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REVIEW ARTICLE

Kallikrein-related peptidases (KLKs) and the hallmarks of cancer

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Abstract

The kallikrein-related peptidases (KLKs) represent the largest family of serine proteases within the human genome and are expressed in various tissues. Although they regulate several important physiological functions, KLKs have also been implicated in numerous pathophysiological processes, including cancer. Growing evidence describing the deregulation of KLK expression and secretion, as well as activation in various malignancies, has uncovered their potential as mediators of cancer progression, biomarkers of disease and as candidate therapeutic targets. The diversity of signalling pathways and proteolytic cascades involving KLKs and their downstream targets appears to affect cancer biology through multiple mechanisms, including those related to the hallmarks of cancer. The aim of this review is to provide an update on the importance of KLK-driven molecular pathways in relation to cancer cell traits associated with the hallmarks of cancer and to highlight their potential in personalized therapeutics.

Keywords

Kallikreins, cancer hallmarks, metastasis, invasion, therapeutics

History

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Abbreviations: Akt/eNOS: Akt/endothelial nitric oxide synthase (eNOS) pathway; **AR**: androgen receptor; **ARA-70**: AR-associated protein 70; **beta-cat**: beta-catenin; **B1R/B2R**: bradykinin receptors 1/2; **BK**: bradykinin; **CTL**: cytotoxic T cells; **ECM proteins**: extracellular matrix proteins; **EMT**: epithelial-mesenchymal transition; **ERKs**: extracellular signal-regulated protein kinases; **bFGF**: basic fibroblast growth factor; **HIF-1**: hypoxia-inducible factor-1; **HKa**: high-molecular-weight kininogen; **IFN-d**: interferon-d; **IGF(BP)**: insulin growth factor (binding protein); **IL-1beta**: interleukin-1beta; **IL-6**: interleukin-6; **KBP**: kallikrein-binding protein; **KLK**: kallikrein-related peptidase; **MAC**: membrane attack complex; **MAPK**: mitogen-activated protein kinase; **mCRPs**: membrane complement regulatory proteins; **MHC-II**: major histocompatibility complex II; **MMPs**: matrix metalloproteinases; **mTOR**: phosphoinositide 3-kinase(PI3K)/protein kinase B/mammalian target of rapamycin, NO/cGMP; **Nrf2**: nuclear factor (erythroid-2)-related factor 2; **PAR**: proteinase-activated receptor; **PDGF-beta/PDGFR-beta**: platelet-derived growth factor-beta and receptor; **PGE2**: prostaglandin E2; **PI3K/Akt**: phosphatidylinositol-3 kinase-Akt; **PKC**: protein kinase C; **PLZF**: promyelocytic leukemia zinc finger protein; **pro-MMP9**: pro-matrix metalloproteinase-9; **RB**: retinoblastoma; **TCR**: T-cell receptor; **TCF**: T cell factor; **TGF-beta/TGFR-beta**: transforming growth factor B and its receptor; **TNF-alpha**: tumor necrosis factor-alpha; **uPA/uPAR**: urokinase-type plasminogen activator and uPA receptor; **VEGF**: vascular endothelial growth factor; **VEGFR**: vascular endothelial growth factor receptor

Introduction

Human kallikrein-related peptidases (KLKs) constitute a subgroup of 15 secreted trypsin- or chymotrypsin-like serine proteases, with diverse tissue expression patterns and biological roles[1]. Although KLKs participate in many physiological processes, including homeostatic control of blood pressure, skin desquamation, semen liquefaction, tooth enamel formation and neural development, their aberrant regulation and excessive protease activity have been linked to diverse diseases, including neurodegeneration, inflammatory skin conditions and cancer[2].

Following protein translation, KLKs are expressed as pre-pro-enzymes, as the signal (pre) sequence is cleaved from the N-terminus prior to secretion. Pro-peptide removal is a prerequisite for enzyme activation[3], which occurs via

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Table 1. KLKs implicated in hallmarks related to cancer cell growth and survival.

Cancer hallmark	Involved KLK	Cancer promoting or inhibiting function	Implicated pathway	Reference
Proliferative signalling	KLK2–5, 14	Promotion	Cleaves IGFBPs	11–13
	KLK14, KLK2, KLK4, KLK6	Promotion	ERK1/2 signalling via PAR	15–19
	KLK1	Promotion	EGFR phosphorylation via KLK–kinin pathway	1,23
	KLK2, KLK4	Promotion	AR and mTOR signalling	24,25
Growth-suppressor evasion	KLK3	Promotion	p53 pathway	26
	KLK10	Inhibition	Promoter hypermethylation	27–29
	KLK3	Inhibition	Suppression of uPA, VEGF; induction of IFN	30
Resistance of cell death	KLK3	Promotion	Enhance AR-dependent p53 suppression	26
	KLK2	Promotion	Bax/bcl2/caspase-3 pathway	24
	KLK1	Promotion	Suppresses caspase-3 and -9, induces Akt phosphorylation, promotes VEGF secretion	35
			Activates PAR1	36,37
	KLK4, KLK6	Promotion	Hormone receptors	41
Replicative immortality	KLK3	Promotion		

