR-specific antibody (*green*) and the rabbit CXCR4-specific antibody (*red*), and analyzed by confocal microscopy. SDF1-treated cells, incubated with nonimmune Ig and secondary antibodies, were shown as control. *Scale bar* 10 μm

a

BASELINE MIGRATION (CELL NUMBER)

	V-293	CXCR4-293	CXCR4/uPAR-293
FN	15±3	21±2	15±4
LM	16±3	24±1	14±4
CG	20±3	20±1	19±2
VN	17±3	20±1	16±4

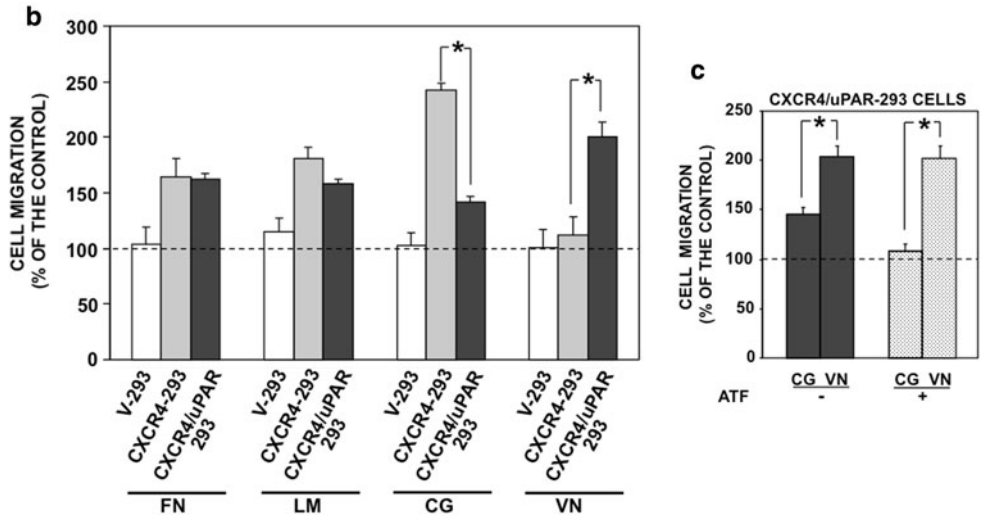

Fig. 3 uPAR expression affects SDF1-induced cell migration on VN and CG. **a** HEK-293 cells transfected with CXCR4 cDNA (CXCR4-293), uPAR and CXCR4 cDNAs, (CXCR4/uPAR-293) or empty vectors (V-293), were plated in Boyden chambers on filters coated with fibronectin (*FN*), laminin (*LM*), collagen (*CG*) or vitronectin (*VN*), and allowed to migrate in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. **b** Transfected cells were plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with FN, LM, CG, or VN. 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. $^*p \leq 0.05$ as determined by Student's *t* test. **c** CXCR4/uPAR-293 cells were plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with CG or VN, in the presence or in the absence of 10 nM of the aminoterminal fragment of uPA (ATF). ATF was added to both upper and lower compartments of the Boyden chamber. 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments. $^*p \leq 0.05$ as determined by Student's *t* test

Control cells, which do not express uPAR and CXCR4, did not migrate to SDF1 on all tested ECM components, as expected.

The observed effect of uPAR expression on cell migration toward SDF1 on CG and VN was due to the contemporaneous coexpression of uPAR and CXCR4, since we previously showed that the same HEK-293 cells used in the present report as recipient cells, transfected only with uPAR cDNA, migrated with similar efficiency on CG and VN toward different ligands [14].

HEK-293 cells do not produce uPA [28] which, upon binding to uPAR through its aminoterminal fragment (ATF), is able to increase uPAR interactions with VN and integrins, likely by promoting the active conformation of uPAR [9, 29]. We then investigated whether ATF could further influence SDF1-induced migration of CXCR4/uPAR-293 cells on VN and CG. ATF can contribute to uPAR effects on CXCR4 activity, further decreasing migration on CG (Fig. 3c).

Thus, uPAR expression does not affect SDF1-induced cell migration on LM and FN, whereas it strongly impairs migration on CG and allows migration on VN.

uPAR effects on CXCR4 activity are mediated by the $uPAR_{84–95}$ region

uPAR involvement in the regulation of CXCR4 activity on VN and CG was confirmed by chemotaxis assays performed in the presence of an anti-uPAR polyclonal antibody. In fact, the anti-uPAR antibody reversed CXCR4/uPAR-293 cell capability to migrate on these specific ECM components, impairing migration on VN and restoring migration on CG (Fig. 4a), thus inducing the same behavior of uPAR-negative CXCR4-293 cells (see Fig. 3b).

Then, we investigated the effect of a polyclonal antibody directed to the $uPAR_{84–95}$ region, which contains the binding sequence for fMLP receptors (residues

