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The CXCL12/CXCR4/ACKR3 Axis in the Tumor Microenvironment: Signaling, Crosstalk, and Therapeutic Targeting

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CXCR4, ACKR3, chemokine receptors, GPCRs, cancer, therapeutics

Abstract

Elevated expression of the chemokine receptors CXCR4 and ACKR3 and of their cognate ligand CXCL12 is detected in a wide range of tumors and the tumor microenvironment (TME). Yet, the molecular mechanisms by which the CXCL12/CXCR4/ACKR3 axis contributes to the pathogenesis are complex and not fully understood. To dissect the role of this axis in cancer, we discuss its ability to impinge on canonical and less conventional signaling networks in different cancer cell types; its bidirectional crosstalk, notably with receptor tyrosine kinase (RTK) and other factors present in the TME; and the infiltration of immune cells that support

tumor progression. We discuss current and emerging avenues that target the CXCL12/CXCR4/ACKR3 axis. Coordinately targeting both RTKs and CXCR4/ACKR3 and/or CXCL12 is an attractive approach to consider in multitargeted cancer therapies. In addition, inhibiting infiltrating immune cells or reactivating the immune system along with modulating the CXCL12/CXCR4/ACKR3 axis in the TME has therapeutic promise.

1. INTRODUCTION

Chemokine receptors, which belong to the superfamily of G protein–coupled receptors (GPCRs), play a prominent role in the homeostasis of the immune system and in developmental functions (1). Their activity is tightly regulated via the expression of ligands and receptors, proteolytic cleavage of chemokines, and posttranslational modification of the receptors. The enhanced expression of CXCR4 and ACKR3, in particular, has been detected in a wide range of tumors and cells residing within the tumor microenvironment (TME) and is most often associated with poor prognosis (2). Although both CXCR4 and ACKR3 are GPCRs that share CXCL12 as a physiological ligand, they possess unique features. CXCR4 signaling is thought to propagate via the canonical G protein–dependent and β-arrestin-dependent signaling paradigm, while ACKR3 [formerly referred to as RDC1 (3) and CXCR7] is classified as an atypical chemokine receptor. ACKR3 lacks the conserved DRY-LAIV motif involved in G protein activation, preferentially signals via β-arrestins, and has a chemokine scavenging role (4–8).

Substantial progress has been made in elucidating crystal structures of GPCRs, including CXCR4 (9). CXCL12 binds with its globular core to the N terminus and extracellular loops of CXCR4 [major binding pocket residues in transdomains 3–7 (TM3–7)], followed by binding of the N terminus of CXCL12, which is critical for agonism, to the 7TM domain (minor binding pocket residues in TM1–3 and TM7) (10). For ACKR3, the first N-terminal residues of CXCL12 are not as critical for receptor activation (11, 12). CXCL12 is the sole chemokine interacting with CXCR4, while ACKR3 also binds CXCL11, the CXCR3 agonist. Various CXCL12 isoforms, including CXCL12α (the main isoform), CXCL12β, and CXCL12γ, have been reported to differentially affect CXCR4 and ACKR3 function (see 13 and 14 for references therein). Similarly, other natural nonchemokine ligands, including macrophage migration inhibitory factor (MIF), ubiquitin, adrenomedullin, and vMIP-II (vCCL2) and opioids (14a), bind CXCR4 and/or ACKR3 (15, 16) (**Table 1**). Besides their differential signaling properties and ligand repertoire, spatial cellular expression is distinct for the two receptors: CXCR4 is usually primarily confined to the plasma membrane, while ACKR3 mainly resides intracellularly in endosomal compartments.

Accumulating evidence suggests that chemokine receptors, including CXCR4 and ACKR3, may associate into dimers forming homodimers, heteromers, or higher oligomeric complexes, which can alter subcellular location/distribution and allosterically modulate signaling properties of each other and other chemokine receptors (17, 18). In addition, CXCR4 and ACKR3 engage in crosstalk with receptor tyrosine kinase (RTK) families, resulting in the convergence of oncogenic signaling pathways. However, the precise molecular mechanisms linking the CXCL12/CXCR4/ACKR3 axis to the modulation of cell proliferation, survival, migration/invasion, angiogenesis, and stemness in a tumor context are not fully elucidated.

In this review, we focus on the contribution of the CXCL12/CXCR4/ACKR3 axis in the activation of oncogenic signaling networks, the bidirectional crosstalk between receptors and with growth factor receptors, the involvement of the TME, and the axis's potential as a drug target.

Table 1 Modulators of CXCR4, ACKR3, and CXCL12

Modulator	Target(s)	Reference(s)
Natural ligands		
CXCL11	ACKR3	50
CXCL12	CXCR4 and ACKR3	3
MIF	CXCR4 and ACKR3	15
Ubiquitin	CXCR4	146
Adrenomedullin	ACKR3	16
vCCL2	CXCR4	16
Opioids	ACKR3	14a
Small molecules/peptides		
AMD3100 (plerixafor/Mozobil)	CXCR4	149
AMD070 (Mavorixafor), AMD11070	CXCR4	152, **https://clinicaltrials.gov/ct2/show/NCT03995108**
IT1t	CXCR4	146
TG-0054 (Burixafor)	CXCR4	151, **https://clinicaltrials.gov/ct2/show/NCT03786094**
POL6326 (Balixafortide)	CXCR4	150
CCX771, CCX777	ACKR3	11, 154–158
TC140 series	CXCR4 and ACKR3	146
FC series	CXCR4 and ACKR3	146
Pepducins	CXCR4	153
LIT-927	CXCL12	165
Biologics		
Ulocuplumab	CXCR4	159
LY2624587, PF-06747143, F50067	CXCR4	147
11G8	ACKR3	160
VUN400-402(-Fc)	CXCR4	162
Nb1-5	ACKR3	163
X7Ab	ACKR3	164
α-CXCL12 Nb	CXCL12	166
Engineered CXCL12	CXCR4 and ACKR3	167
vCCL2 N terminus mimokines	CXCR4 and ACKR3	168
NOX-A12 (Olaptesed pegol)	CXCL12	**https://clinicaltrials.gov/ct2/show/NCT03168139**

2. FROM PHYSIOLOGY TO PATHOLOGY

Tissue association and cellular expression of CXCR4, ACKR3, and their chemokine and nonchemokine ligands have been recently reviewed, including with respect to the crosstalk and biological functions of these receptors (2, 19). Briefly, while CXCR4 is ubiquitously expressed in nonhematopoietic cells and in virtually all leukocytes, the expression pattern of the ACKR3 protein is poorly characterized and still a matter of debate (20, 21), at least in part because of the technical challenge raised by the predominant intracellular localization of this receptor. ACKR3 and CXCR4 share the singular distinction among chemokine receptors of being essential for life (22–26), which is indicative of the nonredundant and finely tuned activities of the two receptors.