autocatalytic activity, activation by other KLKs or by cross-activation by other proteases through a network of protease relays that have been termed ''proteolytic cascades''. Proteolytic activity is irreversible and is tightly regulated by post-translational mechanisms, including endogenous inhibitors, such as serpins and Kazal-type inhibitors[4]. As a result, uncontrolled proteolytic activity is often observed in various diseases.

During cancer, KLKs are capable of exerting both tumor-promoting and tumor-suppressive effects. In many studies, KLKs have been shown to function as tumor suppressors, as in the case of *KLK10*, which is frequently down-regulated in acute lymphoblastic leukaemia and breast cancer[5,6], and *KLK3*, whose down-regulation may be associated with more aggressive forms of prostate cancer[7]. On the other hand, KLKs may contribute to the malignant phenotype by regulating angiogenesis, invasiveness and metastasis of malignant cells, either directly or indirectly through remodelling of extracellular matrix (ECM) components and activation of signal transduction cascades[8]. Additionally, KLKs may function through cross-talk with other signal transduction pathways, such as, urokinase-type plasminogen activator (uPA), protease-activated receptors (PARs) and matrix metalloproteinases (MMPs), thus participating in an extensive molecular network[9]. As such, KLKs may be involved in the acquisition of biological traits that allow cancer cells to survive, proliferate and disseminate, otherwise known as the hallmarks of cancer[10].

The hallmarks of cancer provide a conceptual framework for understanding the biological diversity of cancer and the multistep processes that transform cells from a normal to neoplastic state. A better understanding of cancer development during the last decade has resulted in an updated list of cancer hallmarks, which include: 1) sustaining proliferative signalling, 2) evading growth suppressors, 3) resisting cell death, 4) enabling replicative immortality, 5) inducing angiogenesis, 6) activating invasion and metastasis, 7) reprogramming of energy metabolism and 8) evading immune destruction[10]. KLKs may promote or inhibit neoplastic progression by acting individually and/or in cascades with other KLKs and other proteases. In this review, we detail for the first time, an overview of molecular strategies elicited by KLKs and their degradome, collectively involved in the acquisition of these traits. We anticipate that the widespread recognition of KLK involvement in cancer may facilitate the development of new cancer therapies that center around targeting these proteases.

KLKs and the hallmarks of cancer

Sustaining proliferative signalling

Uncontrollable cell proliferation and evasion of growth-suppressing signals are key factors for the development and progression of neoplastic diseases. Cancer cells can acquire these capabilities in various ways, mainly through mechanisms involving autocrine or paracrine growth factor signalling[10]. Interestingly, KLKs have been documented for their role in sustaining proliferative signalling (Table 1)[1]. Here, we revisit both traditional and novel molecular mechanisms involved in this process, as illustrated in Figure 1.

Kallikrein-mediated proliferation/growth signalling through conventional pathways

KLKs may participate in early neoplastic progression by regulating tumor cell proliferation and growth, mainly by modulating key signalling elements, such as insulin-like growth factors (IGFs)[3]. For instance, certain KLKs, such as KLK2, -3, -4, -5 and -14 may cleave a number of IGF-binding proteins (IGFBPs), leading to increased availability of IGFs that can bind to, and activate corresponding receptors, which in turn, modulate cell survival, mitogenesis and differentiation[11,12]. More specifically, in prostate cancer, KLK3 was shown to cleave IGFBP3, resulting in the dissociation of the IGF1–IGFBP3 complex. This allows IGF1 to bind to the IGF1 receptor, which stimulates cancer cell proliferation[13].

Kallikrein-mediated proliferation/growth signalling through PARs

KLKs may also serve as signalling molecules of specific membrane receptors, namely the PARs. PARs belong to the G protein-coupled family of receptors, and upon activation, can mediate tumorigenic signalling in prostate and colon cancer[14]. Interestingly, during colon cancer proliferation, KLK14