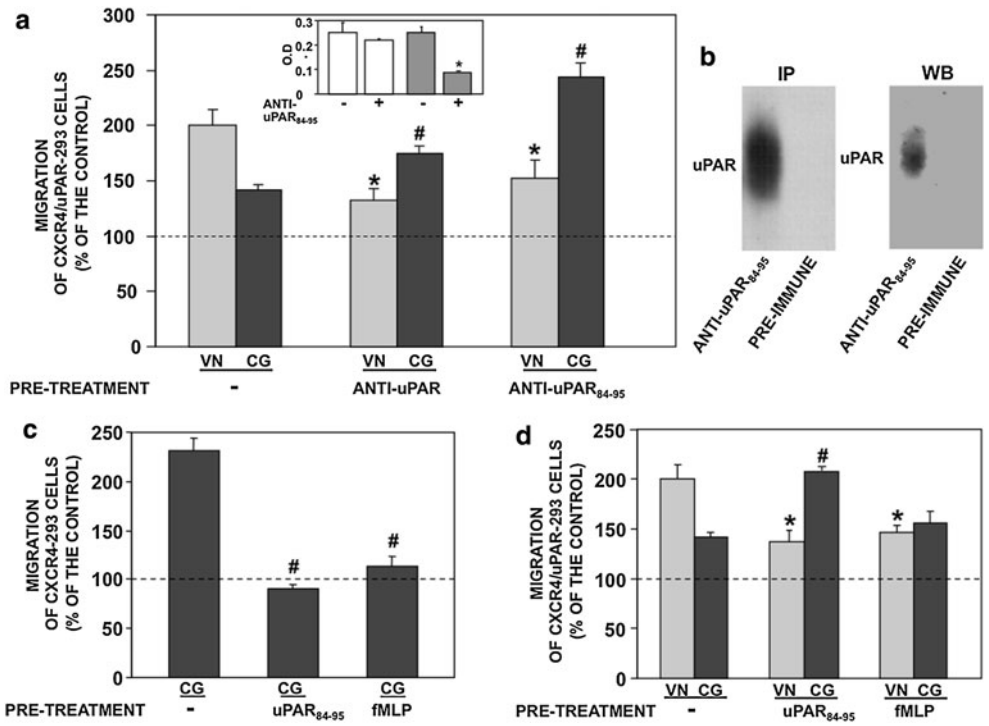

Fig. 4 uPAR effects on SDF1-induced migration involve the $uPAR_{84–95}$ epitope and fMLP-receptors. **a** CXCR4/uPAR-293 cells were incubated with nonimmune immunoglobulins (−), anti-uPAR antibodies or anti-$uPAR_{84–95}$ antibodies, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with collagen (CG) and vitronectin (VN). 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. $^{*}p \leq 0.05$, as determined by Student's *t* test, compared to migration on VN of cells treated with nonimmune Ig. $^{\#}p \leq 0.05$, as determined by Student's *t* test, compared to migration on CG of cells treated with nonimmune Ig. *Inset* CXCR4/uPAR-293 cells were plated on VN-coated wells in the presence of 5 μg/ml of nonimmune immunoglobulins (−) or the anti-$uPAR_{84–95}$ polyclonal antibody (*white columns*) or of 50 μg/ml of nonimmune immunoglobulins (−) or the anti-$uPAR_{84–95}$ polyclonal antibody (*grey columns*). The attached cells were fixed and stained with crystal violet. The stain was eluted, and the absorbance at 540 nm was measured with a spectrophotometer. The values represent the means ± SD of three experiments performed in triplicate. $^{*}p \leq 0.05$, as determined by Student's *t* test. **b** Characterization of the anti-$uPAR_{84–95}$ polyclonal antibody: cell-surface proteins of uPAR-transfected 293 cells were biotinylated; cells were lysed and 500 μg of proteins were immunoprecipitated (IP) with 10 μg/ml of the anti-$uPAR_{84–95}$ polyclonal antibody or pre-immune immunoglobulins (*left*). uPAR-293 cells were lysed in TRITON X-100 and 5 μg of proteins were analyzed by Western blot (WB) with 10 μg/ml of the anti-$uPAR_{84–95}$ polyclonal antibody (*right*). **c** CXCR4-293 cells were pre-incubated with buffer (−), 100 nM $uPAR_{84–95}$ peptide or 100 nM fMLP, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on CG-coated filters. 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. $^{\#}p \leq 0.05$, as determined by Student's *t* test, compared to migration on CG of untreated cells. **d** CXCR4/uPAR-293 cells were pre-incubated with buffer (−), 100 nM $uPAR_{84–95}$ peptide or 100 nM fMLP, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with CG or VN. 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. $^{*}p \leq 0.05$, as determined by Student's *t* test, compared to migration on VN of untreated cells. $^{\#}p \leq 0.05$, as determined by Student's *t* test, compared to migration on CG of untreated cells

88–92) [13, 16]. This specific antibody efficiently recognizes uPAR in Western blot and immunoprecipitation (Fig. 4b). Interestingly, the anti-$uPAR_{84–95}$ antibody induced the same effect of the anti-uPAR polyclonal antibody in a more pronounced manner, thus suggesting the involvement of this specific uPAR epitope in the regulation of CXCR4 activity (Fig. 4a). However, since the $uPAR_{84–95}$ region also contains two of the five residues implicated in uPAR binding to VN, i.e., Arg(91) and Tyr(92) [28, 30], we assessed whether the observed effect of the anti-$uPAR_{84–95}$ polyclonal antibody on the migration of CXCR4/uPAR-293 cells could be due to the impairment of uPAR-VN interaction. A total of 5 μg/ml of the anti-$uPAR_{84–95}$ antibody, able to impair CXCR4/uPAR-293 cell migration on VN and to restore their migration on CG, did not affect CXCR4/uPAR 293 cell adhesion to plastic-bound VN; an higher antibody concentration (50 μg/ml), used as a positive control, impaired CXCR4/uPAR 293 cell adhesion to VN, as expected (Fig. 4a, inset).

These results suggest that the $uPAR_{84–95}$ region is implicated in uPAR-dependent regulation of CXCR4 activity likely through its interaction with fMLP-Rs.

uPAR effects on SDF1-induced migration involve fMLP-receptors

The $uPAR_{84–95}$ epitope, involved in the regulation of CXCR4 activity, interacts with fMLP-Rs [13, 14]. HEK-293 cells express the high-affinity fMLP receptor (FPR), whereas they do not express the low affinity receptor (FPRL-1) [14]; therefore, we explored the involvement of FPR in the uPAR-dependent regulation of CXCR4 activity. FPR can be desensitized by treating cells with its ligands before migration [31]. fMLP and the soluble uPAR-derived peptide, covering the $uPAR_{84–95}$ region ($uPAR_{84–95}$), are ligands of fMLP-Rs, including FPR [13, 14]. Thus, we firstly examined the involvement of FPR in the migration on CG of CXCR4-293 cells, which do not express uPAR, desensitizing FPR. CXCR4-293 cells, following treatment with $uPAR_{84–95}$ peptide or fMLP, were unable to migrate toward SDF1 on CG (Fig. 4c), suggesting that SDF1-induced migration on CG of CXCR4-293 cells requires FPR activity, or, alternatively, that fMLP induces heterologous desensitization [31] of CXCR4, as shown in other cell systems [19–22]. Migration on VN, following FPR desensitization, was not performed since CXCR4-293 cells do not migrate on VN (see Fig. 3b).

We then examined FPR involvement in the migration on CG and VN of CXCR4/uPAR-293 cells (Fig. 4d). Pre-treatment of CXCR4/uPAR-293 cells with the $uPAR_{84–95}$ peptide impaired migration on VN and restored migration on CG (Fig. 4d), fully neutralizing the effect of uPAR expression, similarly to the anti-$uPAR_{84–95}$ antibody (Fig. 4a). fMLP cell-treatment reduced CXCR4/uPAR-293 cell migration on VN, as the $uPAR_{84–95}$ peptide, whereas it did not significantly restore migration on CG. These results prompted us to assess whether both fMLP and the $uPAR_{84–95}$ peptide target FPR in CXCR4/uPAR-293 cells. To this end, we evaluated the effect of $uPAR_{84–95}$ and fMLP on agonist-dependent FPR internalization [32]. The exposure of CXCR4/uPAR-293 cells to the fluorescent FPR-agonist *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys at 37°C induced FPR internalization, as indicated by punctuate green fluorescent intra-cytoplasmic spots (Fig. 5a, left panels), according with previous reports [33]. The internalization of the fluorescent FPR-agonist was prevented by cell pre-incubation with 100 nM fMLP as well as by cell pre-incubation with the $uPAR_{84–95}$ peptide (Fig. 5a, right panels). These results demonstrate that both ligands specifically bind FPR in CXCR4/uPAR-293 cells, even they show a different effect on the SDF1-induced migration on CG.

