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THE CXCR7/CXCR4/CXCL12 AXIS IN HUMAN NEUROBLASTOMA: INVOLVEMENT IN MALIGNANT PROGRESSION

Thèse de doctorat ès sciences de la vie (PhD)

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par

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THE CXCR7/CXR4/CXCL12 AXIS IN HUMAN NEUROBLASTOMA: INVOLVEMENT IN MALIGNANT PROGRESSION

Lausanne, le 9 mars 2012

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof, Thierry Pedrazzini

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Résumé

Le neuroblastome (NB) est la tumeur maligne solide extra-crânienne la plus fréquente chez le jeune enfant. L'évolution clinique est très hétérogène, et les NBs de haut risque échappent encore aux traitements les plus agressifs. Diverses études ont montré que les chimiokines et leurs récepteurs, particulièrement l'axe CXCR4/CXCL12, sont impliqués dans la progression tumorale. Dans le NB, l'expression de CXCR4 est corrélée à un pronostic défavorable. De récentes études ont identifié l'expression d'un autre récepteur, CXCR7, présentant une forte affinité pour le ligand CXCL12. Cependant, son implication potentielle dans l'agressivité des NBs reste encore inconnue.

Notre étude a pour objectif d'analyser le rôle de CXCR7 dans le comportement malin du NB, et son influence sur la fonctionnalité de l'axe CXCR4/CXCL12. Les profils d'expression de CXCR7 et CXCL12 ont d'abord été évalués sur un large échantillonnage de tissus de NB, incluant des tissus de tumeurs primaires et de métastases, provenant de 156 patients. CXCL12 est fortement détecté dans les vaisseaux et le stroma des tumeurs. Contrairement à CXCR4, CXCR7 n'est que très faiblement exprimé par les tumeurs indifférenciées. Néanmoins, l'expression de CXCR7 augmente dans les tumeurs matures, et se trouve spécifiquement associée aux cellules neurales différentiées, telles que les cellules ganglionnaires. L'expression de CXCR7 est faiblement détectée dans un nombre réduit de lignées de NB, mais peut-être induite suite à des traitements avec des agents de différenciation *in vitro*.

La surexpression de CXCR7, CXCR4 et une combinaison des deux récepteurs dans les lignées IGR-NB8 et SH-SY5Y a permis l'analyse de leur fonction respective. En réponse à leur ligand commun, chaque récepteur induit l'activation de la voie ERK 1/2, mais pas celle de la voie Akt. Contrairement à CXCR4, l'expression exogène de CXCR7 réduit fortement la prolifération des cellules de NB *in vitro*, et *in vivo* dans un modèle d'injection sous-cutanée de souris immunodéprimées. CXCR7 altère également la migration des cellules induite par l'axe CXCR4/CXCL12. De plus, l'utilisation d'un modèle orthotopique murin a démontré que la croissance tumorale induite par CXCR4 peut être fortement retardée lorsque les deux récepteurs sont co-exprimés dans les cellules de NB. Aucune induction de métastases n'a pu être observée dans ce modèle.

Cette étude a permis d'identifier un profil d'expression opposé et des rôles distincts pour CXCR7 et CXCR4 dans le NB. En effet, contrairement à CXCR4, CXCR7 présente des propriétés non tumorigéniques et peut être associé au processus de différenciation du NB. De plus, nos analyses suggèrent que CXCR7 peut réguler les mécanismes induits par CXCR4. Ces données ouvrent donc de nouvelles perspectives de recherche quant au rôle de l'axe CXCR7/CXCR4/CXCL12 dans la biologie des NBs.

Summary

Neuroblastoma (NB) is a typical childhood and heterogeneous neoplasm for which efficient targeted therapy for high-risk tumours is not yet identified. The chemokine CXCL12, and its receptors CXCR4 and CXCR7 have been involved in tumour progression and dissemination in various cancer models. In the context of NB, CXCR4 expression is associated to undifferentiated tumours and poor prognosis, while the role of CXCR7, the recently identified second CXCL12 receptor, has not yet been elucidated.

In this report, CXCR7 and CXCL12 expression were evaluated using a tissue micro-array (TMA) including 156 primary and 56 metastatic NB tissues. CXCL12 was found to be highly associated to NB vascular and stromal structures. In opposite to the CXCR4 expression pattern, the neural-associated CXCR7 expression was extremely low in undifferentiated tumours, while its expression increased in maturated tissues and was specifically associated to the differentiated neural tumour cells. As determined by RT-PCR, CXCR7 expression was only found in a minority of NB cell lines. Moreover, its expression in two CXCR7-negative NB cell lines was further induce upon treatment with differentiation agents *in vitro*.

The relative roles of the two CXCL12 receptors was further assessed by overexpressing individual CXCR7 or CXCR4 receptors, or a combination of both, in the IGR-NB8 and SH-SY5Y NB cell lines. *In vitro* functional analyses indicated that, in response to their common ligand, both receptors induced activation of ERK 1/2 cascade, but not Akt signaling pathway. CXCR7 strongly reduced *in vitro* growth, in contrast to CXCR4. Sub-cutaneous implantations of CXCR7-expressing NB cells showed that CXCR7 also drastically reduced *in vivo* growth. Moreover, CXCR7 impaired CXCR4-mediated chemotaxis, and altered CXCR4-mediated growth when CXCR4/CXCR7-expressing NB cells were engrafted orthotopically in mouse adrenal gland, a CXCL12-producing environment. In such model, CXCR7 alone, or in association with CXCR4, did not induce NB cell metastatic dissemination.

In conclusion, the CXCL12 receptors, CXCR7 and CXCR4, revealed opposite expression patterns and distinct functional roles in NB. While CXCR4 favours NB growth and chemotaxis, CXCR7 elicits anti-tumorigenic properties and may be associated with NB differentiation. Importantly, CXCR7 may act as a negative modulator of CXCR4 signaling, further opening new research perspectives for the role of the global CXCR7/CXCR4/CXCL12 axis in NB.

Abbreviations

Akt:	"Ak" transforming
AG:	Adrenal gland
BrdU:	Bromodeoxyuridine
cAMP:	Cyclic adenosine monophosphate
COG:	Children's oncology group
CXCR:	CXC receptor
CXCL:	CXC ligand
DMEM:	Dubelcco's modified eagle's medium
DNA:	Deoxyribonucleic acid
dNTP:	Desoxyribonucleotides triphosphate
ECL:	Enhanced chemiluminescence
ECM:	Extracellular matrix
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal growth factor
ERK 1/2:	Extracellular signal-regulated kinase 1 and 2
FACS:	Fluorescence activated cell sorting
FCS:	Fetal calf serum
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GFP:	Green fluorescent protein
GGN:	Ganglioneuroma
GGNB:	Ganglioneuroblastoma
GPCR:	G protein-coupled receptor
H/E:	Haematoxylin/Eosin
HPRT1:	Hypoxanthine phosphoribosyltransferase-1
HRP:	Horse radish peroxydase
IFN:	Interferon
IGR:	Institut Gustave Roussy
IGF-1:	Insulin growth factor-1
IL:	Interleukin
INSS:	International neuroblastoma staging system

I-TAC:	Interferon-inducible T cell chemoattractant
LOH:	Loss of heterozygosity
MAPK:	Mitogen-activated protein kinases
MS:	Mass screening
MTS/PMS:	3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H tetrazolium/ phenazine methosulfate
NB :	Neuroblastoma
NBnd :	Not differentiated neuroblastoma
NSE :	Neuron specific enolase
OD :	Optical density
PBS :	Phosphate buffer solution
PE:	Phycoerythrin
PFA:	Paraformaldehyde
PI3K:	Phosphatidylinositol 3-kinases
PT:	Primary tumour
RA:	(all-trans) Retinoic acid
RNA:	Ribonucleic acid
RT:	Room temperature
RT-PCR:	Reverse-transcriptase PCR
SD:	Standard deviation
SDF-1:	Stromal cell-derived factor-1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM:	Standard error of the mean
SG:	Sympathetic ganglion
TBS:	Tris buffer solution
TNF-α:	Tumour necrosis factor-α
TGF-β:	Transforming growth factor-β
UnNB:	Undifferentiated neuroblastoma
VEGF:	Vascular endothelial growth factor

Introduction

Oncogenesis

Oncogenesis or carcinogenesis is literally the creation of cancer. From the last decades, pivotal advances have enriched our understanding of processes by which normal cells are transformed into cancer cells. In particular, cancer research have pointed out “cancer to be a disease involving dynamic changes in the genome” (1). These modifications involve gene mutations, which ultimately reprogram a normal cell to undergo uncontrolled cell division and deregulated homeostasis, thus forming a malignant tumour mass. Such mutations may result from the action of endogenous chemical products generated during physiological processes such as cell death, or exogenous factors including chemicals, radiation, and viruses. Moreover, germ-line transmission of already mutated genes also contributes to the occurrence of aggressively growing tumour cells, and may be responsible for rare familial cancer syndromes (2;3). Various types of human cancers, as well as their associated tumour subtypes in specific organs, have been described, revealing a considerable and complex list of cancer cell genotypes. Beyond genetic alterations, a multistep approach has been proposed to characterize human oncogenesis. This model reflects the acquisition of eight essential alterations, the so-called « hallmarks of cancer », that affect normal cell physiology (phenotype) and collectively dictate malignant growth of transformed cells (1;4):

- Sustaining proliferative signaling and evading anti-growth signals,
- Resisting programmed cell death (apoptosis),
- Enabling replicative immortality,
- Deregulating cellular energetics (metabolism),
- Avoiding immune destruction,
- Inducing angiogenesis,
- Activating invasion,
- Inducing metastasis

To date, cancer research has been focusing on either hallmark, with the aim to elucidate new anti-cancer drugs able to prevent cancer formation, or to cure those that already developed.

Tumour microenvironment

Besides the diversity of cancer cell genotypes and phenotypes, the complexity of carcinogenesis lies as well in the fact that a tumour is not just a mass of individual infinitely growing cancer cells, but it is also strikingly linked to its specific surrounding tissues (4). Indeed, tumour environment has recently gained considerable interest, as it has been observed that the tumour and its microenvironment form a functional entity, which continuously evolves as the tumour progresses (5;6). Tumour cells are embedded in a microenvironment which is composed of stromal cells, such as fibroblasts, endothelial cells, infiltrating immune cells, and of non-cellular compartments, including secreted soluble factors and the solid-state structural extracellular matrix (ECM) (7). It has been proposed that tumour cells receive signals from the microenvironment and bilaterally communicate with host stromal cells (8).

During cancer progression, these cellular communications dramatically alter the cellular and molecular composition of a particular tumour microenvironment to support cancer cell proliferation, migration, and invasion (8;9). In particular, it has been recently proposed that anti-tumorigenic functions mediated by immune effector cells, recruited to the tumour site, were downregulated in response to tumour-derived signals (7). Therefore, the constant and mutual exchange of various mitogenic and trophic factors between each cell type within the tumour vicinity, results in a functional interdependence between tumour cells and their microenvironment.

Interestingly, tumour microenvironment may also display both pro- and anti-tumorigenic properties, thus acting either as a tumour suppressor or enhancer. In particular, host-derived soluble cytokines, such as interferon-γ (IFN-γ), has been proposed to reduce tumour formation by controlling inflammation and immunity (10). On the other hand, tumour cells have been shown to exploit host-derived cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), to increase resistance to apoptosis, and to promote or enhance angiogenesis and tumour dissemination (11;12).

The process of tumour dissemination, or metastasis, consists of a long series of sequential, interrelated selective steps that only few cells are able to complete and is tightly link to the tumour microenvironment. Globally, the multistep process of metastasis starts with tumour cell detachment from the primary tumour, followed by invasion of the ECM and surrounding vessels (intravasation), and finally with tumour cell escape from vessel

(extravasation) to invasion of secondary organs (13;14). Accumulated evidences indicate that molecular factors present in the microenvironment of specific secondary organs may influence the implantation, growth and neoangiogenesis of various types of cancer cells, thus increasing aggressive cancer cell invasion potential (metastasis) in those tissues (15-17). For instance, a recent study reported that transient interactions between breast carcinoma cells with normal fibroblasts may increase tumour cell malignancy and expand the tumour cell metastatic capacity, through a transforming growth factor β (TGF-β)-dependent mechanism (18). The acquisition of motility and invasive properties allowing tumour cells to invade adjacent tissues is a key process of tumour dissemination. Importantly, soluble proteins other than growth factors, such as chemokines, secreted by the tumour itself and/or by its environment, have been shown to enhance invasive tumour cell motility potential (19).

Chemokines and their receptors

Chemokines are a super family of chemoattracting, small cytokine-like proteins. Based on the positions of the two conserved cystein residues in their N- termini, chemokines are divided into four families: CC, CXC, CX3C, and C (20-22). The two main families are the CXC and CC chemokines, which interact with seven-transmembrane CXC and CC receptors, respectively (CXCR and CCR, respectively) (23). To date, at least 50 chemokines and 20 chemokine receptors have been identified (22). Chemokines bind with the extracellular domain of their receptors, which comprises the N-terminus and three extracellular loops (24). On the other hand, the chemokine receptor intracellular domain couples with heterotrimeric G proteins which, upon binding of chemokines, mediate biological responses such as cytoskeletal rearrangement, firm integrin-dependent adhesion to endothelial cells and directional migration. Association of chemokine(s) with their corresponding receptor(s) is ubiquitous as some chemokines bind to multiple receptors, while some receptors may recognize multiple chemokine ligands (Annexe).

Chemokine/chemokine receptor axes: complex players in tumorigenesis

Chemokines and their receptors have been originally described as essential mediators of leukocyte directional migration, particularly during infection and inflammation,

and further emerged as crucial players in all stages of tumour development (19;20;25;26). Both tumour and stromal cells express a large pattern of chemokines/chemokine receptors axes (27;28), suggesting that chemokine receptors, associated to their corresponding chemokine ligands, represent major paracrine/autocrine complex players within the tumour and its microenvironment. For instance, the expression of chemokines, such as CCL5, CXCL1, CXCL3, CXCL8, CXCL10, CXCL12, and their corresponding receptors on tumour cells have been associated with both autocrine and paracrine tumour growth stimulation (27).

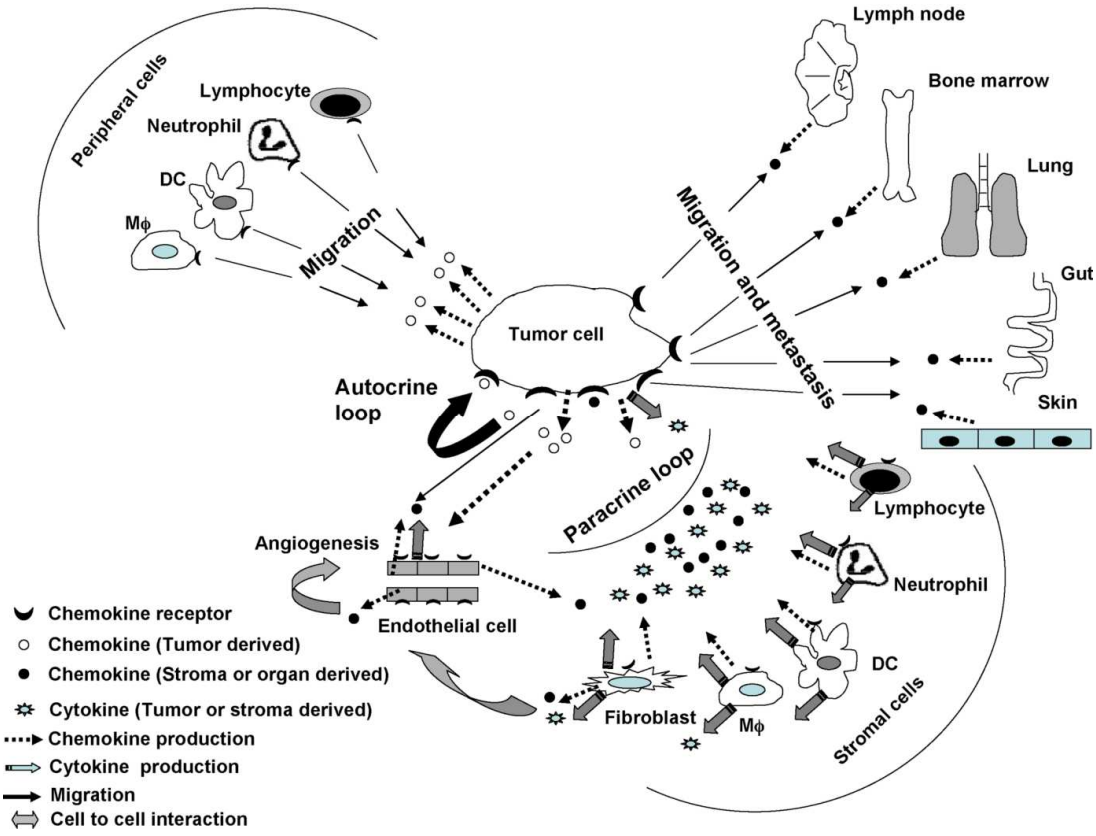
The binding of chemokines to their cognate G protein-coupled receptors (GPCRs) elicits typical cellular responses essential in tumour biology, such as directional migration, through activation of classical mitogen-activated protein kinases (MAPK) or phosphatidylinositol 3-kinases (PI3K) signaling cascades (28;29). In particular, recent studies have shown that tumour cells express patterns of chemokine receptors that "match" chemokines which are specifically expressed in organs to which these cancers commonly metastasize, suggesting that chemokine/chemokine receptor axes may play a pivotal role in determining the metastatic destination of tumour cells (30-34). For instance, the CXCR4/CXCL12 and CCR7/CCL21 axes have been mentioned as essential players in predicting breast cancer cell dissemination to lung and lymph node, respectively (35). In melanoma, the CXCR4/CXCL12 axis was highly associated with pulmonary and liver metastasis formation, whereas the CXCR3/CXCL9, CCR7/CCL21, and CCR10/CCL27 axes were preferentially implicated in lymph node and skin metastases, respectively (36;37).

Beside their implication in promoting tumour growth and dissemination, some chemokine/chemokine receptor axes may be directly or indirectly, via complex interaction with stromal cells or tumour/host-secreted cytokines, involved in tumour angiogenesis (27;38). Angiogenesis is a biological process of new blood vessel formation from pre-existing ones, and is fundamental to many physiological as well as pathological processes, such as tumorigenesis (39;40). The process of angiogenesis is regulated by many angiogenic growth factors and proteins, including the chemokine superfamily. Specific members of the chemokine superfamily can act as pro-angiogenic molecules and support the formation of new blood vessels. For instance, CXCL8 and CCL2 have been proposed as potent mediators of angiogenesis, by enabling survival and proliferation of endothelial cells in tumours (41;42).

The role played by chemokines and their receptors in tumour physiopathology is complex as some chemokines favour tumour growth, dissemination and/or angiogenesis, as

mentioned above, while others may elicit anti-tumour properties. For instance, the chemokine CXCL11 has been shown to elicit anti-tumorigenic property *in vivo,* in the context of lymphoma, by promoting CXCR3-positive immune T cell infiltration to the tumour site (43). In addition, the chemokine CXCL10 was shown to present angiostatic activities, limiting tumour progression (44).

Globally, chemokine-mediated pro-tumorigenic, or inversely anti-tumorigenic effects are part of a complex signaling network involving multiple soluble factors, such as cytokines, which may be specific to the type of tumour and to its microenvironment (Figure 1).



From R.Somasundaram, D. Herlyn/Seminars in Cancer Biology (2009)

Figure 1: Role of chemokines and chemokine receptors in tumorigenesis
Chemokines secreted by tumour cells can induce autocrine tumour growth stimulation by binding to chemokine receptors on tumour cells, can promote angiogenesis by activating endothelial cells, or can attract leukocytes such as dendritic cells (DC), lymphocytes, macrophages (Mϕ) and neutrophils from the periphery to the tumour site. Stromal cells within the tumour, including fibroblasts, DC, lymphocytes, Mϕ and neutrophils, may be activated by tumour cells through cytokines or chemokines secretion. The activated stromal cells may then secrete cytokines (such as TNF-α, TGF-β, IL-1, IL-10) and chemokines (such as CCL2, CXCL8, CXCL12) that can directly or indirectly promote tumour growth (paracrine tumour growth stimulation), angiogenesis, and metastasis. Thus, tumour cell growth stimulation can occur through autocrine and/or paracrine loops. Chemokine receptor-positive tumour cells can migrate toward stromal derived chemokines produced in distant organs (bone marrow, gut, lung, lymph node and skin) resulting in disease progression and metastasis.

The CXCR4/CXCL12 axis in tumorigenesis

CXCL12, also known as stromal cell-derived factor-1 (SDF-1), is a homeostatic chemokine that binds to the CXCR4 chemokine receptor. CXCL12 was initially cloned from bone marrow-derived stromal cells and further characterized as a pre–B-cell growth-stimulating factor, as recombinant CXCL12 supported the proliferation of a stromal cell–dependent B-cell line (45). Initial studies reported a pivotal role for the CXCR4 chemokine receptor in the pathogenesis of HIV infection, particularly as a co-receptor for entry of T-tropic (X4) HIV viruses into CD4-positive T cells (46). In parallel, various reports have shown that CXCL12 supports the survival and growth of a variety of normal cell types, such as hematopoietic progenitors and germ cells, and, in association with its receptor CXCR4, regulate migration of leukocytes and hematopoietic progenitors cells in physiological processes (22;47-49). Further investigation clearly showed that the CXCL12/CXCR4 axis plays a pivotal role in hematopoiesis, development, and organization of the immune system (50). Indeed, deletion of either the *cxcl12* or *cxcr4* gene is lethal at a relatively late stage of mouse embryogenesis. In addition, the *cxcr4* and *cxcl12*-deficient mice exhibited common particular phenotypes presenting defects in the cardiovascular, gastrointestinal, central nervous, and in the immune systems (46-49).

CXCL12 is a highly conserved chemokine that has 99% homology between mouse and human, enabling CXCL12 to act across species barriers what further facilitates human CXCL12 chemokine receptor investigation in mouse models.

Once CXCL12 binds to CXCR4, the receptor may form a complex with the G protein subunits, leading to activation of multiple downstream targets (such as MAPK, and Akt effectors), and resulting in part, in inhibition of cyclic adenosine monophosphate (cAMP) production and intracellular calcium mobilization (22) (Figure 2).

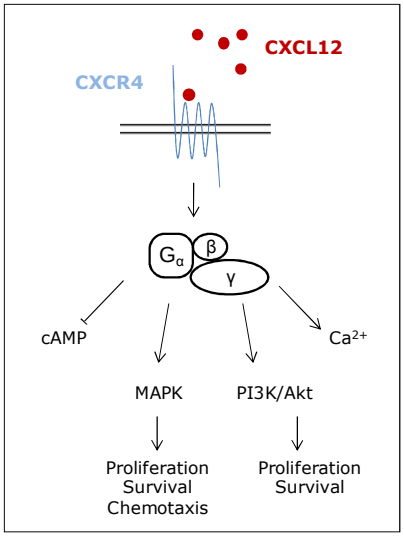


Figure 2 : Ligand-bound CXCR4 signaling.
Binding of CXCL12 to CXCR4 activates G protein subunits (Gα,Gβ and Gγ), leading to inhibition of cAMP production, and activation of calcium (Ca^{2+}) flux, MAPK and PI3K/Akt signaling cascades. These signaling pathway activations mediate tumour proliferation, survival and chemotaxis.

In the context of cancer, CXCR4 is the most commonly found chemokine receptor on tumour cells. At least 23 different types of cancer, including breast, ovarian, colon and prostate cancers and melanoma, express a functional axis (28;31). Besides its critical role in tumour cell growth, survival and angiogenesis in multiple cancers, including breast, lung, colon and prostate carcinomas (22), this receptor/ligand pair has been particularly shown to mediate organ-specific cancer cells homing, particularly in CXCL12-producing organs such as liver and bone marrow (22;51;52). Consequently, the CXCR4/CXCL12 axis represents to date a major investigation target in tumorigenesis.

The CXCR7 receptor in tumorigenesis

CXCR4 has long been considered as the unique receptor for CXCL12, and as the only mediator of CXCL12-induced biological effects. However, CXCR7 formerly called RDC1, has been recently identified as an alternate receptor for CXCL12. This new chemokine receptor has been demonstrated to bind with high affinity to CXCL12 and with low affinity to a second chemokine, interferon-inducible T cell chemoattractant (I-TAC; also known as CXCL11). CXCR7 was shown to be expressed on activated endothelial cells, fetal liver cells, T lymphocyte, neuronal cells and on few other cell types (53-56). Moreover, *in vivo* investigation have detailed that *Cxcr7*-mice died in the first week after birth, and mainly presented cardiovascular defects (57-59).

Despite its phylogenetic relation and ligand binding properties, CXCR7 does not mediate typical chemokine responses such as G protein-coupled receptor-mediated calcium

mobilization (53;54;60;61). Although coupling of the CXCR7 receptor with G proteins is still under debate (61;62), the possibility that the receptor is able to induce signal transduction is suggested by reports demonstrating MAPK and Akt pathway activation upon CXCR7-expressing cell exposure to CXCL12 (63-65). On the other hand, a G protein-independent β-arrestin-mediated pathway has been shown to play a pivotal role in the biology of GPCRs (66). Recently, it has been demonstrated that CXCR7 interacts with β-arrestin in a ligand-dependent manner (67) (Figure 3).

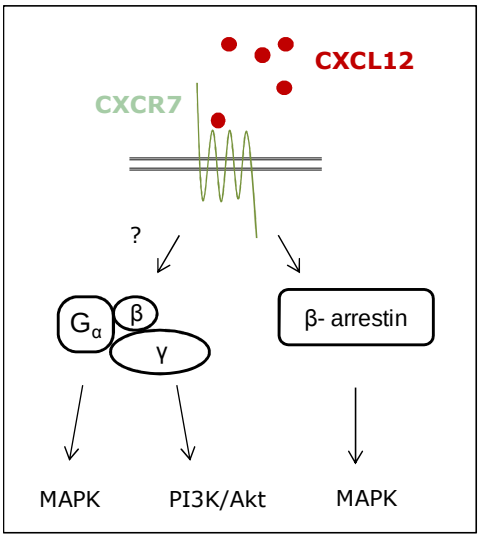


Figure 3: Ligand-bound CXCR7 signaling.
Binding of CXCL12 to CXCR7 activates MAPK, Akt, and β-arrestin-dependent pathways. Whether the CXCL12/CXCR7 axis signals through G protein-mediated pathways is still under debate.

Recent studies particularly identified a specific role for CXCR7 as a CXCL12 scavenger. Indeed, by sequestrating CXCL12, CXCR7 allowed rapid dynamic changes in the ligand distribution and thereby imposed a control of CXCR4/CXCL12-mediated migration (68-71). In addition, CXCR7 was shown to mediate similar function regarding its other chemokine ligand CXCL11 (72) (Annexe). CXCR7 association with T lymphocyte migration, B cell survival, and increased adhesion property of renal progenitor cells into injured renal tissue, was also reported (22;60;73), further arising debate whether CXCR7 functions like a classical GPCR or a decoy receptor.

CXCR7 has been reported to be highly expressed in several tumours, and particularly in the endothelial cells-associated vasculature (22;33;65), suggesting a role in regulating immunity, angiogenesis, and organ-specific metastasis (22). *In vivo* growth assessment in different mouse models revealed a pivotal role for CXCR7 in enhancing growth and neovascularisation of prostate and colon cell-derived tumours, and enabling organ-specific dissemination of breast and lung cancer cells (33;65;74). Moreover, in a human Burkitt's lymphoma cell model, CXCR7 was shown to favour CXCL12-mediated migration of

CXCR4/CXCR7-expressing tumour cells into lymph nodes, suggesting that, in association, the two CXCL12 receptors may enhance tumour dissemination (75). Combined CXCR4/CXCR7 expression has been detected in T- and B-cell subsets, endothelial cells, human renal progenitor cells, as well as in primary human tumours and tumour cell lines (53;60;75;76). In human rhabdomyosarcomas, either CXCR7 or CXCR4 receptor was shown to enhance *in vivo* invasive tumour cell potential, suggesting that targeting the CXCR4/CXCL12 axis alone without simultaneous blockage of CXCR7 would be an inefficient strategy for inhibiting CXCL12-mediated pro-metastatic tumour cell responses (77). In addition, CXCR7 receptor expression was proposed to be related with poor prognosis in breast, lung, and pancreatic carcinomas (22).

Neuroblastoma: Clinical and biological characterizations

Among childhood solid tumours, neuroectodermal tumours originate from the multipotent neural crest cells, which are generated in the early embryo and give rise to the central and peripheral nervous systems. Neuroectodermal tumours include several paediatric cancers such as neuroblastomas (NBs), Ewing sarcomas, medulloblastomas, and melanomas (78).