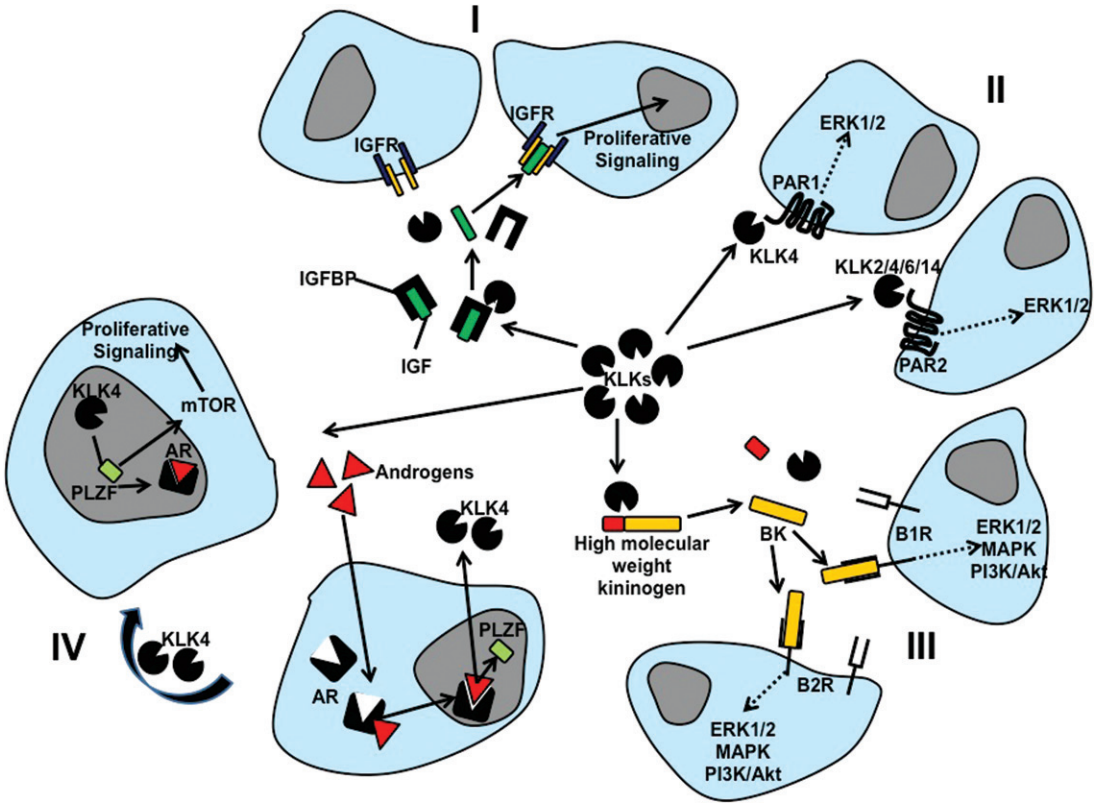

Figure 1. Kallikrein-mediated proliferation/growth signalling through: (I) conventional pathways (i.e. IGFs), (II) PAR signalling, (III) kallikrein/kinin signalling and (IV) steroid hormone signalling. See abbreviation list and text for more details. Molecules are not drawn to scale.

displays similar activity to known PAR activators. As a potent promoter of PAR2 signalling, KLK14 may lead to extracellular signal-regulated kinase-1 and -2 (ERK1/2) activation[15]. Likewise, it has also been demonstrated that KLK4 can induce proliferative signalling through different PARs in colon and prostate cancer[16,17]. The importance of this signalling cascade in prostate cancer development has been further underscored by the fact that the prostate-specific kallikreins, KLK2 and KLK4, directly stimulate prostate cancer cell proliferation through PAR1 and/or PAR2[18]. Moreover, recombinant KLK2 and KLK4 can also activate ERK1/2 signalling of PAR1- and PAR2-expressing prostate cancer cells *in vitro*. Additionally, KLK6 may also promote the proliferation of lung tumoral cells via PAR-2 signalling activation[19].

Kallikrein-mediated proliferation/growth signalling through kallikrein–kinin signalling

In general, kinins are locally released from their parental molecules, the kininogens, through limited proteolysis by kallikreins[20]. Bradykinin (BK), an active peptide produced by the kallikrein–kinin system, exerts diverse functions via BK receptors-1 (B1R) and -2 (B2R)[20]. Certain cancer types (i.e. prostate, breast and lung) largely depend on an autocrine BK signalling loop to stimulate their growth[21]. BK was proposed as a potent mitogen in MCF7 breast cancer cells, utilizing traditional downstream signalling relays, such as protein kinase C (PKC), phosphatidylinositol-3 kinase-Akt (PI3K/Akt) and signal-regulated kinase ERK1/2 pathways, as well as through the activation of the mitogen-activated protein kinase (MAPK) pathways[22].

Given that KLK1 regulates the kallikrein–kinin pathway, it may be an important modulator of cell proliferation[1]. It was previously shown in a human neuroblastoma cell line that EGFR phosphorylation may be mediated by the KLK1-induced kallikrein–kinin system and may result in subsequent activation of downstream signalling cascades that modulate cell growth and proliferation signalling cascades (i.e. ERK1/2)[23].

