

Regional Expression of CXCL12/CXCR4 in Liver and Hepatocellular Carcinoma and Cell-cycle Variation during *in vitro* Differentiation

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The CXCL12/CXCR4 system may be important in carcinoma. Expression of the α -chemokine SDF-1 α (stromal cell derived factor-1 α)/CXCL12 mRNA is reduced in many carcinomas, yet its tissue protein expression may guide metastasis. Here we first compare the mRNA and protein expression of CXCL12 and its receptor CXCR4 in human liver, hepatocellular carcinoma, and malignant cell lines, and then assess cell cycle variation in CXCR4 expression. CXCR4 mRNA was present in most normal human tissues and malignant cell lines; it was only marginally reduced in hepatomas, while CXCL12 was markedly reduced, $P < 0.0001$. Immuno-histochemical staining of adjacent non-malignant liver showed regional CXCR4 cytoplasmic and cell-surface staining, limited to those hepatocytes around the central vein, a distribution resembling that of CXCL12. CXCL12 protein was not present in hepatocellular carcinoma cells *in vivo*, nor was cytoplasmic CXCR4 staining; nuclear CXCR4 protein expression in some malignant hepatocytes and CXCR4 staining of capillary endothelial cells around tumor cells were noted. In some malignant cell lines that had no CXCL12 on northern blots CXCL12 was weakly detectable by RT-PCR or protein staining in the cytoplasm of a few cells. With a view to future manipulation of CXCL12/CXCR4 expression and growth we noted that in HT-29 cells CXCR4 protein expression was less on confluent than on non-confluent cells and varied during the cell cycle. Higher expression was associated most closely with the percentage of cells in the S-phase and inversely with the percentage of cells in the G1-phase. Treatment of HT-29 cells with butyrate reduced CXCR4 cell surface expression and reduced the percentage of cells in S-phase. In summary, CXCL12 protein expression parallels its mRNA, being markedly reduced in malignant cell lines and hepatomas; in liver, the regional distributions of CXCL12 and cytoplasmic CXCR4 are similar; finally, in HT-29, CXCR4 expression correlates with the S-phase of the cell cycle and is reduced during butyrate-induced differentiation.

Key words: CXCR4 — SDF-1 α — hIRH — CXCL12 — Hepatocellular carcinoma

CXCL12, also called stromal cell-derived factor (SDF-1 α), or pre-B cell stimulating factor (PBSF) is the α -chemokine ligand for the receptor LESTR/fusin/CXCR4, which is important for HIV entry and AIDS pathogenesis.^{1,2} Using differential displays of HCC (hepatocellular carcinoma) tumor/normal pairs,³ we isolated a cDNA hIRH (human intercrine reduced in hepatomas), whose mRNA expression on northern blots was lost in 11 of 12 HCC samples compared to normal liver.^{4,5} hIRH was predicted to be an α -intercrine/chemokine based on its deduced amino acid sequence and after full cloning it was noted to be equivalent to the then published SDF-1 α /PBSF,^{6,7} now named CXCL12.^{8,9} CXCL12 mRNA expression was also lost in premalignant colonic adenomas and 27 malignant human cell lines.⁴ We recently described the reduced expression of CXCL12 mRNA in other human malignant

tissues such as colon, esophageal and gastric cancers.⁵ There have been few reports on the biological significance of CXCR4 expression in human cancers. An increased expression of CXCR4 was noted in glioblastomas,¹⁰ and in pancreatic cancer.¹¹ It was recently suggested that breast cancer cells over-expressing CXCR4 may target to specific sites of metastasis, including liver, based on their CXCL12 expression.⁹ Similarly, ovarian cancer cells may spread to the peritoneum utilizing their CXCR4 receptor expression¹² and high CXCR4 expression predicts extramedullary organ infiltration in childhood acute lymphoblastic leukemia.¹³ Given the emerging importance of the CXCL12/CXCR4 system in tumor spread, and that CXCL12 may down-regulate CXCR4 expression in several systems^{14,15} and affect tumor growth,^{10,16} one aim was the definition of the distribution of this ligand and receptor in the liver, a common site of tumor spread. A second aim, with a longer term goal of manipulation of tumor growth, was to assess the relationship between

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CXCR4 expression and cell cycle phase. The present study describes the regional expression of CXCL12 and CXCR4 in HCC, adjacent non-malignant liver *in vivo*, and investigates the cell cycle variation in CXCR4 expression in an *in vitro* carcinoma model system in which cell differentiation can be altered.

MATERIALS AND METHODS

Extraction of total RNAs Thirty fresh surgical pairs of hepatocellular carcinoma and corresponding adjacent non-malignant tissue were resected at the Medical Institute of Bioregulation Hospital, Kyushu University and Oita Prefectural Hospital, between 1995 and 1996 and processed as previously described,³⁾ after informed consent had been obtained. Total cellular RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method or by cesium chloride density centrifugation.³⁾

Reverse transcription-PCR cDNA was synthesized from 5 µg of total RNA and then amplified by PCR exactly as described.⁵⁾ Oligonucleotide primers amplified a 307 bp open-reading-frame (ORF) sequence of CXCR4 at 58°C annealing temperature and a 270 bp hIRH/SDF-1α unique 3'-non-coding region fragment at 56°C annealing temperature as described.¹⁷⁾ The PCR for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), which was used as a control to correct the values of CXCR4 expression and simultaneously to ensure that the used RNA was not degraded, was performed as described¹⁷⁾ on the same cDNA samples, to give a 540 bp product.

Gel electrophoresis and quantification of the products A semi-quantitative RT-PCR method was used to estimate mRNA expression.^{5,18)} The optimal PCR cycle numbers giving a linear increase in radioactivity were 28, 30 and 25 cycles for CXCR4, CXCL12 and G3PDH, respectively. The PCR products were separated on 2% agarose gels, cut out, extracted and counted as described.⁵⁾ The tumor-normal ratio (T/N ratio) was corrected for that of G3PDH expression.

Antibodies The following antibodies were used in this study: mouse monoclonal antibody (MAb) against human CXCR4/fusin (12G5, and mouse isotype-matched control antibody, Genzyme/Techne, Cambridge, MA or PharMingen, San Diego, CA), *R*-phycoerythrin-labeled rat anti-mouse IgG2a (Becton Dickinson, San Jose, CA) and rabbit polyclonal antibody against human CXCL12 (Pepro Tech EC, London, England). Controls omitted the primary antibody.

