# Hypoxia enhances CXCR4 expression in human microvascular endothelial cells and human melanoma cells

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**ABSTRACT. The influence of environmental factors (cytokines, matrix components, serum factors and $O_2$ level) on expression of receptors for angiogenic versus angiostatic CXC chemokines in human microvascular endothelial cells has not been extensively investigated. Our semi-quantitative RT-PCR analysis demonstrated that TNF-α and IFN-γ repressed CXCR4 mRNA levels in immortalized human microvascular endothelial HMEC-1 cells after 4 h, whereas only TNF-α displayed inhibitory activity in primary human microvascular endothelial cells (HMVEC). CXCR4 mRNA expression was not affected by VEGF, GM-CSF, IL-1β or various basal membrane matrix components, but was significantly up-regulated after serum starvation and/or hypoxic treatment of the microvascular endothelial cells. The alternative CXCL12 receptor, CXCR7/RDC1, was also up-regulated by hypoxia in HMEC-1 cells, although less consistently than CXCR4. Furthermore, hypoxia and serum starvation were required for cell surface display of CXCR4 and CXCL12 induction of ERK activation in HMEC-1 cells. In contrast, CXCR2 and CXCR3 mRNA levels remained, respectively, low and undetectable under all the conditions tested, and surface expression of CXCR2, CXCR3 and CXCR7 on the HMEC- 1 cells could not be demonstrated by FACS. In the human SK-MEL-5 melanoma cell line, CXCR4 mRNA expression was also increased under hypoxic conditions, whereas CXCR2 mRNA levels remained low and levels of CXCR3 and CXCR7 were undetectable. However, immunohistochemical staining of human metastatic melanoma sections demonstrated that CXCR2, CXCR3, CXCR4 and CXCR7 are expressed on tumor cells and, to a lesser extent, on endothelial cells. These results demonstrate that the tumor microenvironment regulates chemokine receptor expression through both cytokine and oxygen levels.**

**Keywords:** chemokine receptor, angiogenesis, CXCR2, CXCR3, CXCR7, RDC1

Angiogenesis is a crucial step in cancer progression and metastasis [1-3]. The formation of new blood vessels is essential for the adequate supply of oxygen and serum factors to the growing tumor. Chemokines constitute a family of chemotactic cytokines that can affect many aspects of cancer biology, including the balance between angiogenesis and angiostasis [3-5]. Chemokines exert their various functions mostly through activation of 7-transmembrane domain G protein-coupled chemokine receptors [4]. Depending on the presence and positioning of NH2-terminal cysteines, chemokines can be grouped into four classes, with the CC and the CXC chemokines being the most prominent. The CXC chemokines are further subdivided into the ELR+ and ELR- CXC chemokines based on the presence or absence of a Glu-Leu-Arg (ELR) motif near the NH2-terminus, respectively. The ELR+ CXC chemokines, such as CXCL1 through CXCL3 and CXCL5 through CXCL8, have been shown to promote the growth of new capillaries, likely through their common receptor, CXCR2 [3, 5, 6]. On the other hand, some ELR- CXC chemokines, including CXCL4, the CXCR3 ligands CXCL9, CXCL10 and CXCL11, the CXCR5 ligand CXCL13 and CXCL14 are angiostatic, although their mechanism of action has not been completely clarified [3, 5, 7-11]. For years, no agonistic receptor could be identified for CXCL4 and CXCL14 and the discussion is still open as to whether the CXCR3 ligands and the CXCR5 ligand CXCL13 exert their angiostatic activity through their respective chemokine receptors [3, 5, 8, 9]. Lasagni *et al.* argued that a splice variant of the classic CXCR3 receptor, namely CXCR3B, might mediate the angiostatic activity of both the CXCR3 ligands and CXCL4 [12]. However, it can still not be excluded that these angiostatic ELR-CXC chemokines could also act receptor-independently by inhibiting angiogenic factors (e.g. by forming heterodimers or by occupying common binding sites on glycosaminoglycans presented on the endothelial surfaces), or through an unknown receptor [3, 10]. Furthermore, the distinction between angiogenic and angiostatic chemokines goes beyond the presence or absence of the

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ELR motif, since the ELR- CXC chemokines CXCL12 (through its receptor CXCR4) [5] and CXCL16 [13] also display angiogenic properties. Finally, some CC chemokines, including CCL1, CCL2 and CCL11, have also been reported to stimulate the growth of blood vessels [5].
Many reports relating chemokines and chemokine receptors to endothelial cells and the angiogenesis/angiostasis balance have utilized human umbilical vein endothelial cells to generate *in vitro* results [14-16]. However, these macrovascular endothelial cells are functionally and phenotypically quite different from microvascular endothelial cells, which are more likely to be involved in physiological and pathological angiogenesis [17, 18]. Even the reported expression patterns of the most intensively studied chemokine receptors in endothelial cells, namely CXCR2, CXCR3 and CXCR4, are rather divergent [5, 14-16, 19-26]. Furthermore, the information on the regulation of the expression of these CXC chemokine receptors in microvascular endothelial cells remains scarce [19, 24, 25]. To gain more insight in this issue, we analysed the *in vitro* expression of CXCR2, CXCR3, CXCR4 and CXCR7/RDC1, recently assigned as an alternative CXCL12 receptor [27], in dermal human microvascular endothelial cells. We evaluated in particular, the impact of environmental factors such as inflammatory cytokines, matrix components, oxygen levels and serum factors. Furthermore, we compared our *in vitro* findings with *in vivo* expression of these receptors in human metastatic melanoma lesions.

## METHODS

### *Cells and reagents*

Primary human dermal microvascular endothelial cells (HMVEC; Cascade Biologics, Portland, OR, USA; below passage 12), were routinely grown on 0.1% gelatin-coated flasks in Medium 131 (Cascade Biologics) supplemented with Microvascular Growth Supplement (MVGS; Cascade Biologics; final concentration of 5% FBS) following the provider's instructions. Immortalized human dermal microvascular endothelial cells (HMEC-1; CDC Atlanta, GA, USA; below passage 25) were routinely cultured in MCDB 131 medium (Invitrogen, Carlsbad, CA, USA) enriched with 10% FBS (Hyclone, Logan, UT, USA), 10 mM Lglutamine (Mediatech Cellgro, Herndon, VA, USA) and Endothelial Cell Growth Supplement (ECGS; Upstate Cell Signaling, Lake Placid, NY, USA; 15 µg/ml) or in Endothelial Basal Medium-2 (EBM-2; Cambrex Bio Science, Walkersville, MD, USA) supplemented with Endothelial Growth Medium (EGM-2MV SingleQuots; Cambrex Bio Science; final concentration of 5% FBS). The human axillary node-derived metastatic melanoma cell line SK-MEL-5 and the pleural effusion-derived metastatic breast adenocarcinoma cell line MCF7 were purchased from the American Type Culture Collection (Manassas, VA, USA). The SK-MEL-5 cells and the MCF7 cells were cultured in DMEM-F12 or DMEM medium (Mediatech Cellgro), respectively, supplemented with 10% FBS and 2 mM L-glutamine. Human peripheral blood mononuclear cells (PBMC) were freshly isolated from single blood donations (blood transfusion center of Leuven, Belgium). Erythrocytes were removed by sedimentation in hydroxyethyl starch (Plasmasteril; Fresenius Hemotechnology, Bad Homburg, Germany) for 30 min at 37°C. PBMC were further purified by density gradient centrifugation on Ficoll-sodium diatrizoate (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway) for 30 min at 400 g. The basement membrane matrix components gelatin and collagen type IV were purchased from Sigma-Aldrich (St. Louis, MO, USA) and growth factor-reduced Matrigel is a basement membrane preparation commercialized by BD Biosciences (San Jose, CA, USA). The cytokines VEGF, GM-CSF, IL-1β and TNF-α and the chemokine CXCL12 were purchased from PeproTech (Rocky Hill, NJ, USA), whereas IFN-γ came from R&D Systems (Minneapolis, MN, USA). The mouse monoclonal anti-CXCR3 antibody clone 49801 (MAB160; 0.5 mg/mL), anti-CXCR4 antibody clone 12G5 (MAB170; 0.5 mg/mL) and anti-CXCR4 antibody clone 44708 (MAB171; 1 mg/mL) were obtained from R&D Systems and the mouse monoclonal anti-CXCR3 antibody clone 1C6 (1 mg/mL) was ordered from BD Biosciences. The rabbit polyclonal anti-CXCR2 antibody was previously characterized [28, 29] and the mouse monoclonal anti-CXCR7 antibody (1.6 mg/mL) was developed by Infantino *et al.* [30]. Mouse IgG isotype control antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-mouse phycoerythrin (PE)-conjugated IgG (Jackson ImmunoResearch, Westgrove, PA, USA) were used during FACS analysis.