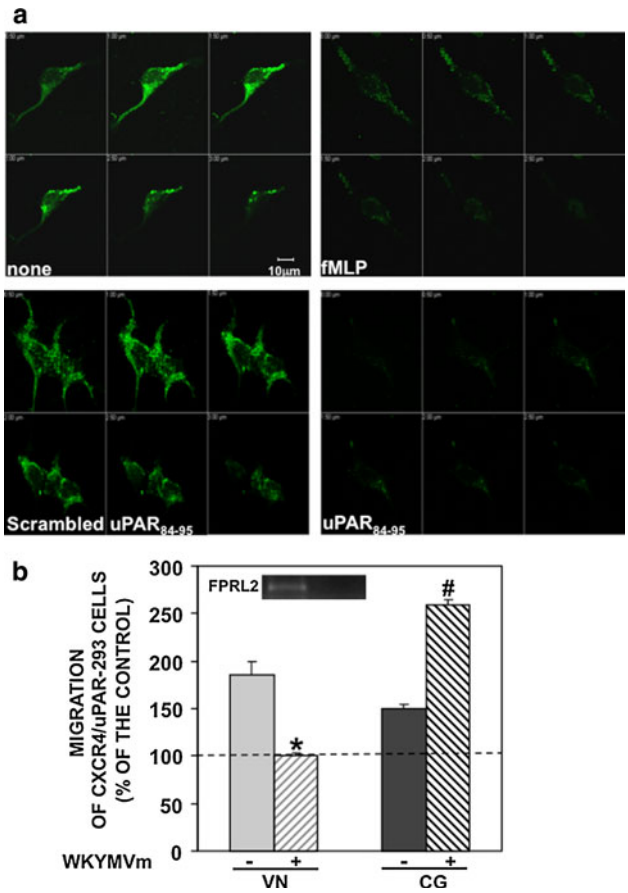

Fig. 5 $uPAR_{84–95}$ peptide and fMLP prevent agonist-FPR interaction. All fMLP-receptors expressed by HEK-293 are involved in uPAR effects on SDF1-induced migration. **a** Representative confocal images of CXCR4/uPAR-293 cells incubated with buffer (none), 100 nM fMLP, 100 nM $uPAR_{84–95}$ peptide or 100 nM scrambled peptide for 30 min at 37°C and exposed for further 30 min at 37°C to 10 nM *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein. *Z*-series images represent focal planes corresponding to 0.5 μm vertical interval. Original magnification 630×. Scale bar 10 μm. **b** CXCR4/uPAR-293 cells were pre-incubated with buffer (−) or 5 nM WKYMVm peptide, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with collagen (CG) or vitronectin (VN). 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments. *$p \leq 0.05$, as determined by Student's *t* test. *Inset* 5 μg of total RNA from CXCR4/uPAR-293 cells was reversely transcribed and amplified using FPRL2-specific primers. The reaction products were analyzed by electrophoresis and stained with ethidium bromide, followed by photography under ultraviolet illumination

Then, we reasoned that fMLP is able to bind only two among the three fMLP receptors, i.e., FPR and FPRL1, while it does not bind FPRL2; vice versa, the $uPAR_{84–95}$ peptide interacts with all three fMLP receptors, including FPRL2. Thus, the $uPAR_{84–95}$ peptide could totally neutralize uPAR effects by targeting also FPRL2, beside FPR, unlike fMLP. Therefore, we assessed FPRL2 expression in CXCR4/uPAR 293-cell (Fig. 5b, inset) and evaluated

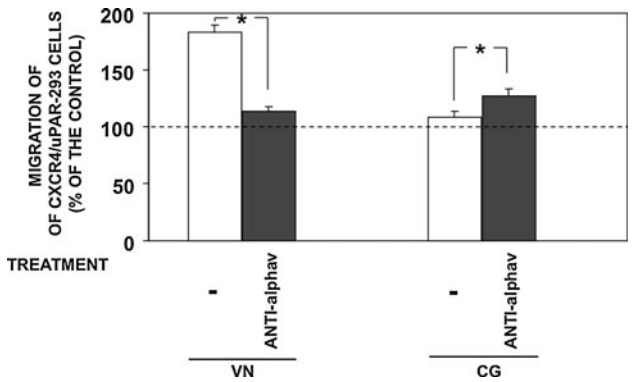

Fig. 6 uPAR effects on SDF1-induced migration involve specific integrins. CXCR4/uPAR-293 cells were incubated with nonimmune Ig (−) or an anti-alpha-v monoclonal antibody, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with collagen (CG) or vitronectin (VN). 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. *$p \leq 0.05$, as determined by Student's t test

CXCR4/uPAR-293 cell migration on CG and VN, following treatment with the synthetic peptide WKYMVm, which is a ligand for all three fMLP receptors [15]. Pre-incubation of CXCR4/uPAR-293 cells with the WKYMVm peptide completely neutralized the effect of uPAR on CXCR4-mediated migration, impairing CXCR4/uPAR-293 cell migration on VN and restoring their migration on CG, similarly to the $uPAR_{84–95}$ peptide (Fig. 5b).

All together, these results indicate that the uPAR-dependent regulation of CXCR4 activity involves all fMLP receptors expressed by HEK-293 cells.

uPAR effects on SDF1-induced migration involve specific integrins

uPAR is a GPI-anchored protein, and requires functional partners provided with cytosolic domains for signal transduction. Integrins interact with uPAR and have been proposed as the best candidates to mediate uPAR signaling [34, 35]. We then also explored the role of alpha-v integrins, which are expressed in HEK-293 cells and are involved in uPAR-related cell signals [36, 37], in uPAR-dependent regulation of CXCR4 activity. CXCR4/uPAR-293 cells were allowed to migrate toward SDF1 on VN or CG in the presence of nonimmune Ig or an anti-alpha-v monoclonal antibody. Specific antibodies significantly reduced migration of CXCR4/uPAR-293 cells on VN, and moderately restored migration on CG (Fig. 6).