Immuno-histochemistry The labeled streptavidin-biotin (LSAB) peroxidase complex technique was used for immuno-histochemical staining. Sections were cut at 4 µm thickness, kept in xylene, rehydrated and washed with water, and microwaved in a citrate buffer solution (0.1 M sodium citrate, pH 6.0) at 500 W for 10 min. After treatment with 3% hydrogen peroxide in water for 5 min to

inhibit endogenous peroxidase, sections were incubated with the primary antibodies against CXCR4 and CXCL12 at 4°C overnight. These specimens were covered with biotinylated anti-mouse and anti-rabbit immunoglobulins in phosphate-buffered saline (PBS) (DAKO LSAB2 kit, Peroxidase; Carpinteria, CA) for 10 min at room temperature and with streptavidin conjugated to horseradish peroxidase in PBS for 10 min at room temperature. Between incubations, sections were washed with PBS. Color was developed with substrate buffer supplemented with 0.04% peroxidase and counterstained with Mayer's hematoxylin (Chroma, Stuttgart, Germany).

Cell culture The human colon adenocarcinoma cell line HT-29 (ATCC HB 38) was grown in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine. Cells were maintained in 95% air/5% CO₂.

Flow cytometric analysis of CXCR4 surface expression HT-29 cells either confluent (8×10⁶ cells) or subconfluent (4×10⁶ cells) were detached with 0.05% trypsin/0.02% EDTA, washed with PBS and single cell suspensions prepared. The suspended cells were incubated for 60 min at room temperature with 10 µg/ml of murine monoclonal antibody against CXCR4 (clone 12G5) or a mouse isotype-matched control antibody, followed by incubation with *R*-phycoerythrin-labeled rat anti-mouse IgG2a. The cells were washed and analyzed by flow cytometry (FACScan, Becton Dickinson, Sunnyvale, CA).

Cell cycle analysis of CXCR4 Cell cycle analysis was

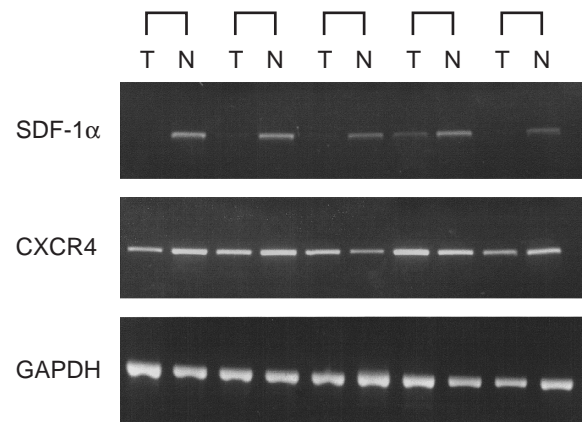


Fig. 1. SDF-1α/CXCL12 ligand and CXCR4 receptor mRNA expression by RT-PCR in five representative cases of hepatocellular carcinoma: total RNA was reverse-transcribed with random primers and then the cDNA amplified with SDF-1α/CXCL12, CXCR4 and G3PDH specific primers to give the anticipated PCR products, which were visualized with ethidium bromide staining. G3PDH mRNA expression is shown as the corresponding control in the lower panel. T, tumor; N, paired non-tumorous portion.

performed by means of DNA-propidium iodide (PI) binding and analysis with a FACS flow cytometer. HT-29 cells (1×10^6) were starved in serum-free medium for 48 h and then incubated in standard medium. At each time point (0, 24, 48, 72, 96 h), the cells were detached with 0.05% trypsin/0.02% EDTA, harvested by centrifugation, counted, and prepared for cell cycle analysis. Cells were suspended in 1 ml of PI solution (50 $\mu\text{g}/\text{ml}$ water), and were incubated in the dark for 30 min at room temperature before analysis. For treating cells with Na butyrate, the starved medium was removed and replaced with standard medium containing 5 mM butyrate that had been dissolved in PBS. The cell histogram FL-2 was divided into 3 regions according to the cell cycle phase: G0/G1, S, G2/M. The data were analyzed with ModFitLT2.0 software (Becton Dickinson, San Jose, CA).

Clinico-pathological findings The criteria for the pathological diagnosis of hepatoma, to include the degree of differentiation, tumor size, additional nodules, portal vein invasion, fibrous capsule formation, existence of capsule invasion and septal formation, were based on the grading system of the Liver Cancer Study Group of Japan.¹⁹⁾

Statistical analysis Student's or Welch's *t* test (two-tailed) was used to compare the distribution of T/N ratios between CXCR4 and CXCL12 and to examine the association of CXCR4 mRNA expression with the clinicopathologic data for hepatomas. A linear regression analysis was used to assess the correlation between CXCR4 mRNA and CXCL12 mRNA. Two-tailed *P* values of less than 0.05 were judged to be statistically significant.

RESULTS

The expression of CXCR4 and SDF-1 α /CXCL12 mRNA, evaluated by RT-PCR, is illustrated in Fig. 1 for 5

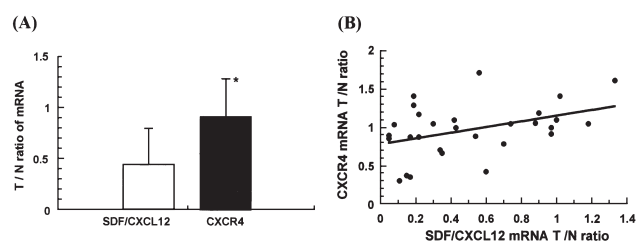


Fig. 2. (A) Distribution of tumor/normal (T/N) ratios for CXCR4 receptor mRNA and SDF-1 α /CXCL12 ligand mRNA in hepatocellular carcinoma. The T/N ratio was corrected for that of G3PDH. Data represent the mean \pm SD for a total of 30 hepatocellular carcinoma samples. The mean CXCR4 T/N ratio was 0.91 ± 0.35 , which was significantly higher than that of SDF-1 α /CXCL12 (0.44 ± 0.37) (*, $P < 0.0001$). (B) Correlation between T/N ratios of CXCR4 mRNA and SDF-1 α /CXCL12 mRNA ($P = 0.014$, $R = 0.40$, $n = 30$).