Kallikrein-mediated proliferation/growth signalling through steroid hormones

It is well established that steroid hormones are implicated in various hormone-related malignancies and are also capable of regulating KLK expression[24]. For instance, *KLK2* gene expression is regulated by androgen receptor (AR) signalling. Moreover, recent evidence suggests that KLK2 may enhance prostate cancer growth by acting in conjunction with the AR coregulator, ARA70, to promote AR transactivation[24]. Meanwhile, other studies have also shown that the activities of AR and mTOR signalling proliferative pathways in prostate cancer are maintained by KLK4 and promyelocytic leukemia zinc finger (PLZF)[25]. Under normal conditions, PLZF acts as a transcription factor to inhibit *AR*, while also preventing mTORC1 signalling by increasing expression of its inhibitor. Through direct interactions, KLK4 associates with PLZF and inhibits its action, thereby keeping AR and mTORC1 signalling active. Consequently, *KLK4* knockdown results in

a decline in prostate cancer cell proliferation *in vitro* and *in vivo*, induces apoptosis, and decreases anchorage-independent growth[25].

Evading growth suppressors

Evasion from suppressive growth signalling is another prominent hallmark of most cancers and is often viewed in close conjunction with the previous hallmark. Although certain tumor suppressors, such as p53 and retinoblastoma (RB) have pivotal roles in cell proliferation, new evidence suggests that they operate as part of a larger network[10]. Here we propose that certain KLKs could have particularly important roles in such networks (Table 1).

Without involving protease activity, KLK3 mediates ARA70-induced AR transactivation via modulating the p53 pathway, thus resulting in decreased apoptosis and increased cell proliferation in prostate cancer cells. Consequently, increased levels of KLK3 could enhance the p53 pathway in prostate cancer to achieve suppression of anti-growth signalling[26].

In addition to regulating traditional tumor suppressors, as in the case of the KLK3/p53 axis, KLKs may also act as tumor suppressors themselves. For instance, *KLK10* is down-regulated in several tumors and has been identified as a candidate tumor-suppressor gene[27]. *KLK10*-transfected breast cancer cells show reduced proliferative activity and diminished potential to generate tumors in nude mice[28]. The acquisition of this hallmark is often mediated through loss of *KLK10* expression in human malignancies by hypermethylation of the *KLK10* promoter region[5,29]. Moreover, KLK3 has also been implicated in tumor growth suppression *in vivo*[30]. The administration of KLK3 in the PC3M prostate cancer cell line led to the suppression of tumor growth promoters [i.e. uPA, vascular endothelial growth factor (VEGF)] and the induction of well-known tumor-suppressor genes [i.e. interferon-d (*IFN-d*)][30].

Nevertheless, evidence to support a definitive role for KLK3 and KLK10 as tumor-defying enzymes is still preliminary, and the extent and nature of the observed suppressive mechanism(s) of action are still obscure. Consequently, the ability of a given *KLK* to act as a tumor suppressor or a tumor-promoting gene may very likely depend on the specific microenvironment where they are released and activated.

Resisting cell death

Programmed cell death is a natural barrier to the development of cancer and as such, molecular strategies developed by cancer cells to evade apoptosis are an important hallmark of the disease[10]. Unfortunately, most aggressive cancers harbor inactivating mutations of *TP53* and *RB*, which are two important regulators of apoptosis[31]. This often results in uncontrolled cell division. Nevertheless, the increased expression of anti-apoptotic regulators (e.g. Bcl-2) or survival signals [e.g. Interleukin-6 (IL6)] and the decreased expression of pro-apoptotic regulators (e.g. Bax and Bak) may also reflect alternative strategies for the acquisition of resistance to cell death. Recent studies have demonstrated that KLKs may play a direct or indirect role in these apoptotic mechanisms (Table 1)[10].

Kallikrein-mediated regulation of pro- and anti-apoptotic pathways

A recent study revealed that the mechanism of action of intermittent androgen deprivation treatment in prostate cancer patients is through the induction of apoptosis. However, androgen independent cancers that become resistant to hormone deprivation are able to survive by targeting this mechanism[32]. Previous studies have suggested that KLKs play a role in acquired resistance by regulating apoptosis via AR pathways. As previously mentioned, KLK3 could enhance AR-dependent p53 suppression, resulting in cell survival via suppression of apoptosis[26]. Moreover, KLK2 combined with the AR coregulator, ARA70, may promote evasion of apoptosis via the bax/bcl2/caspase-3 pathway, thus leading to a higher rate of cell proliferation. The addition of functional *KLK2* cDNA into high passaged LNCaP cells led to increased cell growth, whereas knockdown of *KLK2* expression resulted in increased cell apoptosis with cell growth arrest at the G1 phase[24]. Protease inhibitors have also been shown to regulate apoptosis in a similar way[33]. For instance, the kallikrein inhibitor, rBbKIm, has a deleterious effect on DU145 and PC3 prostate cancer cell lines[34], suggesting interference with cell survival. Mechanistically, caspase-3, but not caspase-9 activation has been proposed, although apoptosis may be activated via mitochondrial release of cytochrome c, as well[34].

