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Potential combinatorial effects of recombinant atypical chemokine receptors in breast cancer cell invasion: A research perspective (Review)

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Abstract. Apart from their major function in the coordination of leukocyte recruitment, chemokines, in cooperation with their receptors, have been implicated in the progression of various diseases including different types of cancer, affecting survival, proliferation and metastasis. A complex network of chemokines and receptors exists in the tumor microenvironment and affects tumor development in various ways where chemokines activate typical signalling pathways by binding to the respective receptors. The identification and characterization of a group of atypical chemokine receptors [D6, Duffy antigen receptor for chemokines (DARC), ChemoCentryx chemokine receptor (CCX-CKR) and CXCR7] which appear to use unique biochemical properties to regulate the biological activities of these chemokines, is useful in the effort to therapeutically manipulate chemokines in a broad spectrum of diseases in which these chemokines play a critical role. The aim of this review was to investigate the combinatorial effect of two reported atypical chemokine receptors, D6 and DARC, on breast cancer cell invasion to understand their role and therapeutic potential in cancer treatment. In this regard, findings of the present review should be confirmed via the construction of recombinant D6 and DARC clones as well as the expression of the respective recombinant proteins using the *Pichia pastoris* (*P. pastoris*) expression system is to be performed in a future study in order to support findings of the current review.

Contents

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1. Introduction

Chemokines are small heparin-binding proteins (~8- to 17-kDa long) with multiple activities. Since their main function is to regulate the migration of cells, particularly leukocytes, to the sites of inflammation, they are known as chemoattractant cytokines. Chemokine biological activities are regulated at several levels and their production can be constitutive or induced by environmental stimuli. Based on this, chemokines are subdivided into homeostatic and inflammatory subsets, with constitutive chemokines usually regulating the homeostatic trafficking of leukocytes and lymphocyte recirculation under normal or steady state conditions, while inflammatory chemokines are produced in response to inflammatory and immune stimuli and direct leukocytes to inflamed peripheral tissues (1). Chemokines are differentially produced in particular tissues either constitutively or after an appropriate stimulus and attract receptor-bearing cells to these locations. It is thought that this process occurs by forming extracellular chemotactic or haptotactic gradients depending on whether they are in solution or bound to extracellular matrix components, respectively (2). Previously, the roles of chemokines and chemokine receptors were identified: i) during leukocyte migration, by acting on firm adhesion, locomotion, diapedesis and chemotaxis; ii) during development, through the regulation of hematopoiesis, cardiogenesis as well as vascular and cerebellar development; and iii) during tumor biology, by controlling angiogenesis, metastasis and cell proliferation (3).

Chemokine structure (Fig. 1) comprises an N-terminal loop region, three-strand antiparallel β-sheets forming the typical core fold of the chemokines and a C-terminal α helix which overlays the β-sheet. The major receptor-binding site is the N-loop region that follows the first two cysteines and connects the N-terminus to the β-sheet region, with the sequence therein conferring receptor specificity (4). Based on the variations in the configuration of a conserved amino-proximal cysteine-containing motif, chemokines are divided into four subfamilies, known as CC, CXC, XC and CX3C (where X is any amino acid). Generally, chemokines belonging

to the CC family act primarily on monocytes, although they are also capable of attracting lymphocytes, basophils and eosinophils, whereas CXC chemokines attract neutrophils and lymphocytes. Additionally, XC and CX3C chemokines act on lymphocytes only and on monocytes/lymphocytes, respectively.