### *RNA isolation and semi-quantitative RT-PCR analysis*

Primary HMVEC (0.2x106 cells/p60 plate) and immortalized HMEC-1 cells (0.5x106 cells/p60 plate) were grown on 0.1% gelatin in their complete culture medium (see above) until 80% confluence was reached (72 h). The cells were then washed, re-suspended in medium containing 0.5% FBS and stimulated for 4 h at 37°C with 10 ng/mL of cytokine or left untreated. In order to investigate the influence of matrix components, serum factors and oxygen concentrations on chemokine receptor mRNA expression, HMEC-1 cells (1x106 cells/p60 plate) were plated on various basement membrane matrix components, namely gelatin (0.1%), collagen type IV (20 µg/mL) or Matrigel (0.25 mg/mL). After 48 h of culturing with their complete culture medium, cells were washed and further incubated for 6 h with 0.5 or 10% FBS under normoxic or hypoxic conditions (in hypoxic GasPak pouches, BD Biosciences). Human melanoma SK-MEL-5 cells (1x106 cells/p60 plate) were grown until confluency in complete culture medium, washed and also further incubated for 6 h under normoxia or hypoxia with 0.5 or 10% FBS. After the appropriate incubation of the endothelial and SK-MEL-5 cells, RNA was isolated with the RNeasy kit (Qiagen, Valencia, CA, USA) with an on-column DNAse treatment (RNAse free DNAse set; Qiagen). Next, 1µg of RNA was reverse transcribed using AMV reverse transcriptase (AMV-RT; 0.2 U/µl; Promega, Madison, WI, USA) and its buffer in the presence of oligod(T)16 primers (1 µM; Applied Biosystems, Foster City, CA, USA), 0.2 mM of each of the dNTPs (Sigma-Aldrich) and RNAse inhibitor (RNAsin, 0.4 U/µL; Promega) in a 25 µL reaction mixture. Prior to adding all these reagents and incubation for 1 h at 42°C, the oligod(T)16 primers had been allowed to anneal

to the poly-A-tail of mRNA strands for 5 min at 70°C. In parallel, reactions were carried out in the absence of reverse transcriptase as negative controls (non-RT), whereas cDNA generated from PBMC and MCF7 cells could serve as positive controls. The PCR reaction mixture (25 µL) contained 1 µL of reverse transcription mixture in the presence of Taq DNA polymerase (0.06 U/µL; Sigma-Aldrich) and its buffer, 0.2 mM of each of the dNTPs and 0.4 µM of the primer pairs. The PCR reaction consisted of 7 min denaturation at 95°C, followed by 20 or 37 cycles (in case of GAPDH or chemokine receptor primers, respectively) of 1 min denaturation at 95°C, 1 min annealing at 58°C and 2 min extension at 72°C, and was concluded by a final extension step of 10 min at 72°C. The primer pair for the housekeeping gene GAPDH (NM_002046; Forward: 5' TCATTGACCTCAACTACATGG; Reverse: 5' GAGTCCTTCCACGATACCAAA) generated a RT-PCR fragment of 413 bp. The CXCR4 primers (NM_003467; Forward: 5' CACTTCAGATAACTACACCG; Reverse: 5' ATCCAGACGCCAACATAGAC) amplified a fragment of 464 bp. The primer pairs for CXCR2 (NM_001557; Forward: 5' CGAAGGACCGTCTACTCATC; Reverse: 5'AGTGTGCCCTGAAGAAGAGC) and for CXCR7 (NM_020311; Forward: 5' AAGAAGATGGTACGCCGTGTCGTCTGCATCCTG; Reverse: 5' CTGCTGTGCTTCTCCTGGTCACTGGACGCCGAG) have been published previously, generating fragments of 519 bp and 281 bp, respectively [31, 32]. Finally, three different CXCR3 primer pairs were tested. Theoretically, the CXCR3 primer pair used by Segerer *et al*. (CXCR3 Seg; Forward: 5' GCCCTCTACAGCCTCCTCTT; Reverse: 5' TGTTCAGGTAGCGGTCAAAGC) [33] should assist in the generation of an RT-PCR fragment of 286 bp, independent of the origin of the CXCR3 template (CXCR3A mRNA, CXCR3B mRNA, CXCR3alt mRNA or genomic DNA; see *table 1*). The primer set from Soejima and Rollins (CXCR3 Soe; Forward: 5' AACCACAAGCACCAAAGCAG; Reverse: 5' TGATGTTGAAGAGGGCACCT) [34] is supposed to distinctively amplify RT-PCR fragments of 466 bp (CXCR3A or CXCR3-alt mRNA), 705 bp (CXCR3B mRNA) or 1444 bp (genomic DNA). Finally, the CXCR3 primer pair applied by Feil and Augustin (CXCR3 Fei; Forward: 5' CCACTGCCAATACAACTTCC; Reverse: 5' GCAAGAGCAGCATCCACATC) [19] leads to an RT-PCR fragment of 401 bp in the presence of CXCR3A mRNA, CXCR3B mRNA or genomic DNA and an RT-PCR fragment of 64 bp with CXCR3-alt as template. To visualize the amplification products, 16 µL of the RT-PCR mixtures were loaded on 2% agarose gels. The specificity of the primers was confirmed by sequencing some RT-PCR fragments after their isolation from these gels with the Qiaquick gel extraction kit (Qiagen). After densitometric analysis of the bands corresponding to the amplified chemokine receptor transcripts and the loading control GAPDH, the ratios of the chemokine receptor relative to the GAPDH PCR products were calculated and normalized against the ratios of control samples. Experiments were independently repeated three to four times and statistically significant differences compared to the control samples were determined with the Mann-Whitney *U* test.

### *FACS analysis*

HMEC-1 cells (3x106 cells/T75) were allowed to attach for 24 h in complete culture medium, washed and incubated under normoxic conditions in the presence of 10% FBS or in a hypoxic GasPak pouch in the presence of 0.5% FBS. After 24 h, cells were washed with DMEM, detached with enzyme-free cell dissociation buffer (Invitrogen) and washed twice with ice-cold FACS buffer (DMEM+0.5% FBS). Subsequently, cells (0.5x106) were labeled with anti-CXCR4 antibody clone 12G5 (1/50 dilution), anti-CXCR4 antibody clone 44708 (1/100 dilution), anti-CXCR7 antibody (1/80 dilution) or the mouse IgG isotype control antibody (1/40 dilution) for 1 h on ice. After washing, cells were incubated with goat anti-mouse PE-conjugated IgG for 1 h on ice, in the dark. Finally, cells were washed three times with ice-cold FACS buffer and analyzed using a FACS Calibur flow cytometer (BD Biosciences).