The importance of the proper regulation of CXCR4 activity is illustrated by its inherited mutations in humans, mostly truncations of its C terminus, which cause receptor dysfunctions (i.e., gain of function and impaired desensitization) and are responsible for the warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome (27). A feature of this

rare immunodeficiency is its selective susceptibility to the pathogenesis induced by the human papillomavirus (HPV) (28), an epithelial commensal with oncogenic potential (29–31). Several lines of evidence support a pro-oncogenic role for CXCR4 dysfunctions in the pathogenesis driven by HPV. These include transformation of keratinocytes upon mutant CXCR4 expression (32, 33) as well as the beneficial outcomes of chronic treatment of mice (34) or patients (35–37) with a selective CXCR4 antagonist marketed as a stem cell mobilizer (AMD3100-plerixafor/Mozobil); this agent also corrects the panleukopenia, which is pathognomonic of the syndrome (36, 38). Additionally, spontaneous deletion of the disease allele in a WHIM patient has been associated with the remission of HPV lesions and repopulation of the myeloid lineage (39). These studies strongly suggest that CXCR4 restricts the oncogenic potential of HPV by contributing to the immune surveillance and keratinocyte-intrinsic responses toward these viruses, which are highly prevalent in healthy epithelium.

Identification of somatic WHIM-like *CXCR4* mutations in the rare non-Hodgkin lymphoma Waldenström macroglobulinemia (40, 41) allows one to envision how the multistep process of oncogenesis can generate CXCR4 dysfunctions. This pathogenic process exploits the abnormal expression of CXCL12 reported in HPV-induced lesions (42) and in a broad range of cancers (43). Preliminary results have suggested that coexpression of ACKR3 and CXCR4 in epithelia could prevent CXCR4 desensitization and thus foster CXCR4-dependent signaling (C. Gallego, G. Schlecht-Louf & F. Bachelerie, unpublished data), as proposed in the context of breast cancer (44) (see Section 3). Of note, pro-oncogenic properties were suggested for ACKR3 in Kaposi's sarcoma-associated herpesvirus (HHV-8)-mediated transformation along with the upregulation of both ACKR3 and CXCR4 in HHV-8 lesions that correlated with the severity of the lesions (45, 46). Understanding the pro-oncogenic roles of the receptors will require additional relevant models.

3. CXCR4/ACKR3 SIGNALING NETWORKS IN CANCER CELLS

The CXCL12/CXCR4/ACKR3 signaling pathways have been primarily described in immune cells or in model systems, such as HEK293 cells. However, emerging evidence using different types of cancer cells with endogenous receptor levels points to a nonconventional and heterogeneous scenario with multiple layers of complexity. These layers include the context-specific modulation of ligand levels, heterogeneous patterns of expression, regulation and cellular/subcellular localization of CXCR4/ACKR3 receptors, cell and tumor type–specific receptor interactomes, and intricate crosstalk mechanisms with other transduction networks acting within the TME (4–8, 19, 47).

3.1. CXCR4 and ACKR3 Receptors Display Differential Signaling and Regulation

Canonical CXCR4 signaling includes both G protein–dependent and β-arrestin-dependent routes (**Figure 1*b***). CXCR4 preferentially couples to pertussis toxin–sensitive Gi proteins, triggering a variety of downstream effectors of Gαi and Gβγ subunits, thus leading to the activation of different signaling pathways such as increased calcium levels or the stimulation of PKC-, MAPK-, Src-, and PI3K/AKT-dependent cascades. These pathways are in turn associated with the modulation of cell proliferation, survival, migration/invasion, angiogenesis, and stemness in specific tumor contexts (reviewed in 4, 7, 8). CXCR4 has also been reported to couple to Gα12/13 in metastatic breast cancer cells or to Gαq in immune cells, resulting in the modulation of Rho-related cascades (see 47 and references therein). Upon ligand binding, CXCR4 is phosphorylated by different G protein–coupled receptor kinases (GRKs) at specific serine/threonine intracellular residues (8, 48), thus eliciting recruitment of β-arrestins, receptor uncoupling from G proteins, and internalization and trafficking of ubiquitinated CXCR4 to lysosomes for degradation (49).