Chemokine receptors are known to be embedded in the lipid bilayer of the cell surface and also to possess seven-transmembrane domains (TM) (Fig. 2) (5). Chemokines bind to the seven-TM spanning G-protein-coupled receptors (GPCRs) to exert their actions and the receptors are classified according to the chemokines they bind. There are eleven CCRs (receptors for CC chemokines) and seven CXCRs (receptors for CXC chemokines) in addition to XCR1 and CX3CR1. Some of these receptors, including CXCR2, CCR1 and CCR3, are highly promiscuous and are crucial during inflammation, while others such as CXCR4, CCR7 and CCR9, are more selective and perform critical homeostatic functions (6). These chemokine receptors are located on the surface of leukocytes and other cell types. Moreover, they sense the extracellular chemokine environment and transmit signals to change cell behaviour. Chemokine responsiveness is generally determined by the expression of these receptors, converting induced or constitutive tissue chemokine expression into appropriate biological responses (6). A typical receptor specifically binds to its ligand leading to typical signalling pathways. Following the chemokine-driven activation of chemokine receptors, these receptors initiate a wide range of intracellular signalling cascades, including those involving G-proteins, phosphoinositide-3-kinases, MAP kinases and small GTPases (7) and typically lead to cell polarisation and directed motility.

However, certain chemokines are subject to natural modulation of their concentrations by proteins to which they bind without leading to typical signalling (8). These proteins may be endogenously encoded or expressed by exogenous sources to modulate chemokine functions. These 'scavenger' or 'decoy' proteins act as 'interceptors' (intercepting receptors) that neutralize the action of the chemokine or by transporting chemokines across endothelial barriers. These decoy receptors, which bind ligands with high affinity but do not elicit signal transduction, include D6, Duffy antigen receptor for chemokines (DARC), ChemoCentryx chemokine receptor (CCX-CKR) and CXCR7 (9).

Chemokines and chemokine receptors play many key roles in physiological and pathological activities in infectious and inflammatory diseases, in the modulation of angiogenesis, in tumor growth as well as stem cell proliferation and have been widely reported to participate in the process of malignant progression (10-13). Cancer cells have been found to produce chemokines and chemokine receptors which are able to respond specifically to these chemokines, thus forming a complex chemokine network which is involved in influencing tumor cell survival, spreading and growth (14).

2. The role of chemokine networks in cancer

Although the major function of chemokines is the coordination of leukocyte recruitment, their involvement is not limited to inflammation and immunity. They are produced by different cell types, including tumor cells, and mediate other

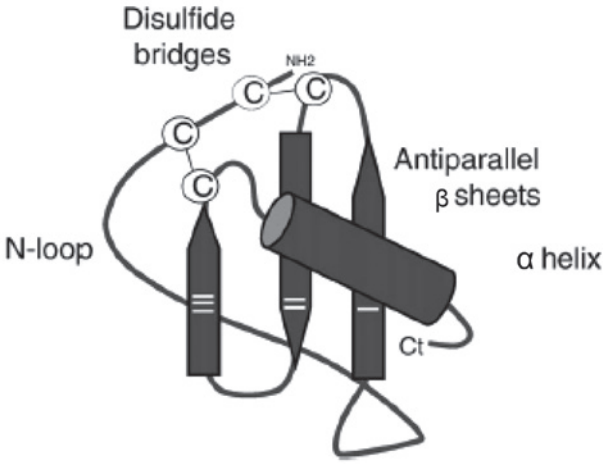

Figure 1. The structure of a chemokine (4). C, cysteine.

biological activities, including the regulation of cell differentiation, proliferation, survival and senescence. The expression of chemokines and their receptors is relevant in several types of human pathologies, including cancer, since the identification of CCL2 in culture supernatants of tumor cell lines (15). In cancer biology, their roles expand from the regulation of leukocyte attraction within the tumor mass to the promotion of tumor cell survival, proliferation and dissemination. Tumors are major producers of chemokines and are an invaluable source for chemokine identification and characterization.