NB accounts for 7 to 10% of all childhood malignancies (79). This neoplasm was shown to recapitulate characteristics and features of its originating pluripotent neural crest cells, with an extensive heterogeneity, pluripotential differentiation and migratory abilities, indicating that NB results from defects in mechanisms that control normal development (80-83). The median age at diagnosis for NB patients is about 18 months, approximately 40% of patients are diagnosed by one year of age, 75% by four years of age and 98% by ten years of age (84). The origin and migration pattern of immature neuroblasts during foetal development explains the multiple anatomic sites where these tumours occur. Tumours may occur in the abdominal cavity (40% adrenal, 25% paraspinal ganglia) or involve other sites (15% thoracic, 5% pelvic, 3% cervical tumours, 12% miscellaneous). Location of NB varies with age of patients, while infants are more likely to have thoracic and cervical tumours, older children more frequently present abdominal NBs (85).

As shown in Figure 4, NB tumours are divided into four different stages according to the International Neuroblastoma Staging System (INSS).

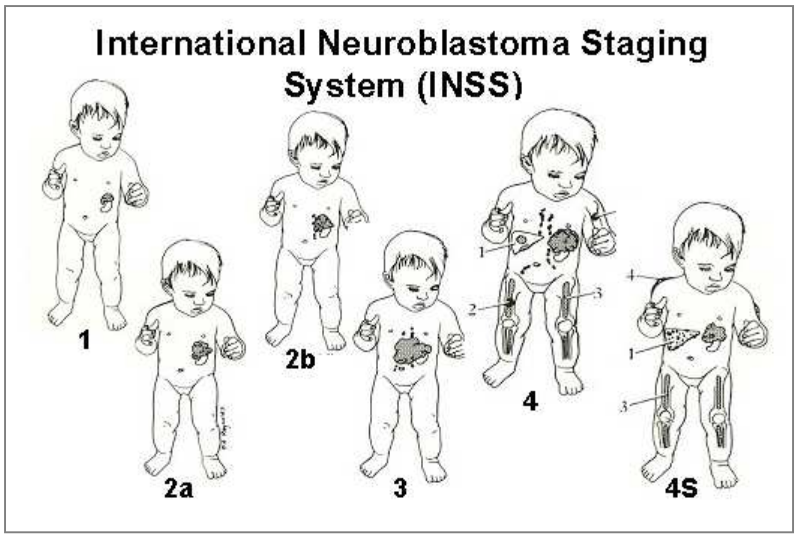


Figure 4: International Neuroblastoma Staging System (INSS)

Stage 1

- Localized tumour with complete gross excision and/or microscopic residual disease
- Ipsilateral lymph nodes negative for tumour (nodes attached to the primary tumour may be positive for tumour)

Stage 2A

- Localized tumour with incomplete gross resection
- Representative ipsilateral non adherent lymph nodes negative for tumour microscopically

Stage 2B

- Localized tumour and/or complete gross excision, with ipsilateral non adherent lymph nodes positive for tumour
- Enlarged contralateral lymph nodes, which are negative for tumour microscopically

Stage 3

- Unresectable unilateral tumour infiltrating across the midline and/or regional lymph node involvement
- Alternately, localized unilateral tumour with contralateral regional lymph node involvement

Stage 4

- Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin, and/or other organs (except as defined for stage 4S)

Stage 4S

- Localized primary tumour (as defined for stages 1, 2A, or 2B) with dissemination limited to skin, liver, and/or bone marrow (<10% involvement)
- Limited to infants

Of note, NB classification is in constant evolution as clinical protocols are systematically updated. Although the INSS classification has been redefined in 2007 by the new International Neuroblastoma Risk Group (INRG) classification composed of four NB categories: L1/L2 for localized tumours, M for metastatic disease (stage 4), and MS for stage 4s, the INSS is still a relevant reference.

NB displays a remarkable clinical diversity, ranging from spontaneous regression to fatal progression and dissemination (80;85). A favourable outcome, essentially due to spontaneous maturations and regressions, is generally associated to low stages and localized tumours, and represents 80-90% 3-year event-free survival rate. However, stage 3 (large, progressing tumours) and stage 4 (metastatic tumours) NBs have bad prognosis and are extremely difficult to treat, due to the rapidly progressive, metastatic and drug-resistant disease. Interestingly, the spontaneous regression of NB is often observed in patients under one year-old presenting a stage 4s NB. Thus, stage 4s NB is associated with a good prognosis even if patients presenting a unique and unexplained pattern of metastatic spread limited to bone marrow, liver, and skin. Inversely, patients older than one year with metastatic disease to privileged sites such as bone, bone-marrow and liver, have very severe prognosis; with long-term survival still less than 40% (86;87).

Histology of NB tumours

Histologically, neural crest tumours can be classified as NB, ganglioneuroblastoma (GGNB), and ganglioneuroma (GGN), depending on the degree of maturation/differentiation of the tumour (88). Most of NBs are undifferentiated and small round blue cell ("neuroblast") tumours, characterized by the presence of neuritic processes, neuropil and/or Homer-Wright rosettes (neuroblasts surrounded by eosinophilic neuropil). The typical tumour shows uniform neuroblasts with scant cytoplasm and hyperchromatic nuclei. GGNBs and GGNs show a higher degree of histological differentiation with a predominant stroma and more mature cells (88). The most differentiated form consists in the completely benign GGN which is typically composed of clusters of mature ganglions cells surrounded by a dense stroma of Schwann cells; whereas GGNB is composed of both mature ganglion cells and immature neuroblasts, Schwann cells, and has an intermediate malignant potential.

Because of the presence of different histological components, the pathologist must evaluate the tumour thoroughly; the regions with different gross appearance may demonstrate a different histology. Neuron specific enolase (NSE), neurofilament protein (NF), chromogranin, synaptophysin, glial fibrillary acidic protein (GFAP), and S-100 are known neuroblastic, glial and shwannian markers usually used for immunohistochemical detection of NB, GGNB, and GGN tissues (89).

Shimada and colleagues have developed a classification system based on histopathological features, such as the presence or absence of schwannian stroma, the degree of differentiation, and the mitosis-karyorrhexis index (MKI) (88).

Clinical prognostic consideration

Stage of tumour at time of diagnosis, age of patient, bone marrow involvement, and clearance have been shown to be the most important clinical prognostic factors (91;92). Moreover, NB was found to secrete catecholamine metabolites excreted in urine, thus offering a non invasive diagnostic technique. Mass screening (MS) with the use of specific catecholamine markers was thus considered for alternative screening for NB in infants. Several studies have been performed for the relevance of MS in NB, showing that most tumours identified by this method revealed to be tumours with a favourable prognosis. However, MS was not shown to reduce either the disease-related mortality or the yearly numbers of aggressive NBs, and is still under debate whether such technique may be adopted as a relevant clinical prognostic factor (87;93).

Beside clinical consideration, biological features encountered in tumour cells are essential prognostic factors being used for NB risk classification and treatment assignment.

Genomic and biologic markers in NB

Chromosome alterations

During the last 2 decades, the genetic alterations of NB tumours have been explored through a panel of techniques including array-CGH. Many chromosomal abnormalities have been identified in NB, and evaluated to determine their value in assigning prognosis (94-97). NB can be classified into subtypes that are predictive of clinical behaviour based on these patterns of genetic changes. The oncogene *MYCN* amplification, the first genetic alteration described in NB, is observed in 25-30% of cases and is strongly associated to advanced-stage disease and poor outcome. Deletion of the short arm of chromosome 1 is one of the most common chromosomal abnormalities present in NB, and is associated with a high risk of relapse and poor prognosis. The 1p chromosome region likely harbours tumour suppressor genes or genes that control neuroblast differentiation. Deletion of 1p is more common in near-diploid tumours and is associated with a more advanced stage of the disease. Most of

the deletions of 1p are located in the 1p36 area of the chromosome (98-100). The other segmental copy number alterations mainly include deletions of chromosome 1p, 3p, 4p, 9p, 11q, 18q and gain of 1q, 2p and 17q (101-103). Partial 17q gain is frequently observed in primary tumours in association with segmental alterations, whatever MYCN status. The recurrent segmental alterations are thought to lead to the loss of putative tumour suppressor genes and/or to the gain of oncogenes (104;105). The expression profiles of these regions, where genetic alterations occur, suggest some candidate genes involved in NB progression (106;107). Tumours from the youngest patients with lower stages of the disease are often hyperdiploid or near-triploid, and numerical chromosomal alterations without structural rearrangements are associated with a favourable outcome (108-110).

Molecular markers

Abnormal patterns of expression for some molecular markers can also distinguish different NB clinical groups. It has been proposed that the three neurotrophin TrkA, TrkB, and TrkC receptors (encoded by *NTRK1*, *NTRK2* and *NTRK3*, respectively) and their ligands nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT3), respectively, are important regulators of survival, growth, and differentiation of neural cells (87). Indeed, the TrkA/NGF signaling have been associated to NB differentiation, while the TrkB/BDNF autocrine pathway has been proposed to contribute to either enhanced angiogenesis and drug resistance (111-114). Interestingly, *NTRK1* gene expression is correlated with lower stage disease and absence of *MYCN* amplification (115;116), while the expression of *NTRK2* gene is strongly associated with *MYCN*-amplified tumours.

Other biological markers have been identified and associated with NB poor prognosis include the increase of the multidrug resistance 1 transporter (MDR1), the multidrug resistance-related protein (MRP), high telomerase activity, as well as the lack of expression of glycoprotein CD44 on the tumour cell surface (117-123).

More recently, studies have implicated various activating *anaplastic lymphoma kinase (ALK)* gene mutations in both familial and sporadic cases of NB. Importantly, somatic *ALK* mutations have been associated with NB aggressive forms. The protein product of *ALK* is a tyrosine kinase, an enzyme that regulates the activity of other proteins through phosphorylation. Abnormal activity of the ALK protein has been implicated in other various solid tumours, including non-small-cell lung cancers (124-127).

Risk assessment

Using both clinical and biological criteria, low, intermediate, and high-risk groups have been defined to adapt patient treatment strategies. In the Children's Oncology Group (COG), risk-group assignment is currently based on INSS stage, age of the patient, *MYCN* copy number, tumour cell ploidy, and Shimada tumour histopathology. Low-risk patients have an excellent event-free survival with surgical excision of tumour only, while high-risk patients require treatment with multi-agent chemotherapy, surgery, and radiotherapy, followed by consolidation with high-dose chemotherapy and peripheral blood stem cell rescue.

Neuroblastoma cell lines

The neural crest is a transitory structure of the vertebrate embryo formed by the lateral borders of the neural tube. Multi-potent neural crest cells migrate ventrally and laterally to contribute to a variety of tissues such as the peripheral nervous system, medullary cells of the adrenal gland, calcitonin producing cells of thyroid, pigmented cells, and ectomesenchymal derivatives. Growth and differentiation specific factors encountered by migratory neural crest cells are thought to influence their development and lineage specific differentiation (90).

Most NB cell lines originate from the more aggressive tumours, and present, like their tumour of origin, an important biological heterogeneity. A model has been proposed leading to a classification of established NB cell lines in the three following morphological types: the neuroblastic (N), the flat or substrate adherent (S) and the intermediate (I) types. These distinct variants were proposed to recapitulate the sympathetic nervous systems development from neural crest cells, by reflecting their derivation from multi-potent neural crest precursors. Indeed, N-type cells display properties of embryonic sympathoblasts, whereas S-type cells harbour those of schwannian, glial or melanocytic progenitor cells or ectomesenchymal derivatives. I-type cells are rather classified as morphologically intermediate cells, regarding their potential to differentiate into N- or S-type cells (80;128;129).

Neuroblastoma and chemokines

Over the last years, a multiple number of chemokine receptors such as CCR1, CCR5, CCR6, CCR9, CXCR1, CXCR2, CXCR4, CXCR5, and CXCR6 were found expressed in NB, suggesting that several chemokine/chemokine receptor axes may either contribute to NB progression or inversely, elicit anti-tumorigenic properties (130).

For instance, the chemokine IL-8 (also known as CXCL8) and its receptors (CXCR1 and CXCR2) were proposed to displayed angiogenic properties in NBs, while CCL5 (also known as RANTES) was shown to induce cell death in CCR5-positive NB cells (131;132).

As efficient targeted therapies for high-risk and metastatic NB tumours are not yet identified, various studies have been focused on the identification of chemokines that either promote or inhibit the metastatic spread of NB cells. For instance, studies have reported that CXCR5-positive neuroblastic cells in aggressive tumours might easily disseminate to CXCL13-producing distal sites, such as the bone marrow (133;134). On the other hand, the CXCL13 ligand was also shown to be specifically produced by schwannian stroma in favourable stroma-rich tumours, and was proposed to potentially limit dissemination of CXCR5-expressing neuroblasts to distal sites of metastasis (134). In parallel, a study demonstrated that, although secreted by the bone marrow stroma cells, the CXCL10 chemokine elicited anti-malignancy functions, such a growth and migration inhibitory potentials, through its interaction with a CXCR3-like receptor in NB cells (135).

The CXCR4 and CXCR7 receptors in neuroblastoma

The CXCR4/CXCL12 axis has been largely shown to participate in tumour development and progression. Indeed, reports have proposed a predominant role of the CXCR4 receptor and its ligand CXCL12 in mediating invasion of malignant tumour cells to sites of metastasis in several cancer models (31).

In NB, the CXCR4 receptor expression was shown to correlate with poor prognosis, thus warranting its implication in the aggressive behaviour of NB tumours (136-138). Moreover, Nevo *et al*. reported that NB cells highly expressing CXCR4, have a different profile of metastasis-associated gene products than cells expressing low levels of this receptor (139). Indeed, high CXCR4-positive NB cells were shown to differentially express genes that are known to enhance tumour progression, such as the oncogene c-kit receptor,

previously reported to increase NB cell proliferation *in vitro* (140), as well as the IL-8 chemokine and the vascular endothelial growth factor (VEGF), two known angiogenic factors (141). Nonetheless, the specific involvement of the CXCR4/CXCL12 axis in cell invasion is still under debate. Even though the receptor CXCR4 has been previously demonstrated as an essential mediator of aggressive NB cell dissemination to the CXCL12-producing bone marrow (138), contradicting reports have been published on the relative contribution of the CXCR4/CXCL12 axis in NB invasion (137;142-145). Moreover, CXCR4 was found on either metastatic stage 4 and non-metastatic stage 3 NB tumours, further supporting a more general and complex role of the receptor in advanced stage disease (137). By using *in vivo* growth and metastasis assessment in an orthotopic NB mouse model (146), we previously showed that CXCR4 strongly increased growth of primary tumours and liver metastases, without altering the frequency or the localisation of metastases (137). Our data thus pointed to a predominant and tumour type-specific growth-promoting influence of CXCR4 in NB. High levels of CXCL12 were detected in the adrenal gland (primary tumour site), as in the liver (preferred site of metastasis), suggesting an essential and paracrine role for CXCL12 in increasing NB tumour growth and survival. These observations strengthened a pivotal role for CXCR4 in NB growth and underlined the impact of a CXCL12-producing microenvironment on NB tumour cell behaviour. However, the mechanisms by which the CXCR4/CXCL12 axis influences NB tumour growth are yet to be identified and may strongly depend on complex signals from the microenvironment.

The identification of CXCR7, as a second receptor for CXCL12, has added considerable complexity and also new perspectives for the implication of the CXCR4/CXL12 axis in tumour biology. The contribution of CXCR7 in tumour progression and dissemination has already been proposed in different tumour systems (22).

In addition, a very recent study reported that CXCR7 was expressed in some NB cell lines and that the receptor might enhance NB migration *in vitro* in presence of CXCL12-producing mesenchymal cells (147). Moreover, this study reported a higher *CXCR7* gene expression in NB tumours (from 19 patients) as compared to control neuronal tissues. As a relevant player in NB pathogenesis, CXCR7 expression in larger panel of NB tissues and its contribution to NB progression should be further elucidated.

Importantly, CXCR7 and CXCR4 share a common ligand, CXCL12 which elicits higher affinity to the newly discovered CXCR7 receptor than to CXCR4 (53). Moreover, several evidences suggest that the two CXCL12 receptors may interact to enhance tumour progression. Consequently, investigating the functional impact of the CXCL12/CXCR7 axis on CXCR4 functionality in NB might help to gain further insight into NB pathogenesis, and more precisely into the particular and atypical role of CXCR4 in NB.

Aims of the project

The CXCR4/CXCL12 axis may have a crucial role in conferring aggressive behaviour to many types of cancer, especially in the context of NB. Nonetheless, the specific involvement of the CXCR4/CXCL12 axis in NB cell proliferation, survival, and invasion is still under debate. The recent identification of CXCR7, as a second CXCL12 high affinity receptor, has shed a new light on the role of the CXCR4/CXL12 axis in tumour biology. As several hypotheses on CXCR7 function(s) and possible interactions between the two CXCL12 receptors have been proposed in different tumour systems, research is now focusing on the roles and relationships between these two very closely related receptors. As few investigations on CXCR7 have been reported so far in NB, it appears essential to explore, not only the individual role of CXCR7, but also to address the global CXCL12/CXCR4/CXCR7 system that may confer malignant behaviour to NB.

Therefore, the present project aims at exploring the functional role and signaling networks mediated by CXCR7, and its impact on the CXCR4/CXCL12-mediated signaling in NB.

We first investigated the expression of CXCR7 and CXCL12 in a large panel of NB tissues by using tissue-microarray technology. Then, individual CXCR7, CXCR4, and a combination of the two receptors were ectopically expressed in two NB cell lines, and the resulting *in vitro* growth, survival and migration properties of CXCR7 or/and CXCR4-expressing NB cells were explored. Proliferative and invasive capacities of these transduced cells were further evaluated *in vivo*, by using either heterotypic or orthotopic NB mouse models.

Materials and methods

Patients and tissue-microarray (TMA)

The TMA is composed of tumour samples from 156 patients with neuroblastic tumours treated and followed in four clinical centers: Bicêtre hospital and Gustave Roussy Institute (Villejuif, France), the American Hospital (Reims, France), CHU Sainte Justine (Montréal, Canada), and Shiga University hospital (Otsu, Japan). These patients were diagnosed between July 1988 and April 2002. On average, four tissue cylinders with a 0.6 mm diameter were obtained and transferred into a recipient paraffin block using a manual tissue arrayer (Alphelys, Plaisir, France) at the Institut Gustave Roussy, France. NB samples consisted of 156 primary tumours, 56 metastases (48 lymph nodes, 6 hepatic and 2 cutaneous metastases) and 65 controls (50 adrenal glands and 15 sympathetic ganglia). Clinical parameters of the patients and associated tumours are detailed in Table 1 (p46). Immunohistochemical study on patient tissues was performed after patients' informed consent and according to the ethical regulations of the institution. All Immunohistochemical procedures on TMA tissues were carried out at the Institute of Pathology of Lausanne. 5-µm sections of the TMA blocks were deparaffinated in a xylol bath for 10 min, rinsed with xylol, rehydrated by transfers in alcohol baths for 5 min with decreasing concentration (100%, 95%, 70%, and 40%), and finally in H_20. Then, sections were washed for 5 min in 3% H_2O_2 to inhibit endogenous peroxydase. For antigen retrieval, slides were incubated in a cooking-pot with a Tris-EDTA buffer pH 9.0 for 1 min and 30 sec as soon as the steamer is under pressure. They were then incubated with the monoclonal mouse anti-human CXCL12 antibody (clone 79018, R&D systems, Minneapolis, MN, USA) and the monoclonal mouse anti-human CXCR7 antibody (clone 9C4, kind gift from Pr. M. Thelen, IRB, Bellinzona, Switzerland) in Dako REAL™ antibody diluent (Dako), overnight at 4°C. Incubation with secondary antibody was performed using EnVision™ HRP-antibodies (Dako, Glostrup, Denmark) for 30 min, followed by treatment with 100 µl DAB (Dako) at 1/50 dilution for 8 min. Slides were then incubated in hematoxylin bath for 10 s, and then dehydrated in baths with increasing alcohol concentration (70%, 95%, and 100%), and finally in xylol. Washes between each step were done in TBS pH 7.6. Slides were mounted using Eukitt Mounting Medium (EMS, Hatfield, PA, USA). Immunostaining scores (0-4) were established for each stained tissue by semi-

quantitative optical analysis by two independent investigators, blinded for clinical data, at the Institute of Pathology of Montreal. The percentage of positive cells in each sample was scored as follows: 0, all cells negative ; 1+, up to 25% of cells were positive ; 2+, 26% to 50% ; 3+, 51% to 75% ; 4+, more than 75%.

Cell lines

Two main cell lines were used in this study: the IGR-NB8 and the SH-SY5Y N-type NB cell lines (90). The IGR-NB8 cell line is derived from a xenotransplanted human stage 3 abdominal NB. IGR-NB8 xenograft and cell line are para-diploid with 1p36 LOH and *MYCN* amplification. The SH-SY5Y cell line originates from the SK-N-SH cell line which derives from highly involved bone marrow of a 4-year-old girl with stage 4 NB and exhibits *MYCN* single copy. Other well-characterized cell lines used in this study include the N-type IGR-N91, IMR-32, SK-N-SH, LAN-1, SJNB-12, GI-M-EN, and CLB-Ber-Lud2; the I-type SK-N-BE(2c), SH-IN and LAN-5; and the S-type SK-N-AS and SH-EP NB cell lines (90); the SW480 colon cancer cell line (148); the PC-3 prostate cancer cell line (149); and the breast cancer cell line MCF-7 (53). Unless specified, all cell lines were cultured in Dubelcco's modified Eagle's medium (D-MEM) (Gibco, Paisley, UK), supplemented with 1% penicillin/streptomycin (Gibco) and 10% heat inactivated Foetal Calf Serum (FCS) (Sigma-Aldrich, S^{t} Louis, MO, USA), and under standard culture conditions in a humidified incubator at 37°C with 5% CO_2.

RNA isolation

1 µg of total RNA was extracted from cell lines using the RNeasy Mini kit (Qiagen, Hilden, Germany), and reverse-transcribed using PrimeScript™ RT reagent Kit according to the manufacturer's instructions (TAKARA Bio Inc., Shiga, Japan). The quality of each RNA sample was verified by a Nanodrop (Agilent Technologies).

Qualitative RT-PCR and semi-quantitative real-time PCR

CXCR4 and *CXCR7* mRNAs relative expressions were measured in cell lines by qualitative RT-PCR (standard) and semi-quantitative real-time PCR.

For standard procedure, 1 µl of cDNA was added to 5 U/µl GoTaq® Hot Start Polymerase (Promega, Madison, MI, USA), specific buffer, 0.2 mM dNTPs and 1 µM specific primer pairs. The PCR reaction consisted of 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at

60°C, and 30 s at 72°C, with a final extension step of 5 min at 72°C. For CXCR3 detection, 35 cycles were processed with a 30 s annealing step at 58°C as described elsewhere (150). To visualize the amplification products, RT-PCR reactions were loaded on 2% agarose gels.

Real-time PCR was performed using the ABI PRISM 7900 HT real-time PCR system (Applied Biosystem) with SYBR Green© detection (Qiagen). The expression levels of *CXCR4* and *CXCR7* transcripts for each sample were calculated relatively to the level of the housekeeping gene *HPRTI*. The $\Delta\Delta C_t$ method was used to evaluate the relative gene expression. PCR program corresponded to: 2 min at 50°C for stabilization, 5 min at 95°C for SYBR activation, 40 cycles of three repeated steps of amplification (10 s at 95°C, 30 s at 60°C, 15 s at 95°C).

Human-specific pairs of primers :

CXCR7 (5'-TGGGCTTTGCCGTTCCCTTC-3' and 5'-TCTTCCGGCTGCTGTGCTTC-3'),

CXCR4 (5'-TATCTGTGACCGCTTCTACC-3' and 5'-GCAGGACAGGATGACAATAC-3'),

CXCR3 (5'-TGCCAATACAACTTCCCACA-3' and 5'-CGGAACTTGACCCCTACAAA-3'),

GAPDH (5'-AGATCATCAGCAATGCCTCC-3' and 5'-GTGGCAGTGATGGCATGGAC-3'),

HPRT1 (5'-TGACACTGGCAAAACAATGCA-3' and 5'-GGTCCTTTTCACCAGCAAGCT-3').

Plasmid constructs and transduction

The complete coding sequence of *CXCR7* (1.089 kb) was amplified by PCR from a pcDNA3 plasmid containing *CXCR7* (kindly provided by Prof. Marcus Thelen, Bellinzona) using 5' and 3' primers containing *XhoI* and *EcoRI* sites as follows: sense: 5'-GCGC*CTCGAG*ATGGATCTGCA-TCTCTTCGACTACT-3'; antisense: 5'-GCGC*GAATTC*TCATTTGGTGCTCTGCTCCA-3'. The amplified cDNA was subcloned into the pMigr vector (kind gift from F. Louache, Institut Gustave Roussy, Villejuif, France) containing IRES-EGFP sequence. The pMigr plasmid containing complete coding region of *CXCR4* (1.1 kb) was already used and described elsewhere (137;146). CXCR7 and CXCR4 expression in vectors were verified by DNA sequencing.

The pMigr-EGFP vectors encoding for EGFP with or without *CXCR4* or *CXCR7* genes was inserted by retroviral-mediated infection into IGR-NB8 and SH-SY5Y cells. For the transfection of the two receptor in the IGR-NB8 cell line, the pMigr-CXCR7 vector was inserted into NB8 cells, preliminary transduced with the pMigr-CXCR4 vector.

The day before transduction, 2×10^6 293T cells were plated on 6-well plates in DMEM, 10% FCS. 250 µl of a DNA solution containing 10 µg of pMIGR vector, 20 µg of pHit60 and 2.5 µg

of pCG, was mixed with 250 µl $CaCl_2$ 0.5 M, and incubated at room temperature (RT) for 10 min. The $CaCl_2$/DNA mix was added to a HBS buffer pH 7.1 (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO4\text{-}2H_2O$, 12 mM Glucose, 50 mM HEPES), incubated for 15 min at RT, and then added onto 293T cells. Cells were incubated at 37°C for 16 h. Viral medium was replaced by fresh culture medium containing 10 mM sodium butyrate (Sigma). After 8 h incubation at 37°C, transfection medium was then replaced by fresh DMEM/10% FCS and cells were incubated again for 20 h at 37°C. Viral supernatant was harvested, supplemented with 8 µg/ml polybrene (Sigma, S^t Louis, MO, USA), filtrated through a 0.45 µm filter (Milian SA, Geneva, Switzerland) and added to NB cells, preliminary seeded in a 6-well plate the day before, at a density of 2×10^5 cell per well. After 20 h incubation of NB cells at 37°C, viral supernatant was replaced by fresh DMEM/10% FCS. This step was repeated every 48 h for at least two weeks. Infection efficiency was then evaluated by fluorescence-activated cell sorting for green fluorescent protein (GFP) expression using the FACS Aria^{TM} cell sorter (BD Biosciences, San Jose, CA, USA).

Cell surface expression of CXCR7 and CXCR4 by flow cytometry

For CXCR4 cell surface detection, NB cells were collected, washed in FACS buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA), and stained with phycoerythrin(PE)-labelled monoclonal mouse anti-human CXCR4 antibody (clone 12G5, BD Pharmingen, San Jose, CA, USA) for 20 min at 4°C. Cells were then washed three times in FACS buffer and analyzed by FACScan (BD Biosciences, San Jose, CA, USA). For the detection of CXCR7 expression levels, cells were collected with 0.02% EDTA in PBS pH 7.2, washed once with PBS, blocked 20 min at 4°C with 10% goat serum (Sigma) in PBS, and then incubated 1 h at 4°C with the monoclonal mouse anti-human CXCR7 antibody clone 9C4 (kind gift of Dr. Marcus Thelen, Bellinzona, Switzerland), diluted in PBS containing 1% goat serum. Then, cells were washed with PBS prior to incubation with Alexa Fluor® 647-labelled secondary antibody (Invitrogen, Carlsbad, CA, USA) for 20 min at 4°C in PBS supplemented with 1% goat serum. Cells were washed three times in PBS before analyses. For each condition, a total of 10^4 events were analysed.

Immunofluorescence

10^5 cells were plated in Lab-Tek^R Chamber Slide™ System (Nunc, Ny, USA), 48 h before immunofluorescence analysis. Cells were processed as previously described (63). Briefly, cells were washed twice with PBS, fixed in 4% PFA (Fluka, Buchs, Switzerland) for 10 min at room temperature (RT), and then permeabilized with SAP buffer (0.1% saponin (sigma)-0.05%NaN_3 in PBS) for 15 min at RT. Cells were washed twice with SAP buffer before 20 min blocking at RT in SAP buffer supplemented with 10% goat serum (Sigma). Incubation of fixed cells with anti-CXCR7 (clone 9C4) or anti human β_3-tubulin antibodies (clone 2G10, Sigma-Aldrich, S^t Louis, MO, USA) in SAP buffer supplemented with 1.5% goat serum (dilution: 1/100 and 1/1000 respectively) was performed. Then, cells were washed three times in SAP buffer, and incubated for 30 min at RT with Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). DAPI (Sigma) was added for nuclear staining, and Slides were mounted using DAKO® Fluorescent mounting medium (Dako, Carpinteria, CA, USA). Imaging was performed using a camera DFC345 FX (Leica Microsystems Schweiz AG, Switzerland) and analysed with the Leica Application Suite (LAS) software.