These results demonstrate that also integrins are involved in uPAR-related activities enabling CXCR4/uPAR-293 cells to migrate on VN and impairing migration on CG.

uPAR expression down-regulates adhesion of CXCR4-transfected cells

We then investigated whether uPAR expression influences also the stable adhesion to ECM of CXCR4-expressing

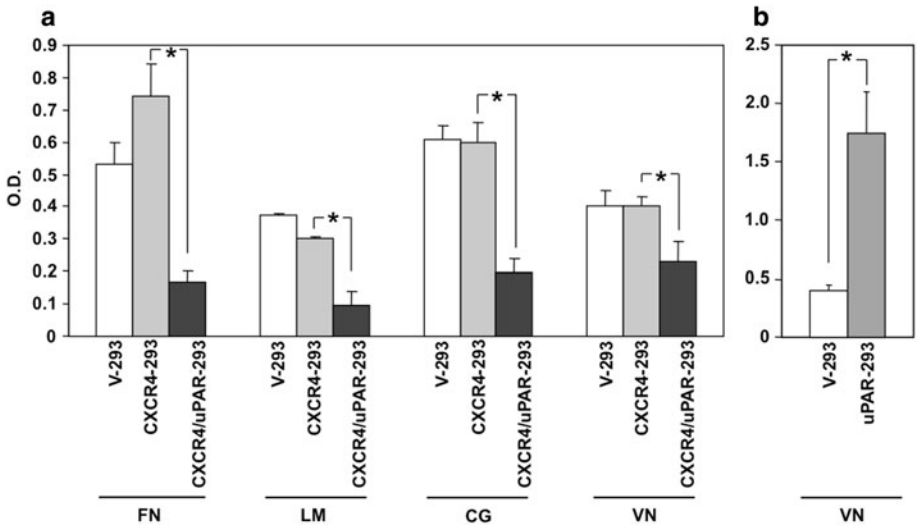

Fig. 7 uPAR expression down-regulates the adhesion of CXCR4-transfected cells to ECM components. **a** CXCR4-293, CXCR4/uPAR-293 or control cells (V-293) were plated on FN-, LM-, CG-, or VN-coated wells. The attached cells were fixed and stained with crystal violet. The stain was eluted, and the absorbance at 540 nm was measured by a spectrophotometer. The values represent the mean ± SD of six experiments performed in triplicate. *$p \leq 0.05$, as determined by Student's t test. **b** HEK-293 cells transfected with uPAR cDNA (uPAR-293) or control cells transfected with the empty vector (V-293) were plated on VN-coated wells. The attached cells were fixed and stained with crystal violet. The stain was eluted, and the absorbance at 540 nm was measured by a spectrophotometer. The values represent the mean ± SD of three experiments performed in triplicate. *$p \leq 0.05$, as determined by Student's t test

cells. The adherence of V-293 control cells, CXCR4-293 and CXCR4/uPAR-293 cells to different components of the ECM was examined (Fig. 7a). Transfected cells were plated on FN, LM, CG, and VN coated wells. Adherent cells were stained by crystal violet and the absorbance of eluted stain was measured. CXCR4-293 cells adhered efficiently to all substrates, as well as control cells, even though no significant variations were observed in their adhesion to FN and LM. uPAR co-expression down-regulated cell adhesion to all substrates, including VN. CXCR4/uPAR-293 cell behavior on VN was unexpected, since uPAR expression had been previously shown to up-regulate HEK-293 cell adhesion to VN [38]; increased adhesion of HEK-293 cells upon transfection with only uPAR cDNA was confirmed in control experiments (Fig. 7b).

uPAR expression reduces integrin association to lipid rafts in CXCR4-expressing cells

uPAR association to lipid rafts has been previously shown [7]. Lipid rafts are cholesterol-rich microdomains of cell membrane that represent anchoring platforms for specific cell-signaling mediators and can be associated to active integrins [8, 39]. Thus, the effect of uPAR expression on the partitioning of integrins to lipid rafts has been examined.

Protein association to lipid rafts is evaluated by ultracentrifugation of cell lysates in a sucrose gradient, in which proteins excluded from lipid rafts remain in the bottom, whereas proteins associated to lipid rafts tend to float to the top, together with lipids. Lysates of transfected cells plated on CG and VN have been loaded on a sucrose gradient and ultracentrifuged. Harvested fractions have been analyzed by Western blot with antibodies directed to beta1 or alpha-v integrins, CXCR4, and uPAR or caveolin as floating controls [7, 40] (Fig. 8). Beta1 distribution was analyzed to investigate the effect of uPAR on integrins with different specificities, since beta1 associates to a wide variety of alpha chains; alpha-v partitioning was examined because this VN-specific alpha chain is a signaling partner for uPAR [37].

In lysates from uPAR-negative CXCR4-293 cells plated on CG and VN, beta1 integrins were present both in the bottom of the gradient, which contains the solubilized non-raft membranes, and in the upper fractions, containing the detergent-resistant lipid rafts. By contrast, in lysates from CXCR4/uPAR-293 cells plated on the same substrates, beta1 integrins were totally confined to the bottom of the gradient, thus associated to non-raft membranes (Fig. 8, upper). Alpha-v integrins appeared associated to non-lipid fractions (bottom fractions) in both CXCR4- and CXCR4/uPAR-293 cells plated on CG; by contrast, they tended to