Table I. Relationship between CXCR4 mRNA Expression and Clinicopathological Features in Patients with Hepatocellular Carcinoma

Variable ^{a)}	No. of subjects	T/N ratio ^{b)}	<i>P</i> value ^{c)}
HBs-Ag			
negative	26	1.1 ± 0.5	0.61
positive	4	1.2 ± 0.2	
HCV			
negative	6	1.2 ± 0.2	0.59
positive	24	1.1 ± 0.5	
Cirrhosis			
absent	19	1.1 ± 0.5	0.84
present	11	1.1 ± 0.3	
Tumor number			
single	22	1.2 ± 0.5	0.25
multiple	8	0.9 ± 0.1	
Tumor size			
≤ 3 cm	15	1.1 ± 0.3	0.88
> 3 cm	15	1.1 ± 0.6	
Histology			
Grade [*] 1 and 2	27	1.1 ± 0.5	0.45
Grade 3	3	0.9 ± 0.1	
Growth pattern			
eg	24	1.1 ± 0.5	0.87
ig	6	1.2 ± 0.3	
fc			
absent	6	1.2 ± 0.2	0.60
present	24	1.1 ± 0.5	
fc-inf			
absent	8	1.2 ± 0.2	0.86
present	22	1.1 ± 0.5	
vp			
absent	18	1.1 ± 0.5	0.96
present	12	1.1 ± 0.3	
im			
absent	23	1.2 ± 0.5	0.40
present	7	0.9 ± 0.1	

a) The criteria used in this study are based on the rules outlined by the Liver Cancer Study Group of Japan.

b) Mean \pm SD.

c) *P* value was calculated according to Student's *t* test.

HBs-Ag, hepatitis B surface antigen; HCV, anti-hepatitis C virus antibody; eg, expansive growth; ig, infiltrative growth; fc, capsular formation; fc-inf, infiltration to capsule; vp, invasion to the portal vein; im, intrahepatic metastasis; *, the Edmondson and Steiner classification.

representative HCC tumor/normal pairs. The distribution of T/N ratios for CXCR4 and SDF-1 α /CXCL12, calculated by semi-quantitative RT-PCR and corrected for that of G3PDH, is shown in Fig. 2A. The range and mean \pm SD T/N ratios for each gene were: CXCR4, 0.23–1.67, 0.91 \pm 0.35 ($n=30$); SDF-1 α /CXCL12, 0.02–1.25, 0.44 \pm 0.37 ($n=30$). The T/N ratio of SDF-1 α /CXCL12 was less than 1.0 in 28/30 cases, and less than 0.5 in 19 cases (63%) of HCC's. In contrast, a closer distribution of CXCR4 T/N ratios was seen; it was less than 0.5 in only 4 samples (13%), and this distribution was significantly different from that of SDF-1 α /CXCL12 ($P<0.0001$) (Fig. 2A). When the SDF-1 α /CXCL12 T/N ratio was compared with the CXCR4 T/N ratio for the same HCC sam-

ples, there was a correlation between the mRNA expression levels of these two genes ($r=0.40$, $P=0.014$, Fig. 2B). The expression levels of CXCR4 mRNA did not correlate with clinical or pathological variables in patients with HCC (Table I). Both CXCR4 and CXCL12 were widely expressed at similar levels in normal human tissues as determined by RT-PCR, though expression of CXCL12 was weaker in brain. The tissues included normal human liver, pancreas, lung, larynx, muscle, breast, skin, uterus, bone marrow, and brain (Ref. 4), and other data not shown).

The distributions of both CXCR4 and CXCL12 proteins in HCC, adjacent non-cancerous liver tissues and malignant cell lines were examined by immuno-histochemical staining (Figs. 3 and 4). Controls with no primary anti-