Although inflammation ensures effective host defence and tissue repair, chronic inflammation has been connected to the development of cancer in mice and humans (16,17). Established tumors develop the mechanisms to autonomously dictate the nature of their inflammatory infiltrate, harnessing pro-tumorigenic leukocyte activity to help tumor survival and progression. In the tumor microenvironment, chemokines are crucial regulators of the levels of tumor-infiltrating leukocytes, especially of macrophages. Strong evidence has suggested that tumor-associated macrophages may promote tumor progression by releasing angiogenic factors, proteolytic enzymes and immunosuppressive molecules, which would enhance tumor growth, dissemination and evasion from immune control. Preneoplastic to neoplastic transformation, tumor growth, invasion as well as metastases depend on the establishment of a proangiogenic environment (18). Angiogenesis is a crucial step in tumor growth and progression. Net angiogenesis in the local microenvironment is determined by the imbalance in the overexpression of angiogenic factors, as compared to angiostatic factors (18). Inflammatory leukocytes are thought to create a supportive environment for early tumor development, possibly through the production of cytokines, proteases and angiogenic factors. Thus, prompt resolution of inflammation prevents the establishment of an inflammatory environment conducive to tumorigenesis. A complex network of chemokines and receptors exists in the tumor microenvironment and affects tumor development. Modification of these chemokine networks modulates leukocyte recruitment, angiogenesis and tumor growth.

The chemokine system is also used by cancer cells to promote cell proliferation, tumor survival and neovascular-

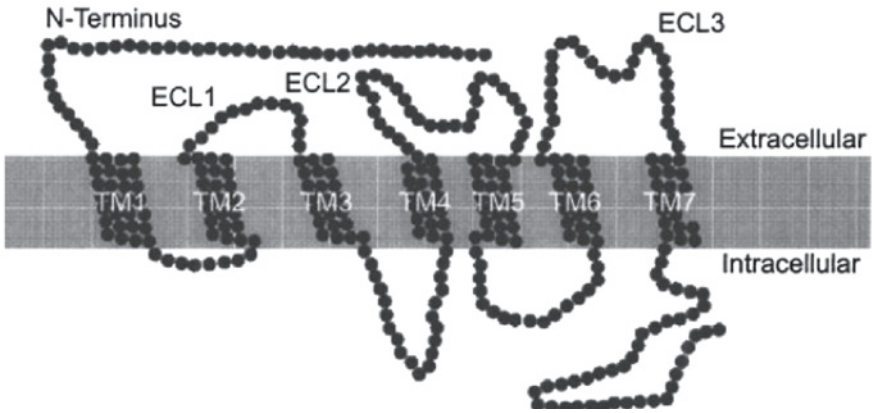

Figure 2. Topology of a typical chemokine receptor (7). ECL, extracellular loop; TM, transmembrane domain.

ization, or to establish metastases at distant but non-random sites (19). These mediators (chemokines) control a variety of biological activities, such as the production and deposition of collagen, the activation of matrix-digesting enzymes, the stimulation of cell growth, the inhibition of apoptosis and the promotion of neo-angiogenesis. Chemokines are powerful inducers of enzymes and receptors that degrade the extracellular matrix and facilitate tumor invasion (20). Tumor-derived proteases are able to cleave the extracellular matrix molecules and lead to the dissolution of the basement membrane, thus facilitating the process of tumor cell invasion. These proteolytic enzymes include the tissue-type plasminogen activator (t-PA), the urokinase-type plasminogen activators (u-PA) and the large family of matrix-metalloproteinases (MMPs). Therefore, the expression of chemokines is of potential advantage for tumor cells, rendering them capable of proliferation and dissemination (21).

Tumors are composed of cancer and stromal cells, where, besides fibroblasts and endothelial cells, leukocytes (macrophages and T lymphocytes, in particular) are the most represented cell types. In the tumor microenvironment, chemokines are produced both by stromal cells (fibroblasts, endothelial cells and infiltrating leukocytes) and by the tumor itself. Immune cells are actively recruited at the tumor site by the chemokines produced by neoplastic and stromal cells, leading to the recruitment of tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), lymphocytes, cancer-associated fibroblasts (CAFs), mesenchymal stem cells (MSCs) and endothelial cells into the tumor microenvironment. These infiltrating cells provide a secondary source of chemokines that may affect tumor growth, cell survival, senescence, angiogenesis and metastasis. For instance, TAMs produce a host of growth factors which affect tumor cell proliferation, angiogenesis, as well as the deposition and dissolution of connective tissues. Uneven vascularisation and hypoxia are characteristics of neoplastic tissues. TAMs accumulate preferentially in the poorly-vascularized regions of tumors with low oxygen tension. Under hypoxic conditions, TAMs are stimulated to express hypoxia-inducible genes, such as the vascular endothelial growth factor (VEGF), the basic fibroblast growth factor (bFGF) and CXCL8. In various studies, TAM accumulation in human cancer has been associated with high neovascularisation and with the production of angiogenic factors such as VEGF, platelet-derived endothelial cell growth factor, fibroblast, epidermal growth factor (EGF), fibroblast growth factor (FGF) and chemokines (22,23).