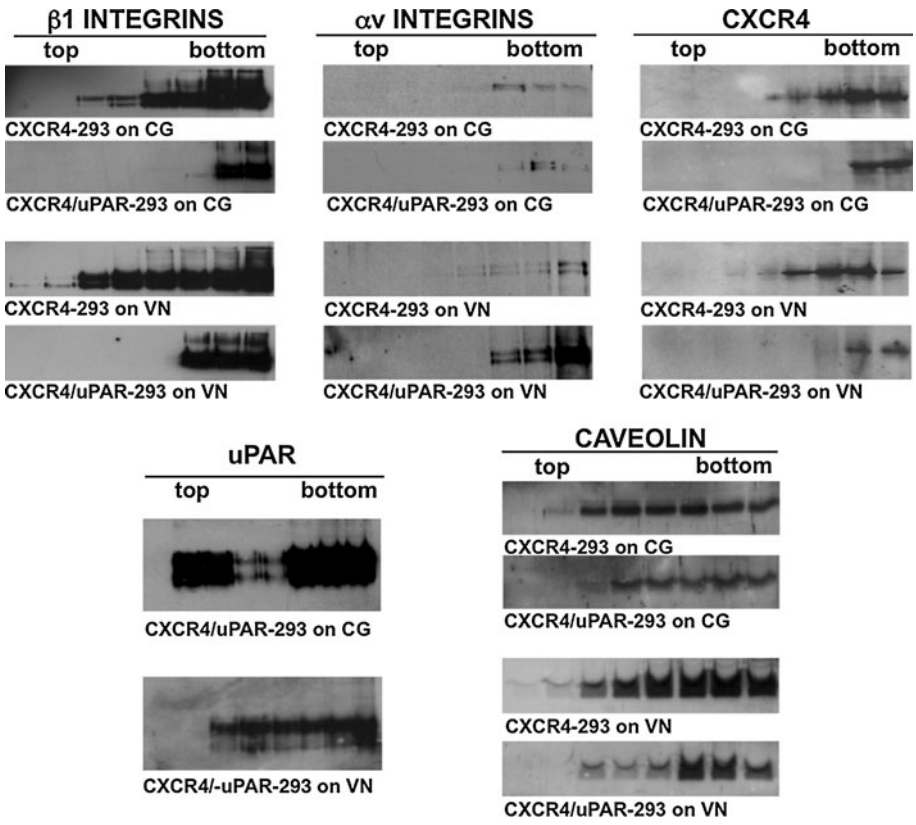

Fig. 8 uPAR expression affects integrin association to lipid rafts. CXCR4-293 and CXCR4/uPAR-293 cells were plated on CG- or VN-coated wells, incubated for 1 h at 37°C and lysed in buffer containing 1% Triton X-100. Cell lysates were subjected to sucrose density gradient ultracentrifugation. After centrifugation, 8 ml was harvested as 1-ml fractions. Equal volumes of each fraction were analyzed by Western blot with anti-beta1 or anti-alpha-v integrin antibodies or with anti-CXCR4 antibodies (*upper panels*) or with anti-uPAR or anti-caveolin antibodies (*lower panels*)

float with lipid rafts in CXCR4-293 cells and to remain in the bottom fractions in CXCR4/uPAR-293 cells plated on VN, similarly to beta1 integrins (Fig. 8, upper). The analysis of other integrins, such as beta3, alpha5, and alpha3, showed a partitioning pattern very similar to that of beta1 integrins (not shown).

CXCR4 partitioned to lipid and non-lipid fractions in uPAR-negative CXCR4-293 cells plated on both CG and VN, as reported in other cells types [41], whereas it was confined, as integrins, in non-lipid fractions in uPAR-expressing cells (Fig. 8, upper).

uPAR, expressed only in CXCR4/uPAR 293 cells, was found both in the bottom of the gradient, and in the top fractions, as previously shown in the same cells transfected only with uPAR [7]. Caveolin, which associates to lipid-rafts [40], was used as a control (Fig. 8, lower).

All together, these results show that uPAR, partitioned to both lipid raft and non lipid raft fractions, affects integrin and CXCR4 association to lipid rafts, confining them in non-raft membranes.

uPAR expression affects ERK 1/2 activation in CXCR4-transfected cells

Integrin confinement in non lipid-raft fractions could suggest modifications in their signaling pathways [39]. Integrin-dependent signaling pathways can converge on various mediators, including extracellular regulated kinases (ERK1 1/2) [42]. Thus, we explored the possibility that the affected partition of integrins to lipid rafts, induced by uPAR co-expression in CXCR4 transfected cells, could coincide with a modification of integrin-mediated signaling.

CXCR4-293 and CXCR4/uPAR-293 cells were serum-starved, plated on CG or VN, harvested at the indicated times and lysed. Western-blot analysis with anti-phospho-ERKs 1/2 antibodies showed ERK1/2 activation in CXCR4-293 cells plated both on CG and VN, even if at different incubation times (Fig. 9, left upper and lower panels, respectively). By contrast, ERK1/2 activation was not detected in CXCR4/uPAR 293 cells plated on the same substrates (Fig. 9, right panels), suggesting a modification of ECM-dependent signaling following uPAR expression in CXCR4-expressing cells.

uPAR regulates SDF1-induced migration in tumor cells

uPAR and CXCR4 are up-regulated in several types of tumor [5, 9]; thus, the interference of uPAR in CXCR4 activity was examined also in tumor cells that constitutively express both receptors. PC3 prostate carcinoma cells express uPA, uPAR, and CXCR4 [43, 44], thus, their migration to SDF1 on CG and VN, in the presence or in the absence of the anti-uPAR$_{84–95}$ polyclonal antibody, was examined. The results showed that PC3 cells migrated efficiently on VN and poorly on CG in a SDF1 gradient; the anti-uPAR$_{84–95}$ polyclonal antibody and the uPAR$_{84–95}$ peptide, ligand of all three fMLP receptors, reduced their migration on VN and allowed a significant migration on CG (Fig. 10a, b), as for CXCR4/uPAR-transfected 293 cells (Fig. 4a).

To further support these results, uPAR expression in PC3 cells was silenced by transfection with a specific siRNA. Migration toward SDF1 of PC3 cells transfected with a uPAR-specific siRNA or a control siRNA was examined, showing that uPAR silencing in PC3 cells induced the same effect of the anti-uPAR$_{84–95}$ polyclonal antibody and of the uPAR$_{84–95}$ peptide (Fig. 10c, left). uPAR silencing was assessed by Western-blot analysis of transfected cell lysates (Fig. 10c, right) and real-time PCR analysis (not shown).

These results confirm in tumor cells the interference of uPAR in CXCR4 activity observed in transfected cells.

Discussion

Chemokine gradients are central to direct the movement of cells in many normal and pathologic processes. We previously found that a soluble form of uPAR, exposing the

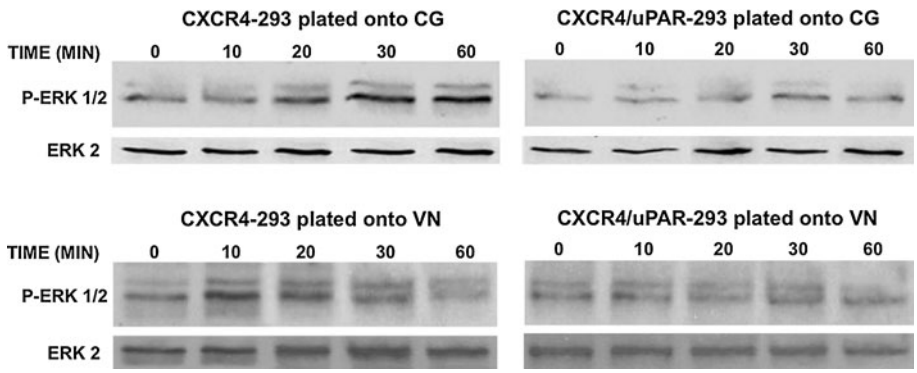

Fig. 9 uPAR expression affects ECM signaling in CXCR4 expressing cells. CXCR4-293 (*left*) and CXCR4/uPAR-293 (*right*) cells were serum-starved and plated on collagen (CG) (*upper panels*) or vitronectin (VN) (*lower panels*). The cells were harvested at the indicated times and lysed for Western-blot analysis with anti-phospho-ERKs and anti-ERK 2 (as a loading control) antibodies