KLK1 gene transfer has also been shown to protect against apoptosis and oxidative stress in non-cancerous cells. The overexpression of *KLK1* inhibits oxidative stress and apoptosis in cultured human endothelial progenitor cells, specifically through suppressing the activation of caspase-3 and -9, inducing Akt phosphorylation and promoting aberrant secretion of VEGF[35]. The potential of a similar mechanism in cancerous cells remains to be investigated.

Kallikrein-mediated regulation of survival pathways

Increasing expression of survival signals in cancer cells is another strategy to evade apoptosis. As already mentioned, KLKs are potential activators of PARs. Notably, it was recently shown that the mechanism by which KLK6 promotes the resistance to apoptosis in glioblastoma multiforme (GBM) is through PAR1-mediated activation. PARs, in turn, may induce the release of numerous cytokines[36], as well as other kallikreins via paracrine signalling. For instance, PAR1 activation by KLK4 leads to an induction of interleukin 6 (IL-6) expression, which is implicated in prostate cancer development and progression. The release of IL-6 into the tumor microenvironment provides a survival advantage to cancer cells and increases the production of KLKs from the adjacent cancer population[37].

Enabling replicative immortality

In normal cells, senescence is associated with the progressive loss of telomeres with each round of cell division. Eventually, they lose the ability to protect the ends of chromosomes from end-to-end fusions, leading to apoptosis. Cancer cells usually overcome telomere attrition by activating telomerase or through an alternative pathway for telomere lengthening

(ALT), thus acquiring replicative immortality[38]. Little can be speculated about the role of KLKs, if any, for this specific hallmark (Table 1).

Hormone-related pathways modulating KLK regulation may also potentially affect chromosome length as estrogen and progesterone have been shown to enhance telomerase activity[39]. Since estrogens and progestins regulate KLKs in a number of model systems[40], it is possible that an estrogen/progesterone–kallikrein–telomerase axis exists in cancer. In support of such hypotheses, recent studies revealed an inverse correlation between relative telomerase activity and KLK3 protein levels, and such differential expression was under the control of steroid hormone receptors in breast cancer[41].

In addition, telomerase has been documented to interfere with extra-telomeric tumor-promoting pathways[42]. For instance, TERT, otherwise known as telomerase reverse transcriptase, can function as a transcriptional modulator of Wnt/beta-catenin signalling for cell development and proliferation, in combination with BRG1 (a SWI/SNF-related chromatin remodeling protein), as part of a beta-catenin transcriptional complex. By activating the beta-catenin/TCF pathway, COX-2/PGE2 signalling might contribute to limitless replicative potential, thus enhancing cell survival and growth. Recent findings also indicated that beta-catenin signalling capacity reflects ligand-dependent AR function in prostate cancer cells. Specifically, beta-catenin/TCF-related transcription can be inhibited by androgen treatment[43]. Therefore, KLK3 might indirectly affect the function of these signalling pathways by controlling the AR levels in androgen-stimulated prostate cancer cells.

Additional evidence supports an alternate role for TERT in the regulation of apoptosis in a telomere-independent manner. The overexpression of TERT suppresses programmed cell death, whereas TERT down-regulation enhances the mitochondrial apoptotic pathway through post-translational activation of Bax[39]. Moreover, mice lacking telomerase activity are resistant to cancer development in the presence of an intact p53 pathway[44], whereas forced TERT overexpression is associated with increased incidence of spontaneous tumors[45]. Other studies have also shown that damaged telomeres activate the canonical DNA damage response, and as such, they trigger p53[38]. p53 may then induce apoptosis or senescence if the telomere damage persists, and can also promote BK B2R transcription, which, in turn, is required to prevent uncontrolled p53 activation and cell death[46]. Consequently, a link between KLKs and p53 via telomerase activity might be the key in controlling cell death and/or senescence, and needs to be further addressed.

Inducing angiogenesis

Angiogenesis is necessary for tumor maintenance and metastasis, and results in major changes in the morphology and function of endothelial cells (ECs). These changes include the interaction of the parental vessel's endothelium with stromal/tumor cells and the basement membrane, ECM remodeling and migration, as well as EC proliferation, differentiation and structural polarity towards formation of new blood vessels[10]. It is well known that various proteolytic enzymes that are critical to ECM degradation and remodelling are also participating in the angiogenic switch[10]. As such, human KLKs are now considered to be powerful modulators of angiogenesis by either promoting or inhibiting its underlying mechanisms (Table 3)[1].