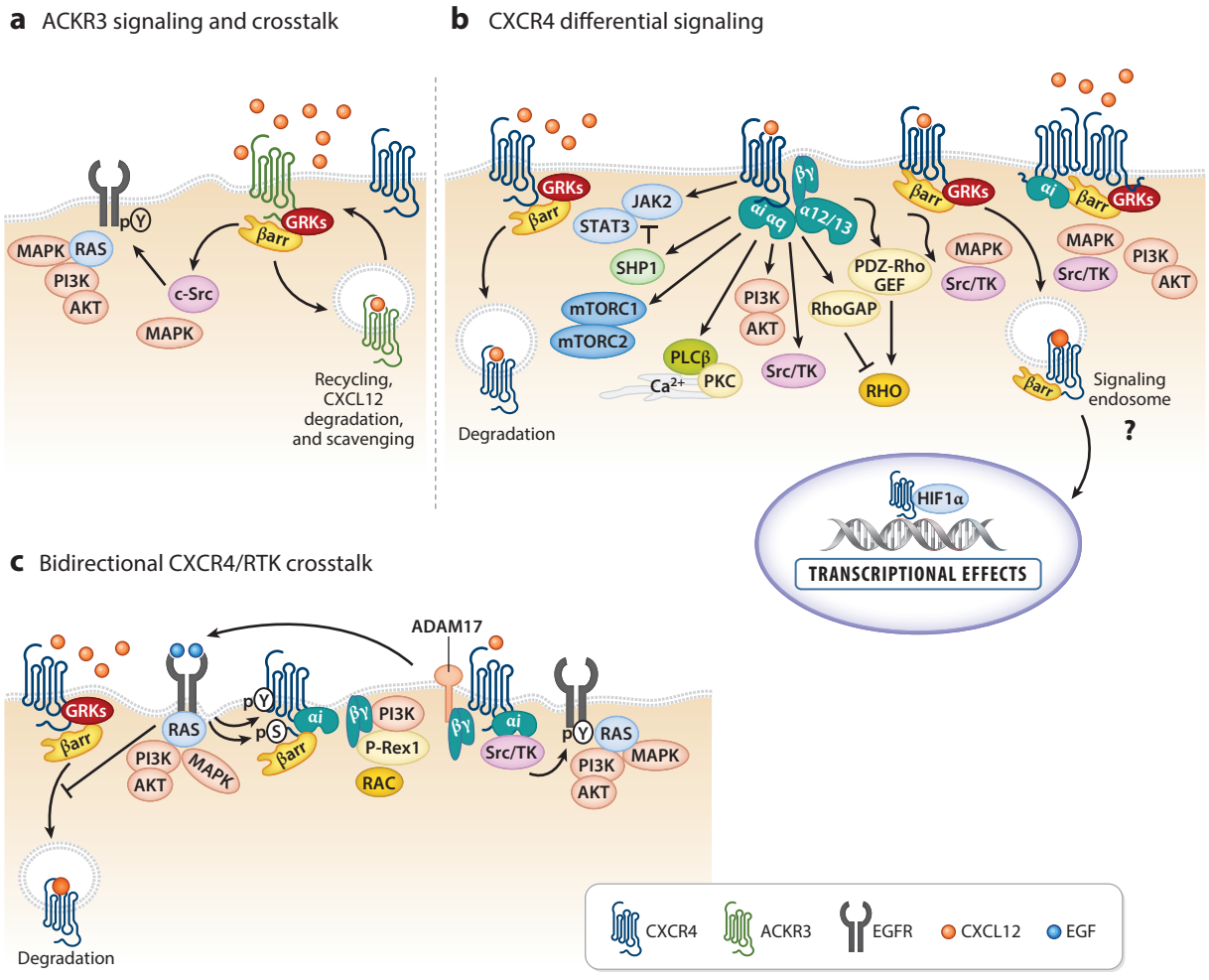

Figure 1

Overview of CXCR4/ACKR3 signaling networks in cancer cells. (*a*,*b*) Upon activation by CXCL12, CXCR4/ACKR3 couple to G proteins and/or the G protein–coupled receptor kinase (GRK)/β-arrestin axis, leading to the activation of major signaling routes such as MAPK, Src tyrosine kinase, and PI3K/AKT or the engagement of cancer type–specific signaling interactomes. Active internalization and intracellular trafficking of these receptors also play a relevant role in the local scavenging of CXCL12 levels, chemokine receptor regulation, and CXCR4/ACKR3 modulatory interactions. The question mark indicates the signaling endosome. (*a*,*c*) The frequent concomitant upregulation of CXCR4/ACKR3 and growth factor receptor tyrosine kinases (RTKs) in several cancer types can create complex bidirectional crosstalk between their transduction networks that can foster hallmarks of cancer. See text for details.

In contrast to CXCR4, ACKR3 is viewed as being unable to activate G proteins (6, 50). Yet ACKR3 can signal through Gi/o proteins in astrocytes and human glioma cells, where these proteins are highly abundant, suggesting that functional interaction occurs in specific contexts (51). ACKR3 triggers β-arrestin-dependent signaling, such as via Src, MAPK, or AKT cascades (52–54). Interestingly, ACKR3, which displays an approximately tenfold higher affinity for CXCL12 compared to CXCR4, shows a marked pattern of constitutive and ligand-dependent internalization and recycling, thus favoring degradation of receptor-bound CXCL12 in different experimental models and in breast cancer cells (50, 55) (**Figure 1*a***). In mouse interneurons,

ACKR3-mediated CXCL12 scavenging is key for cell migration by preventing desensitization and degradation of nearby coexpressed CXCR4, thus fostering CXCR4-dependent cascades (56, 57). Uptake of CXCL12 by ACKR3-positive breast cancer cells increases proliferation and metastatic potential of CXCR4-positive ($CXCR4^+$) cells (44). These data suggest that the impact of CXCL12 scavenging by ACKR3 on surrounding CXCR4 receptors depends on their relative expression levels and whether CXCR4 and ACKR3 are expressed in the same or distinct subpopulations of cancer or stromal cells (6), which is a question for future research.

The mechanisms underlying constitutive and ligand-dependent ACKR3 internalization remain controversial. Although ACKR3 ligands can trigger β-arrestin recruitment in different cell systems, and ACKR3 internalization and chemokine scavenging are dependent on β-arrestin in breast cancer cells (55), constitutive ACKR3 recycling can also occur in a ligand- or β-arrestin-independent manner (47, 58). A recent report showed that in neurons, ACKR3 phosphorylation by GRK2, but not β-arrestin recruitment, is the key event triggering ACKR3-mediated CXCL12 scavenging and subsequent CXCR4 functional regulation (59).

The GRK/β-arrestin axis is key for CXCR4/ACKR3 signaling in cancer cells. ACKR3 phosphorylation by GRK2 is essential for the activation of ERK1/2 and AKT pathways in glial cells (60). The ACKR3/β-arrestin 2 cascade induces cell migration in cholangiocarcinoma cells (61) and melanoma tumorigenesis via activation of c-Src and subsequent VEGF secretion (62). In castration-resistant prostate cancer cells, ACKR3 fosters MAPK signaling in a ligand-independent but β-arrestin 2–dependent way, leading to resistance to androgen receptor antagonists (53). β-Arrestin 2 recruitment to ACKR3 might also affect CXCR4 signaling by triggering ACKR3/CXCR4 cointernalization in breast cancer cells (63).

Given the established role of both β-arrestins and GRKs as signaling hubs relevant to cancer (64–66) and evidence indicating altered expression or function of these proteins in several tumor contexts (67, 68), thorough investigation of their functional interactions with ACKR3/CXCR4 emerges as a very interesting research field. In this regard, GRK3 suppression may contribute to abnormally sustained CXCR4 signaling in glioblastoma (69), some WHIM patient–derived cells (70), and triple-negative breast cancer cells, thus potentiating CXCR4-dependent migration, invasion, and metastasis (71). Of note, while both CXCR4 and GRK2 levels are increased in breast cancer cells (72), GRK3 is decreased, suggesting a differential role for GRK2 and GRK3 and supporting the need for a better characterization of specific CXCR4-GRK interactions in different tumor contexts.