Differentiation assay

In vitro neuronal or glial differentiation assays was performed by treating NB cells with All-Trans Retinoic Acid (RA) or Bromodeoxyuridine (BrdU), respectively, as previously described (151-153). RA (Sigma, S^t Louis, USA) was dissolved in DMSO to a concentration of 3.5 mg/ml and stored in light protected vials at -20°C. Aliquots of stock solution were freshly thawed for each experiment and diluted in DMEM, 10% FCS. NB cells were plated 24 h before treatment with either 10 µM RA or BrdU. Untreated cells or cells cultured in DMSO were used as controls. Treated and untreated cells, as well as supernatants, were collected at day 3, 7, 14, and 30 for RNA, protein extractions and CXCL12 ELISA assay. Medium containing each differentiating agent was renewed every three days.

Apoptosis assay

Cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in 1 ml of ice-cold PBS, and fixed with 3 ml of 100% ice-cold ethanol for 1 h at 4°C. For staining with propidium iodide (PI) (Sigma), cells were washed twice in ice-cold PBS and incubated for at

least 30 min at RT in 0.2 ml of PBS containing 200 µg/ml RNase A and 10 µg/ml PI. The stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson). In parallel, NB cells were treated for 48h with 1 µg/ml Doxorubicin (Sigma) as positive control.

CXCL12 ELISA

Supernatant of cultured NB cell was harvested and filtrated through a 0.45 µm filter (Milian SA, Geneva, Switzerland), to quantify the release of the ligand CXCL12 by NB cell lines. The CXCL12 production was measured as well in NB cell lines, normal mouse adrenal gland tissue, and sub-cutaneous xenografts. Cultured NB cells were washed once with PBS pH 7.2 and suspended in 400 µl lysis buffer (Sigma), supplemented with a protease inhibitor cocktail (Complete mini, EDTA-free, Roche, Mannheim, Germany). Snap frozen tumours and mouse tissues were cut in small pieces and suspended in the above described lysis buffer. Sonication for 30 s followed by centrifugation for 15 min at 20'000 g were performed. Total protein amount was quantified using the Bradford method (Biorad Laboratories, Richmond, CA, USA). CXCL12 extra- and intra-cellular levels were measured using an ELISA kit (R&D Systems, Minnesota, MN, USA) in triplicates according to the manufacturer's guide.

Immunoblotting

Cultured cells were harvested by trypsinization, washed once with PBS pH 7.2, and then suspended in 30 µl of Lysis buffer (Sigma), supplemented with a protease inhibitor cocktail (Complete mini, EDTA-free, Roche, Mannheim, Germany). Cell extracts were then agitated for 15 min at 4°C and centrifugated at 20'000 g for 10 min. Total protein amount was quantified using the Bradford method (Biorad Laboratories, Richmond, CA, USA). Protein extracts (25–50 µg) were loaded on 10% SDS-PAGE and transferred on nitrocellulose membranes. Blots were saturated with 5% milk, 0.1 % Tween 20 in TBS and revealed using the polyclonal rabbit anti-human enolase-2 (Cell Signaling) at a dilution of 1/1000, and the monoclonal mouse anti-human vimentin (clone RV202, GeneTex®, Irvine, CA, USA) at a dilution of 1/500. Binding of the first antibody was revealed by incubation with either goat anti-mouse IgG (Jackson ImmunoResearch) or goat anti-rabbit IgG (Nordic Immunological Laboratories). Bound antibodies were detected using the Lumi-light western blotting substrate (Roche) according manufacturer's instructions.

ERK 1/2 and Akt phosphorylation

Following overnight serum starvation, cells were either unstimulated or stimulated with 100 ng/ml human recombinant CXCL12 or CXCL11 (PeproTech, Rocky Hill, NJ, USA) for indicated time, or pre-treated with 1 µM of the specific CXCR4 blocker 4F-benzoyl TN14003 (kind gift of N.Fujii, Kyto, Japan) prior to ligand stimulation. Cells were washed once in cold PBS and then lysed by the addition of 200 µl 4x concentrated sample buffer (250 mM Tris-HCl at pH 6.8, 10% SDS, 40% Glycerol, 16% β-mercaptoethanol, 0.04% Bromo-phenol-blue). Lysates were sonicated for 15 s in cold water to shear DNA and reduce sample viscosity, and heated at 95°C for 5 min to denaturate proteins. Diluted samples were separated on SDS-PAGE gels, and then transferred to Immobilon-P membranes (Millipore, Volketswil, Switzerland). Membranes were sequentially blocked for 1 h in TBS-Tween 0.01% containing 2% ECL Advance™ Blocking Agent (Amersham™ ECL Advance™ Western Blotting Detection Kit, GE Healthcare, Buckinghamshire, UK) at RT, incubated overnight at 4°C with the phospho-p44/42 MAPK (thr202/Tyr204) or Phospho-Akt (Ser 473) antibodies (Cell Signaling, Danvers, MA, USA) followed by 30 min incubation with HRP-labelled rabbit secondary antibody (Dako) at RT. The ECL system (GE Healthcare, Buckinghamshire, UK) was used for detection. The first antibody was removed by incubation of the membrane for 20 min at 50°C in a stripping solution containing 2% SDS, 65.5 mM Tris-HCL pH 6.8, and 100 mM β-mercaptoethanol. Membranes were then blocked and probed with the antibodies against total p44/42 MAPK or total Akt (Cell Signaling, Danvers, MA, USA) for 1 h at RT followed by 30 min incubation with the secondary antibody and proteins were revelated using the ECL system. When stipulated, cells were treated with 10 ng/ml recombinant human IGF-1 (PrepoTech) for 1 h, or 50 ng/ml recombinant human EGF (R&D Systems) for 5 min as positive controls for AKT or ERK 1/2 cascade activation, respectively (154;155).

Cell viability

10^4 cells in 100 µl DMEM containing 2% or 10% FCS were plated in triplicates in a 96-well plate (Corning Inc, Corning, NY, USA). Cell viability after 0, 24, 48, 72 and 96 h was assessed using the MTS/PMS cell proliferation kit (Promega) according to the manufacturer's protocol. When specified, cells were treated with 100 ng/ml CXCL12 or CXCL11 (Peprotech) in 2% FCS. OD was measured using an ELISA reader (Dynatech MRX Microplate Reader,

Dynatech Laboratories, Chantilly, VA, USA). Values were set to an OD 0 as a baseline (time point 0 h).

Soft agar assay

Anchorage-independent colony formation assay, modified from a previously described bioassay of human tumour stem cells (156), was performed using double-layer soft agar in 6-well plates (Corning) with a top layer of 0.175% agar (Difco™ Agar Noble, BD Biosciences, MA, USA) and a bottom layer of 0.35% agar. Briefly, 5×10^4 NB cells were suspended in 0.175% agar diluted in DMEM/10% FCS, and laid on the top of the supporting agar layer. 100 µl per well of fresh medium were added weekly. When stipulated, fresh medium supplemented with 100 ng/ml CXCL12 was weekly added to the top of the double-layer soft agar. Colonies were allowed to form in an incubator at 37°C for at least two weeks. Colony cell viability was assessed using the MTS/PMS cell proliferation kit (Promega), and viable colonies were counted using light microscopy (Leica Laborluc D).

Chemotaxis assay

Cell migration was measured using Transwell Costar® cell culture chambers with polycarbonate filters of 8 µm porosity (BD Biosciences), as previously described (137). Briefly, 2×10^5 cells suspended in DMEM/2% FCS were seeded in the upper compartment of the chambers system. The lower compartment was filled with DMEM/2% FCS supplemented or not with 100 ng/ml CXCL12 (PrepoTech). The cells were allowed to settle down for 4 h. After washing with PBS, membranes were fixed for 10 min in 4% PFA (Fluka, Buchs, Switzerland) in PBS at RT. After three washing steps in PBS, membranes were stained with haematoxylin (Polysciences, Warrington, PA, USA) for 3 min at RT. The non-migrated cells were carefully scraped from the upper side of the filter, and migrated cells on the lower side were counted by light microscopy (Leica Laborluc D).

In vivo studies

All animal experiments were carried out with Swiss athymic nude mice (Balb/C nu/nu), in accordance with established guidelines for animal care of the Swiss veterinary services. For surgical and ultrasonic procedures, mice were anaesthetized using isoflurane (Baxter) and received paracetamol as analgesia the day before the surgery.

For heterotypic assays, groups of three mice were subcutaneously injected in the flank with 2×10^5 cells suspended in 200 µl 1:1 mix of DMEM and BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Bedford, MA, USA). The grafted animals were then weekly monitored with calipers for tumour growth assessment. The tumour volume was calculated using the formula: volume = (length×width^2)/2. Standard H/E and immunostaining procedures on *s.c* tumours were performed at the Mouse Pathology Facility of Epalinges. To evaluate cell proliferation and vascular structure of tumours, 4 µm-thick paraffin-embedded tissues were incubated with the monoclonal anti-human Ki67 (clone MIB-1, Dako, Carpinteria, CA, USA) at a dilution of 1/50, and the polyclonal anti-human CD31 (Thermo Fisher Scientific, Waltham, MA, USA) at a dilution of 1/200. Immunostaining was analysed at the Institute of Pathology of Lausanne.

For orthotopic assays, seven animals per cell line were engrafted with NB cells directly in the left adrenal gland, as previously described (137;146). Briefly, the implantation was performed through a midline incision practiced under microscope. A total of 5×10^5 cells in 15 µl DMEM were injected in the adrenal gland using a 22G needle connected to a Hamilton syringe. The abdominal wall and skin were closed with a continuous suture of 4/0 Safil® Quick$^+$ (B Braun). Tumour take and growth were followed by ultrasound imaging every 10 days at the Lausanne Cardiovascular Assessment Facilities. Macroscopic metastases were assessed by gross examination.

Statistical analyses

TMA analyses were performed using SAS software, version 8.2 (SAS Institute). The Student's t-test was used to assess correlation between the expression of CXCR7 and CXCL12, and clinical data. Event-free survival was computed from the time of surgery of the primary tumour to the time of first event (local relapse, metastasis, or death) or at last follow-up. Overall survival was computed from the time of surgery to the time of death or last follow-up. Differences in survival between patients with a low (inferior to the median score) versus a high (superior to the median score) level of CXCR7 and CXCL12 expression were assessed on a log-rank test, and displayed using the Kaplan-Meier procedure.

For *in vitro* and *in vivo* experiments, statistical analyses (Student's t-test, Mann-Whitney test, Two-way ANOVA) were performed using GraphPadPrism 5.0 (GraphPad Software Inc., San

Diego, CA, USA). *p<0.05 was considered to represent significance, **p≤0.01 and ***p≤0.001 were interpreted to be highly significant.

Results

Expression of CXCR7, and its ligand CXCL12 in NB tissues

There is now a body of evidences that chemokine receptors, associated to their corresponding chemokine ligands, represent major paracrine/autocrine complex players within the tumour and its microenvironment (8). Defining the precise pattern of expression of chemokine/chemokine receptor in NB appears thus essential to elucidate their functional role within the tumour. To that purpose we screened a NB TMA, including a panel of 156 primary NB tumours, 56 metastatic and 65 control normal tissues, such as normal adrenal gland (AG) and normal sympathetic ganglion (SG), for CXCR7 and CXCL12 expression. Clinical parameters of the patients and associated tumours are detailed in Table 1.

Expression of the receptor and its ligand were semi-quantitatively assessed as an immunostaining score (0-4), representing percentage of positive cells, in three distinct cell populations within each tissue sample: neural, endothelial and stromal compartments. Neuroblasts and tumour ganglion cells were included in the neural compartment of NBs, while adrenal medulla and normal ganglion cells represented the neural part of AG and SG, respectively. Fibroblasts in tumours and AG, and Schwann cells in tumours and SG were attributed to the stroma.

Table 1. TMA : clinical characteristics

Patient at diagnosis	(N = 156)
Age (mo)	
Median (range)	26 (0-151)
< 12 mo, n (%)	78 (50)
≥ 12 mo, n (%)	78 (50)
Follow-up (mo)	
Median (range)	101 (1-243)
Survival	
Alive at time of last follow-up, n (%)	117 (75)
INSS stage	
1, n (%)	31 (20)
2, n (%)	19 (12)
3, n (%)	32 (21)
4, n (%)	58 (37)
4S, n (%)	16 (10)
N-*myc* oncogene analysis	
Amplified, n (%)	19 (14)
Non amplified, n (%)	114 (86)
Unknown, n (%)	23 (14)
Children's Oncology Group Risk Classification	
Low, n (%)	54 (35)
Intermediate, n (%)	44 (28)
High, n (%)	58 (37)
Neuroblastoma type	
Standard, n (%)	101 (65)
Mass screening, n (%)	55 (35)
Sample type	
Primary tumour, n	156
Metastasis, n	56
Control normal tissues, n	65
Adrenal Gland, n (%)	50 (77)
Sympathetic ganglion, n (%)	15 (23)
Differentiation Stage	
NBnd, n (%)	130 (83)
UnNB, n (%)	36 (23)
GGNB, n (%)	20 (12)
GGN, n (%)	6 (3)

mo : month
n : number of cases
INSS : International Neuroblastoma Staging System
NB : neuroblastoma
NBnd : not differentiated NB
UnNB : undifferentiated NB
GGNB : ganglioneuroblastoma
GGN : ganglioneuroma

CXCR7 is preferentially expressed by mature neural cells in differentiated NB tumours

As shown in Figure 5A and in Table 2, a low CXCR7 expression (median score of 0.92) was globally observed in neural cells in 76% of primary tumours (PTs), while an even lower staining was measured in the vascular structure, and in the stroma of 33% and 58% of PTs, respectively (median score of 0.15 and 0.48, respectively). Thus, CXCR7 staining, albeit low, was generally localized in the neural compartment of NBs.

No significant variations of the neural CXCR7 expression were noted between NB PTs, metastases and control tissues (median score of 0.92, 0.93 and 0.78, respectively, Table 2).

The neural-associated CXCR7 expression was then further evaluated in different NB tissues regarding their pattern of differentiation. To that extent, we analysed the receptor expression in undifferentiated tumours (UnNBs), in differentiated tumours such as ganglioneuroblastomas (GGNBs) and ganglioneuromas (GGNs), and finally in not differentiated (NBnd) NBs which include all NB tumours except GGNBs and GGNs. Our data first showed that CXCR7 expression was particularly associated to GGNBs and GGNs, as compared to UnNBs (Figure 5B). Moreover, CXCR7 staining was almost restricted to the more mature neural elements of GGNBs and GGNs, such as tumour ganglion cells, while no similar staining was observed in normal ganglion cells in SG tissues. As shown in Figure 5C, the neural-associated CXCR7 staining score was significantly enhanced in NBnd tumours (median score of 0.81±0.43, $p<0.05$), in GGNB (median score of 0.93±0.65, $p<0.05$), and in GGN (median score of 1.62±0.64, $p<0.01$) tissues, as compared to that of UnNB tumours (median score of 0.57±0.37). In addition, all GGN tissues expressed CXCR7 as compared to other tumours. Thus, CXCR7 is preferentially expressed by the more mature elements in differentiated tumours.

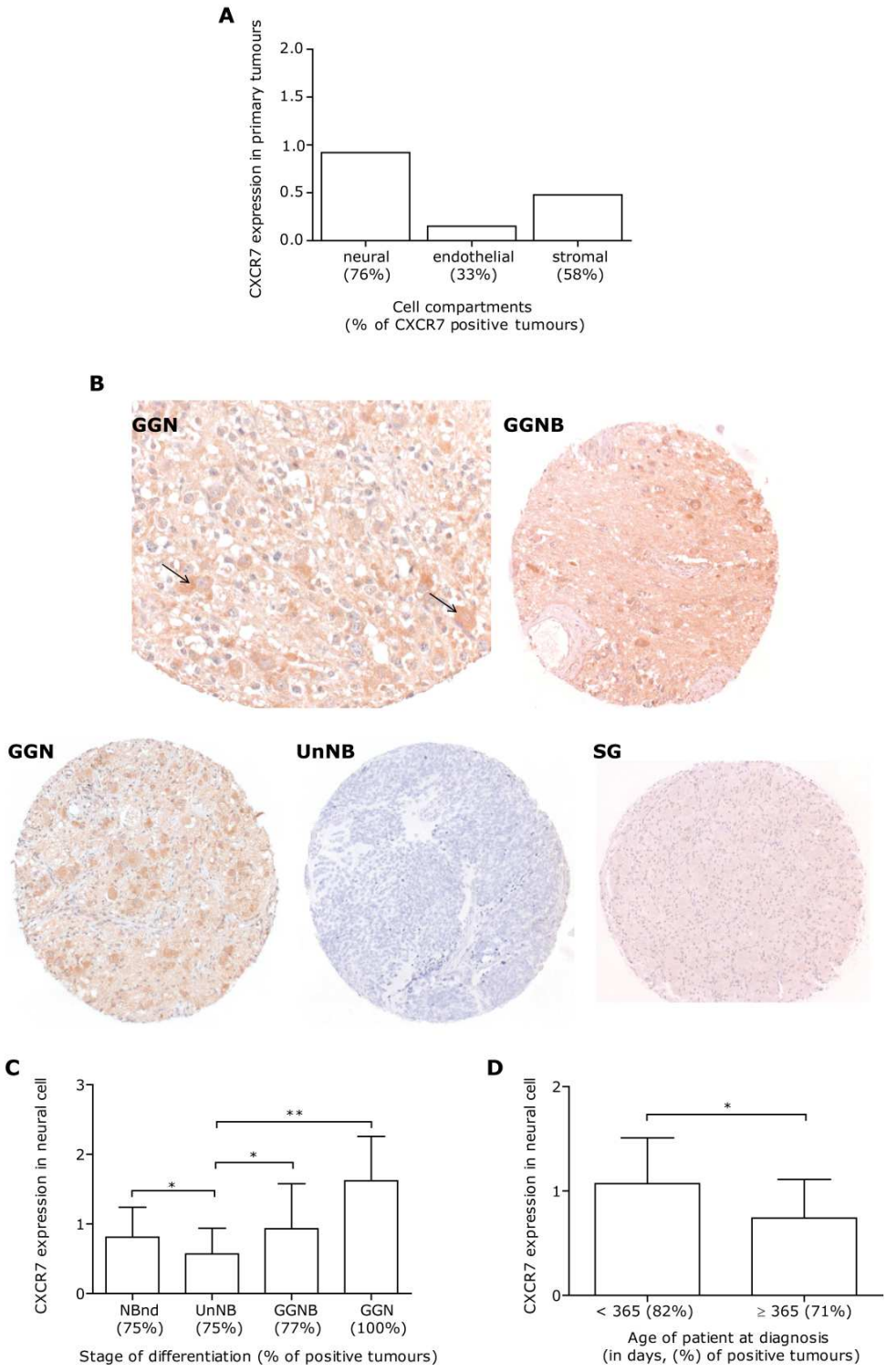


Figure 5: Expression of CXCR7 in NB tissues by TMA analyses

(A) Semi-quantitative assessment of CXCR7 expression in the neural, endothelial and stromal cell compartments of NB primary tumours. Columns represent the average of the immunostaining score of each cell compartment. Percentage (%) of chemokine receptor positive tumours indicates the % of positive tissues. (B) Immunohistochemical analysis of CXCR7 in Undifferentiated tumour (UnNB), Ganglioneuroblastoma (GGNB), Ganglioneuroma (GGN) and control normal sympathetic ganglion (SG) tissues. Black arrows represent CXCR7-positive tumour ganglion cells. (C) CXCR7 expression level (median score) in not differentiated tumours (NBnd), UnNBs, differentiated tumour tissues, (D) and in tumours of patient according to the age of patient at diagnosis. Student's t-test: *p<0.05, **p<0.01.

Table 2. Expression of CXCR7 and CXCL12 in NB primary tumours, metastases and control tissues

	Neural cells			Endothelial cells			Stromal cells		
	Primary tumour	Metastasis	Control	Primary tumour	Metastasis	Control	Primary tumour	Metastasis	Control
CXCR7									
Positive tissues (%)	76	75	63	33	33	26	58	53	24
Number of cases	119	42	41	52	19	17	92	30	16
Median score	0.92	0.93	0.78	0.15	0.18	0.14	0.48	0.45	0.2
p-value		*ns*	*ns*		*ns*	*ns*		*ns*	*0.01*
CXCL12									
Positive tissues (%)	77	92	70	100	100	100	98	98	93
Number of cases	121	52	46	156	56	65	153	55	61
Median score	0.82	1.04	0.59	3.12	3.13	3	1.97	1.93	1.98
p-value		*0.02*	*0.01*		*ns*	*0.01*		*ns*	*ns*

Control represents normal adrenal gland and sympathetic ganglion tissues

Median score means average tumour score, established by semiquantitative analysis of the immunostaining

p-value (Student's t-test) refers to primary tumour. ns : not significant ; $p < 0.05$: significant ; $p \leq 0.01$: very significant

We next analysed CXCR7 expression in different NB clinical groups (Table 3). The neuroblast-associated CXCR7 expression did not vary between NB stages 1-2 versus 3-4, as well as in NB stages 4 versus 4S ($p=0.54$ and $p=0.07$, respectively). Thus, CXCR7 expression in neural compartment of tumours is independent of NB clinical stages. However, the receptor expression significantly increased in tumours from less than 1 year-old patients ($p=0.02$, Figure 5D). Most children of less than 1 year of age present tumours with the potential to regress spontaneously, or to mature into benign differentiated tumours such as GGNs. Our analyses thus suggest an association of CXCR7 expression with a favourable outcome. *MYCN* oncogene amplification is linked with more aggressive tumours and poor prognosis (85). However, when the MYCN status in NB tumours was considered, no correlation with CXCR7 expression was statistically found (data not shown). Similarly, CXCR7 expression did not correlate with overall nor even-free survival of patients (Figure 6). Therefore, even though CXCR7 is expressed by mature tumour cells in differentiated GGNB and GGN tissues, our TMA analyses did not allow us to assign CXCR7 a statistically significant and favourable prognosis value.

Table 3. Expression of CXCR7 and CXCL12 in NB clinical groups

	Neural cells					Endothelial cells					Stromal cells				
INSS stage	1	2	3	4	4S	1	2	3	4	4S	1	2	3	4	4S
CXCR7															
Positive tumours (%)	61	78	87	67	93	41	26	53	43	18	54	52	75	48	50
Number of cases	19	15	28	39	15	13	5	17	25	3	17	10	24	28	8
Median score	0.94	1.04	0.98	0.87	0.94	0.14	0.05	0.23	0.21	0.01	0.4	0.39	0.63	0.55	0.29
p-value		*0.54*$^{(+)}$			*0.07*$^{(-)}$		*0.208*$^{(+)}$			*0.004*$^{(-)}$	*0.001*$^{(\S)}$		*0.157*$^{(+)}$		*0.0002*$^{(-)}$
CXCL12															
Positive tumours (%)	77	89	93	84	87	100	100	100	100	100	100	100	100	96	93
Number of cases	24	17	29	49	14	31	19	32	58	16	31	19	32	56	15
Median score	0.77	0.89	0.84	0.79	0.98	3.09	3.05	3.16	3.16	3.06	1.95	2.45	1.97	1.93	1.91
p-value		*0.83*$^{(+)}$			*0.25*$^{(-)}$		*0.14*$^{(+)}$			*0.42*$^{(-)}$		*0.25*$^{(+)}$			*0.99*$^{(-)}$

INSS : International Neuroblastoma Staging System

Median score means average tumour score, established by semiquantitative analysis of the immunostaining

p-value (Student's t-test) : $^{(+)}$ represents p-value between stages 1-2 and stages 3-4 ; $^{(-)}$ represents p-value between stage 4 and stage 4s ;

$^{(\S)}$ represents p-value between stage 1 and stage 4 ; $p < 0.05$ (significant); $p \leq 0.01$ and $p \leq 0.001$(highly significant)

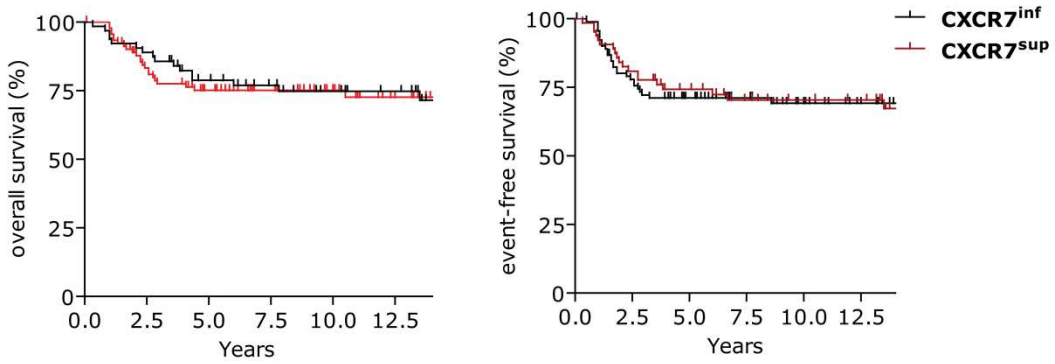


Figure 6: Survival of patients grouped by NB tumour cell expression of CXCR7.
Graphs represent Kaplan-Meier analyses of overall survival (p=0.9) and event-free survival (p=0.83) of patients. All NB tissues were classified regarding their neural-associated pattern of CXCR7 immunostaining score: $CXCR7^{inf}$ represents NB tumours with a CXCR7 score inferior to the median, while $CXCR7^{sup}$ represents NB tumours with a staining score superior to the median.

CXCR7 is not expressed in the microenvironment of NB tumours and metastases

As mentioned above, CXCR7 expression was almost undetectable in the stromal and vascular compartments of NB PTs and metastases (Table 2). Indeed, as all median scores were inferior to 0.5 in NB PTs, metastases and in control normal tissues, no relevant CXCR7 expressions were taken into account (statistical variations in Table 2). Similarly, no relevant variations of receptor expression were observed in the different NB clinical groups (Table 3).

CXCL12 is predominantly associated to the vascular and stromal structures of NBs

In parallel, our TMA analyses revealed that the CXCL12 ligand was strongly expressed in endothelial cells in all PTs (median score of 3.12), and highly associated to the stroma (median score of 1.97), while weakly expressed in the neural compartment (median score of 0.82) in NB PTs (Figure 7A, 7B, Table 2). Thus, these data showed that the CXCR7 ligand is likely associated to the tumour microenvironment.

In particular, tumour endothelial cells expressed higher levels of CXCL12 when compared to normal tissues (p=0.01, Table 2). Although vascular CXCL12 expression was found independent of NB clinical stages (Table 3), it increased in tumours from patients over one year-old (p=0.02, Figure 7C).

In parallel, even though intense basal CXCL12 levels were observed in the stromal compartment of tumours and controls (mean scores of 1.9, Table 2), its expression was

further enhanced in the schwannian stroma of GGNBs and GGNs (albeit not statistically significant probably due to the low number of analysed GGN tissues in our TMA), as compared to stromal elements present in UnNBs (Figure 7D). No particular pattern of stroma-associated CXCL12 expression was noted in the different NB clinical stages (Table 3).

Finally, neural-associated ligand expression, albeit low, enhanced in 92% of metastatic samples as compared to PTs, as well as in 77% of PTs as compared to controls (Table 2). However, it was not correlated with NB clinical (Table 3) nor differentiation stages (data not shown).