### *ERK1/2 phosphorylation*

HMEC-1 cells (0.5x106 cells/well) were grown in a 6-well plate for 24 h in complete culture medium until 70% confluency was reached. After washing, cells were serum-starved (0.5% FBS) for 24 h under normoxic or hypoxic (GasPak pouch) conditions. Subsequently, cells were refreshed with 900 µL EBM-2 medium containing 0.5% FBS and preincubated at 37°C for 15 min prior to treatment with 100 µL CXCL12 solution (final concentration 100 ng/ml) for 1, 5 or 20 min or with 100 µL control medium for 5 min (Co) at 37°C. Cell treatment was stopped by chilling the cells on ice, adding ice-cold PBS and washing the cells twice with ice-cold PBS. Cells were lysed by incubation with ice-cold PBS (100 µL/well) containing 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea and protease inhibitor cocktail for mammalian tissues and phosphatase inhibitor cocktails 1 and 2 (all 1/100; Sigma-Aldrich). After 10 min, the cells were scraped off and the cell extracts were collected, incubated for another 45 min on ice and clarified (10 min, 1200 g). The total

**Table 1**
Expected size of RT-PCR fragments depending on the CXCR3 primers used (CXCR3fei, CXCR3soe, CXCR3seg) and on the origin of the CXCR3 template

| | CXCR3A | CXCR3B | CXCR3alt | Genomic |
|---|---|---|---|---|
| | mRNA | mRNA | mRNA | DNA |
| CXCR3fei [19] | 401 bp | 401 bp | 64 bp | 401 bp |
| CXCR3soe [34] | 466 bp | 705 bp | 466 bp | 1444 bp |
| CXCR3seg[33] | 286 bp | 286 bp | 286 bp | 286 bp |

protein content in the cell extracts was measured with the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The amount of phosphorylated ERK1 and phosphorylated ERK2 (p-ERK1/ERK2) was detected with the DuoSet p-ERK1/ERK2 sandwich ELISA (R&D Systems). The ratios of the amount of p- ERK1/2 to the total protein content were calculated and normalized against the ratio of the buffer-treated control sample. Statistically significant differences compared to the control cells were determined with the Mann-Whitney *U* test.

### *Immunohistochemical staining of melanoma patient samples*

Tumor tissue samples were collected from 12 melanoma patients using protocols approved by the Vanderbilt University Institutional Review Board. The majority of the melanoma samples were from metastatic origin (five lymph node metastases, one visceral metastasis, one skin/soft tissue metastasis, and four without additional information), whereas one sample was derived from a primary melanoma lesion. After fixation, the tumors were embedded in paraffin, cut into slices and placed onto glass slides. The sections were first deparaffinized/hydrated by washing the slides three times for 10 min with xylene, twice for 10 min with 100% ethanol and twice for 10 min with 95% ethanol. The sections were then washed twice in $dH_2O$ for 5 min, followed by a wash step with PBS for 5 min. For antigen unmasking, sections were heated in 10 mM sodium citrate buffer (pH 6.0) for 1 min at full power, followed by 9 min at medium power. After allowing the sections to cool down for 20 min, they were washed three times with $dH_2O$ for 5 min and incubated for 10 min in 1% $H_2O_2$ to quench endogenous peroxidases. Subsequently, sections were washed three times with $dH_2O$ for 5 min, once with PBS for 5 min and then blocked with 5% normal horse serum (RTU Vectastain Universal ABC kit; Vector Laboratories, Burlingame, CA, USA) in PBS for 1 h at room temperature. The sections were then incubated overnight at 4°C with the primary antibodies diluted in 2% normal horse serum in PBS, namely anti- CXCR2 (1/150), anti-CXCR3 from R&D (1/100), anti-CXCR3 from BD Biosciences (1/25), anti-CXCR4 clone 12G5 (1/10), anti-CXCR4 clone 44708 (1/40) or anti-CXCR7 (1/100). After washing three times for 5 min with PBS, the secondary antibodies (RTU Vectastain Universal ABC kit) were added. After 30 min of incubation at room temperature, the sections were washed three times for 5 min with PBS and incubated for 30 min at room temperature with ABC reagent (RTU Vectastain Universal ABC kit). After removal of the ABC reagent, sections were washed three times with PBS for 5 min and incubated at room temperature with AEC substrate (Vector Laboratories). As soon as the sections turned red (after 10 to 30 min), they were immersed in $dH_2O$, counterstained for 1 min in hematoxylin, washed in $dH_2O$ and finally mounted with coverslips. Stained melanoma sections were analysed with a Nikon Labophot microscope (Melville, NY, USA). For each of the anti-chemokine receptor antibodies tested, at least 10 of the 12 melanoma sections were investigated. Sections in which the primary antibody was omitted, served as negative controls.

## RESULTS

### *Transcriptional regulation of CXCR2, CXCR3 and CXCR4 by inflammatory cytokines in microvascular endothelial cells*

In order to study the regulated expression of the CXC chemokine receptors CXCR2, CXCR3 and CXCR4 in microvascular endothelial cells, we performed semi-quantitative RT-PCR experiments on RNA that was isolated from primary (HMVEC cells) or from immortalized dermal human microvascular endothelial cells (HMEC-1 cell line). The housekeeping gene GAPDH was used as an internal control. The specificity of the various primers was initially demonstrated by the appearance of single DNA bands following RT-PCR analysis of RNA obtained from freshly isolated peripheral blood mononuclear cells (PBMC) and the lack of any bands in the absence of reverse transcriptase *(figure 1A)*. Sequence analysis of the RT-PCR fragments obtained after gel extraction confirmed their identity. To evaluate the regulation of chemokine receptor expression, microvascular endothelial cells were stimulated for 4 h with 10 ng/mL VEGF, GM-CSF, IFN-γ, IL-1β or TNF-α in the presence of 0.5% FBS and RNA was subsequently isolated. *Figure 1B* depicts the results obtained for CXCR4 as the mean of four separate sets of semi-quantitative RT-PCR experiments with primary HMVEC and immortalized HMEC-1 cells. VEGF, GM-CSF and IL-1 β did not considerably affect the amounts of CXCR4 transcripts in these microvascular endothelial cells. However, TNF-α significantly reduced the CXCR4 mRNA levels in both cell types, and IFN-γ caused only downregulation of these transcripts in immortalized HMEC-1 cells. Even in the presence of these stimuli, the CXCR2 mRNA levels remained below the detection level in primary HMVEC cells and were too low for firm conclusions to be drawn in HMEC-1 cells (data not shown). Various splicing variants have been reported for CXCR3 in different cell types, namely the classic CXCR3A, the potentially angiostatic CXCR3B [12] and finally CXCR3alt [35]. We analysed the regulated expression of CXCR3 by human microvascular endothelial cells using three sets of previously published primers *(table 1)* [19, 33, 34]. As a control, RT-PCR analysis of PBMC-derived RNA generated fragments of 401 bp, 466 bp and 286 bp using the CXCR3 Fei, CXCR3 Soe and CXCR3 Seg primer sets, respectively, that could not be due to genomic contamination *(figure 1A)*. However, no CXCR3-derived RT-PCR fragments could be detected in the microvascular HMVEC or HMEC-1 cells with any of these CXCR3 primer pairs, not even after stimulation by cytokines (*figure 2* and data not shown).