A subgroup of chemokines, the CXC family, is important in angiogenesis, as well as in physiologic and pathologic contexts, including chronic inflammation, fibrosis and malignancy (24). With regard to tumor growth, chemokines of the CXC branch have been shown to exert either angiogenic or angiostatic activities depending on whether or not amino acid sequence Glu-Leu-Arg (ELR motif) is present or absent (25). The NH_2 terminus of several CXC chemokines contain three amino acid residues (Glu-Leu-Arg or ELR motif), which precede the first cysteine amino acid residue of the primary structure of these chemokines. Depending on whether or not the ELR motif is present or absent prior to the first cysteine residue in their structure, CXC chemokines are subdivided into ELR^+ and ELR^- (24). On the basis of their structure and receptor binding, individual ligands exhibit either angiogenic or angiostatic biological activities in the regulation of angiogenesis. The balance of ELR^+/angiogenic vs. non-ELR/angiostatic chemokines produced in the tumor microenvironment is likely to determine the degree of angiogenesis surrounding and inside the tumor tissue, and the consequent tumor progression (21).

Chemokines may be involved in tumor progression through the direct stimulation of neoplastic growth, promotion of inflammation and induction of angiogenesis. Apart from indirectly recruiting leukocytes that provide angiogenic factors, chemokines regulate angiogenesis directly, through receptors expressed on endothelial cells (26). Tumor cells upregulate the expression of widely distributed chemokine receptors (CXCR4, in particular) and indicate an unexpected, tissue-of-origin unrelated, chemokine receptor repertoire, which possibly supports tumor cell survival and invasion. The most common receptor is CXCR4, expressed by different tumor types of epithelial, hematological and mesenchymal origin (14). The expression of chemokine receptors influences diverse aspects of cancer cell behaviour, with chemokines serving as cues for the secondary localization of tumors (27). Malignant cells bearing chemokine receptors on the cell surface are capable of responding to the chemokine gradient and selectively migrating to specific organs where the chemokine is present, meaning that chemokines were able to direct tumor cell migration *in vivo*. In addition to affecting tumor

cell proliferation, angiogenesis and metastasis, chemokines also seem to regulate senescence and cell survival. Thus, the chemokine system is a valuable target for the development of innovative therapeutic strategies (27).

3. Atypical chemokine receptors

Numerous studies have reported findings on the atypical action of chemokine receptor proteins. These receptor proteins are known as 'decoy' or 'scavenger proteins' due to the fact that the binding of these proteins to the respective ligands does not lead to a typical signalling pathway, but intercepts the respective pathway and neutralizes chemokine action (4,28-30). Therefore, these chemokine receptor proteins are also regarded as 'intercepting receptors' and are able to bind a broader spectrum of chemokines. The ability of these receptors to couple to signal transduction has been principally analysed by examining intracellular calcium ion mobilization and chemotaxis in transfected cells following ligand binding, where they appeared to be functionally 'silent'. In addition, they are predominantly expressed on non-leukocytic cell types and are unlikely to be directly involved in leukocyte migration. It has been proposed that atypical receptors may act in several ways: i) competing for ligand binding and, thus, inhibiting the migration of cells bearing typical receptors; ii) internalizing and degrading ligands and, therefore, depleting bioavailable chemokine levels in a particular microenvironment in order to reduce the cell recruitment to that site; iii) in the transcytosis of chemokines in order to then transfer ligands across certain barriers; or iv) retaining or presenting chemokines (2). Four endogenous proteins are reportedly included in this group: pro-inflammatory CC chemokine receptors (D6 and DARC), the homeostatic CC chemokine receptor (CCX-CKR) and CXCR7, a second receptor for CXCL11 and CXCL12, with critical roles in development and tumorigenesis. These proteins are crucial in inflammation and chemokine-associated diseases, such as cancer, since some of them act to trap the chemokine, internalize it as well as direct it towards degradation and transport the bound chemokine across the plasma membrane. The ability of these proteins to block the function of chemokines in cancer cells has been widely described (31).