3.2. Molecular Interactions Between CXCR4 and ACKR3

Chemokine receptor output, including that of CXCR4, is controlled by the receptors' monomeric, dimeric, or higher-order oligomeric state in so-called nanoclusters, thus diversifying their functioning and pharmacological tractability (17, 18). CXCR4 and ACKR3 can form homodimers in both ligand-dependent and ligand-independent manners in heterologous expression systems (73–75). CXCR4/ACKR3 heterodimerization modulates signaling via CXCL12 scavenging, affecting the migratory potential of receptors expressed both on the same cells and in *trans* (55, 76), or by modifying CXCR4 signaling output (74, 77). Since overexpression of both CXCR4 and ACKR3 takes place in various tumor cell types, it is tempting to suggest that increased levels of both receptors will promote oligomerization in the two-dimensional confinement of the membrane, which may strengthen its cellular responses and possibly favor alternative coupling to other cascades (78, 79). Insight into the formation of oligomeric states of CXCR4 and ACKR3, and concomitant signaling output thereof, may allow specific targeting of these chemokine receptors in particular phases of oncogenesis.

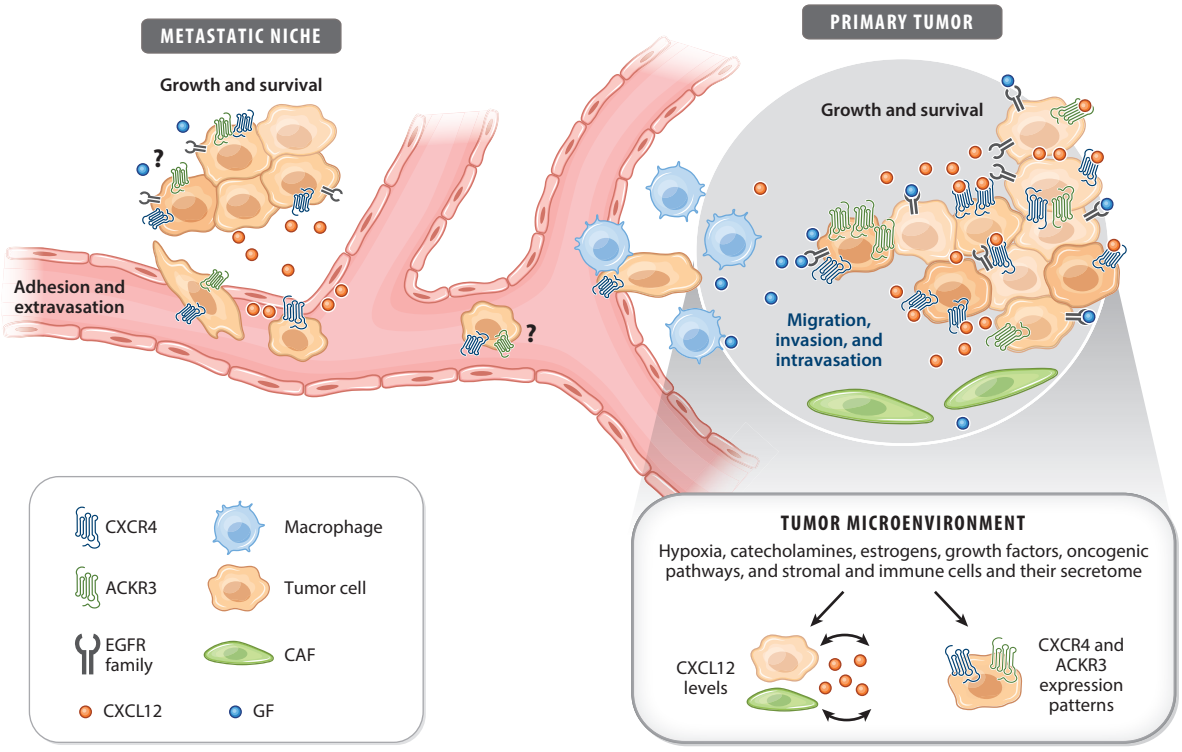

Figure 2

The role of the CXCL12/CXCR4/ACKR3 axis in different stages of tumor progression. CXCL12/CXCR4/ACKR3 signaling is implicated in different phases of tumor progression and involves an array of connecting signaling cascades and complex crosstalk with other cells and factors present in the tumor microenvironment. A variety of signals emanating from tumor type–specific microenvironments can modulate local levels of CXCL12 as well as the heterogeneous patterns of expression and cellular/subcellular localization of CXCR4 and ACKR3 receptors (*lower right box*). The specific CXCL12/CXCR4/ACKR3/RTK/microenvironment signaling networks may change during the different phases of tumor progression and in the diverse tumor niches (primary versus metastatic) in order to promote cell proliferation; angiogenesis; survival or stemness at the primary tumor; local invasion and intravasation; and adhesion/extravasation, growth, and survival at metastatic niches. The potential presence of the ACKR3 receptor in circulating cells and in the metastatic niche is indicated with a question mark. Abbreviations: CAF, cancer-associated fibroblast; EGFR, epidermal growth factor receptor; GF, growth factor.

3.3. Crosstalk of CXCR4/ACKR3 with Oncogenic Signal Transduction Networks

The downstream CXCR4/ACKR3 signaling cascades, G proteins, and/or β-arrestins summarized above and detailed in several comprehensive reviews (4, 5, 8, 80) do not fully explain the complex array of actions of these receptors in promoting cancer hallmarks in different tumor types and oncogenic phases (**Figure 2**). Accumulating evidence points to a role for bidirectional crosstalk with growth factor receptors and other elements present in the TME and of emerging connections with noncanonical signaling cascades.

3.3.1. Bidirectional crosstalk of CXCR4/ACKR3 with receptor tyrosine kinases and tumor microenvironment factors. CXCR4 expression is transcriptionally upregulated by several tumorigenic transcription factors such as Slug, NF-κB, and c-myc in the presence of TGFβ1, TNFα, and estradiol and posttranscriptionally by different microRNAs (miRNAs) in specific cell

types (see 4 and 81 and references therein) (**Figure 2**, *lower right box*). Pathogenic IgGs secreted by tumor-educated B cells trigger CXCR4 expression via a complex HSPA4/ITGB5/Src/NFκB pathway in breast cancer cells, which is critical for lymph node metastasis (82). The HIF1α pathway, which is triggered by tumor-associated hypoxia (83) or downstream of the oncogenic heregulin/Erbb3 cascade (84), enhances *CXCR4* gene transcription in breast cancer cells. Interestingly, in renal cell carcinoma, both HIF1α and CXCL12 promote the nuclear localization of CXCR4 and subsequent CXCR4/HIF1α interaction, thus fostering a hypoxia-like transcriptional program (including CXCL12, CXCR4, and MMP9 metalloprotease expression) and triggering a feed-forward signaling network leading to metastasis (85). Since its presence in cancer cell nuclei has been observed in different tumor types, these data open the way for novel CXCR4 signaling cascades emanating from such intranuclear localization. The functional connection between CXCR4 and hypoxia-governed networks is an interesting venue for future research.