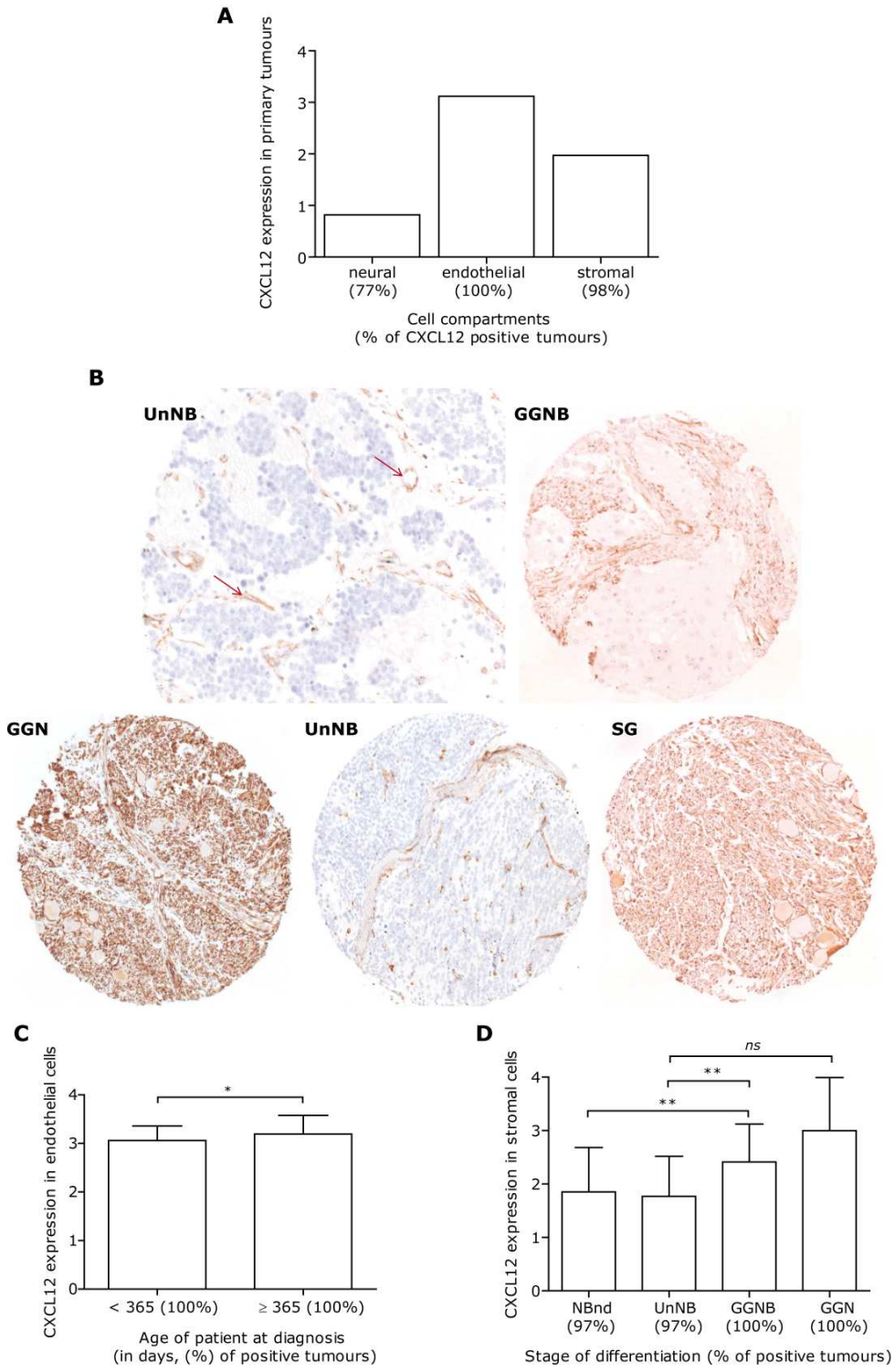


Figure 7: Expression of CXCL12 in NB tissues by TMA analyses

(A) Semi-quantitative assessment of CXCL12 expression in the neural, endothelial and stromal cell compartments of NB tissues. Columns represent the average of the CXCL12 immunostaining score in each cell compartment. Percentage (%) of chemokine positive tumours indicates the % of positive tissues. (B) Immunohistochemical analysis of CXCL12 in undifferentiated tumour (UnNB), Ganglioneuroblastoma (GGNB), Ganglioneuroma (GGN) and control normal sympathetic ganglion (SG) tissues. Red arrows represent CXCL12-positive endothelial cells. (C) Ligand expression level (median score) in tumours of patient according to the age of patient at diagnosis, and (D) in not differentiated tumours (NBnd), UnNBs, and differentiated tumour tissues. Student's t-test: *p<0.05, **p<0.01.

Expression of the CXCR7 and CXCR4 receptors in NB cell lines

To corroborate our TMA analyses, we next assessed CXCR7 expression in a panel of well-characterized N-, I- and S-type NB cell lines (90). As illustrated in Figure 8, RT-PCR analyses did not reveal any *CXCR7* expression in all selected I-type NB cell lines (SK-N-Be(2c), LAN-5, SH-IN), while 5/9 N-type cell lines (IGR-N91, LAN-1, IMR-32, CLB-Ber-Lud2, SJN-B12) and 1/2 S-type cell line (SH-EP) expressed the receptor. In contrast, *CXCR4* expression was expressed in almost all selected NB cell lines.

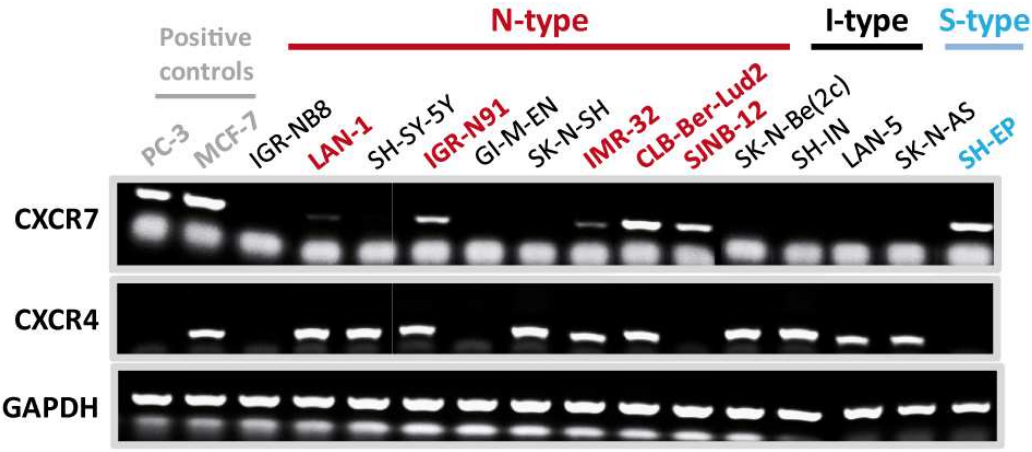


Figure 8: Expression of the CXCL12 receptors in NB cell lines
Qualitative RT-PCR analyses for *CXCR7* and *CXCR4* mRNA expression level in a panel of NB cell lines. *GAPDH* was used as gene of reference. The prostate cancer cell line PC-3 and the breast cancer cell line MCF-7 were used as positive controls for *CXCR7* expression.

As only a minority of NB cell lines expressed *CXCR7*, we further asked whether external stimuli, such as serum deprivation or presence of the ligand CXCL12, could regulate the receptor expression (Figure 9).

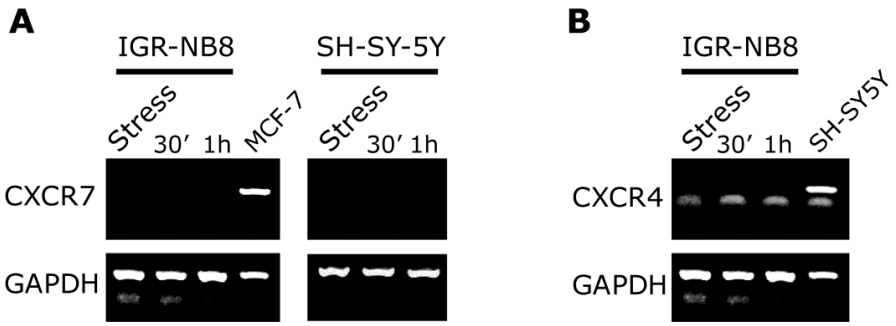


Figure 9: Induction of both *CXCR7* and *CXCR4* expression in NB cell lines upon exposure to CXCL12
The IGR- NB8 and SH-SY-5Y NB cell lines were cultured in stressful condition (2% serum) and exposed to 100 ng/ml CXCL12, for indicated time points. (A) Qualitative RT-PCR analyses for *CXCR7* mRNA expression level in the IGR- NB8 and SH-SY-5Y NB cell lines. (B) RT-PCR analyses for *CXCR4* mRNA expression level in the IGR-NB8 and SH-SY-5Y NB cell lines. *GAPDH* was used as gene of reference.

As shown in Figure 9A, *CXCR7* expression was not further induced in the CXCR7-negative IGR-NB8 and SH-SY-5Y NB cell lines in either stressful culture condition (2% serum) or in presence of CXCL12. Similar observation was noted when NB cells were exposed to the other CXCR7 ligand, CXCL11 (data not shown). Moreover, no induction of *CXCR4* expression in the CXCR4-negative IGR-NB8 cell line was observed in 2% serum, and in presence of CXCL12 (Figure 9B).

The two CXCL12 receptors seem to be differentially expressed by NB cell lines. To evaluate functional implications of both CXCR7 and CXCR4 receptors, we next quantified and compared CXCR7 and CXCR4 surface expressions in selected RT-PCR-positive NB cells by flow cytometry (Figure 10).

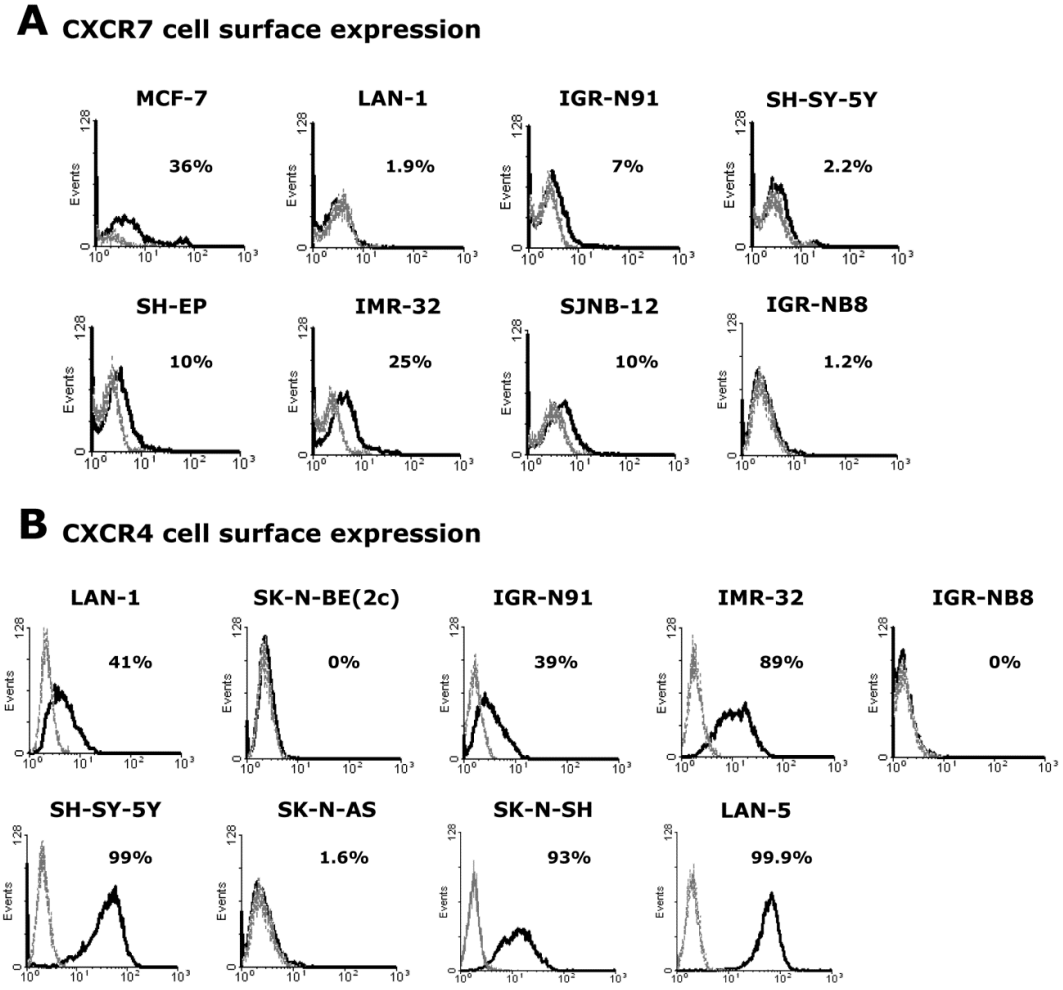


Figure 10: Expression of the CXCL12 receptors in NB cell lines
(A) CXCR7 cell surface expression was analysed by flow cytometry. Grey dotted line : cells stained without the primary Ab. Black line : cells stained with anti-CXCR7 antibody clone 9C4. (B) CXCR4 cell surface expression. Grey dotted line: cells stained with PE-conjugated secondary antibody. Black line: cells stained with anti-CXCR4 antibody clone 12G5. Percent represent CXCR7 or CXCR4 positive cells.

Very low cell surface expression of the CXCR7 receptor was detected in most of the selected NB cells, while 25% of IMR-32 cells harboured a moderate CXCR7 cell surface expression (Figure 10A). In addition, heterogeneous CXCR4 surface expression was measured in NB cell lines, for which rather moderate or intense expression levels were detected (Figure 10B), as already described (137;157).

Taken together, our data showed a distinct expression pattern of the two CXCL12 receptors in NB cell lines.

CXCR7 and NB differentiation in vitro

Our TMA and *in vitro* observations showed that the CXCR7 receptor was preferentially expressed in the more mature elements of tumours, and in a minority of NB cell lines. Taken together, these data thus suggest that CXCR7 may be associated to NB cell differentiation.

To further confirm this hypothesis, we performed NB differentiation assay *in vitro.* All-trans retinoic acid (RA) and bromodeoxyuridine (BrdU) have been fully characterized as primordial tools to evaluate the *in vitro* capacity of NB cell lines to differentiate towards the neuronal and glial lineages, respectively (151;158-161). As N- and I-type NB cell subtypes were shown to progress towards neuronal and glial fates upon either all-trans retinoic acid (RA) or Bromodeoxyuridine (BrdU) treatment, respectively (158), the N-type IGR-NB8 and the I-type SK-N-Be(2c) NB cell lines were used in this study.

Differentiation of NB cells by treatment with all-trans retinoic acid and bromodeoxyuridine

The IGR-NB8 and the SK-N-Be(2c) cell lines were exposed to 10 µM of either RA or BrdU for 30 days. To detect morphological changes in NB cells, we used an antibody directed against β_3-tubulin (162). NB cell morphology changes appeared as early as 3 days after both RA or BrdU treatment, and persisted during all the differentiation induction experiment (30 days), as previously observed (158) (Figure 11).

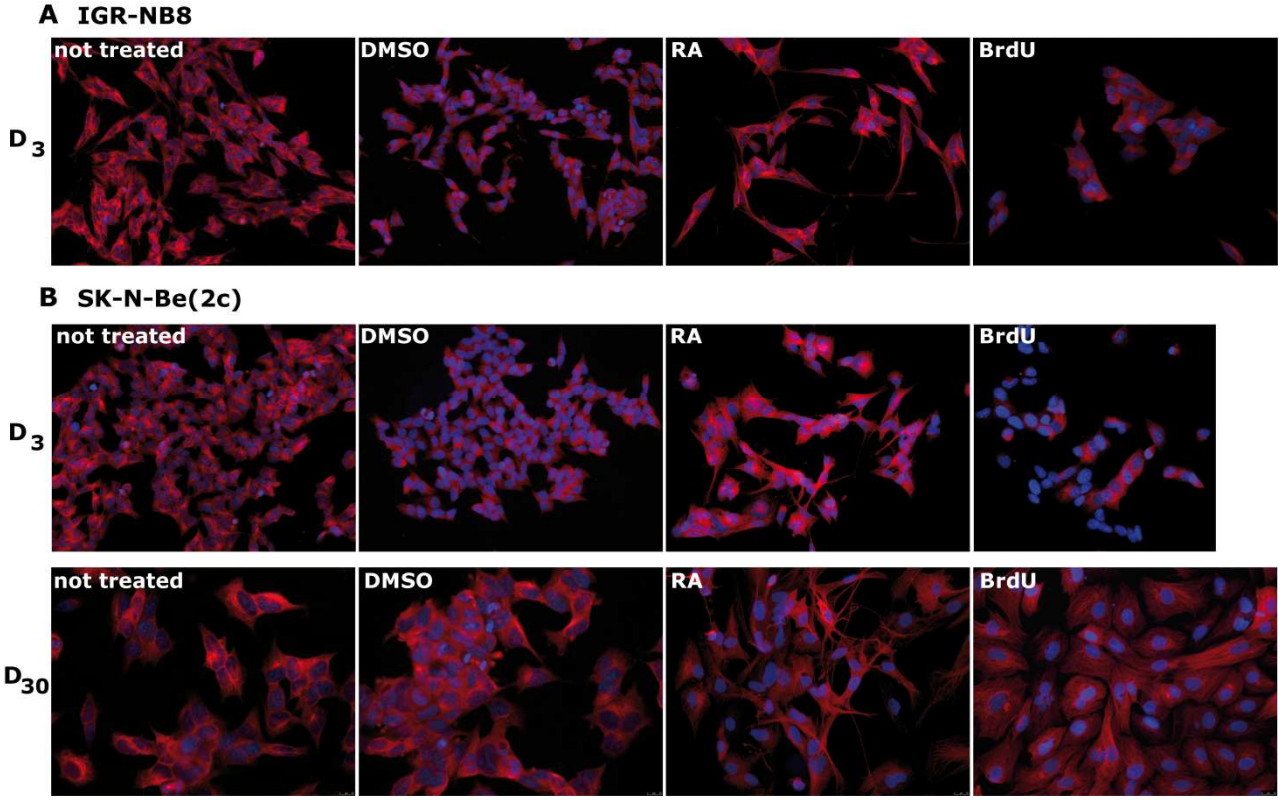


Figure 11: Morphological features of NB cells upon RA or BrdU treatment *in vitro*
The IGR-NB8 and the SK-N-Be(2c) cell lines were exposed to 10 µM all-trans Retinoic Acid (RA) or 10 µM 5-bromo-2-deoxyuridine (BrdU). Pictures represent immunofluoresence staining of β_3-tubulin (red) and DAPI (blue). (A) 3 day-treatment of the IGR-NB8 cell line ; (B) 3 day-(upper panel) and 30 day-(lower panel) treatment of the SK-N-Be(2c) cell line.

Indeed, RA-treated NB cells elaborated enhanced neuritic processes and proliferated by forming interconnected cell clumps (Figures 11A and 11B). During BrdU treatment, NB cells presented glial-like morphology, with large flat cytoplasm and enhanced adherence capacity to the flask surface, which was fully acquired after 30 days of treatment (Figure 11B). Morphological changes were not detected in the untreated (DMEM, 10%FCS), nor in the DMSO-treated control cell lines.

To further confirm the RA and BrdU-induced differentiation *in vitro*, we assessed the influence of differentiation agents on NB growth and apoptosis (Figure 12).

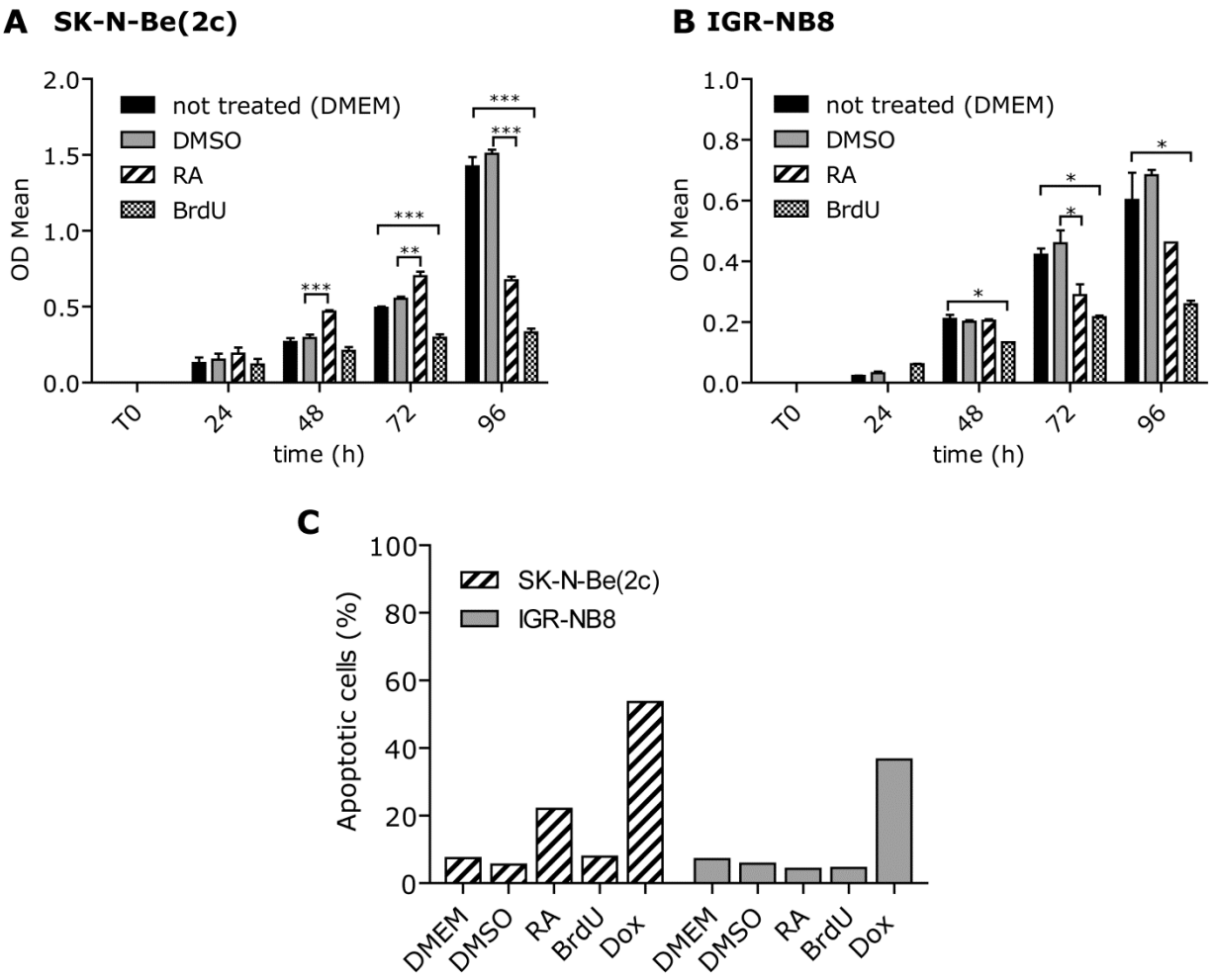


Figure 12: Growth and apoptosis assays upon NB cell differentiation *in vitro*
(A,B) Growth of the SK-N-Be(2c) and the IGR-NB8 cell lines was followed upon treatment with differentiation agents for 96 h. Columns represent OD mean ± SEM of two independent experiments. (C) Apoptosis was measured by detection of the sub-G_1 apoptotic cell using the PI staining method. Such assay was performed after 7 days of treatment with both 10 µM RA and BrdU. Treatment of NB cells with 1 µg/ml doxorubycin (Dox) for 48 h was used as positive control.

As previously described (158), RA enhanced SK-N-Be(2c) cell growth for 72 h, as compared to cells exposed to DMSO, before inducing a growth arrest in those cells at 96 h (Figure 12A). In parallel, the proliferation of IGR-NB8 cells already slowed down after 72 h of RA treatment, as compared to DMSO-treated control cells (Figure 12B). BrdU-treatment induced a reduction of both SK-N-Be(2c) and IGR-NB8 cell growth, as compared to non treated cells.

Then, we evaluated the percentage of apoptotic cells after exposure of NB cells to either RA or BrdU. Doxorubicin (dox) is a cytotoxic drug known to induce NB cell death *in vitro*. As positive control, cells were thus treated in parallel with 1 µg/ml doxorubicin, which is known to induce apoptosis in approximatively 50% of NB cells (118). A slight induction of

mortality was noted for the SK-N-Be(2c) cell line when treated with RA, which was also reported in a previous study (158), while no effect was observed upon treatment with BrdU, as compared to control cells. None of the treatments induced apoptosis of IGR-NB8 cells (Figure 12C).

Taken together, these data showed that both RA and BrdU treatments affected NB cell growth, without promoting apoptosis, further confirming the *in vitro* RA/BrdU-induced differentiation capacity of NB cells.

Expression of early neuronal and glial differentiation markers

Neurone specific enolase (NSE), also known as enolase-2, and vimentin have been proposed as early markers of neuronal and glial phenotypes, respectively (159;163). Finally, to further characterize the neuronal- and glial-like profile of *in vitro* differentiated NB cells, we evaluated the expression levels of both proteins after 3, 7 and 30 days of RA and BrdU treatments (Figure 13).

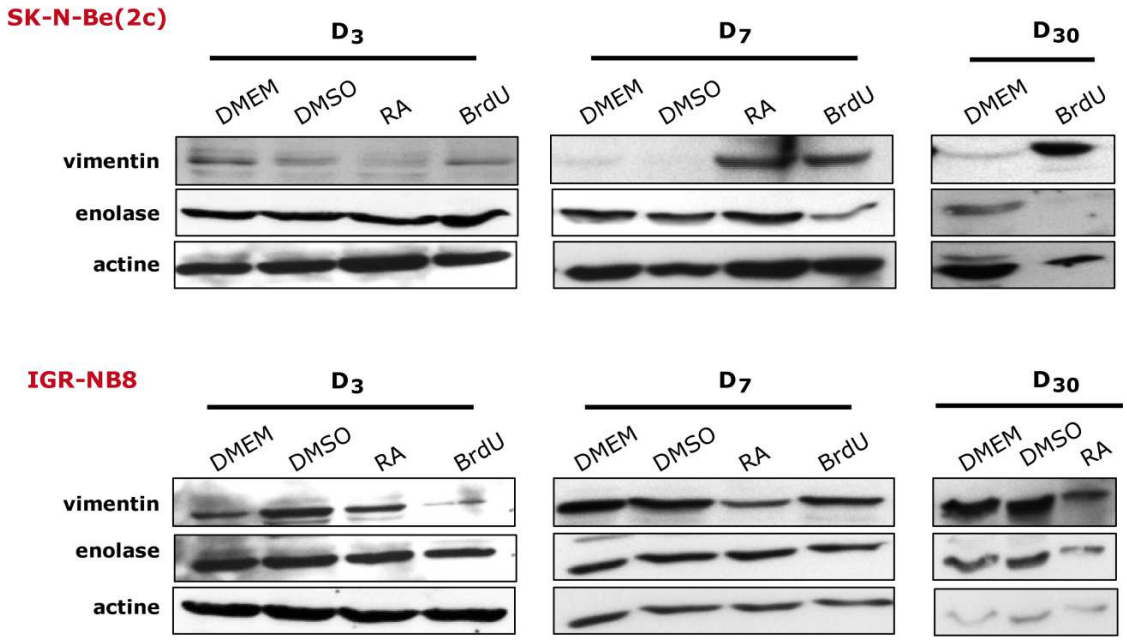


Figure 13: Expression of differentiation marker in NB cell lines after differentiation *in vitro*
Expression level of vimentin and enolase-2 were assessed by immunoblotting in the SK-N-Be(2c) and in the IGR-NB8 cell lines after 3, 7 and 30 days of treatment with 10 µM RA or BrdU.

As shown in Figure 13 (upper panel), the expression of vimentin in SK-N-Be(2c) cells did not change at day 3, but enhanced at day 7 until day 30 of BrdU treatment, when compared to not treated cells. Inversely, NSE expression in SK-N-Be(2c) cells decreased from

day 7 in presence of BrdU, and was totally abolished after 30 days of treatment. However, RA-induced differentiation did not further enhance NSE expression, due to the already high endogenous protein expression levels in those cells.

The IGR-NB8 cell line expresses high levels of both enolase-2 and vimentin (Figure 13, lower panel). Then, we could not detect any increase in both protein expressions upon RA or BrdU treatment, respectively. Nonetheless, a slight reduced expression of vimentin was observed after 7 and 30 days of RA treatment.

Expression of CXCR7 upon neuronal and glial differentiation of NB cells in vitro

With the aim to evaluate the putative implication/association of the CXCR7 receptor in NB differentiation, we evaluated whether *CXCR7* expression changed upon neuronal and/or glial differentiation of NB cells *in vitro*. As shown in Figure 14, *CXCR7* expression was induced in IGR-NB8 cells after 3 days of RA treatment ($p<0.01$), whereas its expression was weakly detectable upon BrdU exposure. Similar RA-induced CXCR7 expression pattern was detected in the SK-N-Be(2c) and the SH-SY5Y cell lines (data not shown). These data suggest that *CXCR7* induction, albeit weak, may occur when NB cells undergo neuronal rather than glial differentiation.