D6. D6 is an atypical receptor that acts as a decoy and scavenger receptor protein for most inflammatory CC chemokines, including CCL2 (9). In humans, D6 is abundant on lymphatic endothelial cells (LECs) of the vessels draining the skin, the gut and the lungs (32) and may be found on trophoblasts, leukocytes (27), tissue mast cells, macrophages and also dendritic cells (31). Previous studies have shown that D6 was found to be expressed by malignant vascular tumors, T-cell large granular lymphocyte leukemia cells, choriocarcinomas (32-34) and also human breast cancer cells (28). D6 overexpression in human breast cancer cells was reported to downregulate CCL2 levels and to subsequently inhibit the proliferation and metastasis of breast cancer cells *in vivo* and *in vitro* (4,27,28,32). D6-deficient mice have demonstrated increased susceptibility to skin carcinogenesis (35) and colitis-associated cancer, the latter being representative of a clinical paradigm of the inflammation-cancer connection (36).

D6 has been shown to be able to undergo ligand-independent constitutive internalization. When D6 binds chemokines, they rapidly enter the cell through endosomal compartments, dissociate from the receptor and internalized chemokines remain trapped in the cell and are targeted for degradation. Simultaneously, D6 recycles back to the cell surface for further chemokine sequestration. Trafficking is unaffected by chemokine exposure, chemokine-induced signalling is not required, and the receptor recycles without causing a reduction in the cell-surface D6 levels (37). Through repeated rounds of chemokine internalization, D6 is capable of removing and destroying large quantities of free extracellular proinflammatory chemokines (38). As a result, D6 constitutive internalization and recycling regulate continuous chemokine sequestration (39).

DARC. DARC is a promiscuous and non-signalling chemokine receptor (40). In humans, this receptor is expressed in red blood cells, endothelial cells as well as neuronal cells (41) and it has been observed to bind to CC and CXC chemokines (4,30). DARC is involved in the transcytosis or neutralization of chemokines at endothelial barriers (42) and on erythrocytes, and it may act to regulate plasma chemokine concentrations (43). An additional noteworthy feature of DARC is that it binds angiogenic (ELR^+) CXC chemokines and certain CC chemokines, but not angiostatic (ELR^-) CXC chemokines. Within the ELR^+ CXC chemokines, DARC was found to bind the angiogenic ELR^+ CXC chemokines CXCL1, CXCL5 and CXCL8 (44,45).

Research data have indicated that DARC expression in tumor or endothelial cells plays a negative role in tumor progression, through the control of angiogenic and inflammatory chemokines, or transmitting a senescence signal to tumor cells through interaction with tumor tetraspanin KAI1/CD82 (30). In a DARC-deficient mouse model with spontaneous prostate cancer, larger and more aggressive tumors were developed (46). This finding was of particular interest since African-American men, of whom 70% lacked erythrocyte DARC, had exhibited increased occurrence and mortality from prostate cancer (47). This has been associated with increased intra-tumor levels of angiogenic ELR^+ CXC chemokines and blood vessel density, supporting the hypothesis that DARC acts as a decoy receptor that sequesters angiogenic chemokines, thereby, inhibiting tumor growth. Therefore, DARC expression on erythrocytes may normally negatively regulate the levels of angiogenic plasma chemokines that promote prostate cancer progression. This receptor is also important in regulating breast cancer growth and metastasis. The expression of CCL2 has been reported to be significantly correlated with the progression of tumor and microvessel density in human breast cancer cells (27,28,48). Wang *et al* (30) have demonstrated that tumor cell lines expressing high levels of ectopic DARC are less able to grow and metastasize compared to wild-type tumor cells. This finding has been associated with a decrease in tumor angiogenesis and lower levels of CCL2 within the tumor. Chemokines, such as CCL2, have been found to accelerate tumor growth and metastasis of cancer cells upon binding with typical specific receptors. The overexpression of DARC in human breast cancer cells has been reported to downregu-

late CCL2 levels and to subsequently inhibit the proliferation and metastasis of breast cancer cells *in vivo* and *in vitro* (4,27,30,34).