The crosstalk between CXCR4/ACKR3 and RTKs such as ErbB2/ErbB3 or the epidermal growth factor receptor (EGFR) is an active research area, given the frequent concomitant upregulation of these receptors in several cancer types. Cancer-related growth factor receptors can modulate CXCR4 signaling pathways by a variety of mechanisms (**Figure 1*c***). Her2/ErbB2, amplified and overexpressed in ∼30% of breast cancers, enhances CXCR4 protein expression by increasing its rate of translation and preventing CXCL12-induced CXCR4 ubiquitination and degradation (86). Similarly, a frequent activating mutation of the EGFR (L858R) in lung adenocarcinomas upregulates CXCR4 surface expression and facilitates malignant invasion (87). EGFR activation promotes CXCR4 phosphorylation and activity in glioblastoma cells (69). Both epidermal growth factor (EGF) and heregulin trigger CXCR4 serine and tyrosine phosphorylation in breast cancer cell lines, leading to β-arrestin 2 association with CXCR4 and activation of the PRex1/Rac1 axis via Gβγ subunits in a CXCL12-independent way (88). VEGFR2 stimulation in myeloma cells also induces CXCR4 tyrosine phosphorylation and activation (89). Conversely, CXCL12 can enhance EGFR/Her2 functionality in a variety of tumor contexts. This can be accomplished indirectly via CXCL12/CXCR4-mediated activation of membrane-bound proteases, e.g., ADAM17, leading to the release of different EGFR ligands (90), as shown in colon cancer cells (91). Alternatively, activated CXCR4 can lead to tyrosine phosphorylation and EGFR transactivation via Gi/Src pathways, as reported in breast (92, 93), leukemic (94), ovarian (95), and prostate (96) cancer cells (**Figure 1*c***). Similarly, ACKR3 colocalizes with and phosphorylates/modulates EGFR in breast, prostate, and non-small-cell lung cancer (NSCLC) tumor contexts (6, 52, 97–99) via cell type–specific mechanisms often involving β-arrestins (**Figure 1*a***). It would be of interest to explore whether such functional interactions take place in defined membrane microdomains such as lipid rafts.

These findings strongly suggest cooperation between CXCR4/ACKR3 and growth factor receptors in cancers, likely facilitated by the simultaneous increased abundance/functionality of these proteins in many tumor types. Such cooperating networks may extend to other cascades that are upregulated in tumors, such as estrogen receptors (ERs) in breast cancer. ACKR3 increases estrogen signaling in ER-positive breast tumor cells and is associated with tamoxifen insensitivity (100), whereas estrogens appear to favor CXCR4-mediated EGFR transactivation (93). A better understanding of the changing nature and microenvironment of such CXCL12/CXCR4/ACKR3/RTK signaling networks during phases of tumor progression and in primary tumors versus metastatic niches is needed to design improved therapeutic approaches (**Figure 2**).

It has been hypothesized that the bidirectional cross-activation mechanisms between CXCR4/ACKR3 and RTKs might help create ligand-independent constitutive proliferation and survival loops. For instance, in certain breast tumor cells, Her2/Neu expression leads to enhanced

CXCR4 expression and ligand-independent stimulation, in turn fostering EGFR and Her2/Neu activation and a sustained positive feed-forward loop (4, 93). This scenario would be consistent with the frequently observed low responsiveness of tumor cells to exogenous CXCL12 versus the pronounced effects of receptor inhibitors.

A recent report using single-cell imaging of breast cancer cells expressing CXCR4 and signaling reporters emphasized the highly heterogeneous response of cell subsets to CXCL12 (from strong to undetectable) and the role of tumor cell environmental inputs in modulating CXCR4 signaling (101). The CXCR4 signaling status is affected by driver mutations present in specific cells and fine-tuned via conditioning of cells by growth factors. Spinosa et al. (101) suggested that MEK or mTORC1 pathway inhibitors may inadvertently foster prometastatic CXCR4 outputs in some cell subsets by rewiring such crosstalk regulatory networks. This heterogeneous pattern of CXCR4 signaling responses and modulation by tumor environmental signals and chemotherapeutic agents provides a very interesting conceptual framework that should be further investigated in more endogenous experimental systems and mouse models.

3.3.2. Emerging signaling interactomes in specific cancer types. Connections of CXCR4/ACKR3 receptors with oncogenic signaling cascades other than the MAPK or PI3K/AKT routes have been increasingly described. CXCR4 activates mTORC1 in HeLa and breast cancer cells, leading to enhanced migration and metastasis (102, 103) as well as enhanced mTORC2/AKT cascades by promoting DEPTOR degradation in HeLa cells (104). Both CXCR4 and ACKR3 stimulate the STAT3 pathway in breast and prostate tumor cells (see 6 and 105 and references therein) and foster epithelial mesenchymal transition and cancer stem cell features (including high proliferative capacity and resistance to therapeutics) in different tumor cell types (6, 103, 105–108). However, the molecular mechanisms linking CXCR4/ACKR3 to these cellular processes are very heterogeneous and cell type dependent or have not been explored in detail. Novel avenues for the control of cancer cells by CXCR4 are being identified, including the modulation of expression of the long noncoding RNA lncRNAXIST (109) or miR15a/16-1 miRNAs (110), the fine-tuning of metabolic networks (111), and direct interaction with signaling proteins such as LASP1 (112) or PI4KIIIα (113), further indicating the modulation of a large array of signaling cascades via these receptors.

3.4. Local CXCL12 Levels Modulated by Signals Emanating from the Tumor Microenvironment

Different cell types (e.g., epithelial, endothelial, fibroblasts, immune) present in the TME can respond in an autocrine or paracrine way to locally released CXCL12, a key player in stromal-cancer cell crosstalk. In addition, high CXCL12 expression in certain niches (e.g., lymph nodes, lungs, liver, bones) can dictate cancer cell seeding at specific locations during the metastatic process (4, 80, 114) (**Figure 2**, *lower right box*). Although cancer-associated fibroblasts (CAFs) are the main source of CXCL12 in the TME, cancer cells can secrete this chemokine in an autocrine manner (4, 5, 80). CXCL12 is upregulated at the transcriptional level by a variety of protumorigenic transcription factors such as β-catenin, c/EBPβ, Slug, or c-myc by the hypoxic conditions characteristic of many tumors (reviewed in 108) and by different miRNAs, whereas epigenetic silencing via promoter hypermethylation decreases CXCL12 expression (see 4 and references therein).