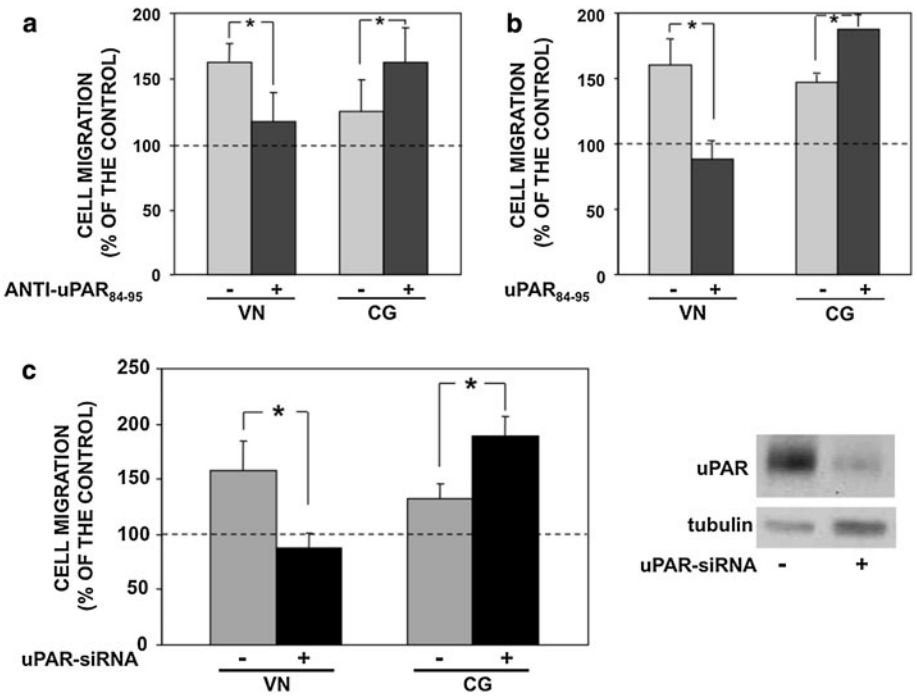

Fig. 10 uPAR expression regulates SDF1-induced migration in tumor cells. **a** PC3 cells were pre-incubated with nonimmune Ig (−) or the polyclonal anti-uPAR$_{84–95}$ antibody, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with collagen (CG) or vitronectin (VN). **b** PC3 cells were pre-incubated with buffer (−) or the uPAR$_{84–95}$ peptide, plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with CG or VN. **c** PC3 cells were transiently transfected with a uPAR-specific siRNA (+) or a control siRNA (−). Transfected cells were plated in Boyden chambers and allowed to migrate toward 100 ng/ml SDF1 on filters coated with CG or VN (*left*). 100% values represent cell migration in the absence of chemoattractants. The values are the mean ± SD of three experiments performed in triplicate. $*p \leq 0.05$, as determined by Student's *t* test. uPAR silencing was assessed by Western-blot analysis of transfected cell lysates with uPAR-specific antibodies (*right*)

chemotactically active SRSRY sequence (aa 88–92), inhibited migration of HSCs toward the SDF1 [17, 25]. On this basis, in the present report, we investigate whether the cell-surface uPAR could interfere with CXCR4 activity, thus playing a new role in chemokine-mediated cancer cell trafficking.

We transfected HEK-293 cells with CXCR4 or with both receptors and observed their colocalization on the cell surface. Then, transfected cells were analyzed in functional assays. We showed that CXCR4-transfected cells efficiently migrated to SDF1 on CG and were unable to migrate on VN. The co-expression of cell-surface uPAR, which is a VN receptor [28], allowed cell migration on VN, a component largely expressed in tumor-associated ECM [10, 11], whereas it strongly impaired migration on CG. Antibodies against alpha-v integrins, or cell pre-treatment with fMLP-receptor (fMLP-R) ligands [15], such as fMLP, the synthetic WKYMVm peptide, the soluble uPAR$_{84–95}$ peptide, impaired SDF1-induced migration on VN of CXCR4/uPAR-293 cells, strongly suggesting that alpha-v integrins and fMLP-receptors are likely uPAR partners in cell-signaling on VN. In fact, uPAR lacks the cytosolic domain, thus it requires cell-surface partners to transduce signals inside the cell.

Interestingly, pre-treatment of CXCR4/uPAR-293 cells with fMLP, able to desensitize FPR and FPRL-1, only impaired CXCR4/uPAR-293 cell migration on VN, but was not able to restore their migration on CG, whereas soluble uPAR$_{84–95}$ and WKYMVm peptides, also targeting FPRL-2, completely restored migration on CG. This observation suggests that also uPAR capability to impair cell migration to SDF1 on CG depends on its interaction with fMLP-Rs, in particular with FPRL-2. Indeed, we previously demonstrated that, in human basophils, uPAR function is mostly mediated by its interaction with FPRL-1 and FPRL-2 [45].

Besides regulating SDF1-induced migration on specific ECM substrates, we also showed that uPAR expression significantly decreased the adherence of CXCR4-expressing cells to various components of the ECM. The decrease in HEK-293 cell adhesion to CG, FN and LM, following uPAR expression, was in agreement with previous results obtained with HEK-293 cells transfected only with the uPAR cDNA [14, 38], although, in other cell types, uPAR up-regulation induces activation of FN-specific integrins

[46]. On the contrary, CXCR4/uPAR-293 cell behavior on VN was unexpected, since uPAR expression had been previously shown to up-regulate HEK-293 cell adhesion to VN [38], thus suggesting a reciprocal interference between uPAR and CXCR4. In agreement with cell-adhesion results, uPAR seems to saturate lipid rafts and to exclude integrins from these membrane microdomains, which would play a proactive function in clustering integrins and in the priming of cells for efficient ligand binding [47]. Integrin confinement in non lipid-raft fractions could suggest a modulation of their ligand-binding activity and, likely, modifications in their signaling pathways. In fact, we found that uPAR expression in CXCR4-expressing cells impairs ERK 1/2 activation induced by ECM components (Fig. 9). Thus, integrin confinement in non lipid-raft fractions and modifications in their signaling pathways could contribute to the impaired adhesion observed in CXCR4/uPAR transfected cells.