DARC has been previously demonstrated to regulate the biological effects of chemokines in three different ways: through scavenging, retention or transportation (2). Erythrocyte DARC may act as a chemokine buffer, sequestering chemokines present at high levels in the serum, while maintaining a residual homeostatic level as their presence subsides. Since plasma chemokines likely desensitize circulating leukocytes, buffering by DARC potentially controls leukocyte sensitivity to pro-inflammatory chemokines, limiting under- or over-responsiveness (2). These different ways that DARC manipulation may affect chemokine biology and cell migration should be considered when DARC is therapeutically targeted.

CCX-CKR. CCX-CKR, also known as CCR11 or CCRL1, is a heptahelical surface protein. It has been demonstrated that CCX-CKR, when expressed in HEK293 cells, is unable to mediate Ca(2+) fluxes upon ligand binding (49). It cannot couple to typical chemokine receptor signalling pathways or mediate chemotaxis. As an atypical chemokine receptor, it is less well-characterized compared to D6 and DARC. Similar to DARC, CCX-CKR binds chemokines of CC and CXC subfamilies. However, unlike DARC and D6, it binds the homeostatic chemokines such as CCL19, CCL21, CCL25 and CXCL13 with high affinity. These chemokines participate in the axis role of CCR7/CCL19 (CCL21), CCR9/CCL25 and CXCR5/CXCL13 in leukocytes and cancer cell migration (2,49) through interaction with the receptors CCR7, CCR9 or CXCR5, respectively. CCR7 and CXCR5 control lymph node organogenesis (50), while CCR7 and CCR9 control thymocyte localization during T-cell development (51-53). CCR7 also regulates the recruitment of dendritic cells, naive T-cells and some memory T-cell subsets into T-cell compartments in secondary lymphoid organs, and contributes to B-cell extravasation. Therefore, CCX-CKR has been predicted to regulate homoeostatic lymphocyte and dendritic cell trafficking, which constitute key migratory events in acquired immune responses directed by CCX-CKR-binding chemokines (39). CCX-CKR, which is expressed exclusively by stromal cells, and not hematopoietic cells, regulates homeostatic leukocyte migration through the control of chemokine availability in the extracellular space (54).

By contrast, previous investigations have indicated that CCR7/CCL19 (CCL21), CCR9/CCL25 and CXCR5/CXCL13 axes are able to promote the growth and metastasis of various tumors, including breast cancer. The CCR7/CCL19 (CCL21) axis was able to promote the pathogenesis and progression of breast cancer, melanoma, non-small cell lung cancer, head and neck, gastrointestinal and hematologic cancer. The CCR9/CCL25 axis has been involved in breast carcinoma, ovarian and prostate cancer as well as cutaneous melanoma, while the CXCR5/CXCL13 axis has been previously demonstrated in various cancers including non-Hodgkin's lymphomas, primary intraocular lymphoma, metastatic neuroblastoma and breast cancer (55). CCX-CKR overexpression in breast cancer cells inhibited proliferation and invasion *in vitro*, as well as tumor growth, lung and lymph node metastasis *in vivo*. It has been shown to be a negative regulator of growth and metastasis in breast cancer mainly through the sequestration of homeostatic chemokines as well as the subsequent inhibition of intratumoral neovascularity. CCX-CKR as a scavenger receptor for chemokines, binds and clears expressed chemokines, including the CC chemokine ligands CCL19, CCL21, CCL25 and CXCL13. CCX-CKR expressing cells sequestered and degraded these cognate chemokines with notable efficiency and also negatively regulated cancer development and progression. CCX-CKR has been demonstrated to be associated with longer patient survival and has been identified as an independent prognostic factor for disease-free survival in breast cancer patients.