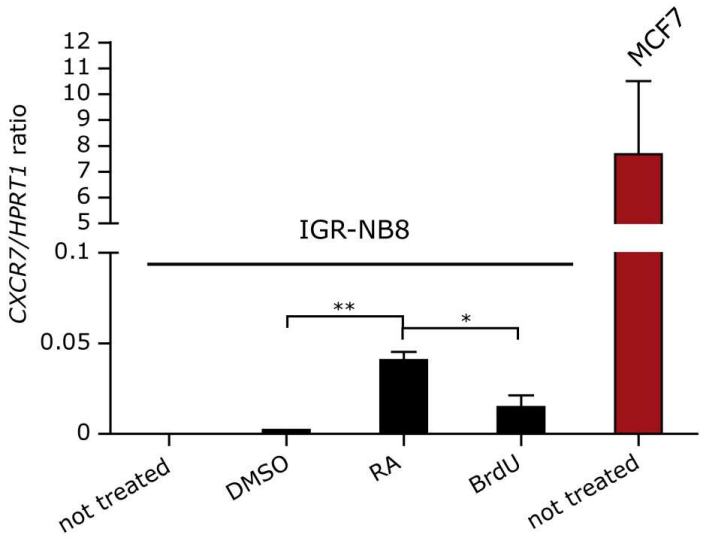


Figure 14: Expression level of *CXCR7* mRNA upon differentiation of NB8 cells *in vitro*
Semi-quantitative real-time PCR analyses of *CXCR7* mRNA expression level in the IGR-NB8 cell line after 3 days of treatment with 10 µM RA or BrdU. Expression levels of *CXCR7* transcripts were calculated relatively to the level of the housekeeping gene *HPRTI*. The breast cancer cell line MCF-7 was used as positive control for *CXCR7* expression. Columns indicate results in triplicates and were representative of two independent experiments. Error bars indicate S.D. Student's t-test: *$p<0.05$, **$p<0.01$.

However, overall induced-receptor expression remained weak, as compared to the *CXCR7* endogenous expression levels in the MCF-7 positive control cell line. Of note, the CXCR7 receptor has been shown to be functional in the MCF-7 cells, particularly in presence of its ligand CXCL12 (164). However, it does not mean that comparable levels of *CXCR7* transcripts are required in NB cells to express a functional receptor. Then, to assess the putative implication of RA-induced *CXCR7* expression, we next measured CXCR7 protein expression in differentiated cells. No CXCR7 expression could be detected during the 30 days of differentiation induction experiment, neither at the surface membrane as assessed by flow cytometry, nor in the intracellular space as measured by immunofluorescence (data not shown).

As detected levels of *CXCR7* expression were low, we asked whether cell culture conditions were adequate to induce receptor expression. Our TMA data showed that CXCL12 was markedly enhanced in the stroma of differentiated tumours (Figure 7D). Consequently, we postulated that a CXCL12-producing environment might favour CXCR7 induction in differentiating NB cells. Induction experiment was thus repeated in presence of the ligand. However, CXCL12 did not further enhanced CXCR7 expression in the SK-N-Be(2c) cell line (Figure 15), nor in the IGR-NB8 cell line (data not shown), after RA treatment.

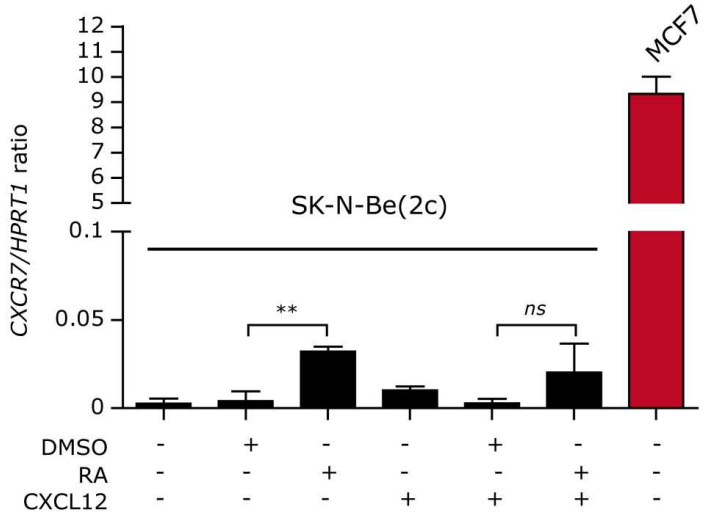


Figure 15: Expression level of *CXCR7* mRNA upon neuronal differentiation of the SK-N-Be(2c) cells *in vitro*

Real-time PCR analyses of *CXCR7* mRNA expression level in the SK-N-Be(2c) cell line after 3 days of treatment with 10 µM RA and BrdU, and in presence or in absence of 100 ng/ml CXCL12. Expression levels of *CXCR7* transcripts were calculated relatively to the level of the housekeeping gene *HPRTI*. The breast cancer cell line MCF-7 was used as positive control for *CXCR7* expression. The experiment was performed in triplicates. Error bars indicate S.D. Student's t-test: **p<0.01.

Importantly, these results were not biased by an autocrine production of the ligand, as no CXCL12 was released by NB cells during *in vitro* differentiation treatment (Figure 16).

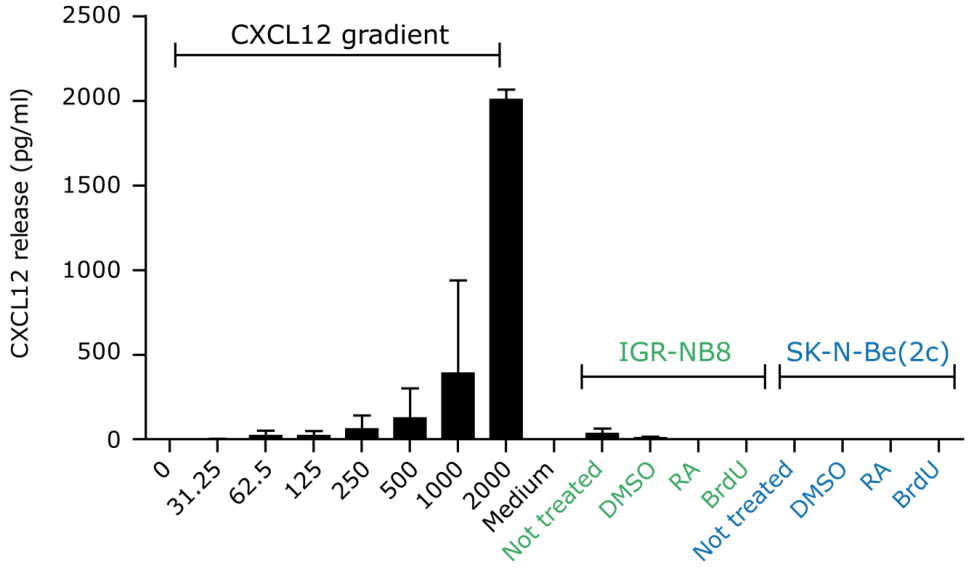


Figure 16: CXCL12 release upon differentiation of NB cells *in vitro*
The release of CXCL12 was measured by ELISA in the IGR-NB8 and the SK-N-Be(2c) cell lines after 3 days of treatment with 10 µM RA or BrdU. A gradient of human recombinant CXCL12 and normal medium corresponding to DMEM+10% FCS were also measured as positive and negative controls respectively. Columns indicate results in triplicates.

Regarding the above results, further investigation will be necessary to determine the putative involvement of CXCR7 in cell maturation and whether it might represent a neural and/or a differentiation marker for NB.

Overexpression of the two CXCL12 receptors in NB cell lines

With the identification of CXCR7, research has focused on interactions between the two CXCL12 receptors. As the functional role of CXCR7 in NB is still elusive, we analyzed the relative contribution of CXCR7 alone, or together with CXCR4 in mediating NB biological functions *in vitro*. In that purpose, we overexpressed CXCR7, CXCR4 or a combination of the two receptors in the CXCR4/CXCR7-negative IGR-NB8 cell line (respectively NB8x7, NB8x4 and NB8x4x7 cell lines). CXCR7 was also ectopically overexpressed in the SH-SY5Y cell line (SHSYx7 cells), which already showed a high CXCR4 endogenous expression (Figure 10B).

Transfection efficiency was analysed by assessing the cell surface and intra-cellular expression of CXCR7 and CXCR4 in transduced cell lines (Figure 17). In parallel, expression

levels of *CXCR7* and *CXCR4* transcripts were measured by semi-quantitative real-time PCR (Figure 18).

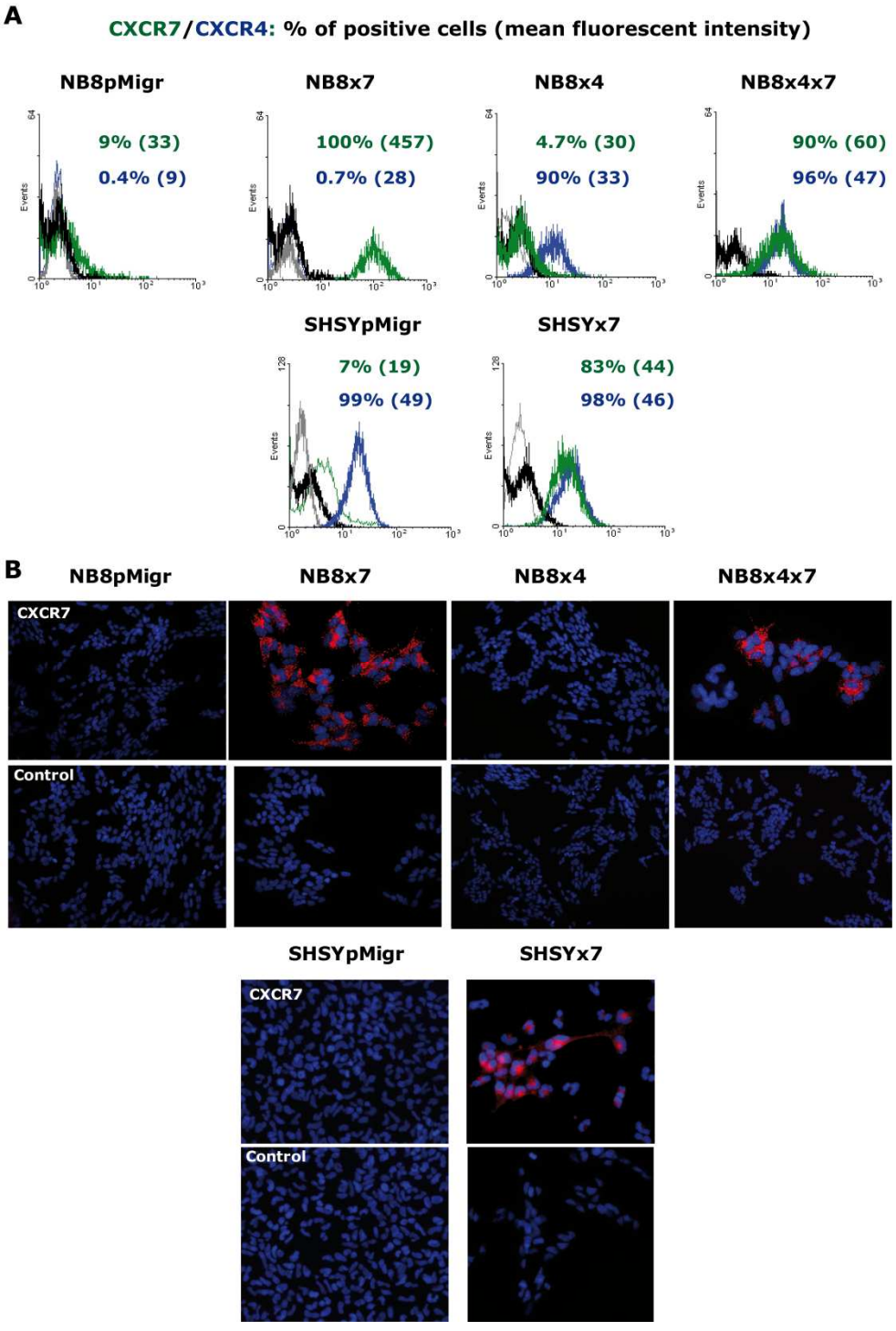


Figure 17: Expression of the CXCL12 receptors in NB transduced cell lines
(A) Both NB8pMigr and SHSYpMigr cell lines represented control cells transduced with the pMigr empty vector. Percent of CXCR7 and CXCR4 positive transduced cells, and the mean fluorescent intensity (brackets) for CXCR7 and CXCR4 staining are indicated. Dark and grey lines : cells stained without anti-CXCR7 and anti-CXCR4 Abs, respectively; Green and blue lines: cells stained with anti-CXCR7 and anti-CXCR4 Abs, respectively. (B) Immunofluorescence for CXCR7 expression in the permeabilized NB8 and SHSY transduced cell lines. Control staining represents incubation of cells without the primary antibody.

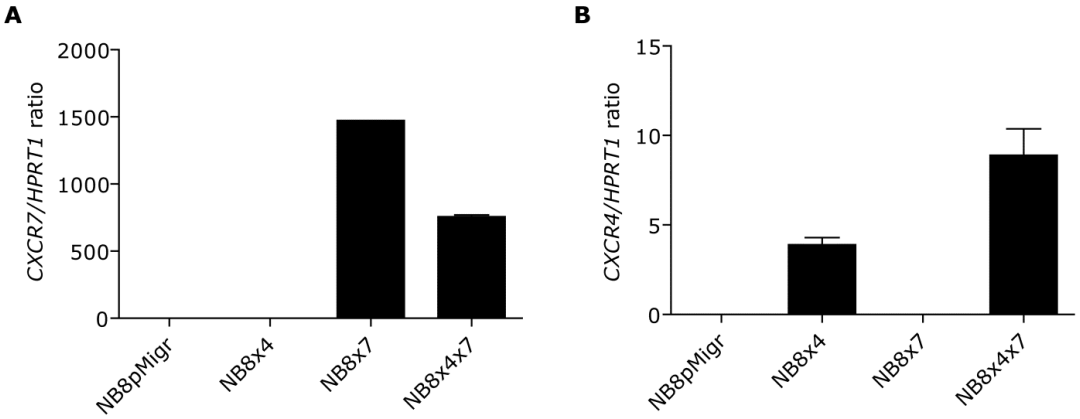


Figure 18: Semi-quantitative real-time PCR analyses for *CXCR7* and *CXCR4* expression
(A, B) Semi-quantitative real-time PCR analyses for *CXCR7* and *CXCR4* mRNA expression level, respectively, in the NB8pMigr, CXCR4-expressing NB8x4, CXCR7-expressing NB8x7, and CXCR7/CXCR4-expressing NB8x4x7 cell lines. Expression levels of *CXCR7* and *CXCR4* transcripts were calculated relatively to the level of the housekeeping gene HPRTI. Experiment was performed in triplicates. Error bars indicate SEM.

CXCR7 was highly expressed at the cell surface of the NB8x7 and the NB8x4x7 cell lines (Figure 17A). Similarly, CXCR4 surface expression was also strongly detected in the NB8x4 and the NB8x4x7 cell lines. In addition, neither *CXCR7* nor *CXCR4* expression was induced in the CXCR4-expressing NB8x4 or in the CXCR7-expressing cell lines, respectively, showing the specificity of receptor transfection (Figure 18).

Interestingly, surface expression of CXCR7 was slightly higher in the NB8x4x7 (mean fluorescent intensity of 60) than in the SHSYx7 cell lines (mean fluorescent intensity of 44), whereas that of CXCR4 was similar in both cell lines (Figure 17A).

Moreover, a reduced CXCR7 surface expression level was detected in the double receptor-positive NB8x4x7 cells (mean fluorescent intensity of 60), as compared to NB8x7 cells (mean fluorescent intensity of 457) (Figure 17A). Intra-cellular CXCR7 staining also decreased in the NB8x4x7 group as compared to the NB8x7 group (Figure 17B). These observations was further confirmed by real-time PCR analyses (Figure 18A), suggesting that the CXCR7 receptor expression might be modulated by a translational control, or affected by transfection process.

In contrast, CXCR4 surface expression was slightly enhanced in the NB8x4x7 cells (mean fluorescent intensity of 47), as compared to NB8x4 cells (mean fluorescent intensity of 33) (Figure 17A). An increase of *CXCR4* mRNA expression was also found by real-time PCR in the NB8x4x7 cells, as compared to NB8x4 cells (Figure 18B).

Taken together, these data suggest a mutual CXCR7 and CXCR4 expression regulation in the NB8x4x7 cell line. Of note, the CXCR4 surface expression was not altered upon ectopic expression of CXCR7 in the CXCR4-positive SHSYx7 cell line (Figure 17A). No alteration of the *CXCR4* endogenous expression was noted in those cells by semi-quantitative real-time PCR analyses (data not shown), suggesting that ectopic CXCR7 expression did not affect CXCR4 endogenous expression in SHSYx7 cells.

The CXCR7/CXCL12/CXCL11 and the CXCR4/CXCL12 axes activate ERK 1/2 cascade in NB cell lines

Extracellular regulated kinases 1 and 2 (ERK 1/2) are known key regulators of pathways involved in the control of growth and survival signals. Several studies strongly link the CXCR4/CXCL12 axis to enhanced NB growth and survival (165), and activation of the ERK 1/2 cascade in response to CXCL12 binding to CXCR4 has been previously reported in NB (137). Although it has been proposed that CXCR7 (alone) does not mediate typical chemokine receptors signals, such as calcium mobilization (61), CXCR7/CXCL12-mediated ERK1/2 activation have been detected in different models (63;64).

Therefore, to assess the functionality of (co-)transduced receptors in NB cells, we first monitored CXCL12-mediated ERK 1/2 phosphorylation in NB transduced cell lines (Figure 19).

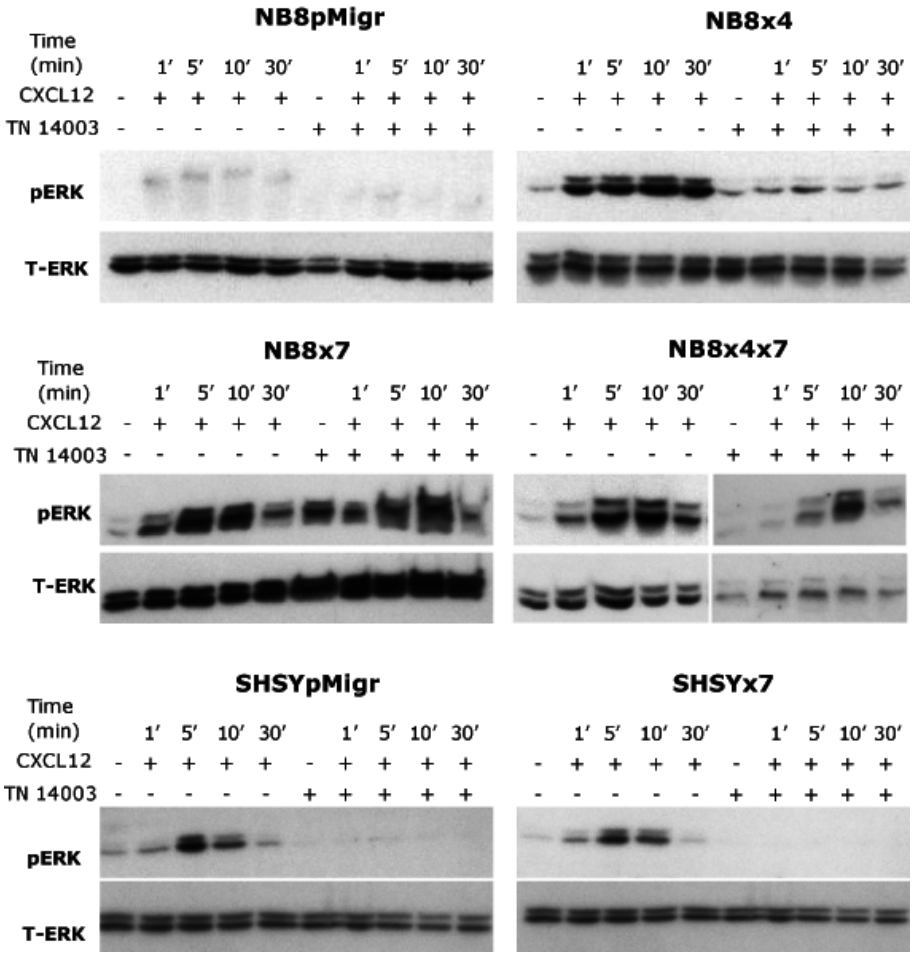


Figure 19: ERK 1/2 signaling in NB cell lines
Immunobloting of phospho-ERK (pERK) and total ERK (T-ERK) in transduced cells, treated with 100 ng/ml CXCL12, in presence or in absence of the CXCR4 blocker TN14003.

As shown in Figure 19, ERK 1/2 cascade was highly activated in all transduced cells after CXCL12 stimulation. Thus, CXCR7, as CXCR4, was able to activate downstream pathways in response to the CXCL12 ligand. Interestingly, a constant ERK 1/2 activation was maintained until 30 min after CXCL12 stimulation in the NB8x4 cells, whereas a peak of intensity from 5 to 10 min followed by a signal decrease was observed in the NB8x7 and NB8x4x7 cell lines. These data suggest that CXCR7 and CXCR4 may mediate different CXCL12-induced downstream pathways, and that CXCR7 may affect CXCR4/CXCL12-mediated signaling in NB8x4x7 cells.

In addition, ERK 1/2 activation was lost in CXCR4-expressing NB8x4 cells upon addition of the specific CXCR4 inhibitor (TN14003), confirming that this activation was specific to the CXCR4/CXCL12 axis in those cells. However, CXCL12-mediated ERK1/2

activation in NB8x4x7 cells was not completely inhibited by TN14003 treatment, suggesting that ERK1/2 activation is partially mediated through CXCR7.

In the SHSYx7 cell line, CXCR7 alone was not able to mediate ERK 1/2 activation, as no signal was detected after addition of the TN14003 inhibitor. However, the receptor slightly weakened the intensity of the CXCR4/CXCL12-mediated ERK 1/2 activation in those cells, as compared to that observed in the SHSYpMigr control cell line.

These data suggest that CXCR7, upon exposure to CXCL12, may not activate ERK 1/2 by its own in SHSYx7 cells in contrast to NB8x4x7 cells, and that the receptor differently affect the *in vitro* CXCR4/CXCL12-mediated ERK 1/2 activation in these two double receptor-positive NB cell lines.

The CXCR7 receptor is also known to bind to another chemokine ligand, CXCL11. Thus, we next addressed the functionality of the receptor by evaluating ERK 1/2 activation in CXCR7-expressing NB transduced cells in presence of CXCL11. As shown in Figure 20, CXCR7 was able to activate ERK 1/2 cascade in the NB8x7 and NB8x4x7 cell lines upon CXCL11 engagement, but not in the CXCR7-expressing SHSYx7 cells. These observations showed that the two CXCR7/CXCR4-expressing SHSYx7 and NB8x4x7 cell lines might differently respond to CXCL11, as observed above after stimulation of these cells with CXCL12.

Interestingly, the CXCL11-induced signaling by CXCR7 was sustained after 30 min of CXCL11 exposure in the NB8x7 cell line, while the signal weakened after 10 min in NB8x4x7 cells. These data suggested that CXCR4 might interfere with CXCR7 response to CXCL11.

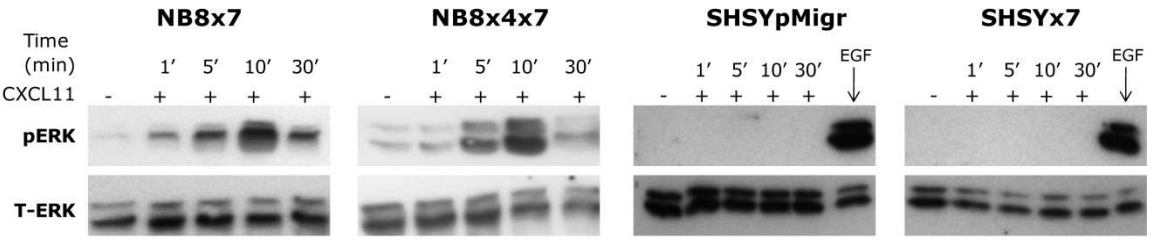


Figure 20: CXCL11 mediated ERK 1/2 activation in NB cell lines
Immunobloting of phospho-ERK (pERK) and total ERK (T-ERK) in transduced cells treated with 100 ng/ml CXCL11. Cells were treated with 50 ng/ml EGF for 5 min as positive control for ERK1/2 activation.

Importantly, CXCR7/CXCL11-mediated ERK 1/2 cascade activation was not biased by the presence of the other CXCL11 receptor, CXCR3, as no detectable mRNA expression level of this receptor was noticed in CXCR7-positive cell lines (Figure 21).

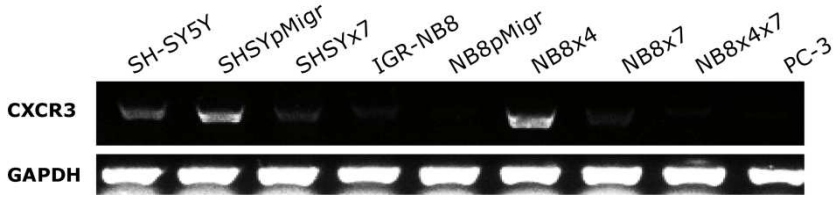


Figure 21: Expression of *CXCR3* in the CXCR7-positive cell lines
Qualitative RT-PCR analyses for *CXCR3* mRNA expression level in the NB8, and the SHSY transduced cell lines. *GAPDH* was used as gene of reference.

Akt activation is neither mediated by CXCR4 nor by CXCR7 in NB cell lines

Akt is a downstream effector of the phosphoinositide 3-kinase (PI3K)–dependent signaling cascade. Activation of Akt mediates diverse cellular functions, including cell proliferation, survival, and its signaling network is considered as a key determinant of the biological aggressiveness of tumours (166). In various models, binding of CXCL12 to its receptors CXCR4 and CXCR7 has been previously reported to activate the Akt pathway (22;63-65;165). The PI3K/Akt pathway is thus a potential candidate to transmit CXCR7/CXCL12/CXCL11 and CXCR4/CXCL12-mediated signals in NB. Consequently, we next measured the activation of Akt by phosphorylation, in NB transduced cell lines upon CXCL12 or CXCL11 exposure.

While IGF-1 induced Akt activation in all NB transduced cell lines (Figure 22A), as previously described (154), no phospho-Akt was detected upon stimulation of NB cells with CXCL12, in contrast to the positive control SW480 cell line (Figure 22B). Therefore, neither CXCR4/CXCL12 nor CXCR7/CXCL12 axes were able to signal through the Akt pathway in NB cells.

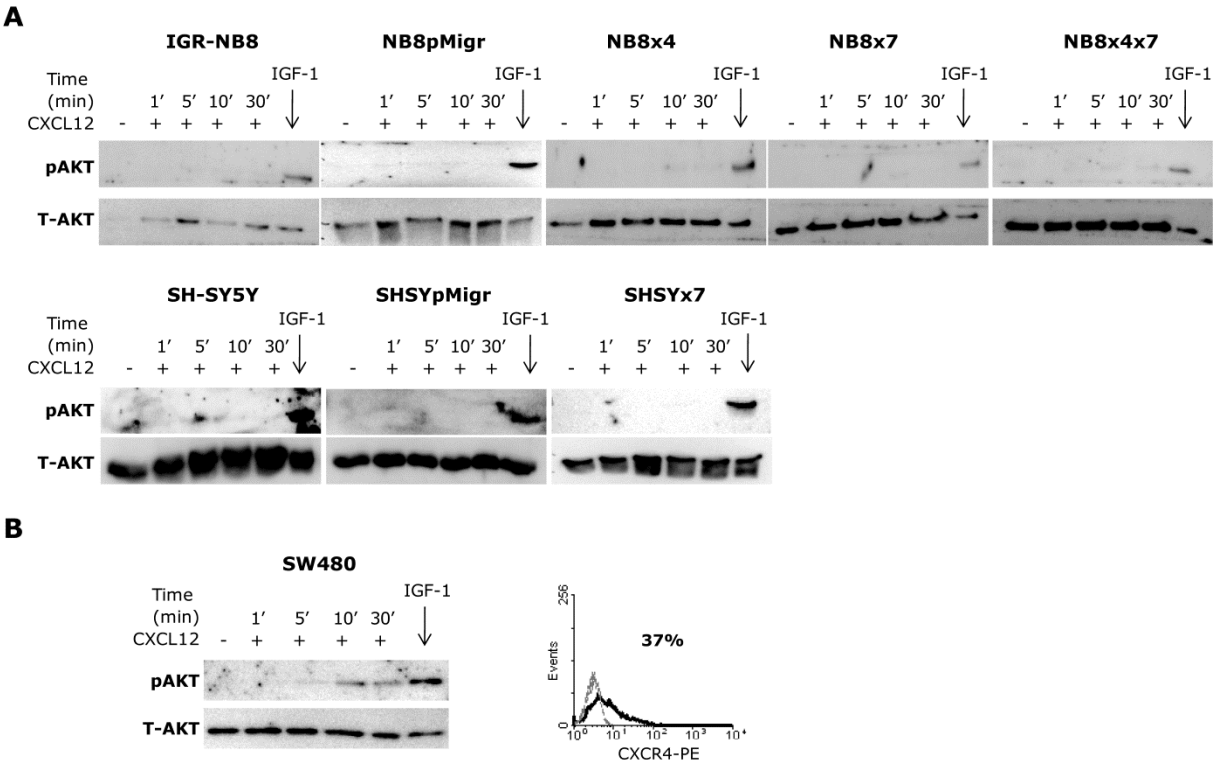


Figure 22: Akt pathway activation upon CXCL12 stimulation
(A) Immunobloting of phospho-Akt (pAKT) and total Akt (T-AKT) in transduced cells stimulated with either 100 ng/ml CXCL12 at indicated time points, or 10 ng/ml IGF-1 for 1 h. (B) Left panel: CXCR4/CXCL12-mediated Akt activation in the CXCR4-positive SW480 colon cancer cell line. Right panel: flow cytometry analyses of the CXCR4 surface expression in the SW480 cell line. Percent represents CXCR4 positive SW480 cells.