Kallikrein-mediated angiogenesis through proteolytic remodeling of ECM

ECM remodelling is necessary for tumour angiogenesis, and is carried out by the concerted functions of proteolytic cascades, i.e. MMPs, and uPA pathways, which, in turn, may be regulated by KLKs. For instance, KLK1 activates the type IV collagenases, pro-MMP-1, -2 and -9, which cleave major components of the basement membrane[47] (Figure 2A). Along the same lines, the activation of the uPA–uPAR system is regulated by KLK2, -4 and -12 and results in the degradation of ECM components through plasmin formation[48,49]. Plasmin degrades a large number of ECM proteins and activates latent collagenases[50], accompanied by the release and/or activation of proangiogenic growth factors (i.e. VEGF) and pro-MMPs (Figure 2A). As a result, proteolytic remodelling of the ECM may not only facilitate EC migration during angiogenesis, but may also modify the activity status and sequestration of growth factors related to angiogenesis within the ECM. Moreover, KLK12 has been shown to cleave several members of the CCN family, which are matricellular proteins that contain multiple domains capable of interacting and modulating the bioavailability and/or activity of various growth factors, such as VEGF[51].

Kallikrein-mediated angiogenesis through conventional angiogenic pathways

One of the major molecular pathways that regulate angiogenesis is the platelet-derived growth factor (PDGF)–beta/PDGFR pathway[52]. Hypoxic conditions may increase KLK12 synthesis in cancer cells, as well as PDGF-beta, through hypoxia-inducible factor (HIF)-1 pathway activation[53,54] (Figure 2B). An analysis of the secreted proteome of ECs treated with KLK12 showed that this protease may convert the ECM- or membrane-bound precursor of PDGF-beta into its soluble form. Subsequent reports showed that the release of PDGF-beta by KLK12 leads to the fibroblast-mediated secretion of VEGF-A (Figure 2B), which is important for EC differentiation and the formation of capillary structures during angiogenesis[54].

In the majority of cases, KLKs promote angiogenesis, however, certain KLKs, such as KLK3 are able to suppress it. Although KLK3 might directly promote angiogenesis by activating the pro-angiogenic growth factor TGF-beta[55] (Figure 2B), it also displays potent anti-angiogenic activity *in vitro* and *in vivo*[56], which occurs through angiostatin generation via enzyme-mediated proteolysis. In addition to KLK3, *in vitro* studies suggest that KLK5, -6 and -13 can potentially generate angiostatin-like fragments from plasminogen[9].

Other less conventional but well-established bypass mediators of angiogenesis, such as galectin-3, are also regulated by KLK activity[57]. For instance, galectin-3 is cleaved by KLK3 and controls VEGF- and bFGF-mediated angiogenic responses[58]. Moreover, previous studies have shown that