CXCR7. CXCR7 (RDC1), a heptahelical receptor with strong phylogenetic similarity to chemokine receptors or GPCRs (56), has recently been described as a second receptor for CXCL12 after CXCR4. CXCL12, a chemokine also known as stromal-derived factor 1 (SDF1) employs CXCR4 and CXCR7 receptors and modulates homeostatic and pathologic processes, such as the development of primary epithelial tumors, where it is known to regulate organogenesis, leukocyte homeostasis, proliferation as well as survival of tumor cells, tumor angiogenesis, and metastasis (57). Besides playing a role in fetal endothelial biology, cardiac development and B-cell localization, CXCR7 potentially modulates CXCR4 functions. It dimerizes with CXCR4, a coreceptor for CXCL12 and studies have suggested the modulation of CXCR4-signalling through heterodimerization with CXCR7. CXCR7 is coexpressed with CXCR4 in primary human T lymphocytes and is known to be involved in the regulation of CXCL12-promoted cell migration (58). CXCR7 expression has also been proven to induce conformational rearrangements within preassembled CXCR4-G protein complexes. This phenomenon may explain findings demonstrating that CXCR7 impairs CXCR4-mediated G protein activation and calcium responses (57).

Binding of CXCR7 to CXCL12 and CXCL11 has recently been reported without triggering chemotaxis or calcium mobilization in response to ligation. It has been shown to bind CXCL12 and CXCL11 with high affinity, while it does not induce typical chemokine receptor-mediated cell responses, such as migration and associated intracellular signal transduction (59,60). However, CXCR7 expression increases cell survival and adhesive properties of transfected cell lines, a fact suggesting that alternative signals originate from this receptor (59). Another study provided contradictory evidence, demonstrating chemotaxis and calcium mobilization in CXCR7 transfectants (58). The function of CXCR7 is controversial with certain studies (61,62) suggesting CXCR7 to possess signalling activity in mammalian cells and zebrafish embryos, while other studies (63) have demonstrated that CXCR7 possesses decoy activity in fish. Further studies are needed in order for these discrepancies to be clarified prior to the inclusion of CXCR7 in the atypical chemokine receptor family.

These receptors therefore exhibit the following critical features: high-affinity binding to chemokines, lack of chemotactic signalling and a marked ability to continuously internalize their ligands. *In vivo*, these receptors function either by regulating the levels of bioavailable chemoattractants,

competing with signalling receptors, or mediating transcytosis of chemoattractants across endothelial and epithelial barriers. Moreover, there appear to be some structural similarities between the above-mentioned receptors. Residues important for initiating signal transduction in typical receptors are often either altered or absent. Thus, generating these proteins in a significant amount in order to investigate their roles in various contexts is crucial.

4. Conclusion and future perspectives

Breast cancer is one of the most frequently diagnosed life-threatening types of cancer in women. Most breast cancer patients succumb to the disease due to cancer invasion and metastasis. Treatment of breast cancer invasion and metastasis is difficult and the survival rate for patients with invasive and metastatic cancer is low. Apart from their role in regulating leukocyte trafficking, chemokines have been shown to be involved in cancer growth and metastasis. The complex network of chemokines and the respective receptors in the tumor microenvironment constitutes the object of intensive investigation aimed at targeting these molecules for therapeutic interventions. Chemokine (C-C motif) ligand 2 (CCL2), also referred to as monocyte chemotactic protein-1 (MCP-1), is primarily secreted by monocytes, macrophages and dendritic cells. An elevated serum level of CCL2 has been found to be associated with breast cancer invasion and metastasis. D6 or DARC overexpression in breast cancer cells was detected to intercept the signalling pathway of CCL2, thereby decreasing the production level of this chemokine. Single administration of either D6 or DARC has been demonstrated to be able to neutralize the action of CCL2 *in vitro* and suppress invasion on MDA-MB-231 cells (30). However, the combinatorial effects of D6 and DARC on MDA-MB-231 cell invasion has yet to be demonstrated. A combination of D6 and DARC may yield better effects on invasive breast cancer cells compared to the single administration of either D6 or DARC. Neutralization of protein ligands, such as chemokines, are likely to lead to the validation of signalling pathways under physiological or pathophysiological conditions, and in certain cases, to the development of novel therapeutic molecules, such as chemokine receptors, to be used in disease treatment in the future.