Moreover, some tumor type–specific factors such as estrogens in breast cancer (93) and PDGF in advanced squamous cell carcinoma (115) induce CXCL12 secretion and subsequent autocrine/paracrine signaling in cancer cells. β_2-adrenergic receptor agonists increase CXCL12 secretion via the HIF1α pathway in osteoblasts, thus contributing to migration and invasion of

prostate cancer cells in a paracrine fashion (116), an action that may underlie the reported effects of catecholamines and chronic stress in tumor progression. Local CXCL12 concentrations might also be modulated by inactivating exopeptidases, as was recently reported by the acceleration of breast cancer metastasis in mouse models upon inhibition of dipeptidyl-peptidase-4 (103).

The constitutive expression of CXCL12 in the TME may contribute to and explain the sometimes modest effects on signaling readouts and tumor hallmarks of exogenously added chemokine in certain isolated and starved cancer cell types versus the clearer effects observed after CXCR4/ACKR3 receptor silencing or pharmacological inhibition, which would impinge on endogenous CXCL12 signaling loops. The sustained presence of CXCL12 in the TME would also favor CXCR4 and ACKR3 internalization and may underlie the frequent presence of these receptors in intracellular compartments in cancer cells (4, 85), thus opening the possibility of localized intracellular/endosomal signaling, as observed for other GPCRs (117) (also see below). Conversely, a local CXCL12 decrease in the primary tumor may favor the spread of cancer cells harboring CXCR4/ACKR3 expression to distant CXCL12-containing niche organs and thus promote metastasis (see 4 and 5 and references therein) (**Figure 2**).

Alternatively, ACKR3 might shape CXCL12 gradients in the process of the CXCR4-promoted dissemination of cancer cells (118, 119) akin to the coordinated expression and function of both receptors during organogenesis, whereby CXCR4-expressing cells migrate through local shaping of CXCL12 extracellular cues (see 56 and 120 and references therein). Additionally, upregulation of ACKR3 in a wide array of cancer cells that abnormally express the receptor at the cell membrane suggests a direct contribution of ACKR3 to tumor formation or spreading. ACKR3 upregulation in tumor-associated endothelial cells (121, 122) might contribute to the *trans*-endothelial migration of CXCR4-expressing cancer cells or the angiogenesis in the TME (reviewed in 123) (**Figure 3**).

4. CXCR4 AND ACKR3 CROSSTALK IN IMMUNE CELLS WITHIN THE TUMOR MICROENVIRONMENT

Studies in the past decade have provided substantial insight into the highly dynamic nature of the TME and its critical importance for the outcome of cancer, including growth, angiogenesis, progression, and metastasis (124). We focus here on the role of the CXCL12/CXCR4 axis in immune cells within the TME, as has been reported in several studies that used CXCR4 inhibitors or modulators.

Several layers of contribution might be envisioned for CXCR4 and CXCL12 in this context, ranging from the recruitment of immune cells to their functional modulation (**Figure 3**). Indeed, CXCL12, via its action on both lymphoid and myeloid $CXCR4^+$ immune cells, might recruit protumorigenic as well as antitumorigenic immune cell populations to the tumor niche (see 125 and references therein). Such CXCR4-mediated recruitment to the TME was observed for B cells, plasmacytoid dendritic cells in ovarian cancer (26, 126), and regulatory T cells (Tregs) in lung adenocarcinoma (127). Of note, intratumoral Tregs express high CXCR4 levels (128, 129), which provides a rationale for blocking this receptor in cancer. Several studies have demonstrated the beneficial effect of CXCR4 antagonists in reducing Treg number and/or suppressive activity while promoting effector antitumor responses and reducing metastasis in models of epithelial ovarian cancer (130, 131) and renal cancer specimens (132), respectively. The demethylation of the Foxp3 promoter was proposed as a possible mechanism by which CXCR4 inhibition modulated Treg function (133).

CXCL12 secreted by multiple myeloma cells was reported to attract $CXCR4^+$ monocytes, which differentiate into M2 macrophages, to the tumor niche, thereby supporting tumor growth (134). In colorectal cancer, the deleterious action of the $Ly6C^{low}$ monocytes subset in the context of