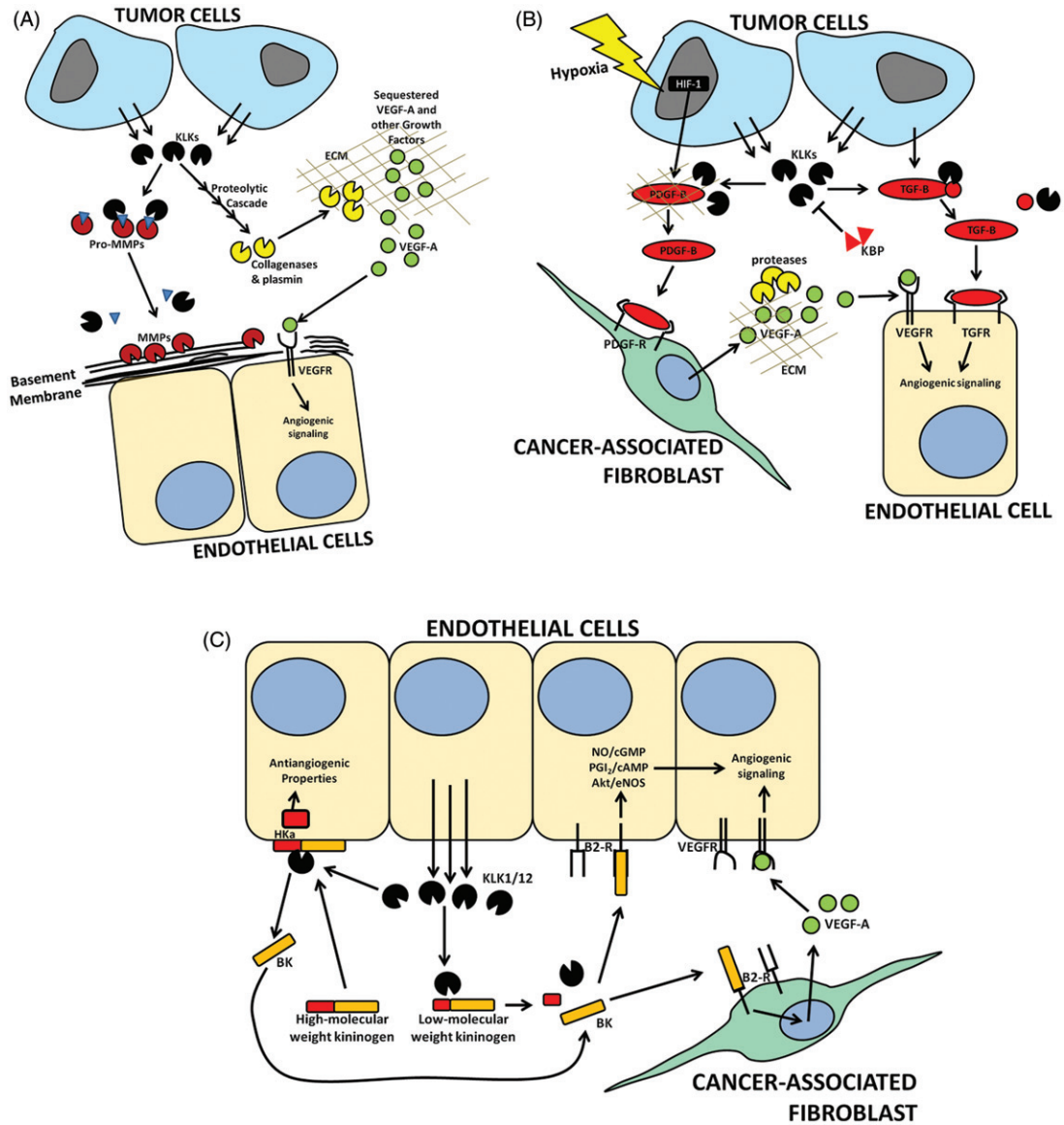

Figure 2. Involvement of KLKs in angiogenesis through (A) the proteolytic remodeling of ECM, (B) the conventional angiogenic pathways and (C) the kinin–kallikrein pathway. See abbreviation list and text for more details. Molecules are not drawn to scale.

kallikrein-binding protein (KBP), an angiogenesis inhibitor, may induce the down-regulation of VEGF in tumor cells via inhibition of HIF-1alpha (Figure 2B) [directly by inhibiting HIF-1alpha expression or indirectly by affecting HIF-1alpha degradation][59], but such mechanisms need to be further elucidated.

Kallikrein-mediated angiogenesis through the kinin–kallikrein pathway

A functional kallikrein–kinin system may also be involved in tumor angiogenesis[60], which includes KLK1, -2 and -12[49]. These members have been shown to cleave low molecular weight kininogen to release Lys-BK[9]. BK stimulates angiogenesis in ECs due to B2-dependent increase in vascular permeability at early stages or B2-dependent up-regulation of VEGF in the stromal fibroblasts at later stages of carcinogenesis (Figure 2C). Therefore, active kinin promotes angiogenesis by up-regulation of bFGF or stimulation of VEGF[61]. The kinin signal transduction is mediated by the B2 receptor in ECs, where the binding of kinins activates intracellular nitric oxide (NO)-cGMP and prostacyclin-cAMP, two prominent angiogenic signalling cascades[62]. Previous studies have demonstrated that KLK1 may cleave kininogen, generating kinins to trigger a VEGF-A-independent activation of the Akt/endothelial nitric oxide synthase (eNOS) pathway[35] (Figure 2C).

High-molecular-weight kininogen (HKa) binds to ECs and is subsequently cleaved by KLK1 to release BK[63]. The remaining portion of the molecule is designated ‘‘cleaved high-molecular-weight kininogen’’ (HKa), which has recently been demonstrated to display antiangiogenic effects, thereby opposing the proangiogenic activity of BK[63] (Figure 2C). More specifically, HKa suppresses angiogenesis with the anti-adhesive activity of domain D5 (kininostatin), which has a potent antiangiogenic effect by inhibiting EC proliferation and vessel formation[64]. New studies have shown that the proangiogenic activity of KLK12 is not directly related to kinin-dependent activation of the B2 receptor. Indeed, upon the release of kinins, the resulting fragmented kininogen

Table 2. The role of KLKs during cancer invasion and metastasis.