### *Insufficient oxygen or serum factor supply increases CXCR4 transcription in microvascular endothelial and melanoma cell lines*

We next investigated whether factors other than cytokines, such as matrix components or the deprivation of serum factors and oxygen, could influence CXC chemokine receptor expression. In the presence of 10% FBS and normal oxygen concentrations (normoxic conditions), no major differences in CXCR4 mRNA levels were observed upon

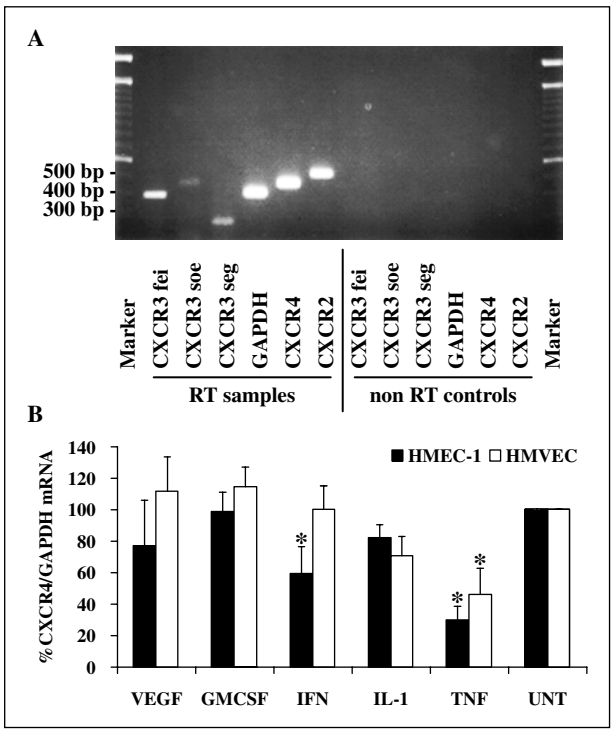


**Figure 1**

TNF-α and IFN-γ down-regulate CXCR4 mRNA levels in human microvascular endothelial cells *Panel A.* The specificity of the primers for CXCR2, CXCR3 (CXCR3fei, CXCR3soe and CXCR3seg; see also *table 1*), CXCR4 and the housekeeping gene GAPDH was first checked by performing RT-PCR analysis on PBMC. After RNA isolation, 1 µg of RNA was incubated in the presence (RT samples) or absence (non-RT controls) of reverse transcriptase (RT). PCR reactions were carried out on 1 µl of RT or non-RT mixtures and 16 µl of the PCR mixtures were finally loaded on 2% agarose gels. The specificity of the primers was confirmed by sequencing the RT-PCR fragments isolated from this gel. Panel B. Immortalized (HMEC-1; filled bars) and primary (HMVEC; open bars) human dermal microvascular endothelial cells were grown on 0.1% gelatin in their complete culture medium containing 5-10% FBS and growth supplements for 72 h (80% confluency). Cells were washed and stimulated with 10 ng/mL of VEGF, GM-CSF, IFN-γ, IL-1β or TNF-α in the presence of 0.5% FBS or were left untreated (UNT). After 4 h of incubation, RNA was isolated and semi-quantitative RT-PCR analysis was started. The ratios of the densitometric intensities of the CXCR4 relative to the GAPDH RT-PCR fragments on the gel were calculated and normalized versus the ratio of the untreated sample. The results represent the mean percentages (± standard error of the mean) of four independent experiments. Statistically significant differences compared to the untreated cells were determined with the Mann-Whitney U test (*, p-value < 0.05).

culturing of HMEC-1 cells for 48 h on various basement membrane matrix components, namely gelatin, collagen type IV or Matrigel, a commercially available basement membrane preparation *(figure 3A, 3B)*. However, if these endothelial cells were then incubated for an additional 6 h under hypoxic versus normoxic conditions, CXCR4 mRNA expression was significantly up-regulated *(figure 3A, 3B)*. The CXCR4 levels were also increased when the HMEC-1 cells were kept under normoxic conditions but serum-starved (0.5% FBS) during these additional 6 h. Furthermore, the CXCR4 up-regulation was reinforced by combining the hypoxic and serum starvation conditions, but remained independent of the basement membrane matrix components used for coating *(figure 3A, 3B)*. In contrast, changing the oxygen or serum concentrations or the coating agents did not consistently influence

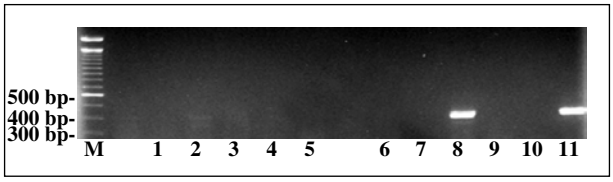


**Figure 2**

Lack of transcriptional regulation of CXCR3 by cytokines in human microvascular endothelial cells.

The influence of cytokines on the expression of CXCR3 mRNA in microvascular endothelial cells was also analysed by semi-quantitative RT-PCR analysis. Primary HMVEC cells were incubated for 4 h with 10 ng/ml VEGF (lane 1), GM-CSF (lane 2), IFN-γ (lane 3), IL-1β (lane 4), TNF-α (lane 5) or were left untreated (lanes 6, 7, 9, 10 and 11). RT-PCR analysis was performed with three different CXCR3 primer sets, namely CXCR3fei (lane 1 to 8), CXCR3soe (lane 9) and CXCR3seg (lane 10) (see also *table 1*). Nevertheless, no CXCR3 could be detected in the HMVEC cells. The successful RT-PCR analysis of PBMC with CXCR3fei primers (lane 8) and of untreated HMVEC with GAPDH primers (lane 11) served as positive controls. Lane 7 was a non-RT control (RT-PCR reaction in the absence of reverse transcriptase). Immortalized HMEC-1 cells also lacked CXCR3 expression, even in the presence of one of the cytokines mentioned previously (data not shown). M, DNA Marker.

the CXCR2 expression levels *(figure 3A)* and left the CXCR3 mRNA levels undetectable in HMEC-1 cells (data not shown).

The hypoxia-dependent CXCR4 up-regulation was not endothelial cell-specific, since we obtained similar results for human melanoma SK-MEL-5 cells. Indeed, CXCR4 mRNA expression was much higher in SK-MEL-5 cells cultured with 0.5% FBS under hypoxic than under normoxic conditions, but was only slightly more elevated compared to SK-MEL-5 cells treated with hypoxia in the presence of 10% FBS *(figure 3A, 3C)*. However, the CXCR2 and CXCR3 expression remained, respectively, very low or undetectable in the melanoma SK-MEL-5 cells under all conditions tested (*figure 3A* and data not shown).

### *Presence of CXCR7 mRNA in human microvascular endothelial but not melanoma cell lines*

For a long time, CXCR4 was believed to be the chemokine receptor unique for CXCL12. Recently however, CXCL12 was nominated as a putative ligand for the orphan receptor RDC1, hence renamed as CXCR7 [27]. We therefore investigated the presence and the hypoxic inducibility of CXCR7 in human microvascular endothelial cells. Although CXCR4 was observed in all the semi-quantitative RT-PCR experiments carried out with the HMEC-1 cell line, CXCR7 transcripts were only detected in 25% of these experiments *(figure 4)*. Nevertheless, sequence analysis confirmed that the RT-PCR fragment of 281 bp obtained with the CXCR7 primers in HMEC-1 cells as well as in PBMC and MCF7 breast carcinoma cells corresponded to the expected CXCR7 sequence. In those experiments where CXCR7 could be demonstrated, more CXCR7 transcripts were detected after hypoxic compared to normoxic treatment of the HMEC-1 cells *(figure 4)*. No CXCR7 transcripts could be observed in the CXCR4-expressing melanoma SK-MEL-5 cells.