Similarly, no activation of the Akt pathway was detected in any CXCR7-positive NB cells upon CXCL11 stimulation (Figure 23A), in contrast to the positive control PC3 cell line (Figure 23B).

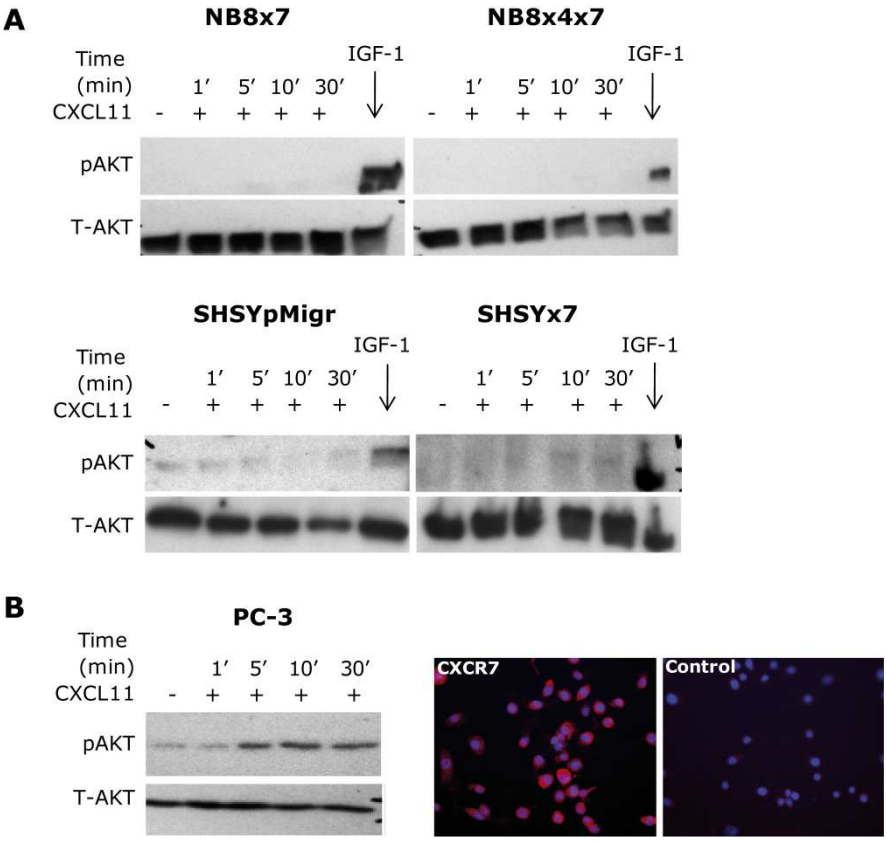


Figure 23: Akt activation unpon stimulation of NB cells with CXCL11
(A) Immunobloting of phospho-Akt (pAKT) and total Akt (T-AKT) in transduced cells treated with either 100 ng/ml CXCL11 or 10 ng/ml IGF-1. (B) CXCR7/CXCL11-mediated AKT activation in the CXCR7-expressing PC-3 prostate cancer cell line (left panel). Immunofluorescence staining for CXCR7 expression in PC-3 cells (right panel); control staining represents incubation of cells without the primary anti-CXCR7 antibody.

Impact of CXCR7 on NB growth in a two dimensional (2D) culture system

As we showed above that the CXCR7/CXCL12 and the CXCR4/CXCL12 axes potentiated ERK 1/2 activation, we next addressed the impact of CXCR7, CXCR4 and the combination of the two receptors in mediating growth of NB cells *in vitro* in absence and in presence of the chemokine ligand.

Ectopic expression of CXCR7 slightly enhanced NB8x7 cell proliferation in 2% serum, as compared to NB8pMigr control cells ($p<0.05$, Figure 24A). However, CXCR7 did not affect the growth of NB8x7 cells in 10% serum, as compared to NB8pMigr cells. In contrast, CXCR4 highly increased both NB8x4 and NB8x4x7 cell growth in 10% and 2% serum, as compared to control NB8pMigr cells ($p<0.001$). Interestingly, a slight but significant additive effect (p-value not shown) of CXCR4 and CXCR7 on NB8x4x7 cell growth was noted in either 10% or 2% serum (mean OD value at 96h of 0.41 and 0.19, respectively), as compared to NB8x4 cell

growth in those culture conditions (mean OD value at 96h of 0.36 and 0.14, respectively). Moreover, CXCL12 stimulated growth of CXCR4-expressing NB8x4 and NB8x4x7 cells ($p<0.001$), but had no impact on CXCR4-non expressing NB8x7 cell growth. Interestingly, an additive growth promoting effect of both receptors in NB8x4x7 cells was also noted in presence of the ligand. Altogether, our data suggest that the CXCR7/CXCL12 axis alone may not mediate *in vitro* NB growth, and that the CXCR7 receptor may further increase the CXCR4-mediated growth promoting effect in such 2D culture system.

In parallel, CXCR7 did not influence the CXCR7/CXCR4-expressing SHSYx7 cell growth in 10% nor in 2% serum, comparing to the mock transduced control SHSYpMigr cells (Figure 24B). Presence of the ligand CXCL12 significantly increased the proliferation of CXCR4-expressing SHSYpMigr cells as compared to stress conditions ($p<0.05$), but such effect was lost in the SHSYx7 cell line ($p<0.05$). In contrast to the growth-promoting effect observed in NB8x4x7 cells, these data reveal the existence of a competition between the CXCL12 receptors, as CXCR4/CXCL12 signaling is impaired when the two receptors are co-expressed in SHSYx7 cells.

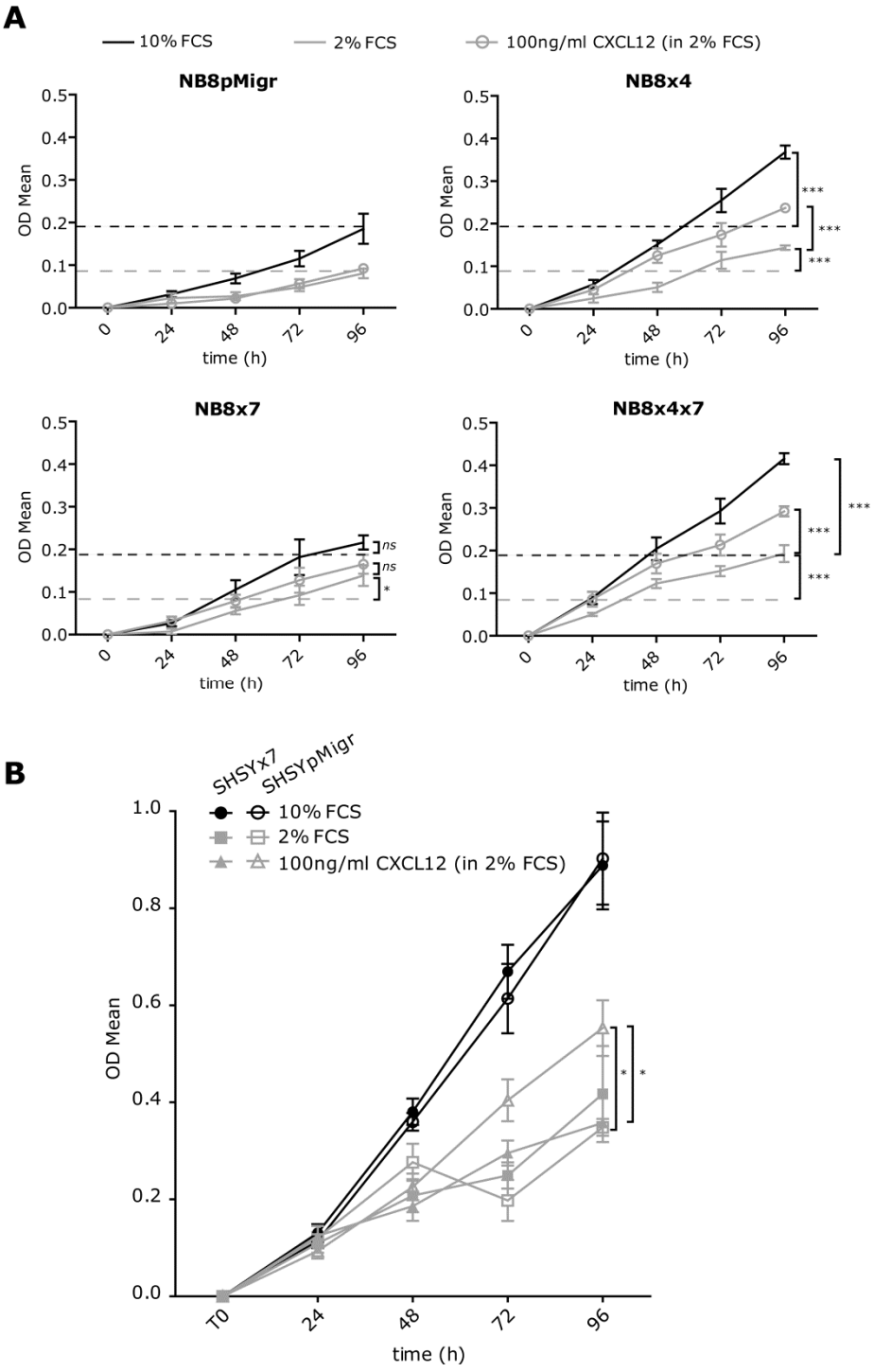


Figure 24: Impact of ectopic CXCR7 expression on NB growth *in vitro*

In vitro growth of (A) NB8 and (B) SH-SY transduced cell lines were measured by PMS/MTS cell proliferation kit in normal (10% FCS), or stress (2% FCS) culture conditions, and in presence of 100 ng/ml CXCL12 (in 2% FCS). Dotted lines represent max OD values at 96 h for NB8pMigr control cell line growth in 10% (black) and 2% (grey) serum. Graphs represent mean OD values ± SEM of at least three independent experiments. Student's t-test: *p<0.05, ***p<0.001.

As the CXCR7/CXCL11 axis was also shown to activate ERK 1/2 cascade, we also evaluated the impact of the other CXCR7 ligand, CXCL11 on CXCR7-expressing NB cell growth

using similar *in vitro* proliferation assay (data not shown). We did not observe any CXCL11 influence on either NB8x7 or NB8x4x7 cell growth, as compared to NB8pMigr or NB8x7 cell growth, respectively, nor on that of SHSYx7 cells, as compared to SHSYpMigr cells, suggesting that the CXCR7/CXCL11 axis is not able to mediate *in vitro* NB growth in 2D culture conditions.

CXCR7 alters in vitro NB growth in a three dimensional (3D) environment

Soft agar assay is an essential tool to evaluate the *in vitro* tumorigenicity of tumour cells, based on their ability to form colonies in an anchorage-independent manner. We therefore examined whether *in vitro* clonogenic properties of NB cell were influenced by exogenous CXCR7, CXCR4 or a combination of the two receptors in such 3D culture condition (Figure 25).

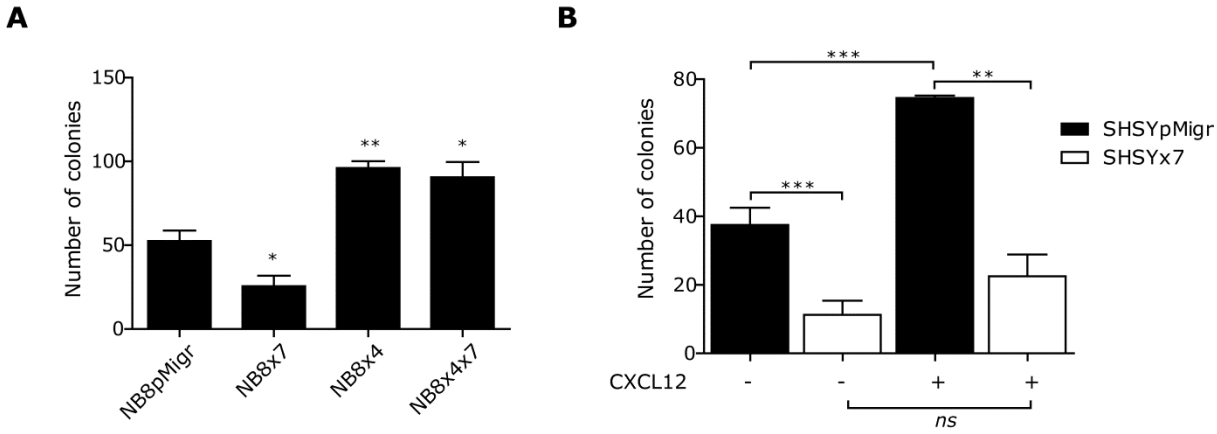


Figure 25: Impact of ectopic CXCR7 expression on NB growth in 3D culture system *in vitro*
Clonogenic growth of the (A) NB8 and the (B) SHSY transduced cell lines in soft agar assay. Columns represent mean values ± SEM of two independent experiments. Student's t-test: *p<0.05, **p<0.01, ***p<0.001.

As shown in Figure 25A, CXCR4 significantly enhanced the clonogenic ability of NB8x4 and NB8x4x7 cell lines ($p<0.01$ and $p<0.05$ respectively), in absence of the ligand, when compared to the NB8pMigr cell line. Inversely, CXCR7 expression in NB8x7 cells resulted in significantly decreased colonies number ($p<0.05$). In contrast to 2D culture system, CXCR7 was not able to influence CXCR4-mediated NB8x4x7 growth in such 3D model, as no significative effects were noted between the clonogenic abilities of NB8x4 and NB8x4x7 cells.

Interestingly, CXCR7 significantly decreased the number of colonies derived from the SHSYx7 cell line, in absence ($p<0.001$) and in presence ($p<0.01$) of CXCL12, as compared to the SHSYpMigr cell line (Figure 25B). Addition of the ligand markedly increased the clonogenic capacity of the CXCR4-positive SHSYpMigr cell line ($p<0.001$), without affecting that of SHSYx7 cells. These data thus suggest that CXCR7 may also affect the CXCR4/CXCL12-mediated SHSYx7 growth in *in vitro* 3D culture.

CXCR7 affects CXCR4-mediated NB cell migration in vitro

Chemotaxis is a major feature of aggressive tumour cells *in vitro*, and a critical indicator of NB cell metastatic dissemination potential *in vivo*. As the CXCR4/CXCL12 axis has been previously shown to mediate chemotaxis of NB cells (137), we next addressed the role of CXCR7 on NB cell migration and its impact on CXCR4/CXCL12 axis mediated chemotaxis. As illustrated in Figure 26, overexpression of both receptors did not modify the migratory capacities of transduced cells in the absence of ligand. The presence of CXCL12 enhanced the migration of both CXCR4-expressing NB8x4 ($p<0.05$, Figure 26A) and SHSYpMigr ($p<0.05$, Figure 26B) cells, while no effect was noted for the CXCR7-expressing NB8x7, NB8x4x7 and SHSYx7 cell lines. These results suggest that CXCR7 may not be involved in NB chemotaxis but may affect CXCR4/CXCL12-mediated migration.

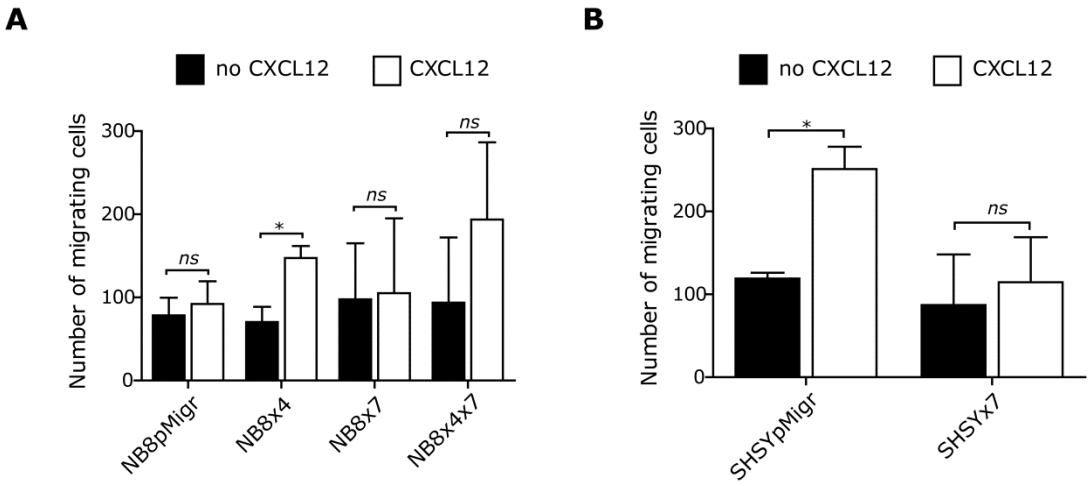


Figure 26: Effect of CXCR7 on cell migration *in vitro*
Chemotaxis of transduced (A) NB8 and (B) SHSY cells toward 100 ng/ml CXCL12. Each experiment was performed in duplicate. Columns represent mean values ± SEM of two independent experiments. Student's t-test: *$p<0.05$.

In addition, we observed that CXCR7 might not promote NB chemotaxis in presence of its other ligand CXCL11 (preliminary data, not shown in this report).

CXCR7 impairs in vivo NB growth upon subcutaneous implantation of NB cells

As the *in vivo* environment is a major player in tumour biology, we first addressed the ability of CXCR7 to regulate and/or impair the CXCR4-mediated NB growth in heterotypic conditions *in vivo*. To that purpose, NB8 transduced cell lines were subcutaneously implanted in the flanks of nude mice. Three animals per cell line were injected in both left and right flanks (n=6) (Figure 27).

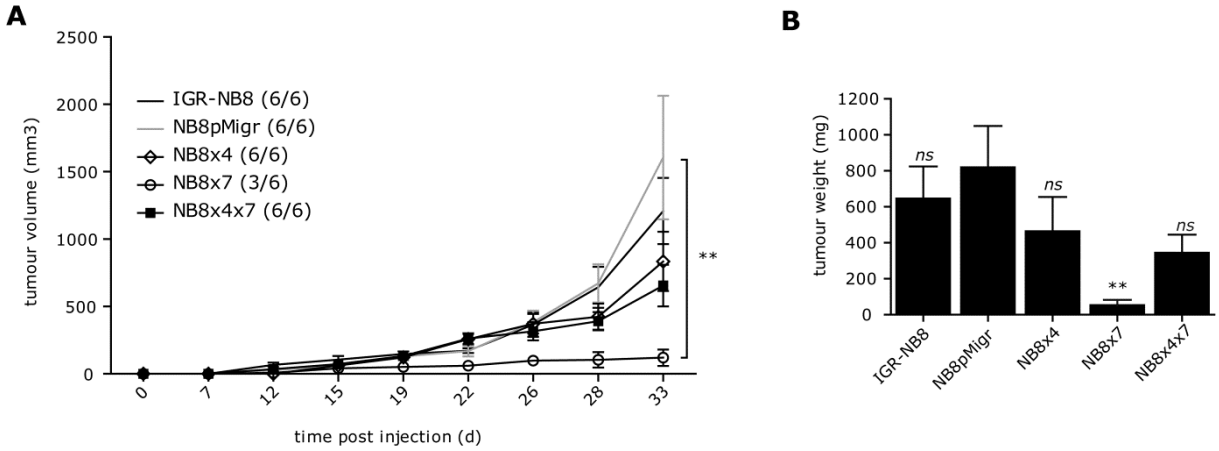


Figure 27: Effect of CXCR7 on NB growth in a model of subcutaneous injection in nude mice
(A) *In vivo* tumour take (number of sites with tumour/total sites) and growth (mean tumour volume ± SEM) after *s.c* implantation of NB cells in nude mice. Two-way ANOVA: **p<0.01. (B) *s.c.* tumour weight at sacrifice (day 33). Student's t-test : **p<0.01.

Overall tumour take was reduced in the group of mice injected with NB8x7, as only 3/6 sites presented a tumour versus 6/6 sites for the other groups. Moreover, CXCR7 ectopic expression in NB8 cells was shown to drastically reduce both the growth and the volume of NB8x7-derived tumours, as compared with tumours derived from the control cell line ($p<0.01$, Figure 27A). At day 33 after implantation, mice in the NB8pMigr, NB8x4 and NB8x4x7–implanted groups had developed tumours with a mean volume of 1605, 835 and 655 mm^3, respectively, which required sacrifice of all animals. At sacrifice, the mean volume of tumours from the group engrafted with NB8x7 was 120 mm^3.

As shown in Figure 27B, tumour weight was significantly reduced in the group of mice engrafted with the NB8x7 cells, as compared to the group implanted with the control NB8pMigr cell line ($p<0.01$). Thus, these data support CXCR7 as a critical actor of NB growth regulation *in vivo*. However, both growth and size of NB8 cell-derived tumours were not significantly affected by the presence of CXCR4 alone, nor in association with CXCR7 in such conditions.

The tumour histology and microenvironment organization was then evaluated by H/E, CD31 and Ki67 staining in paraffin-embedded sections of *s.c.* tumours (Figure 28).

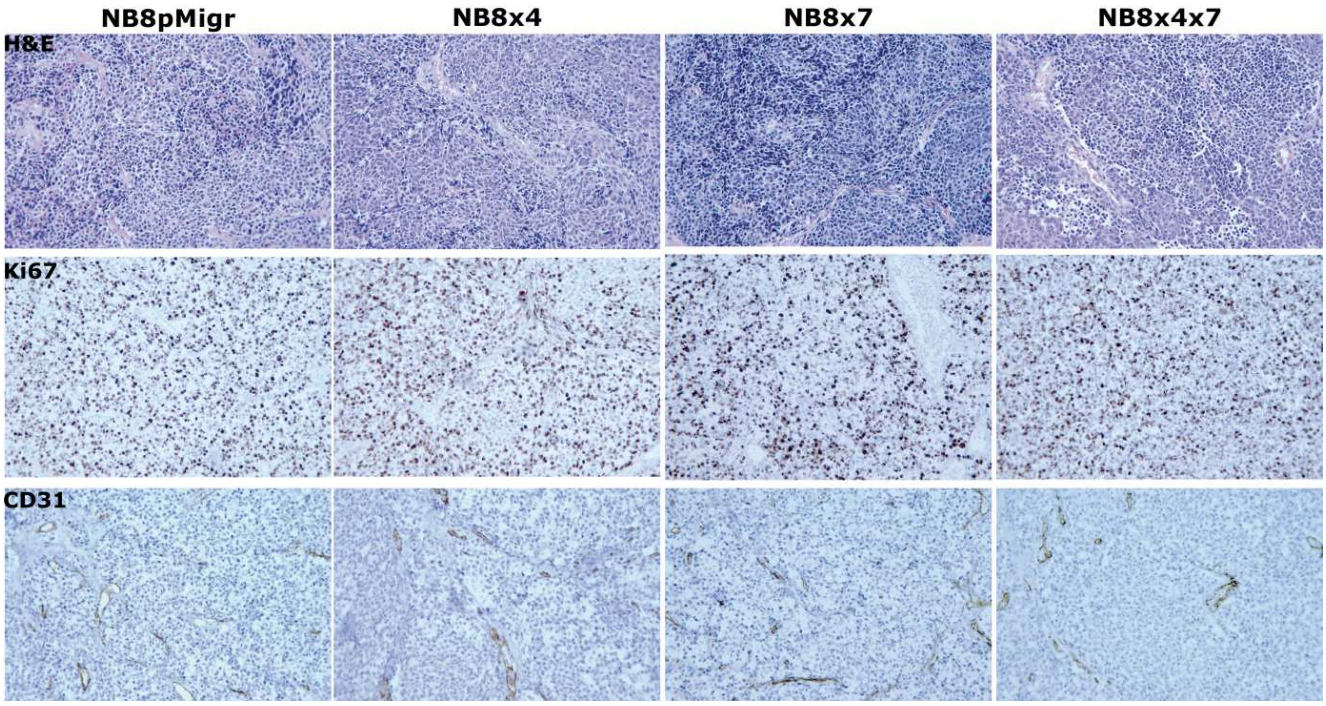


Figure 28: Histology of *s.c.* tumours in nude mice
Paraffin-embedded sections of *s.c.* tumours, derived from the NB8pMigr, NB8x4, NB8x7 and NB8x4x7 cell lines, were stained with H&E. To evaluate cell proliferation and vascular structure, tumours were stained with anti-Ki67 and anti-CD31 antibodies.

Although CXCR7 strongly reduced volume and growth of *s.c* NB8x7-derived xenografts, no particular phenotype (such as induced-differentiation or necrosis) was noted in those tumours as compared to those of the other groups. Moreover, the histology of all tumours, with undifferentiated neuroblasts (small blue round cells), presented similar pattern of vascularisation and proliferation rates.

CXCR7 anti-proliferative effect may not require the presence of CXCL12 in a heterotypic environment

To evaluate a putative functionality of the CXCL12 ligand in CXCR7-mediated effect in our heterotypic mouse model, we measured the concentration of CXCL12 in NB transduced cell lines, associated *s.c* xenografts, and in a normal mouse adrenal gland tissue. As previously demonstrated (137), high levels of CXCL12 were found in the adrenal gland (Figure 29).

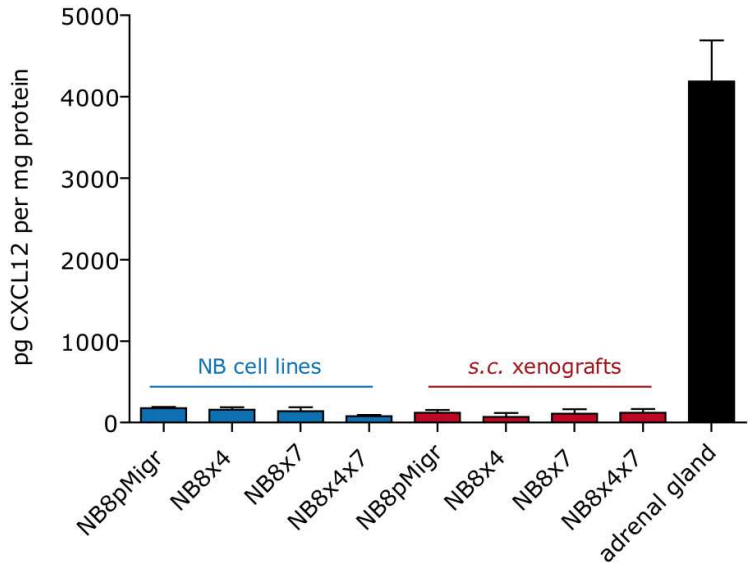


Figure 29: CXCL12 production in *s.c.* NB tumours
The production of CXCL12 was measured by ELISA in normal nude mouse adrenal gland, as well as in NB8 transduced cell lines, and derived *s.c* tumours. Results are expressed in triplicates as pg of CXCL12 per mg of extracted protein. Error bars indicate S.D. of triplicates.

However, CXCL12 production in NB cell lines and xenografts was low (mean concentration of CXCL12 < 180 pg/mg of protein) and did not vary between either cell lines or derived tumours. Thus, these data suggest that the CXCR7-mediated anti-proliferative effect, as observed in *in vivo* heterotypic conditions, is unlikely due to the presence of its ligand CXCL12. As CXCL11 production was not assessed in cell lines and *s.c.* xenografts, we cannot exclude a putative role of the second CXCR7 chemokine ligand in NB growth regulation, which should be further evaluated.