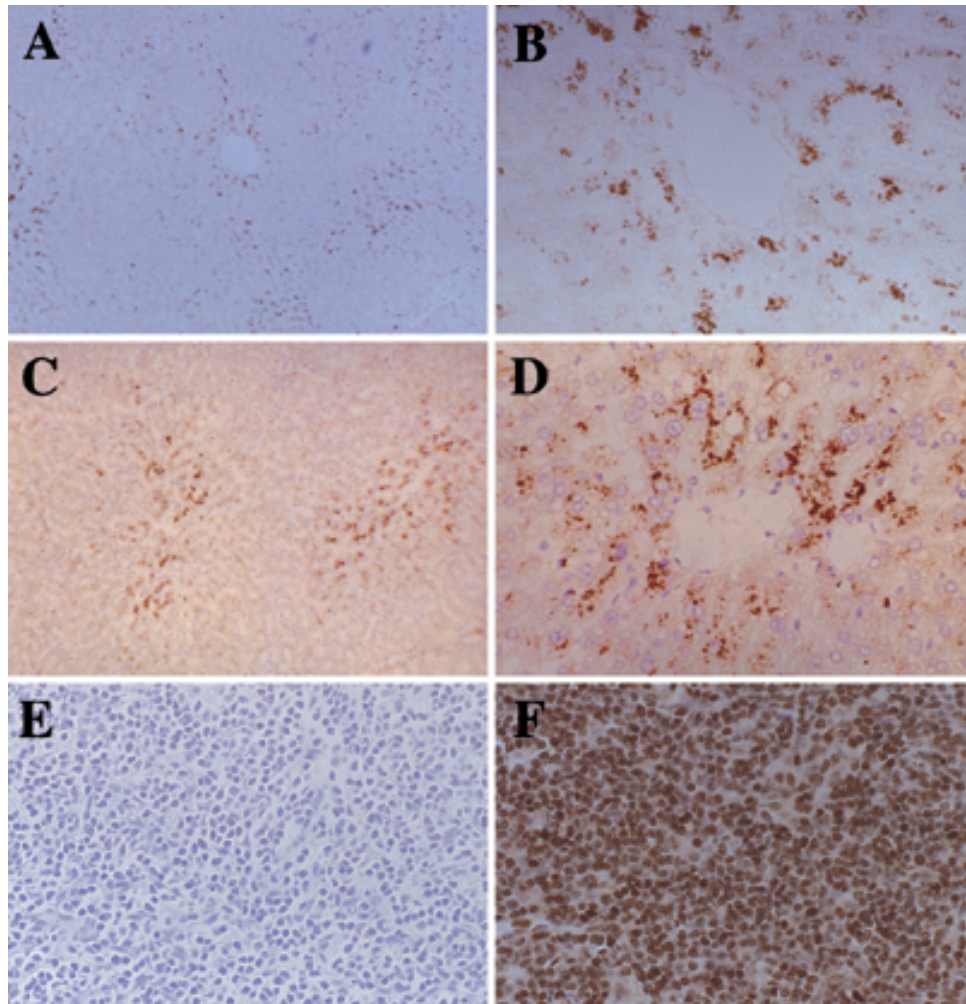


Fig. 3. Immunohistochemical staining of non-cancerous liver tissue with antibody against CXCR4, magnification $\times 100$, (A); $\times 400$, (B); and with antibody against SDF-1 α /CXCL12 $\times 100$, (C); and $\times 400$, (D). The expression of CXCR4 was demonstrated in the cytoplasm and cell surface of hepatocytes mostly around the central vein. SDF-1 α /CXCL12 was also expressed in the cytoplasm of the hepatocytes mostly around the central vein. Mouse thymus tissue negative controls with no primary antibody showed no staining (E); positive controls for CXCR4 staining (illustrated, and for CXCL12, not illustrated) showed strong uniform staining (F).

body showed no significant staining. CXCR4 staining in non-cancerous hepatocytes was observed close to the central vein in a cytoplasmic location (indicative of an accumulation of CXCR4 protein in intracellular storage granules), and on the cell surface, but not in the nucleus (Fig. 3, A and B). In contrast, in cancer tissues, strong CXCR4 staining was limited to the nucleus and nuclear surface of some but not all of the cancer cells, and it was not seen in the cytoplasm (Fig. 4A). CXCR4 staining in the tumors was also noted in capillary endothelial lining cells as a thin line around cells. CXCL12 staining in non-cancerous liver tissues was detected in a granular pattern in the cytoplasm of hepatocytes (Fig. 3, C and D), and not in the nucleus; this cytoplasmic staining of CXCL12 was distinctly limited to hepatocytes around the central vein. This pattern resembled the cytoplasmic staining pattern and the distribution of CXCR4 in non-cancerous tissue. The hepatoma cells showed no CXCL12 protein expression (Fig. 4B), this being similar to the mRNA results by northern blot.

The effects of CXC chemokines on tumor proliferation are different in different tumor types; for example, interleukin (IL)-8 may inhibit the proliferation of non-small

cell lung carcinoma *in vitro*,²⁰ but promote melanoma proliferation *in vitro*²¹ as well as its metastatic potential *in vivo*.²² We examined IL-8 mRNA expression in cell lines using RT-PCR (data not shown), but its expression did not parallel that of CXCL12 or CXCR4. The mRNA expression of CXCR4 and CXCL12 in 27 malignant cell lines *in vitro* was analyzed by RT-PCR. Although the ligand CXCL12 was not expressed in malignant cell lines on northern blots, as we have reported,⁴ some malignant cells weakly expressed CXCL12 mRNA on RT-PCR analysis (e.g. HT-29, a malignant human colon adenocarcinoma cell line, and Hep3B, a hepatocellular carcinoma cell line). In contrast, the receptor CXCR4 was detected by RT-PCR in all malignant cell lines examined (e.g. esophageal lines: KY150, KY200, KY410; gastric lines: KatoIII, MKN-1, NS8; colon lines: colo201, colo205, HT-29; hepatocellular lines: HepG2, Hep3B, huH7). There are some reports of cell lines lacking CXCR4, based on RT-PCR analysis, e.g. PC12, CHO, A549,²³ K562,²⁴ U251,²⁵ and a minority of breast cancer cell lines.⁹ Protein staining was carried out for the cell lines that demonstrated weak CXCL12 and CXCR4 mRNA by RT-PCR analysis. A representative cell line, HT-29, showed CXCR4 protein expression that

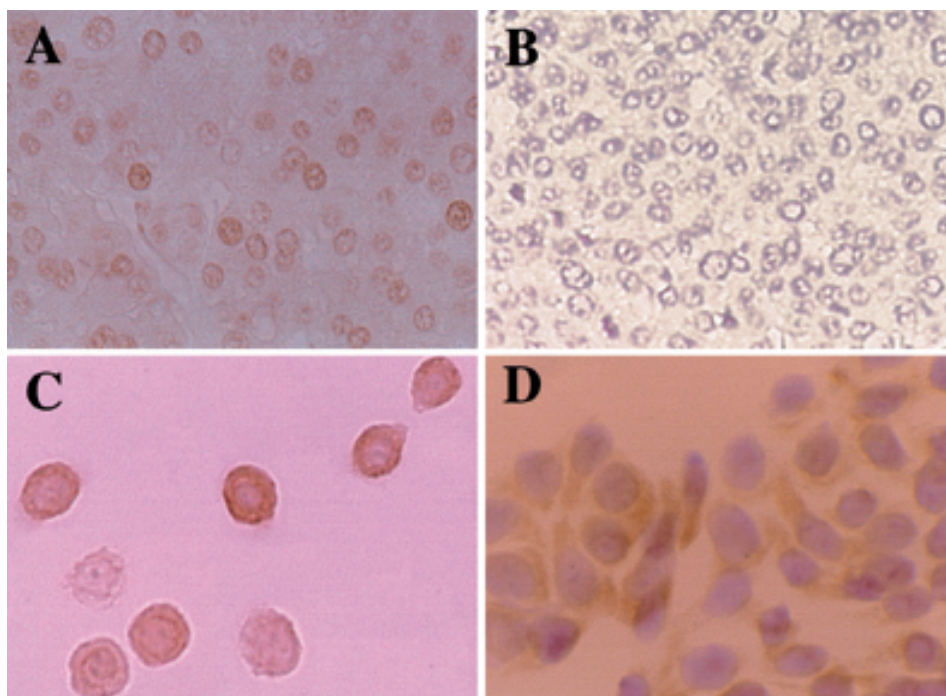


Fig. 4. Immunohistochemical staining of hepatocellular carcinoma tissue and HT-29 cells (colon adenocarcinoma cell line) with antibody against CXCR4 and SDF-1 α /CXCL12. In HCC the expression of CXCR4 was demonstrated on the nucleus of some cancer cells, but not in the cytoplasm ($\times 400$), (A). SDF-1 α /CXCL12 was not detected in cancer tissue ($\times 400$), (B); CXCR4 was demonstrated on the cell surface and in the cytoplasm of non-confluent or sparse HT-29 cells ($\times 400$), (C); SDF-1 α /CXCL12 was weakly expressed in the cytoplasm of a small number of HT-29 cells; this field was selected to show positive staining in a cluster of cells ($\times 1000$), (D).