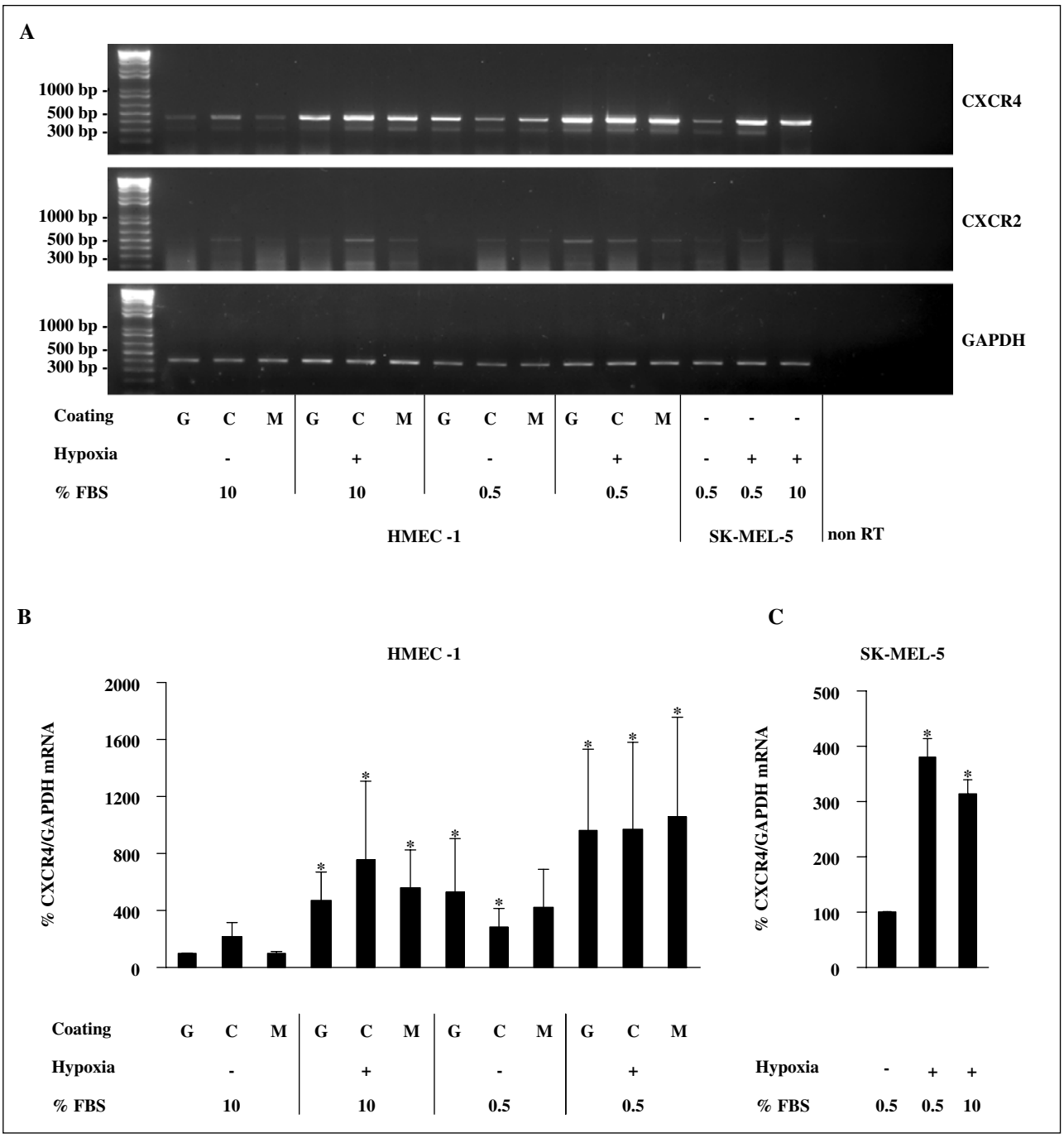


**Figure 3**

Transcriptional regulation of CXCR4 but not CXCR2 by oxygen and serum factor delivery in human microvascular endothelial and melanoma cells.

Immortalized HMEC-1 cells were plated on various basement membrane matrix components, namely gelatin (G), collagen type IV (C) or the commercially available basement membrane preparation Matrigel (M). After 48 h of culturing with medium containing 10% FBS and growth supplements, cells were washed and incubated for a further 6 h under normoxia or hypoxia (in hypoxic GasPak pouches, BD Biosciences) with 0.5% or 10% FBS. Human melanoma SK-MEL-5 cells were grown until confluency in complete culture medium, washed and also further incubated for 6 h under normoxia or hypoxia with 0.5 or 10% FBS. RNA was isolated and semi-quantitative RT-PCR analysis was performed with CXCR4, CXCR2 and GAPDH primers. One representative experiment out of three is shown (**A**). The ratios of the densitometric intensities of the CXCR4 relative to the GAPDH RT-PCR fragments for the HMEC-1 cells (**B**) and SK-MEL-5 cells (**C**) were calculated. Normalization was carried out versus the ratio of the HMEC-1 cells plated on gelatin and incubated with 10% FBS under normoxia (**B**), or the ratio of the SK-MEL-5 cells incubated with 0.5% FBS under normoxia (**C**). The results represent the mean percentages (± standard error of the mean) of three independent experiments. Statistically significant differences compared to the conditions used for normalization were determined with the Mann-Whitney *U* test (*, p-value < 0.05).

### *Low oxygen/serum factor levels up-regulate CXCR4 but not CXCR7 surface expression in HMEC-1 cells*

We next investigated whether the hypoxia/starvation regulated-CXCR4 and -CXCR7 expression had any impact on the presence of these receptors at the endothelial cell surface. Flow cytometry demonstrated that HMEC-1 cells did not display CXCR4 or CXCR7 proteins on their surface when cultured under normoxic conditions in the

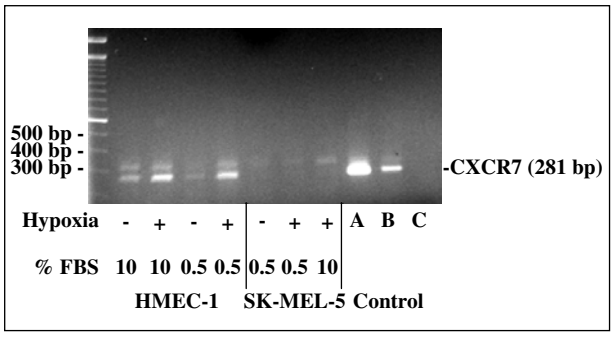


**Figure 4**
Presence of CXCR7 mRNA in human microvascular endothelial cells.
In order to analyse CXCR7 expression, HMEC-1 cells (lane 1 to 4) and human melanoma SK-MEL-5 cells (lane 5 to 7) were cultured for 48 h in the presence of 10% FBS, washed and incubated for additional 6 h under normoxia or hypoxia (in hypoxic GasPak pouches) with 0.5% or 10% FBS. The specificity of the CXCR7 primers was confirmed by sequence analysis of the RT-PCR fragments obtained for HMEC-1 cells (10%FBS/hypoxia for 6h; lower band), human breast carcinoma MCF7 cells (lane A) and human PBMC (lane B). The upper band obtained with the CXCR7 primers for the HMEC-1 cells (10%FBS/hypoxia for 6h) was not related to CXCR7 since sequence analysis of this upper RT-PCR fragment only generated a background signal. Lane C contains the non-RT control.

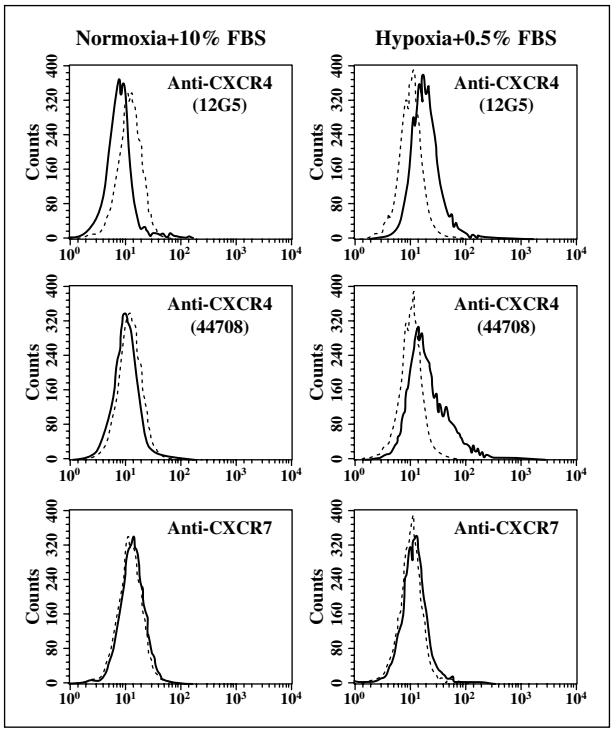


**Figure 5**
Low oxygen/serum factor supply increases CXCR4 but not CXCR7 expression on HMEC-1 cell surface.
HMEC-1 cells were allowed to attach for 24 h in normal culture medium. They were then washed and incubated for 24 h under normoxic conditions in the presence of 10% FBS (*left panels*) or in an hypoxic GasPak pouch in the presence of 0.5% FBS (*right panels*). FACS analysis was performed with different CXCR-specific antibodies, (___), namely anti-CXCR4 antibody clone 12G5 (*upper panels*) and clone 44708 (*middle panels*) from R&D Systems and the anti-CXCR7 antibody developed by Infantino *et al*. [30] (*lower panels*). For the two different treatment conditions, staining of the HMEC-1 cells with a mouse IgG antibody served as negative control (- - -). One representative experiment out of 3 is shown.