CXCR7 delays CXCR4-mediated in vivo growth in an orthotopic environment

Orthotopic animal model is a critical and essential tool to investigate the influence of tumour microenvironment in chemokine/receptor-induced proliferative and invasive NB behaviour (146). Indeed, CXCR4 was previously shown to tremendously increase growth of NB cells only by using our CXCL12-producing orthotopic NB mouse model (137). In addition, CXCR7 was shown to drastically reduce NB growth in a heterotypic mouse model (Figure 27), and to affect CXCR4/CXCL12-mediated migration in NB8x4x7 cells (Figure 26). Therefore, we further evaluated the extent to which CXCR7 would affect the *in vivo* CXCR4-mediated growth signaling in a CXCL12-producing orthotopic environment. To that purpose, the NB8x4, NB8x7 and NB8x4x7 cell lines were directly implanted in the adrenal gland of nude mice (seven animals per group), as previously described (146) (Figure 30).

Tumour take

	Week 3	*Week 5*	*Week 6*
NB8x4	2/6 (33%)	5/6 (83%)	-
NB8x7	0/7 (0%)	3/7 (43%)	3/7 (43%)
NB8x4x7	0/7 (0%)	4/7 (57%)	6/7 (86%)

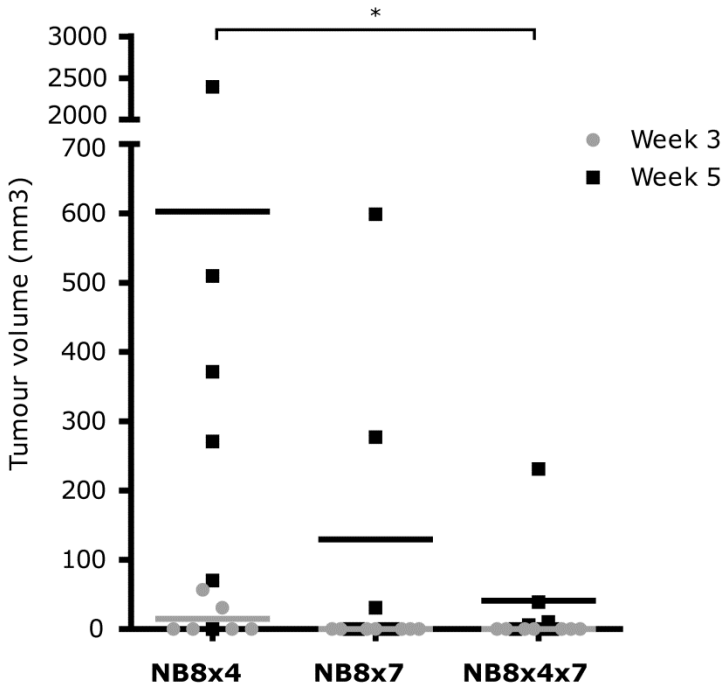


Figure 30: CXCR7 impact on *in vivo* tumour growth in a NB mouse orthotopic model
NB8 transduced cell lines were orthotopically implanted in the adrenal gland of nude mice. Tumour growth was followed by ultrasound every 10 days. Upper panel: tumour take represented as fraction and percentage of tumour-bearing mice at week 3, 5 and 6. Lower panel: kinetics of tumour volume for each mouse at week 3 and 5. Bar: mean tumour volume. Mann-Whitney test: *p<0.05.

One animal of the NB8x4 group died within 24 h after the procedure, due to perioperative complications. All mice were examined by echography at week 3, 5 and 6 for tumour growth measurement. At week 3, two out of six animals (33%) in the group engrafted with NB8x4 cells developed a tumour at the site of injection, whereas no tumours were detected in the group of mice engrafted with NB8x7 and NB8x4x7 cells. However, at week 5, 43% and 57% of animals engrafted with NB8x7 and NB8x4x7 cells, respectively, presented tumours as compared to the 83% of mice of the NB8x4 group.

Tumours in each group presented a high heterogeneity in their growth behaviour, especially in the group implanted with NB8x4 and NB8x7 cells. Indeed, NB8x4–implanted mice had developed tumours with a volume between 77 and 2400 mm^3, while tumours with a volume between 30 and 605 mm^3 were detected in the group of NB8x7.

Interestingly, the growth of NB8x4x7 cell-derived tumours was significantly reduced as compared to that of the NB8x4 cell-derived tumour ($p<0.05$), suggesting that CXCR7 might alter *in vivo* CXCR4-mediated growth promoting effect. Due to excessive volume of tumours in the NB8x4 group, mice had to be sacrificed at earlier time point (week 5) compared to those injected with NB8x7 and NB8x4x7 cell lines (week 6). At week 6, 3/7 mice of the group engrafted with NB8x7 presented a tumour with a mean volume of 353 mm^3, while 6/7 mice of the group engrafted with NB8x4x7 harboured a tumour with a mean volume 607 mm^3. No macroscopic metastases were observed after tumour resection in each group.

Thus, in contrast with CXCR4, CXCR7 may decrease the ability of NB cells to seed and to proliferate in the adrenal gland. Indeed, CXCR7, in association with CXCR4, delayed the proliferative CXCR4-mediated effect in orthotopic conditions. Therefore, present observations support a functional implication of the global CXCR7/CXCR4/CXCL12 axis in the NB8x4x7 cell line, and suggest a critical role for CXCR7 in regulating the CXCR4-mediated NB growth in a CXCL12-producing orthotopic environment. Importantly, CXCR7 co-expression with CXCR4 did not further induce NB dissemination.

Discussion

Background of the study

The CXCR4/CXCL12 axis has been largely shown to participate in tumour development and progression. Indeed, reports have proposed a predominant role of the CXCR4 receptor and its ligand CXCL12 in mediating invasion of malignant tumour cells to sites of metastasis in several cancer models (31). In NB, CXCR4 receptor expression was shown to correlate with poor prognosis (136;137;157). Nonetheless, the specific involvement of the CXCR4/CXCL12 axis in cell proliferation, survival, and invasion is still under debate. Even though the CXCR4 receptor has been previously demonstrated as an essential mediator of aggressive NB cell dissemination to CXCL12-producing bone marrow (138), contradicting reports have been published on the relative contribution of the CXCR4/CXCL12 axis in NB growth and survival (137;142-145).

The identification of CXCR7, as a second receptor for CXCL12, has added considerable complexity and also new perspectives for the implication of the CXCR4/CXCL12 axis in tumour biology. Although several hypotheses on the role of CXCR7 and possible interactions between the two CXCL12 receptors have been proposed in different tumour systems (22), the implication of the CXCL12/CXCR7 axis and its impact on CXCR4 functionality in NB is still unknown.

Distinct expression patterns for the CXCR7 and CXCR4 receptors in NB tumours

The pattern of CXCR4 expression in many tumour types, including NB, has been already shown to be related to advanced stage disease (136;137). In the present study, we reported the pattern of CXCR7/CXCL12 expression in NB, by using an array of different clinical stage, histological type of tumours and control tissues (Table 1). Our TMA analyses revealed a weak CXCR7 expression in NB (Figure 5). Moreover, in contrast to breast, lung and hepatocellular carcinomas (33;167), CXCR7 was not expressed in NB vasculature but rather preferentially associated to its neural compartment. Furthermore, we observed a strong CXCR7 expression in tumour ganglion cells in 77% of GGNB and in all GGN tissues, while 75% of UnNBs presented a poor neuroblast-associated CXCR7 staining. Thus, our data reveal that

CXCR7 expression is specifically associated to mature neural cells in stroma-rich GGNB and GGN tumours.

The presence of neuroblastic maturation toward ganglion cells and a high proportion of Schwannian stroma are recognized as a favourable histological feature and good prognosis (152). The CXCR7 receptor expression significantly increased in tumours from less than 1 year-old patients, whom are known to present tumours with the potential to regress spontaneously, or to mature into benign differentiated tumours, such as GGNs (85). Despite these observations, the TMA analyses did not allow to assign CXCR7 a statistically significant favourable prognosis value. Indeed, no correlations between MYCN status, overall or event-free survivals, and CXCR7 expression were noted (Figure 6). The absence of significance may be due to the low levels of CXCR7 expression, as well as a limited panel of matured tumour tissues included in the TMA. On the other hand, CXCR7 intensity staining associated to tumour ganglion cells in differentiated tumours was higher compared to neuroblast-associated staining in undifferentiated NBs. However, as assessing positive percentage of cells is more precise than quantifying associated immunostaining intensity in TMA analyses, percent of CXCR7 positive cells rather than CXCR7 staining intensity was evaluated in this study. Although this method is more relevant, it might explain as well the lack of significance in investigating the putative CXCR7 prognostic value.

As previous studies have reported that the CXCR4 receptor expression is associated to undifferentiated and high stage NB tumours (136;137), our data revealed an inversely correlated expression pattern for the CXCR4 and the CXCR7 receptors.

Interestingly, the TMA analyses also revealed a strong CXCL12 expression in endothelial and stromal cells in NB tumours (Figure 7). As both CXCR7 and CXCR4 receptors are expressed in tumour neural cells, these data suggest a paracrine role of the chemokine ligand in NB. Moreover, CXCL12 expression levels in tumour endothelial cells slightly increased in patients over one year-old, whom are known to present most aggressive NBs as compared to infants (85). Although endothelial-associated CXCL12 expression did not vary in the different NB clinical stages (Table 3), our data suggest an implication of the ligand in NB aggressive behaviour. In particular, a putative implication of the ligand in NB angiogenesis is likely and should be further investigated, as already reported in the context of ovarian and colon cancers (168;169). In addition, neural-associated CXCL12 expression, albeit low

compared to ligand expression levels in the endothelial and stromal compartments, enhanced in metastatic samples as compared to primary NB tumours (PTs), and in PTs as compared to control tissues (Table 2). Although neural CXCL12 expression in tumour cells was found independent of NB stages, we cannot exclude a putative autocrine function of the ligand in NB progression, which should be further evaluated.

Taken together, TMA analyses suggest a complex contribution of the two CXCR7 and CXCR4 receptors in NB pathogenesis, which may be tightly modulated by a permanent cross-talk with their common ligand CXCL12, highly produced by tumour microenvironment.

Differential expression of the two CXCL12 receptors in NB cell lines

To further corroborate our TMA analyses, we next assessed the expression pattern of both CXCL12 receptors in a panel of well-characterized N-, I- and S-type NB cell lines (90). RT-PCR analyses revealed that *CXCR7* expression was detected in 6/14 NB cell lines (Figure 8). Moreover, CXCR7 cell surface expression was very weak in most NB cell lines (Figure 10). These results supported our TMA analyses showing that some of undifferentiated tumours (UnNBs) poorly expressed CXCR7.

In contrast, *CXCR4* expression was detected in almost all NB cell lines, and heterogeneous CXCR4 surface expression levels were observed in selected RT-PCR-positive NB cells, as already detailed elsewhere (157). Altogether, these observations showed a low and more discrete CXCR7 expression in NB cell lines, as compared to CXCR4.

Furthermore, CXCR7 was detected in N-type and S-type NB cell lines, rather than in the most undifferentiated I-type cells. Although most NB cell lines originate from the more aggressive clones *in vitro* (especially N-type cell lines), our data suggest an association of CXCR7 with neuronal and glial/schwannian lineage specific differentiation *in vitro*.

CXCR7 association with NB differentiation

Interestingly, a link between CXCR7 expression and cell maturation/differentiation has already been reported in immune cells. Indeed, CXCR7 expression was proposed to correlate with dendritic cell maturation, and described as a potential maker of

differentiating memory B cells (73). In the context of cancer, CXCR7 expression has been shown to drastically increase in FCS-induced differentiation of glioma cells *in vitro* (76).

Together with the particular CXCR7 expression pattern observed on mature tumour cells, our data suggest an association of CXCR7 with NB differentiation. To further evaluate the putative implication of the CXCR7 receptor in NB differentiation, we quantified its expression upon RA and BrdU-induced differentiation of the IGR-NB8 and SK-N-Be(2c) cell lines *in vitro*. In agreement with a previous report, treated NB cells elicited morphological changes, and global reduced proliferation (without enhanced apoptosis) starting from the third day after exposure to both RA and BrdU treatments (158) (Figures 11-12). Characteristics of differentiating NB cell profile was further confirmed by assessing the expression pattern of neurone specific enolase (NSE) and vimentin, two early neuronal and glial markers, respectively (159;163). As already noted elsewhere (158), dysregulation of both marker expression was observed later than the occurrence of morphological changes, which was however fully acquired at day 30 of treatment (Figure 13). Together, these observations further confirm the potential of IGR-NB8 and SK-N-Be(2c) cell lines to differentiate toward different lineage *in vitro*. A weak induced *CXCR7* expression was observed by quantitative real-time PCR analyses in NB cells exposed to RA, but not to BrdU, suggesting that CXCR7 is associated with neuronal rather than glial differentiation (Figure 14). These data thus correlated our TMA analyses showing that tumour ganglion cells, rather than schwannian stroma, specifically expressed CXCR7 (Figure 5).

However, CXCR7 could be neither detected at the surface, nor in the intra-cellular space of NB cells during all the differentiation induction experiment. In addition, treatment with both RA and BrdU did not further enhance CXCR7 expression at the surface of the CXCR7-positive IMR32 cell line (data not shown). These observations suggest that the receptor expression may be modulated by potential post-translational modifications, or that the putative induced protein expression is too low to be detected by the antibody used in this study.

In addition, CXCR7 expression was particularly found in mature neural cells, such as ganglion cells, in GGNBs and in GGNs (Figure 5). As we drove immature NB cell line subtypes to early neuronal fate, we cannot exclude a later induction of CXCR7 expression during final stages of NB differentiation.

Our TMA analyses revealed an enhanced CXCL12 expression in the stroma of differentiated tumours, as compared to undifferentiated NBs (Figure 7). Consequently, we asked whether the presence of the ligand could have an impact on *CXCR7* expression during *in vitro* differentiation. Even in presence of CXCL12, no further increased of the receptor expression was noted in RA/BrdU-treated NB cells (Figure 15). The potential role of CXCL12 in NB differentiation in the early steps of NB differentiation may then require further investigation.

M.J. Bissell and others have proposed that « three-dimensional (3D) context may produce distinct cellular morphology and signaling events, as compared with a rigid 2D culture system, which may thus result in important changes on the fate of the culturing cells », especially in differentiation processes (170-172). Moreover, it has been proposed that a 3D environment may play a pivotal role in regulating gene expression (173). Importantly, a recent study reported that the expression of *CXCR4* in human mesenchymal stromal cells was tightly linked to the cell culture context, as a markedly difference in the receptor expression was found between the 3D and monolayer cultures (174). Therefore, the lack of induced CXCR7 expression in RA/BrdU-treated NB cells may also results from an inappropriate culture environment. Additional experiment in 3D culture conditions, such as in soft agar, might be considered to further evaluate the putative association of CXCR7 in NB differentiation.

Interestingly, exogenous CXCR7 did not induce differentiation of slow proliferating-tumours in our heterotypic mouse model, as no ganglion-like cells and no differentiating neuroblasts were detected in *s.c.* tumours derived from CXCR7-overexpressing NB8x7 cells (Figure 28). These observations suggest that CXCR7 may not directly induce NB differentiation or maturation by its own. However, additional *in vivo* experiment should be performed using NB cell lines expressing endogenous CXCR7 expression levels, to further corroborate these observations.

In conclusion, further investigation will be necessary to determine whether the CXCR7 receptor (or the CXCR7/CXCL12 axis) is associated to, or sufficient to induce NB cell

differentiation/maturation, and whether it might represent a neural and/or differentiation marker for NB.

Individual role of the CXCR7 receptor in NB

Our data showed distinct expression pattern for the CXCR7 and the CXCR4 receptors in NB tissues and cell lines, suggesting distinct and probably opposed roles for the two CXCL12 receptors in NB aggressive behaviour. Thus, we examined in an initial step the function of CXCR7 compared to CXCR4 in NB by overexpressing either CXCR7 or CXCR4 receptor in the CXCR4/CXCR7-negative IGR-NB8 cell line (the NB8x7 and NB8x4 cell lines, respectively) (Figures 17-18).

CXCR7 and CXCR4 signaling cascade

We first assessed the ability of each chemokine receptor to activate ERK 1/2 cascade in the NB transduced cell lines (Figures 19-20). We showed that CXCR7 was able to induce downstream signaling pathway on its own, as the ERK 1/2 cascade was activated in the CXCR7-expressing NB8x7 cell line upon either CXCL12 or CXCL11 stimulation. In NB8x4 cells, a CXCR4/CXCL12-mediated ERK 1/2 pathway activation was also highly detected, as previously demonstrated (137).

It has been shown that activation of Akt mediates diverse cellular functions, including cell proliferation, survival, and its signaling network is considered as a key determinant of the biological aggressiveness of tumours (166). Activation of Akt has been reported in NB upon stimulation of NB cells with insulin growth factor 1 (IGF-1) (154). Here, neither the CXCR4/CXCL12 nor the CXCR7/CXCL12/CXCL11 axes were able to signal through the Akt pathway in NB8x7 and NB8x4 cells, respectively (Figures 22-23).

Cell signaling is a complex network, which in many systems turns out to be cell type-dependent. It has been shown that activation of CXCL12–induced Akt and ERK 1/2 cascades in HeLa cells were independent and arised from different signaling pathways (175). In addition, other results obtained with human glioblastoma and glioma cells showed that ERK 1/2 activation did not require that of the PI3K/Akt pathway (76;176). Consequently, the lack of Akt activation upon CXCL12 or CXCL11 binding to CXCR7 (or CXCR4) in NB may be a feature of NB cell lines.

Ligand-dependent role for the CXCR7 receptor in NB growth and chemotaxis

As CXCR7/CXCL12 and CXCR7/CXCL11 axes were able to activate ERK1/2 pathway, we examined whether CXCR7 would elicit particular functions in response to its two ligands, especially in NB growth and migration processes.

We showed that the presence of either CXCL12 (Figure 24) or CXCL11 (data not shown) did not influence CXCR7-expressing NB8x7 cell proliferation in 2D culture conditions. In contrast, CXCL12 highly increased growth of NB8x4 cells, as previously noted (137). Our data were further supported by a recent study showing that proliferation of CXCR7-positive glioma cells was not affected by CXCL12 (76). Similarly, the CXCR7/CXCL11 axis, albeit functional, was not shown to be implicated in rhabdomyosarcoma and glioblastoma cell proliferation *in vitro*, as detailed elsewhere (177).

In addition, we observed that CXCR7 ectopic expression did not favour migration of NB cells toward CXCL12, in contrast to CXCR4 (137) (Figure 26). Inversely, a very recent study reported that CXCR7 enhanced chemotaxis of CXCR7-expressing NB cells in a conditioned medium obtained from CXCL12-producing mesenchymal stromal cells (147). However, it was not clear whether additional factors released by stromal cells were required for truly activating CXCR7/CXCL12-mediated NB chemotaxis. Our mouse orthotopic model is a powerful tool to examine specific NB organ-specific dissemination, upon engraftment of malignant NB cells into CXCL12-producing mouse adrenal gland (137;146). Using such model, we did not observe any macroscopic metastases upon engraftment of NB8x7 cells. Thus, our data suggest that the CXCR7/CXCL12 axis may not promote NB cell migration *in vivo*, even in presence of specific stromal factors.

In parallel, CXCR7 might not promote NB chemotaxis in presence of its other ligand CXCL11 (preliminary data, not shown in this report). Although under current investigation, these preliminary data are nonetheless consistent with a recent study proposing that the CXCR7/CXCL11 axis may not directly favour chemotaxis in various tumour cell lines (177).

Altogether, our analyses suggest that the CXCR7/CXCL12 and the CXCR7/CXCL11 axes do not mediate NB growth in 2D culture, or chemotaxis *in vitro*.

Opposite ligand-independent functions for the CXCR7 and CXCR4 receptors in NB

Interestingly, our data also showed a functional role of the CXCR7 receptor, independently of its ligands CXCL12 and CXCL11. Indeed, as shown by our 2D culture system assay, CXCR7 slightly enhanced CXCR7-expressing NB8x7 cell growth in stress condition (2% serum), in absence of its ligands (Figure 24). This observation is consistent with previous reports showing a growth promoting effect of CXCR7 independently of its ligand in breast and lung tumour cell lines (22;53). Surprisingly, CXCR7 alone was shown to significantly reduce clonogenicity of NB cells in a 3D culture system (Figure 25). Thus, different effects were noted in 2D and in 3D, further highlighting the critical impact of the culture system in investigating chemokine receptor function. Supporting *in vitro* 3D analyses, CXCR7 drastically reduced both kinetics and volume of CXCR7-expressing NB8x7 cell-derived tumours, in our subcutaneous model of injection in nude mice (Figure 27). Such effect was likely independent of CXCL12, as the ligand was only poorly produced by *s.c* xenograft (Figure 29). A ligand-independent proliferative role for CXCR7 has been recently demonstrated in a prostate cancer model (178). Although CXCR7 rather reduced NB cell proliferation in our model, *s.c* NB8x7 cell-derived tumours presented an undifferentiated phenotype, with proliferation index and vascularisation rate similar to those of NB8pMigr/NB8x4 cell-derived xenografts (Figure 28). Thus, we cannot totally exclude the possibility that the NB8x7-implanted mice would have later developed tumours similar to the other groups, what would have suggested a role for CXCR7 in regulating tumour take rather than overall tumour growth.

In contrast to CXCR7, CXCR4 significantly increased *in vitro* proliferation/survival in absence of its ligand, as previously detailed (137). In 3D, CXCR4 was also shown to enhance clonogenic abilities of CXCR4-expressing NB cells in absence of CXCL12. Supporting our *in vitro* observations, CXCR4 has been proposed elsewhere to regulate *in vivo* growth of colon cancer cells independently of CXCL12 (74). However, we did not observe a particular proliferative-promoting role for CXCR4, upon *s.c.* engraftment (non producing CXCL12 environment) of NB cells (Figure 27), further supporting our previous *in vivo* study showing a critical and exclusive NB growth promoting role for CXCR4 in a CXCL12-producing and orthotopic environment (137).

Distinct roles for the two CXCL12 receptors in NB

Taken together, our data suggest that CXCR4 and CXCR7 may display two distinct roles in NB, either in presence and in absence of their common ligand CXCL12. As shown in Figure 31, CXCR7 decreases NB growth and chemotaxis, in contrast to CXCR4. In addition, CXCR4 has been linked with NB aggressive behaviour, while CXCR7 may be associated to NB differentiation.

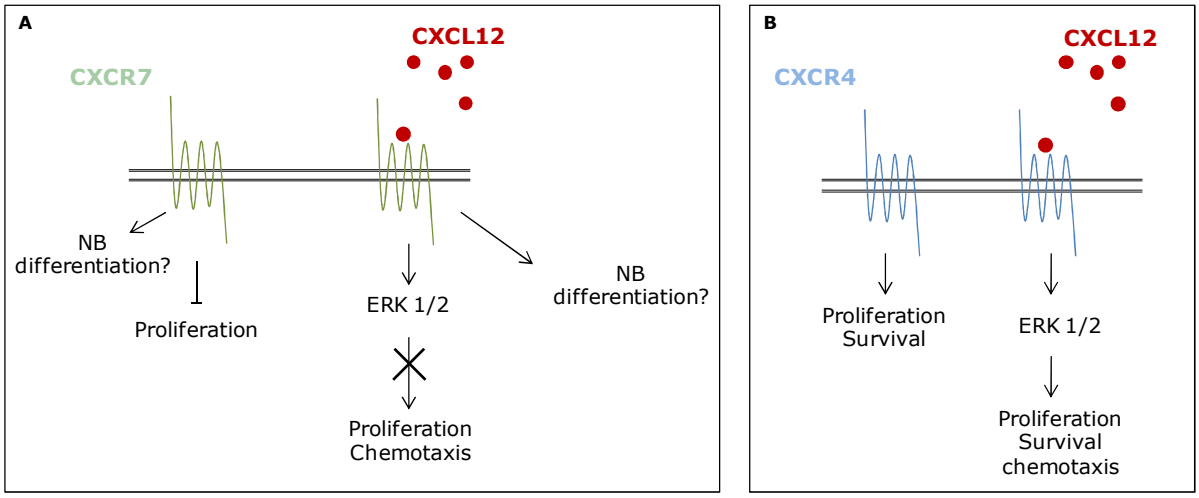


Figure 31: Putative roles for the CXCR7 and CXCR4 receptors in NB
(A) In contrast to other cancer models, CXCR7 displays anti-tumorigenic properties in NB and may be implicated in NB differentiation. (B) CXCR4 promotes NB proliferation, survival in presence and in absence of CXCL12, and is required for CXCL12-induced NB chemotaxis.

Impact of CXCR7 on CXCR4/CXCL12-mediated signaling in NB

We further examined the function of CXCR7 by focusing more precisely on its impact on CXCR4-mediated signaling. To that extent, we co-expressed CXCR7 and CXCR4 in the CXCR4/CXCR7-negative IGR-NB8 cell line (NB8x4x7 cells). As a second model for our study, CXCR7 was also ectopically expressed in the SH-SY5Y cell line (SHSYx7 cells), which expresses already high endogeneous levels of CXCR4 (Figures 17-18).

CXCR7, in association with CXCR4, affects ERK 1/2 cascade activation

Both receptors were functional in NB8x4x7 cells, as specific CXCR4/CXCL12-, CXCR7/CXCL12 and CXCR7/CXCL11-mediated ERK 1/2 activations were reported in those cells (Figures 19-20). Interestingly, we observed a shorter signal with an enhanced intensity for the CXCR4/CXCL12-mediated ERK 1/2 activation in the NB8x4x7 cell line, when compared

to the signal measured in the CXCR4-expressing NB8x4 cell line. Similar observation was also noted for the CXCR7/CXCL11-mediated ERK 1/2 activation in NB8x4x7 cells as compared to NB8x7 cells. Thus, our data highly suggest that, in association in NB8x4x7 cells, the CXCR7 and CXCR4 receptors may display reciprocal modulation of the ERK1/2 pathway in response to their ligands.

In contrast, only the CXCR4/CXCL12 axis was able to signal through ERK 1/2 pathway in the SHSYx7 cell line. Indeed, CXCR7 alone could not activate ERK 1/2 cascade in those cells, either in presence of CXCL12 or CXCL11. However, SHSYx7 cells displayed a particular and functional behaviour *in vitro*, as compared to control SHSYpMigr cells (Figures 24-26), supporting that the CXCR7 receptor is functional in those cells but may signal through other downstream signaling cascade. In addition, a modulation of the CXCL12-induced ERK 1/2 activation was observed in the CXCR7/CXCR4-expressing SHSYx7 cell line, as compared to that of CXCR4-expressing SHSYpMigr cell line. Such modulation suggests that CXCR7 may also affect CXCR4/CXCL12-mediated signaling in those cells. However, the impact of CXCR7 was different to that observed in NB8x4x7 cells, as ERK 1/2 activation was only slightly weakened in SHSYx7 cells.

Although both NB8x4x7 and SHSYx7 cell lines expressed similar CXCR4 expression levels, discrepancy in ERK 1/2 activation between those cell lines might result from the endogenous CXCR4 expression in SHSYx7 cells, as compared to the exogenous CXCR4 levels in NB8x4x7 cells. In addition, it could be due to a different pattern of CXCR7 expression in those cells, as higher CXCR7 expression levels were observed in NB8x4x7 cells as compared to SHSYx7 cells (Figures 17-18).

CXCR7 affects CXCR4/CXCL12-mediated growth promoting effects in vitro

A significant reduction of the CXCR4-mediated NB cell proliferation was observed in 2D culture, in the CXCR7/CXCR4-expressing SHSYx7 cell line, only in the presence of the ligand (Figure 24). A decrease of CXCR4-mediated clonogenicity was also observed in SHSYx7 cells in a 3D soft agar assay (Figure 25). However, such alteration was observed in presence and in absence of CXCL12. Thus, the regulation of CXCR4-mediated growth promoting effect by CXCR7 might not necessarily require the presence of CXCL12 *in vitro.*

Interestingly, the ectopic expression of CXCR7 further enhanced CXCR4–mediated growth promoting effect in the NB8x4x7 cell line, in presence and in absence of CXCL12, as

compared to the NB8x4 cell line in our 2D culture system. However, such additive growth-promoting effect was not observed in our 3D culture model (Figure 25) nor *in vivo* (Figure 27), suggesting that such 2D culture system may affect chemokine receptor function(s), as already mentioned in this report. Importantly, and in contrast to the SHSY transduced cell model, 3D soft agar assay revealed that CXCR7 was not sufficient to affect CXCR4-mediated growth of NB8x4x7 cells in absence of the ligand. An increase of CXCR4 expression together with a decrease of that of CXCR7 was quantified in the NB8x4x7 cells, as compared to NB8x4 and NB8x7 cells, respectively (Figures 17-18). Therefore, these observations suggest a predominant role of the CXCR4 receptor over that of CXCR7 in absence of CXCL12 in the NB8x4x7 cells, which would explain why NB8x4x7 cells behave as similarly as NB8x4 cells *in vitro*.