was uniformly detected only on the cell surface and in the cytoplasm of non-confluent cells (Fig. 4C), and CXCL12 protein was weakly expressed in the cytoplasm of only a small number of cells (Fig. 4D, selected view). After staining HT-29 cells with CXCR4 antibody, we noted that non-confluent cells had a stronger staining for CXCR4 than confluent cells. This was confirmed by FACS analysis with anti-CXCR4 (Fig. 5). We thus wondered whether CXCR4 expression varied with growth or the phase of the cell cycle. HT-29 cells were grown in regular medium and the surface expression of CXCR4 determined by FACS analysis at different times after release of the cells from synchronization (Fig. 6). After release of synchronization, CXCR4 surface expression was higher at the time of release (t_0) than at ~48 h, and it was lower at 120 h, just before the change of medium, then it increased again after medium change. The increase and pattern of CXCR4 expression paralleled most closely the increase and pattern of the percentage of cells in the S-phase of the cell cycle and was related inversely to the proportion of cells in the G1-phase (Fig. 6A). Sodium butyrate treatment induces differentiation of HT-29 cells; it caused a reduction in cell surface expression of CXCR4 at all time points (Fig. 6B); this change was associated with a decline in the percentage of cells in the S-phase and an increase in the proportion of cells in the G1-phase (Fig. 6C).

DISCUSSION

The CXC chemokine SDF-1 α /PBSF/hIRH/CXCL12 is the unique biological ligand for CXCR4. We have reported that CXCL12 mRNA expression is lost from human hepatocellular carcinomas,^{4,5} and is markedly reduced in the majority of gastrointestinal tumors.⁵ Microarray cDNA analysis has recently confirmed the down-regulation of CXCL12 in HCC samples.²⁶ We also reported that the

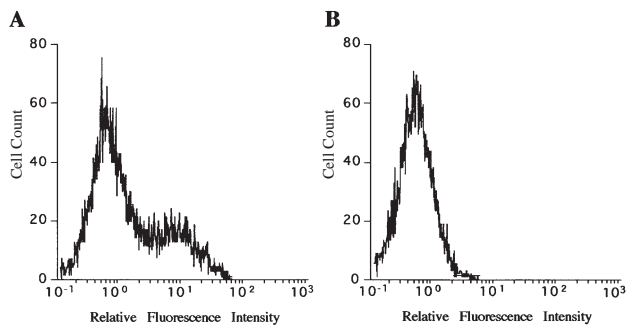


Fig. 5. Surface expression of CXCR4 in sub-confluent and confluent HT-29 cells. Flow cytometric analysis of CXCR4 expression in HT-29 showed an up-regulated surface expression of CXCR4 in sub-confluent cells (A) compared to confluent cells (B).