presence of 10% FBS *(figure 5)*. However, 24 h under hypoxic conditions in the presence of 0.5% FBS allowed for the detection of CXCR4 on the HMEC-1 cell surface *(figure 5)*. This up-regulated CXCR4 surface expression was clearly demonstrated using the anti- CXCR4 antibody clone 44708, whereas the anti-CXCR4 antibody clone 12G5 was generally less sensitive in the FACS analysis. This treatment of HMEC-1 cells with low oxygen and low serum concentrations did not however lead to a detectable increase in CXCR7 surface expression levels using the anti-CXCR7 antibody developed by Infantino *et al*. *(figure 5)* [30]. Furthermore, no CXCR2 or CXCR3 membrane expression could be observed in the HMEC-1 cells under these conditions (data not shown).

### *Hypoxia enhances CXCL12-mediated ERK signaling in HMEC-1 cells*

To evaluate the biological relevance of the hypoxic regulation of the CXCR4 expression in HMEC-1 cells, we investigated the *in vitro* activation of the mitogen-activated protein kinase ERK1/2 (extracellular signal-regulated kinase 1/2) in response to CXCL12. No ERK1/2 phosphorylation could be observed unless the HMEC-1 cells underwent hypoxic treatment for 24 h in the presence of 0.5% FBS prior to CXCL12 stimulation *(figure 6)*.
This indicates that hypoxia promoted CXCR4 transcription and functional CXCR4 expression leading to CXCL12-mediated signal transduction in serum starved HMEC-1 cells.

### *CXCR2, CXCR3, CXCR4 and CXCR7 immunoreactivity on endothelial and tumor cells in melanoma tissue*

Since *in vitro* expression profiles do not automatically reflect the *in vivo* situation, we analyzed the presence of CXCR2, CXCR3, CXCR4 and CXCR7 in human melanoma tissues. Immunohistochemical staining of at least 10 different melanoma samples for each chemokine receptor demonstrated that all these receptors were often expressed on the melanoma tumor cells and to a lesser extent on the endothelial cells *(figure 7)*. The tumor cells were positively stained with our previously characterized anti-CXCR2 antibody in 100% of the melanoma samples, and in 50% of these samples some endothelial cells also displayed CXCR2 immunoreactivity *(figure 7A)* [28, 29]. Strong CXCR3 staining was obtained with the anti-CXCR3 antibody from R&D Systems in 100% of the melanoma samples, and 90% of those tumor samples also showed CXCR3 immunoreactivity in the blood vessels *(figure 7B)*. CXCR4 was also detected in 100% of the tumor samples, with 80% of those tissues showing CXCR4 positive blood vessels using anti-CXCR4 antibody clone 44708 *(figure 7E)*. However, depending upon the antibody used for staining, the levels of detection were variable. Less immunostaining was observed with the anti-CXCR3 antibody from BD Biosciences *(figure 7C)* and with anti-CXCR4 antibody clone 12G5 *(figure 7D)*. Although anti-CXCR3 antibody from BD Biosciences still stained 80% of the tumor samples of which 75% contained CXCR3 positive blood vessels, the staining was less intense than with anti-CXCR3 antibody from R&D Systems. Positive

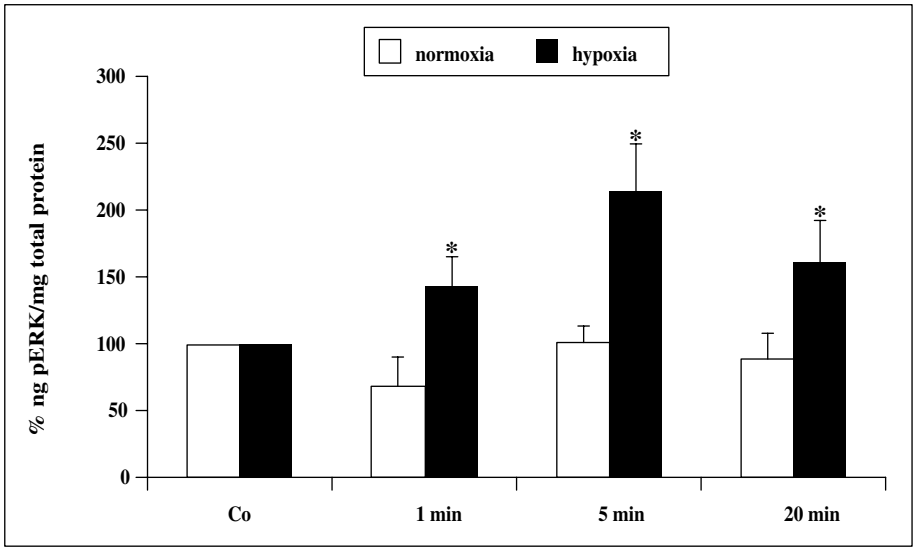


**Figure 6**
Hypoxia enhances CXCL12-mediated ERK1/2 activation in HMEC-1 cells. Immortalized HMEC-1 cells were grown in complete culture medium for 24 h, washed and serum-starved for 24 h under normoxic (empty bars) or hypoxic (filled bars) conditions, and subsequently treated with CXCL12 (100 ng/mL) for 1, 5 or 20 min or with control buffer for 5 min (Co). Cell extracts were analysed with a DuoSet p-ERK1/ERK2 ELISA and a BCA Protein Assay to measure the amount of p-ERK1/2 and total protein content, respectively. The ratios of the amount of p-ERK1/2 and the total protein content were calculated and normalized against the ratio of the buffer-treated control sample. The experiment was repeated four times. Statistically significant differences compared to the control cells were determined with the Mann-Whitney *U* test (*, p-value < 0.05).

immunoreactivity with the anti-CXCR4 antibody clone 12G5 *(figure 7D)* was only obtained in 40% of the melanoma sections, and in 50% of these positive samples, some endothelial cells were also slightly stained for CXCR4. Finally, CXCR7 was expressed in 70% of the melanoma samples and blood vessels. In 50% of these positive samples there was also positive staining for CXCR7 using the anti-CXCR7 antibody developed by Infantino *et al. (figure 7F)* [30].