Although attention has been focused on the expression of chemokines in human and experimental tumors, the expression of chemokine receptors has been investigated to a much lesser extent. Since chemokine decoy receptor proteins have been reported to have inhibitory effects on invasive and metastatic human breast cancer cells, the attempt of generating functional recombinant clones in research laboratories is highly attractive in order for future studies to clarify the role of these proteins in cancer studies, and particularly in breast cancer *in vitro*. An understanding of the function and mechanism of chemokine receptors underlying chemokine action in cancer requires the availability of a significant amount of highly purified and biologically active chemokine receptors. Subsequently, sufficient quantities of D6 and DARC are to be generated from MDA-MB-231 cell RNA in future studies compared to the commonly reported blood serum receptors. As such, clones are to be constructed and the recombinant chemokine decoy receptor proteins of D6 and DARC are to be expressed using the *Pichia pastoris* (*P. pastoris*) expression system. The recombinant proteins produced are to be applied in various combinations (compared to single-compound administration) to a highly invasive breast cancer cell line in order to investigate their effects on the tumorigenesis and metastatic potential of breast cancer cells *in vitro*.

P. pastoris is a non-pathogenic, methylotrophic yeast. It is a versatile microorganism capable of efficient secretion (64) and possesses the ability to produce soluble and correctly folded recombinant proteins (65). It also offers numerous benefits such as the strong expression promoter (AOX1) and the ability to stably integrate expression plasmids at a specific location (66-68). Compared to *Escherichia coli* (*E. coli*), *P. pastoris* shares some advantages of this bacterial expression system, such as the simplicity of genetic manipulation, its cost effectiveness and the high heterologous protein expression efficiency (69). Moreover, *P. pastoris* does not produce high levels of intrinsic proteins, thus making the expressed foreign proteins easy to be isolated. Furthermore, unlike the *E. coli* expression system, *P. pastoris* can produce foreign proteins intracellulary or extracellulary, depending on the signal sequence used in the system (70). The ability to be cultured at a high cell density, as well as the folding, processing of proteins, reduced heterologous proteins degradation and a much easier heterologous protein purification procedure compared to other bacterial expression systems, render *P. pastoris* more attractive as an excellent alternative to the *E. coli* expression system (71). Moreover, heterologous proteins expressed by the *P. pastoris* strain were able to be post-translationally modified as observed in mammalian expression systems without the use of expensive culture media and cell culture equipment, which always significantly raise the cost of the mammalian cell expression systems (67,68,72). Besides, *P. pastoris*-expressed proteins are free from endotoxins or pyrogens, a problem always faced in bacterial-derived proteins (64), as well as viral inclusions, a problem always faced in mammalian cell-derived proteins (73). This constitutes the expressed proteins safe for human use and suitable for clinical and therapeutic applications.

This study provides useful information on the effects of D6 and DARC on the tumorigenesis and metastatic potential of breast cancer cells and may lead to novel therapeutic strategies against breast cancer. Notably, in future studies, recombinant chemokine decoy receptor proteins are expected to be produced rather than using commercially available proteins, which are are considered to be expensive, in order to facilitate the basic requirement of studies such as this one. The use of the *P. pastoris* expression system in producing D6 and DARC is considered to constitute an attractive option for scale-up production in the future, since it is cost-effective and highly efficient in protein expression. The ability to generate the proteins of interest at increased concentration levels would allow significant improvements in study approaches and related applications. Integrating in-house protein production and translational research constitute potential challenges in facilitating research in low-resource settings in developing and under-developed countries. In the future, similar protocols may be utilized to produce other recombinant proteins in order to understand their roles in cancer biology as part of the ongoing investigation for novel therapeutic interventions.

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