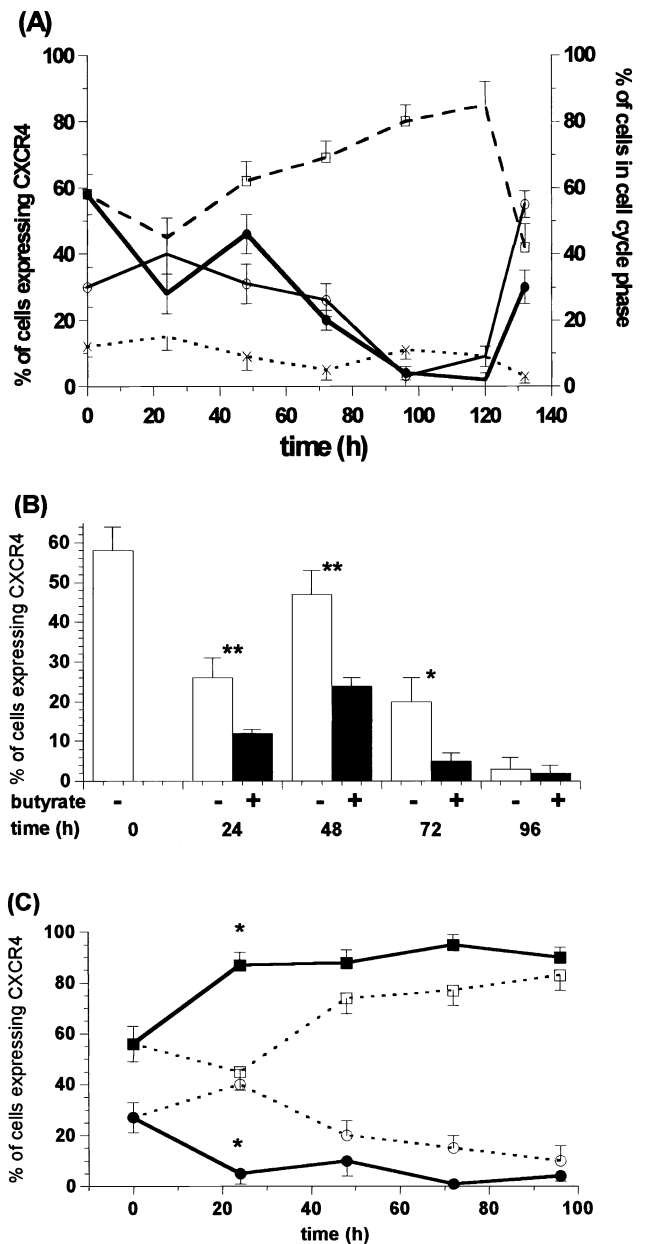


Fig. 6. Cell cycle variation in surface expression of CXCR4 protein in HT-29 cells. HT-29 cells were synchronized with serum-free medium for 48 h and then released by the addition of serum. Cells were analyzed by FACS at various times after release. Results are plotted as the percentage of cells expressing CXCR4 (●) or as the percentage of cells in each phase (G1 (□) vs. S (○) vs. G2/M (×)) of the cell cycle as a function of time (h). CXCR4 expression parallels most closely the percentage of cells in the S-phase, (A); Na butyrate (5 mM) added to sub-confluent cells reduced the percentage of cells expressing CXCR4 at all time points, ** $P < 0.01$, * $P < 0.05$, (B); Na butyrate (5 mM) reduced the percentage of cells in the S-phase (control, ○; butyrate treated, ●) and increased the percentage of cells in the G1-phase (control, □; butyrate treated, ■) of the cell cycle, * $P < 0.05$, (C).

reduction of CXCL12 expression probably occurs at an early step of the adenoma-carcinoma sequence in colon carcinogenesis.⁴⁾ Recent reports suggest an important role for the CXCL12/CXCR4 system later in the cancer sequence in targeting metastasis of breast cancer cells that strongly express CXCR4 to the liver and other organs that have a relatively high CXCL12 expression,⁹⁾ and also in facilitating peritoneal spread of ovarian cancer.¹²⁾ On northern blots we had noted a relatively strong CXCL12 signal in human liver, lung and even breast tissue, a relatively weak signal from larynx, skeletal muscle, colon, and a very weak/absent signal from brain tissue.⁴⁾

CXCR4 expression was detectable in every HCC sample and its degree of expression did not correlate with the clinicopathological features of HCC. This is similar to what was reported in pancreatic cancers,¹¹⁾ but in contrast to glioblastomas, in which CXCR4 expression correlated with tumor grade.²⁷⁾ CXCR4 mRNA expression was not reduced, or significantly increased, in other digestive tract cancers based on RT-PCR.¹⁸⁾ The minimal reduction in CXCR4 receptor expression in HCC in this study (mean CXCR4 T/N ratio=0.9, $n=30$) was not as noticeable as in our prior study¹⁷⁾ (mean T/N=0.65, $n=10$), perhaps because of a bias from the previous smaller sample size. The marked reduction in CXCL12 ligand mRNA expression was confirmed in this larger sample and corroborated at the protein level. CXCR4 mRNA levels correlated with those of CXCL12 mRNA in the same HCC samples. An inverse correlation might have been anticipated because of the possibility of down-regulation of CXCR4 by CXCL12 seen in some systems.^{14, 15)}

To further clarify the relationship between the chemokine CXCL12 and its receptor CXCR4 expression in tissues, we analyzed their protein distributions. There have been no detailed reports regarding the location of chemokine CXCL12 or its receptor CXCR4 protein expression in malignant and non-malignant adult liver tissue. Primary cultures of human liver sinusoidal endothelial cells and Kupffer cells were permissive to HIV-1 replication,²⁸⁾ suggesting that these cell types express CXCR4. The epithelial cell line HepG2 could be infected by HIV²⁹⁾ suggesting a functional role for its CXCR4 in HIV infection, although it may have a defect in CXCR4 cell signaling pathways.³⁰⁾ RT-PCR has been used to detect CXCR4 mRNA in liver in some studies, e.g. from rhesus monkeys,³¹⁾ and our prior human studies,¹⁷⁾ but other studies failed to detect the mRNA.^{7, 10)} During liver development hepatocytes did not show CXCR4 or CXCL12 by *in-situ* hybridization, but signals were noted that correlated with liver hematopoietic cells.³²⁾ In fetal liver, mesothelial and biliary epithelial cells were the only cells noted to contain CXCL12 by monoclonal antibody staining.³³⁾ CXCL12 mRNA has been detected by northern blots in mouse,³⁴⁾ and human tissues, including liver.^{4, 6)} In our study,

CXCR4 was expressed on the nuclear surface and in the nucleus of some hepatoma cancer cells, but not in non-cancerous hepatocyte nuclei. Further, both CXCR4 and CXCL12 protein were expressed intracellularly and on the cell surface only of those non-cancerous hepatocytes located close to a central vein, the site of more mature, zone 3, hepatocytes. This is also a region of lower oxygen tension which might induce cytokine expression, e.g. IL-8.³⁵⁾ One might predict metastatic cells would be preferentially retained in these sites if guiding by SDF expression is a limiting factor.⁹⁾ We noted CXCR4 staining in the endothelial cells of capillary vessels around the cancer cells. The expression of CXCR4 was reported in cultured endothelial cells, with a differential expression in migrating compared to resting endothelial cells,³⁶⁾ and in arterial or venous endothelium.^{32, 37)} CXCR4 was expressed in mesenteric endothelial cells, while CXCL12 mRNA was expressed in surrounding peri-endothelial cells as determined by *in-situ* hybridization.³⁸⁾ Thus, in blood vessel tissue, there is a specific overlapping localization pattern for the expression of this ligand and its receptor, just as we observed in the liver.

Colorectal cancer cell line HT-29 provides a model in which the differentiation status can be manipulated and in which we observed a weak cytoplasmic staining for CXCL12 in a few cells. The expression of surface CXCR4 on HT-29 cells varied with the culture conditions. It seemed greatest in association with rapid growth, being highest in non-confluent cells, correlating with the S-phase of the cell cycle, and less differentiated state; conversely, its expression was lowest in confluent cells, during the G1-phase, and when differentiation was induced with butyrate. A decline with butyrate-induced differentiation has been noted previously.³⁹⁾ A cell cycle variation of ligand CXCL12 expression was noted in another modeling system, that of murine TPAR1, and this was greatest in the S-G2/M-phases in mouse fibroblasts.³⁴⁾ Recent evidence indicates that CXCL12 may arrest the cycling of primitive hematopoietic cells,⁴⁰⁾ although other work suggests it may make precursor cells progress through the S+G2/M-phases.⁴¹⁾ Thus, the exact relationship of CXCL12/CXCR4 to cell cycling is uncertain. We anticipate that the addition of CXCL12 would down-regulate cell surface expression of CXCR4 in HT-29 cells or cancers (as speculated for intestinal lymphocytes⁴²⁾ to reduce mucosal HIV infection), and the percentage of cells in the S-phase, but this will need to be verified. An increased CXCR4 expression may be related to glioblastoma tumor grade²⁷⁾ and metastatic homing of tumor cells,^{9, 12)} while down-regulation of CXCR4 could have important consequences such as driving differentiation of cells or reducing tumor spread. Certainly, when HT-29 cells were induced to differentiate by treating them with butyrate, the expression of CXCR4 was reduced and the percentage of cells in the S-phase decreased.