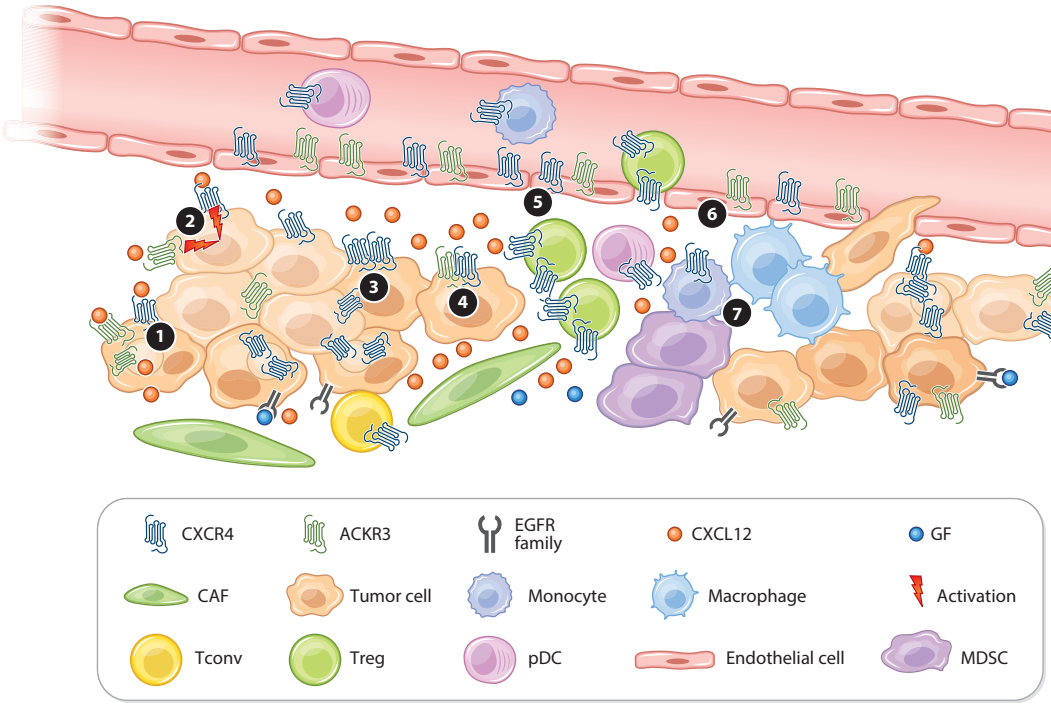

Figure 3

CXCR4/ACKR3 crosstalk in the tumor microenvironment (TME). The main sources of CXCL12 in the TME are the cancer-associated fibroblasts (CAFs) and the cancer cells. CXCR4 and ACKR3 can be highly expressed in cancer cells, either in distinct cell subsets or in the same cell. CXCL12/CXCR4 interactions promote cancer cell stemness, survival, proliferation, and migration. ACKR3 can modulate CXCL12/CXCR4 signaling upon CXCL12 scavenging (①) as well as mediate direct effects on cancer cell stemness and metastasis upon activation of signaling pathways downstream of β-arrestins (②). Whether CXCR4 homodimers (③) or CXCR4/ACKR3 heterodimers (④) can be present in cancer cells is an open question. Tumor endothelial cells can express ACKR3 and may contribute to shaping CXCL12 gradients for cancer and immune cell migration (⑤). T cells [including regulatory (Treg) and conventional (Tconv) populations], plasmacytoid dendritic cells (pDCs), and myeloid-derived suppressor cells (MDSCs) can be recruited into the tumor niche upon CXCR4 engagement (⑥) and may be further skewed toward tolerogenic differentiation (⑦). The extent of ACKR3 expression in immune cells from the TME and its possible role remain to be investigated.

antiangiogenic therapies could be relieved by inhibiting the recruitment of these cells to the tumor niche via CXCR4 blockade (89, 135). In breast cancer, CXCR4 promoted the differentiation of newly arrived motile tumor-associated macrophages into sessile perivascular ones; both subsets collaborated to support cancer cell intravasation (136).

In ovarian cancer, CXCL12 that was produced in response to prostaglandin E2 enhanced the accumulation of myeloid-derived suppressor cells (MDSCs) (137). CXCL12 secreted by CAFs in hepatoma was shown to favor cancer progression upon the induction of MDSCs (138), while the beneficial effect of CXCR4 blockade on liver metastases of colorectal carcinoma was associated with reduced MDSCs in the metastases (139). In glioblastoma, the beneficial effect of combined anti-CXCR4 and anti-PD-1 immunotherapy, associated with reduced MDSCs infiltration (140), highlighted the added value of such a therapeutic strategy (141, 142).

Additional mechanisms may involve CXCL12-induced production of EGF by mononuclear phagocytes, which can favor cancer cell survival and proliferation (143). Moreover, pharmacological agents such as imatinib and nilotinib, tyrosine kinase inhibitors used as first-line treatment

of chronic myeloid leukemia, can selectively increase the cell surface expression of CXCR4 by natural killer cells and monocytes from neuroblastoma patients in vitro (144), a phenomenon also observed for ACKR3 in NSCLC cells upon gefitinib treatment (98), which suggests that such impact should be considered.

Whether and how ACKR3 contributes to the effects attributed to CXCL12 in the tumor niche has largely been overlooked, probably because the encouraging results of CXCR4 blockade supported focused efforts on the CXCL12/CXCR4 axis. Thus, besides the need for characterizing its expression and function in the TME, the role of ACKR3 should also be considered in light of this receptor's ability to modulate CXCL11 levels and recruit CXCR3-dependent cells (145), which is beyond the focus of this review.

5. THERAPEUTIC MODULATORS OF THE CXCR4/ACKR3 AXIS

Small-molecule and biological modulators of CXCR4 and ACKR3 are useful tools to elucidate the role of these receptors in cancer and are potential therapeutics (146, 147) (**Table 1**). Analysis of the structures of chemokine receptors, CXCR4, CCR2, CCR5, CCR9, and US28, cocrystalized with ligands has highlighted the ability of ligands with distinct chemical properties to bind different druggable binding sites on the extracellular and intracellular sites of the receptors (9, 10).

5.1. Small Molecules and Peptide-Based CXCR4 and ACKR3 Modulators

The small molecules AMD3100, AMD11070, and IT1t are currently the most widely used CXCR4 antagonists. The bicyclam AMD3100, identified as an inhibitor of HIV entry, was the first (in 2008) US Food and Drug Administration–approved chemokine receptor ligand for hematopoietic stem cell mobilization in the context of autologous transplantation in patients with non-Hodgkin lymphoma and multiple myeloma (148). Currently, AMD3100 (plerixafor/Mozobil) is in clinical trials for the treatment of WHIM syndrome (37). Besides the AMD3100-like compounds, AMD070 (Mavorixafor), AMD11070, nonmacrocyclic TG-0054 (Burixafor), and cyclo-peptide POL6326 (Balixafortide), which are CXCR4 modulators that display increased bioavailability in vivo, are all in clinical trials for metastatic prostate cancer as sensitizers and/or for the treatment of HIV, WHIM syndrome, and/or breast cancer (149–152; **https://clinicaltrials.gov/ct2/show/NCT03786094**, **https://clinicaltrials.gov/ct2/show/NCT03995108**). While the aforementioned molecules primarily target the receptor's orthosteric binding site, CXCR4-targeting pepducins, which are cell-penetrating lipopeptides mimicking one of the intracellular loops, appear to be allosteric modulators that act as biased agonists and activate G protein–mediated signaling (153).

With respect to ACKR3, a series of small molecules of the CCX series were generated that compete with CXCL12 for ACKR3 binding, induce β-arrestin recruitment, and reduce ACKR3 surface expression (see 146 and references therein). These molecules (mainly CCX771), classified as ACKR3 agonists, inhibit CXCL12-dependent ACKR3 function and decrease tumor growth in various xenograft tumor models (154–158). In addition, ACKR3 antagonists have been patented (WO2018019929), but currently no information has been reported on these molecules in disease-relevant models. Several CXCR4 antagonists (cyclic peptidomimetics and pentapeptides), which bind to ACKR3, albeit with reduced affinities in recruiting β-arrestin, were modified to increase their affinities toward ACKR3 (146). No crystal structure of a ligand:ACKR3 complex has been reported. Nevertheless, the druggable binding pocket of ACKR3 has been visualized using the partial agonist CCX777 (11), which interacts with both the minor and major chemokine binding pockets.

5.2. Biologics Targeting CXCR4 and ACKR3

Monoclonal antibodies targeting RTKs and other targets have been successfully used to treat various types of cancer. In view of their high specificity, bioavailability, immune-based effector functions [i.e., antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)], and the modular function of antibodies fragments, biologics targeting CXCR4 and ACKR3 serve as important tools in fundamental research and are attractive candidates for cancer therapy.