CXCR7 impairs CXCR4/CXCL12-mediated NB chemotaxis

As the role of CXCR4 in enhancing motility of NB cells has been previously described (137), we asked whether CXCR7 could also regulate CXCR4-mediated NB function *in vitro*, such as chemotaxis, in presence of CXCL12. Indeed, CXCR7 significantly altered the CXCR4-mediated chemotaxis of both CXCR7/CXCR4-expressing NB8x4x7 and SHSYx7 cell lines toward CXCL12, further suggesting the existence of a putative competition between the two receptors for their common ligand (Figure 26).

CXCR7 delays CXCR4/CXCL12-mediated growth promoting effects in vivo

Using our mouse orthotopic model, we also showed that CXCR7 delayed tumour take and growth of NB8x4x7-derived tumours, as compared to NB8x4-derived tumours (Figure 30). Our TMA analyses revealed a high ligand expression in the vascular and stromal structures of control tissues, such as the normal adrenal gland of patients (Figure 7). Similarly, strong CXCL12 expression was detected in the adrenal gland of nude mice (Figure 29), as previously described (137). Thus, these data suggest that a regulation of the CXCR4-mediated growth promoting effect by CXCR7 in NB8 transduced cell model may occur and may be dependent on the presence of CXCL12 *in vivo*.

Many studies agree that signals from the tumour microenvironment may play pivotal contributions to cancer progression (8;16;17). For example, hypoxia-induced pathways have been reported to enhance the function of the CXCR4 receptor in lung and breast tumours, as

well as in hematopoietic malignancies, by up-regulating its expression (50). In hepato-and rhabdomyo-sarcomas models, a dysregulation of CXCR7 expression was also induced in hypoxic conditions (77;167). Moreover, cytokines (such as IL-17, IL-1β and IFN-γ) have been shown to dysregulate CXCR7 expression in brain endothelial cells, and to influence receptor function *in vivo* (179). In contrast to orthotopic conditions, no CXCR7-mediated regulation was observed when the NB8x4 and the NB8x4x7 cell lines were subcutaneously engrafted in mouse flanks (Figure 27). Thus, regulation of CXCR4 signaling by CXCR7 may be tightly dependent on the presence of CXCL12 and additional stromal factors. As we also showed a critical and exclusive NB growth promoting role for CXCR4 in a CXCL12-producing and orthotopic environment (137), these remarks suggest a complex role for CXCR7 and CXCR4 in NB tumorigenesis, in which receptor expressions and associated function(s), may be influenced by stromal stimuli other than the ligand.

Finally, CXCR7 in association with CXCR4 did not enhance the invasive potential of NB8x4x7 cells compared to NB8x4 cells *in vivo*, as no metastases were detected in our orthotopic mouse model. In contrast to other tumour models (75;77), cooperation of the two CXCL12 receptors in enhancing NB dissemination is then unlikely.

Two hypothetic models for the negative regulation of the CXCR4/CXCL12 axis-mediated signaling by CXCR7 in NB

In this, study, we showed that CXCR7 was sufficient to affect CXCR4-mediated signaling and functions, especially in presence of CXCL12. Two models have been proposed in the literature and may uncover mechanisms implicated in the regulation of CXCR4 signaling by CXCR7 in NB.

CXCR7 as a ligand scavenger in NB?

It has recently been hypothesized that CXCR7 may not promote classical G protein-coupled receptor (GPCR)-mediated functions in response to its ligands (72;180). Indeed, some « altered » GPCR responsiveness can occur as a result of the sequestration/internalization of the receptor, that « enables either its recycling to a responsive form or facilitates receptor loss by lysosomal degradation » (66). In particular,

Luker *et al* proposed that a balance between constitutive internalization and recycling of the receptor CXCR7 to the cell surface occurred after binding to CXCL12, in a dose-dependent manner (68). In this context, CXCR7 was shown to control CXCL12 distribution by sequestrating the ligand present in the extracellular space. Such scavenger function for CXCR7 resulted in limiting acute CXCR4/CXCL12-mediated signaling (68;69). For instance, recent studies showed a critical role for CXCR7, as a ligand scavenger, in controlling cell migration in a zebrafish model (69-71). Moreover, the scavenging role of CXCR7 has been clearly demonstrated to regulate migratory advantage provided by CXCR4 in CXCR4/CXCR7-expressing primary T cells (180). As CXCR7 was shown to affect CXCR4-mediated chemotaxis of NB8x4x7 and SHSYx7 cells (Figure 26), a role for CXCR7 as a CXCL12 scavenger is likely in those cells. In addition, CXCR7 was proposed to delay CXCR4-mediated NB8x4x7 orthotopic growth (Figure 30), supporting that the CXCR7/CXCL12 axis may limit acute CXCR4/CXCL12-mediated growth promoting effect *in vivo*, thus further suggesting a role for CXCR7 as a ligand scavenger in NB8x4x7 cells.

Interestingly, ligand binding to the "decoy receptor CXCR7" has been shown to activate MAP kinase pathways, such as ERK1/2, through β-arrestin rather than G protein signaling (67) (Figure 3). Moreover, CXCR4 is known to predominantly signal through classical G-mediated pathways (31) (Figure 2). In this study, different CXCR7/CXCL12-mediated ERK 1/2 cascade activations were noted in the NB8x7 and NB8x4x7 cell lines, as compared to the signal mediated by the CXCR4/CXCL12 axis in NB8x4 cells (Figure 19). These observations suggest that the CXCL12-induced ERK1/2 activation in those cells might not be mediated by G-proteins, but rather by other actors, such as β-arrestins, further supporting a scavenger role for CXCR7 in NB8x7 and NB8x4x7 cells.

However, the CXCR7/CXCL12/CXCL11 axes did not activate ERK 1/2 in SHSYx7 cells, suggesting that another mechanism may occur in those cells (Figure 19-20). Moreover, *in vitro* soft agar assay showed that CXCR7 regulated CXCR4-mediated SHSYx7 cell growth in absence of CXCL12 (Figure 25), further excluding a unique ligand scavenger function for the CXCR7 receptor in those cells.

Heterodimerization of CXCR7 and CXCR4 in NB cell lines?

Evidences to date suggest that many, if not all, GPCRs function as either mono- or hetero-oligomers. Such oligomeric complexes have been shown to involve mechanistic

implication on receptor efficacy and represent a key paradigm in GPCR biology (181). There is now a body of evidence that GPCR heterodimerization can alter G protein specificity, thus influencing activation of specific downstream pathways (182). Combined CXCR4/CXCR7 expression has been detected in T- and B-cell subsets, endothelial cells, human renal progenitor cells, as well as in primary human tumours and tumour cell lines (53;60;75;76). Importantly, it has been hypothesized that CXCR7, once engaged in heterodimers with CXCR4, may regulate CXCR4 functions through an allosteric mechanism (independently of CXCL12), further affecting ligand binding to CXCR4 and/or its downstream signaling (180). However, the direct impact of CXCR7/CXCR4 heterodimer activity in enhancing or conversely decreasing CXCR4-mediated proliferation/survival/migration in tumours is still elusive (183). As CXCR7 affects CXCR4-mediated signaling in SHSYx7 cells independently of CXCL12, we postulate that the negative regulation of CXCR4-induced signaling by CXCR7 in NB might also result from the heterodimeric association of both receptors in those cells.

In addition, it has been proposed that heterodimers "can synergistically increase signaling", "or conversely, negatively interact" thus decreasing the response initially mediated by interaction of respective monomers with their ligand(s) (184;185). Constitutive association of CXCR4 and CXCR7 as heterodimers has been also reported to alter CXCR4 signaling, in a CXCL12 dependent manner (70;180;183). As CXCR7 modulated CXCR4/CXCL12-mediated ERK 1/2 activation in the SHSYx7 cells, it further supports an association of CXCR7 and CXCR4 as heterodimeric complexes in those cells (Figure 19). In such context, the putative heterodimeric form of these receptors may also influence efficacy of the CXCR7 receptor in response to CXCL12 or CXCL11, which may explain why both CXCR7/CXCL12 and CXCR7/CXCL11 axes failed to activate ERK 1/2 cascade in SHSYx7 cells.

Two distinct or combined models?

In this study, an "antagonist effect" between the two CXCL12 receptors in NB was strongly suggested. However, whether CXCR7 modulates CXCR4 signaling as a result of their heterodimeric expression or/and by scavenging the ligand needs further analyses. Our data suggest that the modulation of the CXCR4-mediated signaling by CXCR7 might be NB cell-line dependent, as important variations between the NB8 and the SHSY transduced cell lines were noted. In regards to results obtained in this study, we hypothesize that CXCR7 may modulate CXCR4 function(s) as detailed in the two following non-exhaustive models:

✓ <u>CXCR7 as a ligand scavenger (model A, Figure 32):</u> The two CXCL12 receptors are co-expressed at the surface of NB cells where they individually mediate their own signaling activation cascade in response to specific stimuli (ligand, stromal factors, and stress culture conditions). In such model, CXCR7 would modulate CXCR4/CXCL12 signaling by acting as a ligand scavenger. This model may be proposed for the regulation of the CXCR4-mediated signaling by CXCR7 in the NB8x4x7 cell line.

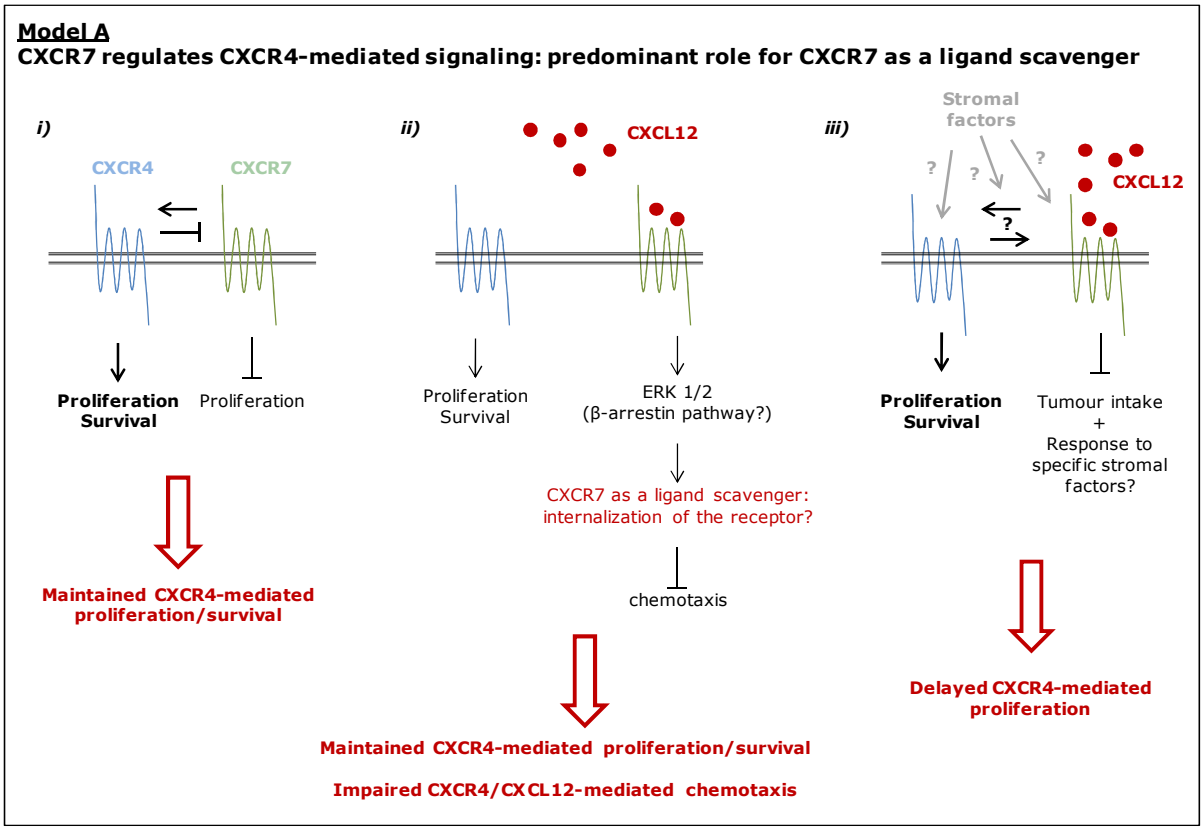


Figure 32: Regulation of CXCR4-mediated NB signaling *in vitro* and *in vivo*: model A
i) Upon co-expression, the two CXCL12 receptors CXCR7 and CXCR4 may form monomers (or homodimers), thus mediating their own function(s). Expression of one receptor may modulate that of the other, as observed in the NB8x4x7 cell line (Figures 17-18). In absence of the ligand, both receptors may individually mediate opposite growth-related function *in vitro*, with a predominant CXCR4-mediated proliferation/survival promoting effect. ii) CXCR7 presents higher affinity for CXCL12 than CXCR4. Thus, in presence of the ligand, CXCR7 may act as a ligand scavenger, preventing CXCL12 binding to CXCR4 and thus resulting in limiting CXCR4/CXCL12-mediated chemotaxis *in vitro*. CXCR4 on its own may additionally elicits a ligand-independent NB growth promoting role. iii) In orthotopic conditions, additional stromal factors may modulate CXCR4, CXCR7, or both receptor expression levels, which in turn may favour CXCR4-mediated NB growth, rather than CXCR7-mediated anti-proliferative function.

✓ Heterodimerization of the two receptors (model B, Figure 33): The two CXCL12 receptors are co-expressed as heterodimers at the surface of NB cells, influencing the individual efficacy, function(s) and ligand selectivity of each receptor. Modulation of the CXCR4 (or CXCR7) signaling would be a direct result of the heterodimeric association of the two receptors. This model may be proposed for the regulation of the CXCR4-mediated signaling by CXCR7 in the SHSYx7 cell line.

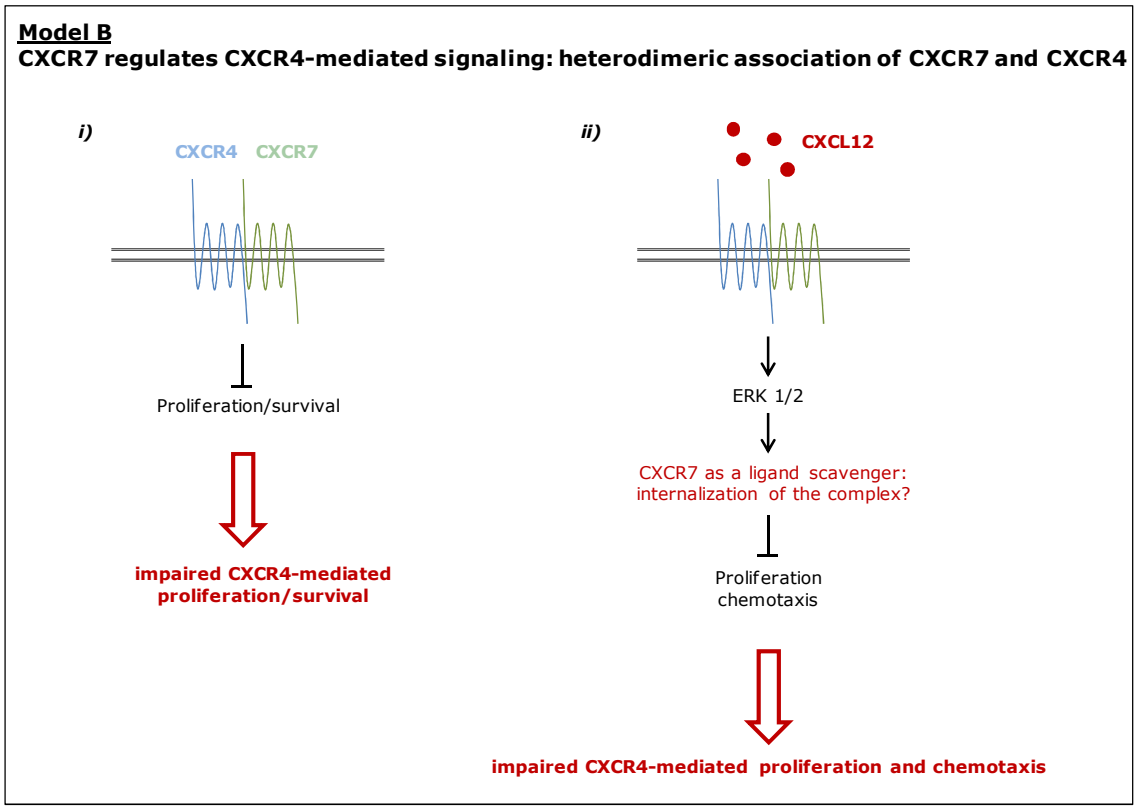


Figure 33: Regulation of CXCR4-mediated NB signaling *in vitro*: model B
i) Upon co-expression, CXCR7 and CXCR4 may form heterodimers at the surface of NB cells. Such chemokine receptor complex may impair CXCR4-mediated growth promoting effect, in absence of the ligand, as observed in SHSYx7 cells. ii) In presence of the ligand, the "CXCR7-CXCR4" complex may be internalized, thus limiting CXCR4/CXCL12-mediated NB proliferation/chemotaxis. As heterodimerization has been proposed to modulate ligand selectivity (183), whether CXCL12 preferentially binds to CXCR7 or to CXCR4 will need further investigations.

In this study, we noted that the expression of CXCR4 also affected the CXCR7/CXCL11-mediated ERK 1/2 activation, especially in NB8x4x7 cells (Figure 20). However, CXCL11 is not a ligand for the CXCR4 receptor, and CXCR3, the other known CXCL11 receptor, is not expressed by NB8x4x7 cells (Figure 21). Except if CXCR4 may elicit remote regulator function on CXCR7 via a more complex signaling network, the model A

cannot provide by itself a suitable explanation for such observations, as the two receptors would not be physically associated at NB8x4x7 cell surface.

Therefore, we cannot exclude the possibility that a more global regulation mechanism may occur in NB cells which may be a combination of these two models.

Conclusion and perspectives

In this study, we propose that CXCR4 and CXCR7 may display two distinct roles in NB: CXCR4 favours NB growth/survival and chemotaxis, while CXCR7 decreases NB growth and may be associated to NB differentiation. Importantly, we postulate that CXCR7, in association with CXCR4, may elicit anti-tumorigenic function by regulating CXCR4-mediated signaling, potentially as a heterodimeric partner or/and as a ligand scavenger.

It has been shown that the two CXCL12 receptors, when co-expressed, may form homodimers as efficiently as heterodimers (180). Thus, whether the different CXCR7 and CXCR4 expression levels detected in NB8x4x7 and SHSYx7 cells may differently influence their conformational association at NB cell surface should be further investigated. Experimental procedures, such as immunoprecipitation and bioluminescence resonance energy transfer (BRET) assays, will help to determine whether CXCR7 and CXCR4 efficiently form hetero- or homo-dimers in NB cell lines. Furthermore, it has been proposed that CXCR4/CXCR7 heterodimers result in attenuation of classical CXCL12-mediated G-activated signaling, by predominantly activating β-arrestin-dependent pathways (64;183). Ligand scavenger function of CXCR7 has also been shown to result from β-arrestin activation (67). Thus, extending our analyses on the activation of the p38 and Jun N-terminal kinases (two β-arrestin-dependent pathway mediators) in NB cells expressing CXCR7, CXCR4 or both receptors, in response to CXCL12 (and CXCL11), may help to further understand mechanisms mediated by individual CXCR7, CXCR4 or by potential heterodimeric complexes of both receptors. In this study, we reported that the IMR-32 NB cell line expresses endogenous levels of both CXCR7 (moderate) and CXCR4 (high) receptors (Figure 10). Thus, the IMR-32 cell line may be a suitable model to further investigate the consequences of CXCR4/CXCR7 co-expression on CXCL12/CXCL11-induced responses in NB.

As postulated in this report, 3D culture systems may be essential tools to go on investigating chemokine receptor function(s) *in vitro*, and particularly to further clarify the putative association of CXCR7 with NB differentiation processes.

Finally, our orthotopic studies suggest a critical role for stromal factors, together with CXCR7, in mediating regulation of CXCR4-mediated growth-promoting signals *in vivo*. Elucidating other chemokine (such as CXCL11), cytokine (such as TNF-α, IFN-γ) and growth factor (such as TGF-β, IGF, VEGF) productions in either normal mouse adrenal gland or after implantation of CXCR4/CXCR7-expressing tumour cells will be essential to further characterize the CXCR7/CXCR4/CXCL12 axis in NB progression.

Altogether, our preliminary findings open new research perspectives for the complex role of the CXCR7 and CXCR4 chemokine receptors and their common ligand CXCL12 in the malignant behaviour of NB. Elucidating the contribution of the CXCR7/CXCR4/CXCL12 axis in NB growth and differentiation, and implicated mechanisms, may help to reveal news targets for novel therapeutic approaches.

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Annexe: Chemokines and their cognate receptors

Most chemokines can bind multiple receptors, and a single receptor can bind multiple chemokines. Decoy receptors can also interact with multiple chemokines. By contrast, a minority of specific chemokine receptors binds to only one ligand (22;27;130;186-188).

	Chemokine Receptors	Chemokine Ligands
Specific receptors	CCR9	CCL25
	CCR6	CCL20
	CXCR5	CXCL13
	CXCR6	CXCL16
	CX3CR1	CX3CL1
	CXCR4	**CXCL12**
Shared receptors	XCR1	XCL1, XCL2
	CXCR1	CXCL6,8
	CXCR2	CXCL1,2,3,5,6,7,8
	CXCR3	CXCL4,9,10,11 CXCL4L1
	CCR1	CCL3,4,5,7,14,15,16,23
	CCR2	CCL2,7,8,12,13,16
	CCR3	CCL5,7,11,13,15,24,26,28
	CCR4	CCL2,3,5,7,17,22
	CCR5	CCL3,4,5,8,11,14,16
	CCR7	CCL19,21
	CCR8	CCL1,4,17
	CCR10	CCL27,28
Decoy receptors	CCX-CKR	CCL19,21,25 CXCL13
	DARC	CCL2,5,11,13,14 CXCL1,2,3,7,8
	D6	CCL2,3,4,5,7,8,12,13,14,17,22
	CXCR7	**CXCL12,11**

CURRICULUM VITAE

PERSONAL

Name:	**Julie Liberman**
Date of birth:	24.09.1983
Place of birth:	Paray-Le-Monial, France
Address:	Avenue de la Vallonnette 17
	1012 Lausanne, Switzerland
Tel.:	Home: 0041 (0)21 311 25 26, Mobile: 0041 (0)76 242 14 61
e-mail:	ju.liberman@gmail.com

EDUCATION

03.2012	**Awarded a doctorate in Life Sciences (PhD)** by the Doctoral School of the Faculty of Biology and Medicine, University of Lausanne, Switzerland.
2007-2012	PhD project entitled "The CXCR7/CXCR4/CXCL12 axis in human neuroblastoma: involvement in malignant progression". Group of Dr. Nicole Gross, Paediatric Oncology Research Unit, Department of Paediatrics, University Hospital (CHUV), University of Lausanne, Lausanne, Switzerland.
07.2007	**Awarded the French elite postgraduate diploma** "Diplôme d'Ingénieur Chimiste", **and the M.Sc. "Bio-Med"**, Montpellier, France
2006-2007	M.Sc. "Bio-Med" in "Biology and Health" (2nd year), at the Ecole Nationale Supérieure de Chimie de Montpellier and the University Montpellier I&II, Montpellier, France
2003-2006	Third to fifth academic years at the Ecole Nationale Supérieure de Chimie de Montpellier (the Top Graduate School of Chemistry, Montpellier), i.e. a French highly selective academic institute of science and technology which educates industry's future top executives, who are both graduate chemists and managers. Specialization: Biochemistry
2001-2003	Post-high school advanced mathematics, physics and chemistry classes in highly selective preparatory classes leading to the competitive entrance examination to French institutes of science and technology, Lycée La Martinière Monplaisir, Lyon, France

06.2001 **French Baccalauréat in sciences**, i.e. **Honours Class High School Leaving Diploma** in chemistry (A), mathematics (A), physics (A), biology (A), English (B), Digoin, France

INTERNSHIPS

2007 (6 months) **M.Sc professional training (2nd year),** in the Group « Tumor identity and Plasticity » of Dr. C. Theillet, Cancer Research Center of Montpellier, Montpellier, France

Objective: Study of the Slug transcription factor involvement in early epithelial differentiation using a 3D model of mammary morphogenesis

2006 (6 months) **Engineer professional training,** in the non-governmental organization (NGO) "Archipelagos", a scientific research institute for conservation of the marine and terrestrial environments of the Greek seas and islands, Ikaria, Greece

Objective: Analyses of soil and water quality

2005 (4 months) **Engineer professional training,** in the department of « Agrotechnology & Food Innovations », Wageningen Food & Biobased Research Center, Wageningen University, Wageningen, Netherlands

Objective: Purification and characterization of mushroom tyrosinase

2004 (3 months) **Technician professional training,** in the department of « Advanced Methods for Innovation in Science", International Research Center for Agricultural and Development issues (« CIRAD »), Montpellier, France

Objective: Synthesis and HPLC analyses of omega 3-enriched oils

2003 (1 month) **Technician professional training**, in the "Laboratory of Aminoacids, Peptides and Proteins" (LAPP), UMR 5810, National Center for Scientific Research (CNRS), Montpellier University I & II, Montpellier, France

Objective: Solid-phase peptide synthesis, purification and HPLC analyses of peptides

SCIENTIFIC SKILLS

Molecular biology:	Protein and RNA extractions from cultured cells and frozen tissues, reverse-transcriptase PCR, real-time PCR (TaqMan© technology), Western Blot, ELISA, DNA cloning and purification, primer design, plasmid construct, lentiviral- and retroviral-mediated infections
Cell biology:	Cell line culture, immunofluorescence staining on living and fixed cells, flow cytometry and cell sorting, cell viability assay, apoptosis (PI staining) assay, drug resistance assay, soft agar assay, migration and wound healing assays
***In vivo* experiment**:	Sub-cutaneous and orthotopic tumour resection (mouse models), preparation of paraffin-embedded sections, sectioning of frozen tissues (cryostat)
Microscopy:	Leica© DM2000 microscope for natural and fluorescence conditions, camera imaging and analysis with LAS 6000 AF software
Chemistry:	Solid-phase peptide synthesis, HPLC, thin layer chromatography, electrophoresis, enzyme kinetics analyses
Computer skills:	Microsoft Office (Word, Excel, Power-Point), Adobe Photoshop, Adobe Illustrator, GraphPad Prism

GRANTS

2007-2012	Research grants from **the National Found for Research (FNS)**, from **FORCE Foundation for Children Cancer Research**, and from the **Hubert Gouin association** (CH)

PUBLICATIONS

- **Involvement of the CXCR7/CXCR4/CXCL12 axis in the malignant progression of human neuroblastoma.** *Liberman J, Sartelet H, Flahaut M, Mühlethaler-Mottet A, Coulon A, Nyalendo C, Vassal G, Joseph JM, Gross N. (in submission)*
- **Functional sphere profiling reveals the complexity of neuroblastoma tumor-initiating cell model.** *Coulon A, Flahaut M, Mühlethaler-Mottet A, Meier R, Liberman J, Balmas-Bourloud K, Nardou K, Yan P, Tercier S, Joseph JM, Sommer L, Gross N.* Neoplasia. 2011 October 13.
- **Individual caspase-10 isoforms play distinct and opposing roles in the initiation of death receptor-mediated tumour cell apoptosis**. *Mühlethaler-Mottet A, Flahaut M, Bourloud KB, Nardou K, Coulon A, Liberman J, Thome M, Gross N.* Cell Death Dis. 2011 March 3.

CONGRESS PARTICIPATIONS

Abstracts selected for oral presentations:

2010 **Swiss Pediatric Oncology group (SPOG) Scientific Meeting, Lugano, Switzerland.** *"The CXCL12/CXCR4/CXCR7 axis in human neuroblastoma: involvement in malignant progression"*. *J. Liberman*

Annual research day of the young researchers in paediatrics', Montana, Switzerland. *"Molecular and Functional Characterization of Neuroblastoma Initiating Cells: Methods and Issues" A. Coulon, M. Flahaut, A. Mülhethaler-Mottet, J.Liberman, G. Kiowski, L. Sommer and N. Gross*

2009 **Swiss Pediatric Oncology group (SPOG) Scientific Meeting, Lugano, Switzerland.** *"The CXCL12/CXCR4/CXCR7 axis in human neuroblastoma: involvement in malignant progression"*. *J. Liberman*.

Annual research day of the young researchers in paediatrics' 2009, St Gallen, Switzerland. *"The CXCL12/CXCR4/CXCR7 axis in human neuroblastoma: involvement in malignant progression"*. *J. Liberman, R. Meier, T. Sengstag, M. Flahaut, A. Mühlethaler-Mottet, A. Coulon, JM. Josep, N. Gross.*

LANGUAGES

French	Native
English	Fluent (read, written and spoken), TOEIC: 915/990 points
Italian	Beginner level