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REFERENCES

- 1) Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J. and Springer, T. A. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature*, **382**, 829–833 (1996).
- 2) Oberlin, E., Amara, A., Bachelier, F., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M. and Moser, B. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature*, **382**, 833–835 (1996).
- 3) Begum, N. A., Mori, M., Matsumata, T., Takenaka, K., Sugimachi, K. and Barnard, G. F. Differential display and integrin alpha six overexpression in hepatocellular carcinoma. *Hepatology*, **22**, 1447–1455 (1995).
- 4) Begum, N. A., Coker, A., Shibuta, K., Swanson, R. S., Chen, L. B., Mori, M. and Barnard, G. F. Loss of hIRH mRNA expression from premalignant adenomas and malignant cell lines. *Biochem. Biophys. Res. Commun.*, **229**, 864–868 (1996).
- 5) Shibuta, K., Begum, N. A., Mori, M., Shimoda, K., Akiyoshi, T. and Barnard, G. F. Reduced expression of hIRH/SDF1a/PBSF in gastrointestinal tract cancers. *Int. J. Cancer*, **73**, 656–662 (1997).
- 6) Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T. and Honjo, T. Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. *Genomics*, **28**, 495–500 (1995).
- 7) Nagasawa, T., Nakajima, T., Tachibana, K., Iizasa, H., Bleul, C. C., Yoshie, O., Matsushima, K., Yoshida, N., Springer, T. A. and Kishimoto, T. Molecular cloning and characterization of a murine PBSF/SDF1 receptor, a murine homolog of the human HIV-1 entry coreceptor fusin. *Proc. Natl. Acad. Sci. USA*, **93**, 14726–14729 (1996).
- 8) Zlotnik, A. and Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity*, **12**, 121–127 (2000).
- 9) Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E. and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature*, **410**, 50–56 (2001).
- 10) Sehgal, A., Keener, C., Boynton, A. L., Warrick, J. and Murphy, G. P. CXCR-4, a chemokine receptor, is overexpressed in and required for proliferation of glioblastoma tumor cells. *J. Surg. Oncol.*, **69**, 99–104 (1998).
- 11) Koshiba, T., Hosotani, R., Miyamoto, Y., Ida, J., Tsuji, S., Nakajima, S., Kawaguchi, M., Kobayashi, H., Doi, R., Hori,

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- T., Fujii, N. and Imamura, M. Expression of SDF and CCR4 in pancreatic cancer: a possible role for tumor progression. *Clin. Cancer Res.*, **6**, 3530–3535 (2000).
- 12) Scotton, C. J., Wilson, J. L., Milliken, D., Stamp, G. and Balkwill, F. R. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res.*, **61**, 4961–4965 (2001).
- 13) Crazzolara, R., Kreczy, A., Mann, G., Heitger, A., Eibl, G., Fink, F. M., Mohle, R. and Meister, B. High expression of the chemokine receptor CXCR4 predicts extramedullary organ infiltration in childhood acute lymphoblastic leukemia. *Br. J. Haematol.*, **115**, 545–553 (2001).
- 14) Aiuti, A., Turchetto, L., Cota, M., Cipponi, A., Brambilla, A., Arcelloni, C., Paroni, R., Vicenzi, E., Bordignon, C. and Poli, G. Human CD34(+) cells express CXCR4 and its ligand stromal cell-derived factor-1. Implications for infection by T-cell tropic human immunodeficiency virus. *Blood*, **94**, 62–73 (1999).
- 15) Yang, O. O., Swanberg, S. L., Lu, Z. J., Dziejman, M., McCoy, J., Luster, A. D., Walker, B. D. and Herrmann, S. H. Enhanced inhibition of human immunodeficiency virus type 1 by Met-Stromal-Derived Factor 1 beta correlates with down-modulation of CXCR4. *J. Virol.*, **73**, 4582–4589 (1999).
- 16) Nomura, T., Hasegawa, H., Kohno, M., Sasaki, M. and Fujita, S. Enhancement of anti-tumor immunity by tumor cells transfected with the secondary lymphoid tissue chemokine EBI-1 and stromal cell derived factor 1 alpha genes. *Int. J. Cancer*, **91**, 597–606 (2001).
- 17) Begum, N. A., Shibuta, K., Mori, M. and Barnard, G. F. Reduced expression of the CXCR4 receptor mRNA in hepatocellular carcinoma and lack of inducibility of its ligand α -chemokine hIRH/SDF1a/PBSF *in vitro*. *Int. J. Oncol.*, **14**, 927–934 (1999).
- 18) Mitra, P., Shibuta, K., Mathai, J., Shimoda, K., Banner, B. F., Mori, M. and Barnard, G. F. CXCR4 mRNA expression in colon, esophageal and gastric cancers and hepatitis C infected liver. *Int. J. Oncol.*, **14**, 917–925 (1999).
- 19) Edmondson, H. A. and Steiner, P. E. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer*, **7**, 462–503 (1954).
- 20) Wang, J., Huang, M., Lee, P., Komanduri, K., Sharma, S., Chen, G. and Dubinett, S. M. Interleukin-8 inhibits non-small cell lung cancer proliferation: a possible role for regulation of tumor growth by autocrine and paracrine pathways. *J. Interferon Cytokine Res.*, **16**, 53–60 (1996).
- 21) Schadendorf, D., Moller, A., Algermissen, B., Worm, M., Sticherling, M. and Czarnetzki, B. IL-8 produced by human malignant melanoma cells *in vitro* is an essential