All together, our results demonstrate that the simultaneous expression of CXCR4 and uPAR influences the adhesive and migratory features of cells. In particular, cells become less adherent to ECM and, at the same time, acquire the capability to migrate toward SDF1 on VN. The mechanism involves both fMLP-receptors and alpha-v integrins, previously proposed as uPAR partners in the activation of cell-signaling pathways. On this basis, we propose that uPAR functionally associates to a supramolecular complex including such molecules; the exclusion of fMLP-Rs or alpha-v integrins from the complex abolishes uPAR capability to regulate CXCR4 activity. The possibility that uPAR, fMLP-Rs, integrins and CXCR4 form a functional complex is based on previous literature. In fact, uPAR can interact, at least functionally, with FPR, through the SRSRY sequence [13, 14], and with integrins, through specific sequence in the D2 and the D3 domains [48, 49]. A cross-talk between CXCR4 and fMLP-Rs is suggested by fMLP capability to desensitize CXCR4 [20, 21], and, on the other hand, by the requirement of fMLP-Rs for the activity of specific chemokine receptors [22]; CXCR4 can regulate the activity of specific integrins and, in turn, its activity can be regulated by integrins [50].

We confirmed uPAR interference in CXCR4 activity in cells derived from prostate carcinomas, in which the expression of uPAR and CXCR4 and the importance of the CXCR4–SDF1 axis have been previously demonstrated [51, 52].

CXCR4 is up-regulated in a large number of common human cancers, such as those of breast, lung, prostate, colon, and in melanoma [2–5]. A variety of strategies have been used to target the CXCR4–SDF1 axis and few compounds are advancing into early stages of clinical development in oncology [53]. uPAR also is strongly up-regulated in various malignancies, including carcinomas [54]. The finding that uPAR can exist also in soluble and cleaved forms and that can regulate the functions of various integrin families and the activity of growth factor and chemokine receptors suggested new potential roles for uPAR in cancer, beside the focusing of uPA proteolytic activity [55]. Several approaches have been proposed in cancer therapy to disrupt ligand-dependent and ligand-independent uPAR activities [56]. Recently, increased expression of fMLP-Rs in some cancers has been reported [24].

The simultaneous expression of CXCR4, fMLP-Rs, and uPAR, observed in various tumors, could thus reinforce a neoplastic phenotype, characterized by decreased adherence to physiologic ECM and by enhanced capability to migrate on VN, suggesting the uPAR-fMLP-Rs-CXCR4 cross-talk as a new molecular target in combating cancer.

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References

1. Murdoch C (2000) CXCR4: chemokine receptor extraordinaire. Immunol Rev 177:175–184
2. Balkwill F (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol 14:171–179
3. Kucia M, Reca R, Miekus K, Wanzeck J, Wojakowski W, Janowska-Wieczorek A, Ratajczak J, Ratajczak MZ (2005) Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem Cells 23:879–894
4. Zlotnik A (2006) Chemokines and cancer. Int J Cancer 119:2026–2029
5. Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107:1761–1767
6. Irigoyen JP, Munoz-Canoves P, Montero L, Koziczak M, Nagamine Y (1999) The plasminogen activator system: biology and regulation. Cell Mol Life Sci 56:104–132
7. Cunningham O, Andolfo A, Santovito ML, Iuzzolino L, Blasi F, Sidenius N (2003) Dimerization controls the lipid raft partitioning of uPAR/CD87 and regulates its biological functions. EMBO J 22:5994–6003
8. Viola A, Gupta N (2007) Tether and trap: regulation of membrane-raft dynamics by actin-binding proteins. Nat Rev Immunol 7:889–896
9. Ragno P (2006) The urokinase receptor: a ligand or a receptor? Story of a sociable molecule. Cell Mol Life Sci 63:1028–1037
10. Felding-Habermann B, Cheresh DA (1993) Vitronectin and its receptors. Curr Opin Cell Biol 5:864–868
11. Henry C, Moitessier N, Chapleur Y (2002) Vitronectin receptor alpha(V)beta(3) integrin antagonists: chemical and structural requirements for activity and selectivity. Mini Rev Med Chem 2:531–534
12. Montuori N, Ragno P (2009) Multiple activities of a multifaceted receptor: roles of cleaved and soluble uPAR. Front Biosci 14:2494–2503
13. Resnati M, Pallavicini I, Wang JM, Oppenheim J, Serhan CN, Romano M, Blasi F (2002) The fibrinolytic receptor for urokinase