## DISCUSSION

The expression of CXCR2, CXCR3 and CXCR4 by endothelial cells has already been well addressed by various research groups, the results pointing toward the potential involvement of these receptors and their ligands in fine-tuning the balance between stimulation and inhibition of new vessel growth [3, 6, 14-16, 20, 22]. However, endothelial cells used for *in vitro* experiments can originate from various species, donors, organs or types of vasculature and often display different phenotypical characteristics [1, 2, 18]. Since microvascular endothelial cells are more likely to participate in angiogenesis and inflammation, they represent a better target for studying the expression of potential angiogenic or angiostatic CXC chemokine receptors than the more often used macrovascular human umbilical vein endothelial cells (HUVEC) [1, 18]. In addition, little is known regarding the influence of environmental factors on the expression of these CXC chemokine receptors in human microvascular endothelial cells. Therefore, we performed semi-quantitative RT-PCR experiments to compare the inducibility of CXCR2, CXCR3 and CXCR4 by various cytokines in primary (HMVEC) and immortalized (HMEC-1) human dermal microvascular endothelial cells. Only TNF-α down-regulated CXCR4 mRNA expression in both HMVEC and HMEC-1 cells after 4 h, whereas IFN-γ reduced the CXCR4 transcript levels only in the HMEC-1 cells. No effect on CXCR4 mRNA levels was observed with VEGF, GM-CSF or IL-1β and the presence of various basement membrane matrix components also did not influence CXCR4 expression. In contrast to the inhibitory role for TNF-α and IFN-γ, CXCR4 mRNA was strongly induced by insufficient oxygen and/or serum, in both HMVEC (data not shown) and HMEC-1 cells. Furthermore, no CXCR2 transcription was detected in HMVEC cells, and the low CXCR2 mRNA levels present in HMEC-1 cells were not consistently affected by any of these factors (cytokines, matrix components, hypoxia or serum starvation). In addition, we did not observe any CXCR3 mRNA expression in any of our experimental settings with the cultured endothelial cells tested. Our FACS data confirmed the hypoxic up-regulation of CXCR4 since we could only observe CXCR4 surface expression in HMEC-1 cells after hypoxic treatment. In contrast to our data, Feil and Augustin reported as data (not shown) that TNF-α down-regulated CXCR3 mRNA but increased the CXCR2 levels upon 4 h treatment of human microvascular endothelial cells [19]. Yoshida *et al.* also showed higher amounts of CXCR2 transcripts in the presence of TNF-α [25], whereas this increase required the co-incubation of TNF-α with LPS in the case of human intestinal microvascular endothelial cells [21]. Furthermore, bFGF and VEGF enhanced surface CXCR4 expression in human microvascular endothelial cells after 24 h of stimulation, mainly through an indirect, prostaglandin E2-mediated pathway [24, 36]. More information is available on the regulated expression of CXCR2, CXCR3 and CXCR4 in the macrovascular HUVEC cells [14, 16, 19, 37, 38]. Importantly, Schioppa *et al.* described that the hypoxia-mediated up-regulation of CXCR4 occurs in HUVEC as well [37]. Furthermore, the CXCR4 mRNA levels in HUVEC were continuously suppressed after stimulation with IFN-γ for 1 h up to 24 h, whereas they decreased after TNF-α treatment for 1 to 5 h, but started to increase

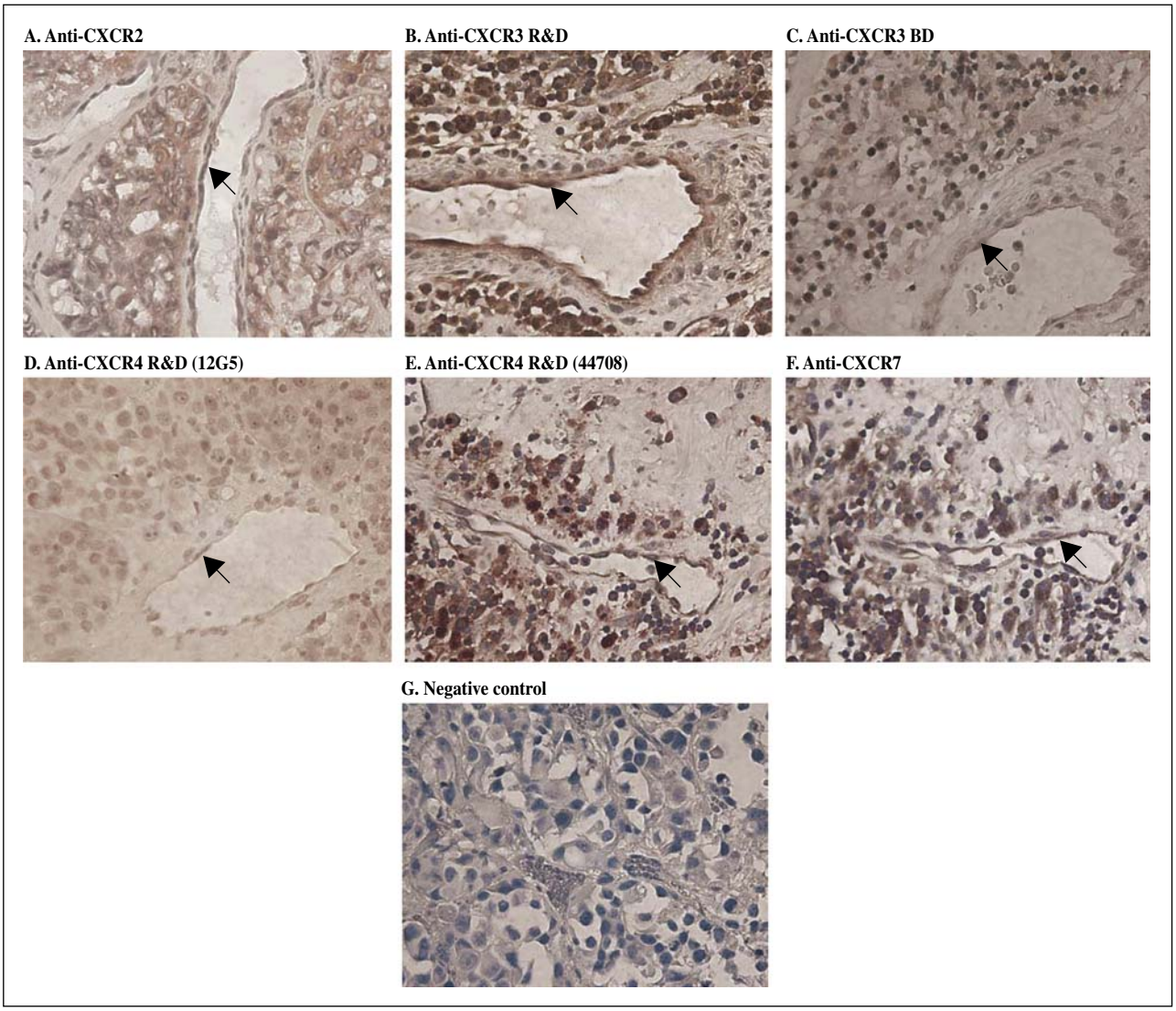


**Figure 7**

Expression of CXCR2, CXCR3, CXCR4 and CXCR7 by endothelial cells and tumor cells in human melanoma.
Immunohistochemical staining of human metastatic melanoma tumor sections for CXCR2, CXCR3, CXCR4 and CXCR7 was performed using the Vectastain ABC Elite (Vector Laboratories) system and NovaRed chromagen (Vector Laboratories). The various antibodies used were our previously characterized anti-CXCR2 antibody [28, 29] (**A**), anti-CXCR3 antibody from R&D Systems (**B**), anti-CXCR3 antibody from BD Biosciences (**C**), anti-CXCR4 antibody clone 12G5 (**D**), anti-CXCR4 antibody clone 44708 (**E**) and anti-CXCR7 antibody developed by Infantino *et al.* [30] (**F**). Mayer's hematoxylin was used as the nuclear counterstain and sections stained in the absence of primary antibody served as negative control (**G**). One representative picture (x40 magnification) is shown out of 10 melanoma biopsies analysed. Black arrows point towards endothelial cells with positive immunoreactivity (reddish color).