- autocrine growth factor. *J. Immunol.*, **151**, 2667–2675 (1993).
- 22) Singh, R. K., Gutman, M., Radinsky, R., Bucana, C. D. and Fidler, I. J. Expression of IL-8 correlates with the metastatic potential of human melanoma cells in nude mice. *Cancer Res.*, **54**, 3242–3247 (1994).
 - 23) Loetscher, M., Geiser, T., O'Reilly, T., Zwahlen, R., Baggiolini, M. and Moser, B. Cloning of the seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. *J. Biol. Chem.*, **269**, 232–237 (1994).
 - 24) Sehgal, A., Ricks, S., Boynton, A. L., Warrick, J. and Murphy, G. P. Molecular characterization of CXCR-4: a potential brain tumor-associated gene. *J. Surg. Oncol.*, **69**, 239–248 (1998).
 - 25) Moriuchi, M., Moriuchi, H., Turner, W. and Fauci, A. S. Cloning and analysis of the promoter region of CXCR4, a coreceptor for HIV-1 entry. *J. Immunol.*, **159**, 4322–4329 (1997).
 - 26) Shirota, Y., Kaneko, S., Honda, M., Kawai, H. F. and Kobayashi, K. Identification of differentially expressed genes in hepatocellular carcinoma with cDNA microarrays. *Hepatology*, **33**, 832–840 (2001).
 - 27) Rempel, S. A., Dudas, S., Ge, S. and Gutierrez, J. A. Identification and localization of the cytokine SDF1 and its receptor CXCR4 to regions of necrosis and angiogenesis in human glioblastoma. *Clin. Cancer Res.*, **6**, 102–111 (2000).
 - 28) Steffan, A. M., Lafon, M. E., Gendrault, J. L., Schweitzer, C., Royer, C., Jaeck, D., Amaud, J. P., Schmitt, M. P., Aubetin, A. M. and Kim, A. Primary cultures of endothelial cells from the human liver sinusoid are permissive for human HIV virus type 1. *Proc. Natl. Acad. Sci. USA*, **89**, 1582–1586 (1992).
 - 29) Banerjee, R., Sperber, K., Pizzella, T. and Mayer, L. Inhibition of HIV-1 productive infection in hepatoblastoma HepG2 cells by recombinant TNF-alpha. *AIDS*, **6**, 1127–1131 (1992).
 - 30) Mitra, P., De, D., Ethier, M. F., Mimori, K., Kodys, K., Shibuta, K., Mori, M., Madison, J. M., Miller-Graziano, C. and Barnard, G. F. Loss of chemokine SDF-1 mediated CXCR4 signalling and receptor internalization in human hepatoma cell line HepG2. *Cell. Signal.*, **13**, 311–319 (2001).
 - 31) Federspiel, B., Melhado, I. G., Duncan, A. M., Delaney, A., Schappert, K., Clark-Lewis, I. and Jirik, F. R. Molecular cloning of the cDNA and chromosomal localization of the gene for a putative seven-transmembrane segment (7-TMS) receptor isolated from human spleen. *Genomics*, **16**, 707–712 (1993).
 - 32) McGrath, K. E., Koniski, A. D., Maltby, K. M., McGann, J. K. and Palis, J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev. Biol.*, **213**, 442–456 (1999).
 - 33) Coulomb-L'Hermine, A., Amara, A., Schiff, C., Durand-Gasselino, I., Foussat, A., Delaunay, T., Chaouat, G., Capron, F., Ledee, N., Galanaud, P., Arenza-Seisdedos, F. and Emilie, D. Stromal cell-derived factor 1 (SDF-1) and antenatal human B cell lymphopoiesis: expression of SDF-1 by mesothelial cells and biliary ductal plate epithelial cells. *Proc. Natl. Acad. Sci. USA*, **96**, 8585–8590 (1999).
 - 34) Jiang, W., Zhou, P., Kahn, S. M., Tomita, N., Johnson, M. D. and Weinstein, I. B. Molecular cloning of TPAR1, a gene whose expression is repressed by the tumor promoter TPA. *Exp. Cell Res.*, **215**, 284–293 (1994).
 - 35) Karakurum, M., Shreenivas, R., Chen, J., Pinsky, D., Yan, S. D., Anderson, M., Sunouchi, K., Major, J., Hamilton, T., Kuwabara, K., Rot, A., Nowygorod, R. and Stern, D. Hypoxic induction of IL-8 expression in human endothelial cells. *J. Clin. Invest.*, **93**, 1564–1670 (1994).
 - 36) Feil, C. and Augustin, H. G. Endothelial cells differentially express functional CXCR4 under the control of autocrine activity and exogenous cytokines. *Biochem. Biophys. Res. Commun.*, **247**, 38–45 (1998).
 - 37) Volin, M. V., Joseph, L., Shockley, M. S. and Davies, P. F. Chemokine receptor CXCR4 expression in endothelium. *Biochem. Biophys. Res. Commun.*, **242**, 46–53 (1998).
 - 38) Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S.-I., Kishimoto, T. and Nagasawa, T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*, **393**, 591–594 (1998).
 - 39) Jordan, N. J., Kolios, G., Abbot, S. E., Sinai, M. A., Thompson, D. A., Petraki, K. and Westwick, J. Expression of functional CXCR4 chemokine receptors on human colonic epithelial cells. *J. Clin. Invest.*, **104**, 1061–1069 (1999).
 - 40) Cashman, J., Clark-Lewis, I., Eaves, A. and Eaves, C. SDF inhibits the cycling of very primitive human hematopoietic cells *in vitro* and in NOD/SCID mice. *Blood*, **99**, 792–799 (2002).
 - 41) Lataillade, J. J., Clay, D., Bourin, P., Herodin, F., Dupuy, C., Jasmin, C. and Bousse-Kerdiles, M. C. SDF regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. *Blood*, **99**, 1117–1129 (2002).
 - 42) Agace, W., Amara, A., Roberts, A. I., Pablos, J. L., Thelen, S., Ugucioni, M., Li, X. Y., Marshal, J., Arenzana-Seisdedos, F., Delaunay, T., Ebert, E. C., Moser, B. and Parker, C. M. Constitutive expression of SDF-1 by mucosal epithelia and its role in HIV transmission/propagation. *Curr. Biol.*, **10**, 325–328 (2000).