activates the G protein-coupled chemotactic receptor FPRL1/LXA4R. Proc Natl Acad Sci USA 99:1359–1364
14. Montuori N, Carriero MV, Salzano S, Rossi G, Ragno P (2002) The cleavage of the urokinase receptor regulates its multiple functions. J Biol Chem 277:46932–46939
15. Le Y, Murphy PM, Wang JM (2002) Formyl-peptide receptors revisited. Trends Immunol 23:541–548
16. Fazioli F, Resnati M, Sidenius N, Higashimoto Y, Appella E, Blasi F (1997) A urokinase-sensitive region of the human urokinase receptor is responsible for its chemotactic activity. EMBO J 16:7279–7286
17. Selleri C, Montuori N, Ricci P, Visconte V, Carriero MV, Sidenius N, Serio B, Blasi F, Rotoli B, Rossi G, Ragno P (2005) Involvement of the urokinase-type plasminogen activator receptor in hematopoietic stem cell mobilization. Blood 105:2198–2205
18. Gargiulo L, Longanesi-Cattani I, Bifulco K, Franco P, Raiola R, Campiglia P, Grieco P, Peluso G, Stoppelli MP, Carriero MV (2005) Cross-talk between fMLP and vitronectin receptors triggered by urokinase receptor-derived SRSRY peptide. J Biol Chem 280:25225–25232
19. Furlan F, Orlando S, Laudanna C, Resnati M, Basso V, Blasi F, Mondino A (2004) The soluble D2D3(88–274) fragment of the urokinase receptor inhibits monocyte chemotaxis and integrin-dependent cell adhesion. J Cell Sci 117:2909–2916
20. Li BQ, Wetzel MA, Mikovits JA, Henderson EE, Rogers TJ, Gong W, Le Y, Ruscetti FW, Wang JM (2001) The synthetic peptide WKYMVm attenuates the function of the chemokine receptors CCR5 and CXCR4 through activation of formyl peptide receptor-like 1. Blood 97:2941–2947
21. Le Y, Shen W, Li B, Gong W, Dunlop NM, Wang JM (1999) A new insight into the role of "old" chemotactic peptide receptors FPR and FPRL1: down-regulation of chemokine receptors CCR5 and CXCR4. Forum (Genova) 9:299–314
22. Svensson L, Redvall E, Johnsson M, Stenfeldt AL, Dahlgren C, Wennerås C (2009) Interplay between signaling via the formyl peptide receptor (FPR) and chemokine receptor 3 (CCR3) in human eosinophils. J Leukoc Biol 86:327–336
23. Chigaev A, Waller A, Amit O, Halip L, Bologa CG, Sklar LA (2009) Real-time analysis of conformation-sensitive antibody binding provides new insights into integrin conformational regulation. J Biol Chem 284:14337–14346
24. Huang J, Chen K, Gong W, Dunlop NM, Wang JM (2008) G-protein coupled chemoattractant receptors and cancer. Front Biosci 13:3352–3363
25. Selleri C, Montuori N, Ricci P, Visconte V, Baiano A, Carriero MV, Rotoli B, Rossi G, Ragno P (2006) In vivo activity of the cleaved form of soluble urokinase receptor: a new hematopoietic stem/progenitor cell mobilizer. Cancer Res 66:10885–10890
26. Madsen CD, Ferraris GM, Andolfo A, Cunningham O, Sidenius N (2007) uPAR-induced cell adhesion and migration: vitronectin provides the key. J Cell Biol 177:927–939
27. Wolfenson H, Henis YI, Geiger B, Bershadsky AD (2009) The heel and toe of the cell's foot: a multifaceted approach for understanding the structure and dynamics of focal adhesions. Cell Motil Cytoskelet 66:1017–1029
28. Wei Y, Waltz DA, Rao N, Drummond RJ, Rosenberg S, Chapman HA (1994) Identification of the urokinase receptor as an adhesion receptor for vitronectin. J Biol Chem 269:32380–32388
29. Yuan C, Huang M (2007) Does the urokinase receptor exist in a latent form? Cell Mol Life Sci 64:1033–1037
30. Gårdsvoll H, Ploug M (2007) Mapping of the vitronectin-binding site on the urokinase receptor: involvement of a coherent receptor interface consisting of residues from both domain I and the flanking interdomain linker region. J Biol Chem 282:13561–13572
31. Ali H, Richardson RM, Haribabu B, Snyderman R (1999) Chemoattractant receptor cross-desensitization. J Biol Chem 274:6027–6030
32. Rabiet MJ, Huet E, Boulay F (2007) The *N*-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. Biochimie 89:1089–1106
33. Bifulco K, Longanesi-Cattani I, Gargiulo L, Maglio O, Cataldi M, De Rosa M, Stoppelli MP, Pavone V, Carriero MV (2008) An urokinase receptor antagonist that inhibits cell migration by blocking the formyl peptide receptor. FEBS Lett 582:1141–1146
34. Blasi F, Carmeliet P (2002) uPAR: a versatile signalling orchestrator. Nat Rev Mol Cell Biol 3:932–943
35. Ossowski L, Aguirre-Ghiso JA (2000) Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. Curr Opin Cell Biol 12:613–620
36. Franco P, Vocca I, Carriero MV, Alfano D, Cito L, Longanesi-Cattani I, Grieco P, Ossowski L, Stoppelli MP (2006) Activation of urokinase receptor by a novel interaction between the connecting peptide region of urokinase and alpha v beta 5 integrin. J Cell Sci 119:3424–3434
37. Carriero MV, Del Vecchio S, Capozzoli M, Franco P, Fontana L, Zannetti A, Botti G, D'Aiuto G, Salvatore M, Stoppelli MP (1999) Urokinase receptor interacts with alpha(v)beta5 vitronectin receptor, promoting urokinase-dependent cell migration in breast cancer. Cancer Res 59:5307–5314
38. Wei Y, Lukashev M, Simon DI, Bodary SC, Rosenberg S, Doyle MV, Chapman HA (1996) Regulation of integrin function by the urokinase receptor. Science 273:1551–1555
39. Del Pozo MA (2004) Integrin signaling and lipid rafts. Cell Cycle 3:725–728
40. Lajoie P, Goetz JG, Dennis JW, Nabi IR (2009) Lattices, rafts, and scaffolds: domain regulation of receptor signaling at the plasma membrane. J Cell Biol 185:381–385
41. Nguyen DH, Taub D (2002) CXCR4 function requires membrane cholesterol: implications for HIV infection. J Immunol 168:4121–4126
42. Harburger DS, Calderwood DA (2009) Integrin signalling at a glance. J Cell Sci 122:159–163
43. Festuccia C, Dolo V, Guerra F, Violini S, Muzi P, Pavan A, Bologna M (1998) Plasminogen activator system modulates invasive capacity and proliferation in prostatic tumor cells. Clin Exp Metastasis 16:513–528
44. Cannio R, Rennie PS, Blasi F (1991) A cell-type specific and enhancer-dependent silencer in the regulation of the expression of the human urokinase plasminogen activator gene. Nucleic Acids Res 19:2303–2308
45. de Paulis A, Montuori N, Prevete N, Fiorentino I, Rossi FW, Visconte V, Rossi G, Marone G, Ragno P (2004) Urokinase induces basophil chemotaxis through a urokinase receptor epitope that is an endogenous ligand for formyl peptide receptor-like 1 and -like 2. J Immunol 173:5739–5748
46. Aguirre Ghiso JA, Kovalski K, Ossowski L (1999) Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. J Cell Biol 147:89–104
47. Luo BH, Carman CV, Springer TA (2007) Structural basis of integrin regulation and signaling. Annu Rev Immunol 25:619–647
48. Degryse B, Resnati M, Czekay RP, Loskutoff DJ, Blasi F (2005) Domain 2 of the urokinase receptor contains an integrin-interacting epitope with intrinsic signaling activity: generation of a new integrin inhibitor. J Biol Chem 280:24792–24803
49. Chaurasia P, Aguirre-Ghiso JA, Liang OD, Gardsvoll H, Ploug M, Ossowski L (2006) A region in urokinase plasminogen receptor domain III controlling a functional association with

alpha5beta1 integrin and tumor growth. J Biol Chem 281:14852–14863
50. Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, Allendorf DJ, Zhang J, Ratajczak J, Ratajczak MZ (2004) CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. J Mol Histol 35:233–245
51. Epstein RJ (2004) The CXCL12-CXCR4 chemotactic pathway as a target of adjuvant breast cancer therapies. Nat Rev Cancer 4:901–909
52. Waugh DJ, Wilson C, Seaton A, Maxwell PJ (2008) Multi-faceted roles for CXC-chemokines in prostate cancer progression. Front Biosci 13:4595–4604
53. Wong D, Korz W (2008) Translating an antagonist of chemokine receptor CXCR4: from bench to bedside. Clin Cancer Res 14:7975–7980
54. de Bock CE, Wang Y (2004) Clinical significance of urokinase-type plasminogen activator receptor (uPAR) expression in cancer. Med Res Rev 24:13–39
55. Sidenius N, Blasi F (2003) The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy. Cancer Metastasis Rev 22:205–222
56. Pillay V, Dass CR, Choong PF (2007) The urokinase plasminogen activator receptor as a gene therapy target for cancer. Trends Biotechnol 25:33–39