The CXCR4 therapeutic antibody Ulocuplumab, which inhibits CXCR4 signaling and induces cell apoptosis, is under study for the treatment of Waldenström macroglobulinemia and acute myeloid leukemia and is the most advanced of such antibodies in clinical trials (159). Several other anti-CXCR4 antibodies (LY2624587, PF-06747143, and F50067) have also included Fc effector functions to induce ADCC and CDC and are in clinical trials (see references in 147). For ACKR3, the commercially available 11G8 antibody has been used in functional studies to inhibit CXCL12 binding without affecting β-arrestin recruitment and was used in xenograft mice models for positron emission tomography (PET) or single-photon emission computed tomography (SPECT) (160).

Besides conventional antibodies, antibody-derived fragments, including nanobodies (heavy chain–only antibodies), have therapeutic potential (161). Such antibody fragments bind to cryptic sites and can be easily produced and administered via multiple routes (injection, nebulization, and oral and ocular administration), which makes them therapeutically attractive. Various CXCR4-targeting nanobodies and i-bodies (human single-domain antibodies) have been described that target different epitopes on CXCR4 (147). As bivalent moieties, they display enhanced affinities, and their bioavailability can be markedly increased. Coupling such nanobodies to a Fc molecule allowed for the inclusion of an ADCC/CDC effector function, targeting only cells with elevated expression of CXCR4 (162). Trivalent, biparatopic [targeting two distinct epitopes and albumin (trivalent)] ACKR3-targeting nanobodies inhibited CXCL12 binding and decreased tumor growth in a head and neck cancer xenograft mouse model (163). The fusion of anti-ACKR3 single-chain variable fragments with Fc (X7Ab) inhibited CXCL12 signaling and mediated ADCC/CCD when administered with temozolomide, significantly reducing tumor growth and improving overall survival in a mouse glioblastoma model (164). In addition, anti-CXCR4 and ACKR3 antibodies serve as excellent imaging tools that can be labeled fluorescently or radioactively to monitor cells expressing increased levels of CXCR4 or ACKR3 in vivo in PET and SPECT studies.

6. CONCLUSIONS AND PERSPECTIVES

The CXCL12/CXCR4/ACKR3 axis plays an important role in the activation of both canonical and noncanonical signaling networks within tumor cells and the TME. Sustained CXCR4 signaling, as in the WHIM syndrome, appears essential to induce and maintain positive feed-forward loops within the tumor niche. Several tumor-specific factors result in the upregulation of CXCR4, ACKR3, and CXCL12 and the infiltration of immune cells into the TME, further supporting tumor growth. Increases in ACKR3 expression may directly activate oncogenic signaling networks and/or indirectly affect CXCR4 function by scavenging CXCL12 or interfering with the CXCR4 interactome. Challenging questions regarding their contribution, the bidirectional crosstalk with RTKs, and changing expression patterns during tumor progression are outlined in the section titled Future Issues.

Considering the significant expression of CXCR4 and ACKR3 in tumor cells and the TME and their prominent contribution to the activation of oncogenic signaling networks as outlined

above, these receptors are potentially important drug targets in cancer therapy. Encouraging results have been obtained in in vivo cancer model systems, and clinical trials targeting CXCR4 have been initiated.

Besides current approaches that solely target CXCR4, other emerging therapeutic avenues should be considered. CXCR4 is widely expressed in various cell types, but its expression is vastly increased in tumor cells concurrently with enhanced levels/activity of different RTKs. In view of the convergence of CXCR4/ACKR3 and RTK signaling and resistance upon RTK inhibition associated with increased expression of CXCR4 and/or ACKR3, targeting the CXCR4/ACKR3 axis along with certain RTKs is an interesting approach. One might engineer antibodies that are chemically or genetically linked to target both CXCR4 and ACKR3 or RTKs, whose expression is elevated, and that reside in the same nanoclusters in the plasma membranes of tumor cells.

In addition, because of the overexpression of CXCR4 and/or ACKR3 in cells of the TME, targeting those cells, which can exacerbate cancer progression, is an attractive alternative. One may consider linking CXCR4/ACKR3-targeting antibodies or molecules for immunotherapeutic purposes via the incorporation of Fc moieties or coupling to immune checkpoint inhibitors (e.g., PD-1/PD-L1, CTLA-4). By these means, one might selectively target tumor cells and their microenvironment and reactivate the immune system, which is crucial for effective cancer therapy. Incorporating dyes into CXCR4/ACKR3-targeting antibodies/antibody fragments to enable photodynamic therapy upon irradiation could selectively kill tumor cells expressing these receptors, as is effectively done for a viral chemokine receptor (161). Moreover, since both CXCR4 and ACKR3 are activated by CXCL12, one may also consider targeting CXCL12 as a means to inhibit the CXCR4/ACKR3 axis. The small molecules neutralizing CXCL12 activity (chalcone 4 derivative LIT-927) (165); neutralizing CXCL12 nanobodies (166) and engineered CXCL12 variants (167) or the N terminus of vCCL2 (mimokines) (168) favoring binding to CXCR4 or ACKR3; and pegylated L-oligoribonucleotide [NOX-A12 (Olaptesed pegol)] interfering with the binding of CXCL12 to cell surface glycosaminoglycans tested in combination with immunotherapies (**https://clinicaltrials.gov/ct2/show/NCT03168139**), which is associated with increased oral availability and proteolytic resistance, have therapeutic promise.

In view of the prominent crosstalk of the CXCL12/CXCR4/ACKR3 axis with RTKs in cancer, targeting these classes of receptors and/or CXCL12 is an attractive approach to consider in multitargeted cancer therapies. Further research is essential to obtain insight into the CXCL12/CXCR4/ACKR3/RTK signaling networks and cellular plasticity in the primary tumor and metastatic niches during different phases of tumor progression. These efforts will be important to designing effective therapeutic approaches.

FUTURE ISSUES

1. What is the spatiotemporal expression of CXCR4/ACKR3 and CXCL12 in the different phases of oncogenesis and within the primary tumor, tumor microenvironment, and metastatic niche?
2. Which factors modulate expression of CXCL12/CXCR4/ACKR3?
3. What are the roles of ACKR3, CXCL11, and other ACKR3 interactors in oncogenic signaling?
4. To what extent is the endosomal/intracellular CXCR4/ACKR3 fraction implicated in oncogenic signaling?

5. How do G protein–coupled receptor kinases differentially regulate CXCR4/ACKR3 function in tumor contexts?
6. How do the oligomeric states of CXCR4 and ACKR3 and concomitant signaling contribute to particular phases of oncogenesis?
7. How do CXCR4/ACKR3 signaling networks and their crosstalk with receptor tyrosine kinases contribute to cancer progression?

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