again afterwards [14]. Our RT-PCR and FACS data are in agreement with most previous reports pointing to CXCR4 as the most abundantly expressed chemokine receptor in microvascular and macrovascular endothelial cells compared to the lower or even undetectable expression levels of CXCR2 and CXCR3 [5, 14-16, 19, 20]. The relatively small degree of chemokine receptor expression at the mRNA and protein level in *in vitro*-cultured endothelial cells could account for the various, contradictory findings by different groups in the past [14-16, 19-26]. The differences in experimental conditions (such as number of cell passages, presence of growth supplements or serum prior to or during experiments, time lapse of experiments) and origin of the micro- and macrovascular endothelial cells, certainly contribute to this variability [17, 18]. It is likely that primary endothelial cells lose their *in vivo* expression pattern of chemokine receptors once they start to be cultured *in vitro,* as shown for CXCR4 in microvascular human bone marrow endothelial cells [17, 18, 26]. This might help clarify our low to undetectable CXCR2 and CXCR3 mRNA levels in HMEC-1 and HMVEC cells. Furthermore, the specificity/efficiency of some of the antibodies widely used in the different reports for Western blotting, FACS staining or immunochemical staining of cells or tissues is an important issue (e.g. monoclonal anti-CXCR3 Clone 49801 from R&D Systems versus Clone 1C6 from BD Pharmingen; antigenically distinct conformations of CXCR4) [7, 12, 33, 35, 39-41]. This antibody issue might also partly explain why microvascular endothelial cells seem to be stained more consistently than expected from the *in vitro* findings for CXCR2, CXCR3 and CXCR4 in immunohistological sections. Our immunohistochemical stainings of melanoma tissue showed that the tumor cells as well as the endothelial vessels, although to a lesser degree, displayed positive immunoreactivity for CXCR2, CXCR3, CXCR4 and

CXCR7. However, we also noticed much stronger staining with the anti-CXCR3 antibody from R&D Systems and the anti-CXCR4 antibody clone 44708 than with the anti-CXCR3 antibody from BD Biosciences and anti-CXCR4 antibody clone 12G5, respectively. Our findings nevertheless also confirm previous reports on the expression of CXCR2, CXCR3 and CXCR4 by melanoma cells [29, 42]. To verify the presence of these receptors on the endothelial cell membranes *in vitro*, some groups permeabilized their cells in order to obtain better FACS results [20, 22] or included confocal immunostainings of the endothelial cells showing clear intracellular, but rather low or unclear membrane-bound localization of the chemokine receptors [20, 22, 37]. Interestingly, a strong intracellular CXCR4 staining in combination with an unexpectedly low membrane localization has also been reported in various other cell types, including melanoma cells [42, 43]. However, even limited surface expression of the chemokine receptors on endothelial or non-endothelial cells does not necessarily preclude their biological responsiveness [20, 37, 42]. Hypoxic treatment of the HMEC-1 cells also induced sufficient CXCR4 surface expression to allow us to observe the activation of the ERK signaling pathway upon CXCL12 stimulation. Furthermore, it cannot be excluded that intracellularly located CXCR4 could still be actively involved in signal reactions. The hypoxic regulation of CXCR4 has already been observed in various cell types [37, 44]. We also demonstrated increased CXCR4 expression in human melanoma cells upon hypoxic treatment. In parallel, the up-regulation of CXCR4 in microvascular endothelial cells caused by the lack of serum factors and oxygen fits nicely with the concept that CXCR4 promotes the outgrowth of new blood vessels from pre-existing vessels (strict definition of angiogenesis) and/or from circulating endothelial progenitor cells (often referred to as vasculogenesis) in order to restore the oxygen and serum factor supply. Indeed, there are many *in vitro* and *in vivo* findings supporting a role for the CXCR4/CXCL12 axis in angiogenesis [16, 24, 38, 45-48]. Studies with CXCR4 and CXCL12 knock-out mice revealed an essential role for CXCR4 in the vascularization of the gastrointestinal tract [49]. Furthermore, it has become clear that not only differentiated endothelial cells but also endothelial cell precursors circulating through the body express CXCR4 [36, 43, 46, 47, 50, 51]. The ERK activation by CXCL12 in our endothelial cells is also in agreement with the stimulation of the MAPK pathway by other angiogenic factors in endothelial cells [13, 21, 52]. Some people however, argue that CXCR4 is more important for angiogenesis-independent metastasis and less relevant for angiogenesis as such, due to the lack of a detectable CXCL12 gradient *in vivo* towards the tumor and the relatively low intratumoral CXCL12 expression [3]. However, other reports show that CXCL12 is nevertheless highly expressed in some tumors [43, 48, 50] and induced by hypoxia in ovarian cancer cells [38]. Furthermore, VEGF, bFGF, hypoxia-inducible factor (HIF) and hypoxia up-regulated CXCL12 expression in endothelial cells [43, 45, 51], and specific blocking of the CXCL12/CXCR4 axis results in reduced angiogenesis *in vivo* [24, 45-47]. CXCL12 has recently been shown to bind both CXCR4 and the orphan receptor RDC1, which was hence renamed CXCR7 [27, 53]. Interestingly, RDC1 has been found to be up-regulated in glioma and colon tumor vasculature compared to non-neoplastic vasculature, and in microvascular endothelial cells after infection with Kaposi's sarcoma-associated Herpes virus [54, 55]. Furthermore, increased RDC1 transcription has been observed in rat brain endothelial cells and in human monocytes [56, 57]. Therefore, we investigated the presence and the hypoxic inducibility of CXCR7 in human microvascular endothelial cells. We found that CXCR7 was also up-regulated by hypoxia in HMEC-1 cells, although its expression was less consistent compared to CXCR4. Futhermore, no CXCR7 protein was observed on the HMEC-1 cell surface, which makes it less likely that this CXCR7 would be involved in the ERK activation by CXCL12 in hypoxia-treated HMEC-1 cells. Nevertheless, CXCR7 was clearly present on both the endothelial and the tumor cells, in human melanoma sections. The CXCR3 ligands are believed to suppress tumor progression through a process called immunoangiostatis, specifically by promoting Th1 immunity through Th1 mononuclear cell recruitment towards the tumor and by simultaneously inhibiting angiogenesis [3, 11, 58]. The way CXCL9, CXCL10 and CXCL11 could exert their angiostatic activity directly on endothelial cells remains an open debate. Besides an angiostatic effect through their interaction with angiogenic factors or glycosaminoglycans, the chemokine receptor CXCR3 has also been postulated to mediate the angiostatic activity of the CXCR3 ligands [3, 7]. Recently, Lasagni *et al*. discovered a new, alternatively spliced variant of CXCR3 and claimed that this so-called CXCR3B and not the classically spliced CXCR3, renamed CXCR3A, was responsible for the angiostatic activity of these ligands [12]. Another CXCR3 splicing variant, called CXCR3-alt, was demonstrated in PBMC as being generated through post-transcriptional exon skipping [35]. Nevertheless, using three different primer sets did not allow us to detect any mRNA for these three CXCR3 variants in primary HMVEC or immortalized HMEC-1 cells. Various groups have also failed to observe any CXCR3 in their endothelial cell preparations *in vitro* [14, 15, 34], despite the detection of endothelial CXCR3 reported by other groups [7, 20, 22]. Nevertheless, our immunohistochemical analysis of melanoma sections clearly showed positive CXCR3 staining on endothelial vessels. Interestingly, for all the angiostatic chemokines known so far, either no receptor has been found (CXCL14) [9], or they have been reported to bind at least one of the known human CXCR3 variants. Indeed, Lasagni *et al*. showed that CXCL4 and the classic CXCR3 ligands CXCL9, CXCL10 and CXCL11 bind CXCR3B [12]. CXCL13 has been reported to bind human CXCR3 in addition to its classic receptor, CXCR5, [5, 8], and murine CCL21 (but not human CCL21) is known to bind CXCR3 and to inhibit angiogenesis in a human lung cancer SCID mouse model [4, 59]. However, Sulpice *et al*. claimed that CXCR3B is probably not responsible for the CXCL4 activity in HUVEC [10] and no CXCR3B has been reported thus far in mice [3]. Nevertheless, CXCR3 ligands do exhibit angiostatic activity in mice [3, 11]. Therefore, it cannot be excluded that there exists only one CXCR3 receptor in mice which is responsible for the inhibition of vessel growth by all the angiostatic chemokines in mice models. In humans on the other hand, the angiostatic activity may be mediated through CXCR3B, through another, not yet identified, CXCR3 variant or through an unknown receptor, possibly with some similarity to CXCR3.

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