Cancer hallmark	Involved KLK	Cancer promoting or inhibiting function	Implicated pathway	Reference
Cancer invasion and metastasis	KLK3	Promotion	Proteolysis Osteoblastic	68,69
	KLK6	Promotion	PAR1 signalling	70
	KLK13	Promotion	Cleaves ECM components	71
	KLK1	Promotion	Activates MMPs, kinin receptors	71–75
	KLK2, KLK4	Promotion	Activates uPA/uPAR system	48
	KLK4–7	Promotion	Cleavage of ECM proteins	11,78,79
	KLK7	Promotion	Fibronectin, Integrin adhesion	79,82
	KLK10	Inhibition	Decreased anchorage-independent growth	84
	KLK3, 4	Promotion	EMT	86
	KLK6–7	Promotion	Cleaves E-cadherin	87,88
	KLK6	Inhibition	MET	93

undergoes extensive conformational changes to expose domain D5, but KLK12 may interfere with kininostatin-related antiangiogenic activity by cleaving this particular domain[65].

Cancer invasion and metastasis

The metastatic cascade is a multistep process involving local invasion, intravasation of cancer cells into nearby blood/lymphatic vessels, transit through the lymphatic/hematogenous circulation, extravasation of cancer cells into the parenchyma of distant tissues, formation of micrometastases and finally colonization[10]. Interestingly, KLKs may play a leading role in the regulation of the aforementioned cell-biological programs, facilitating cancer progression, particularly through extracellular hydrolysis of crucial mediators such as cell–cell adhesion proteins, membrane-bound proteins and receptors, cytokines and growth factors, ECM proteins, as well as other KLKs (Table 2)[66].

Kallikrein-mediated regulation of cancer cell invasion and migration

Local proteolysis during the transition from *in situ* to invasive carcinoma is facilitated by proteases outside the tumor cells that are either bound to the tumor cell surface or produced by the host after induction by tumor cells[67]. These proteases degrade various components of the basement membrane, thus aiding in cancer cell invasion[67]. The involvement of KLK3 in prostate cancer cell invasion was demonstrated using a neutralizing KLK3 antibody to decrease matrigel invasion of LNCaP cells[68]. Further analysis suggested that different forms of secreted KLK3 might decrease or increase the invasive properties of prostate cancer cells. *In vivo* studies further indicated that KLK3 in the bone microenvironment may contribute to the osteoblastic phenotype of the lesions[69]. Moreover, the KLK6-PAR1 axis may contribute to melanoma cell invasion, by regulating the active interaction between melanoma cells and microenvironment[70]. As such, KLKs may function differently depending on the surrounding tumor microenvironment (i.e. primary/secondary tumor site).

KLK13 may play a role in tissue remodeling and/or tumor invasion and metastasis. It was previously shown that KLK13 is able to cleave major components of ECM in ovarian cancer cell lines. Moreover, cancer cells secreting KLK13 migrated less when treated with a KLK13 neutralizing antibody[71]. Along the same lines, KLK1 has also been implicated in the growth and invasiveness of many cancer types[72], mainly through two molecular strategies. First, KLK1 directly regulates the activation of critical MMPs important for tumor progression[73]. Second, KLK1 regulates kinin receptor activation, either directly[74] or through the generation of kinins[75], thereby promoting metastatic behavior.

Along with the human tissue KLK family, the serine protease uPA and its inhibitor PAI-1, have been identified as key players in tumor invasion and metastasis within a complex proteolytic network[76]. Interestingly, tumor cells that are shed and migrate during metastasis may be preferentially associated with specific levels of KLK, uPA and/or PAI-1, which may in turn dictate aggressiveness. Certain KLKs, including KLK2 and KLK4, can cleave precursor uPA to generate its active form[48]. Subsequently, uPA-mediated MMP-9 activation and increased invasion may be partly attributed to the plasminogen/plasmin pathway[77]. Interestingly, neutralization of uPA and MMP-9 activities with specific antibodies attenuated cancer cell invasion[77]. Consequently, the diverse functions of KLKs in regulating the various steps of the metastatic cascade are strongly dependent on the ''proteolysis'' context within a given microenvironment. The mechanisms mentioned above are illustrated in Figure 3(A).

Kallikrein-mediated regulation of cell–cell and cell–ECM adhesion

The importance of KLK-mediated cell–ECM adhesion machinery during the metastatic cascade has been shown during the peritoneal dissemination of ovarian cancer cells, where kallikreins 4–7 cleave a subset of ECM proteins [fibronectin (FN), vitronectin (VN), laminin and Type-I and IV collagens[11,78,79]] in the submesothelial matrix (Figure 3B). The resulting cleaved ECM products can contribute to an altered cellular phenotype and cell function by promoting the formation and invasion of ovarian cancer spheroids[80]. Stable overexpression of *KLK4–7* in ovarian cancer cells attenuates cell adhesion to ECM proteins and integrin expression[81]. However, in other studies, it has been reported that *KLK7* overexpression in SKOV3 ovarian cancer cells induced their adhesion to the KLK7 substrate, FN[79], and the level of the corresponding integrin-a5b1 receptor increased[82] (Figure 3B). Moreover, purified human recombinant KLK6 cleaves gelatin

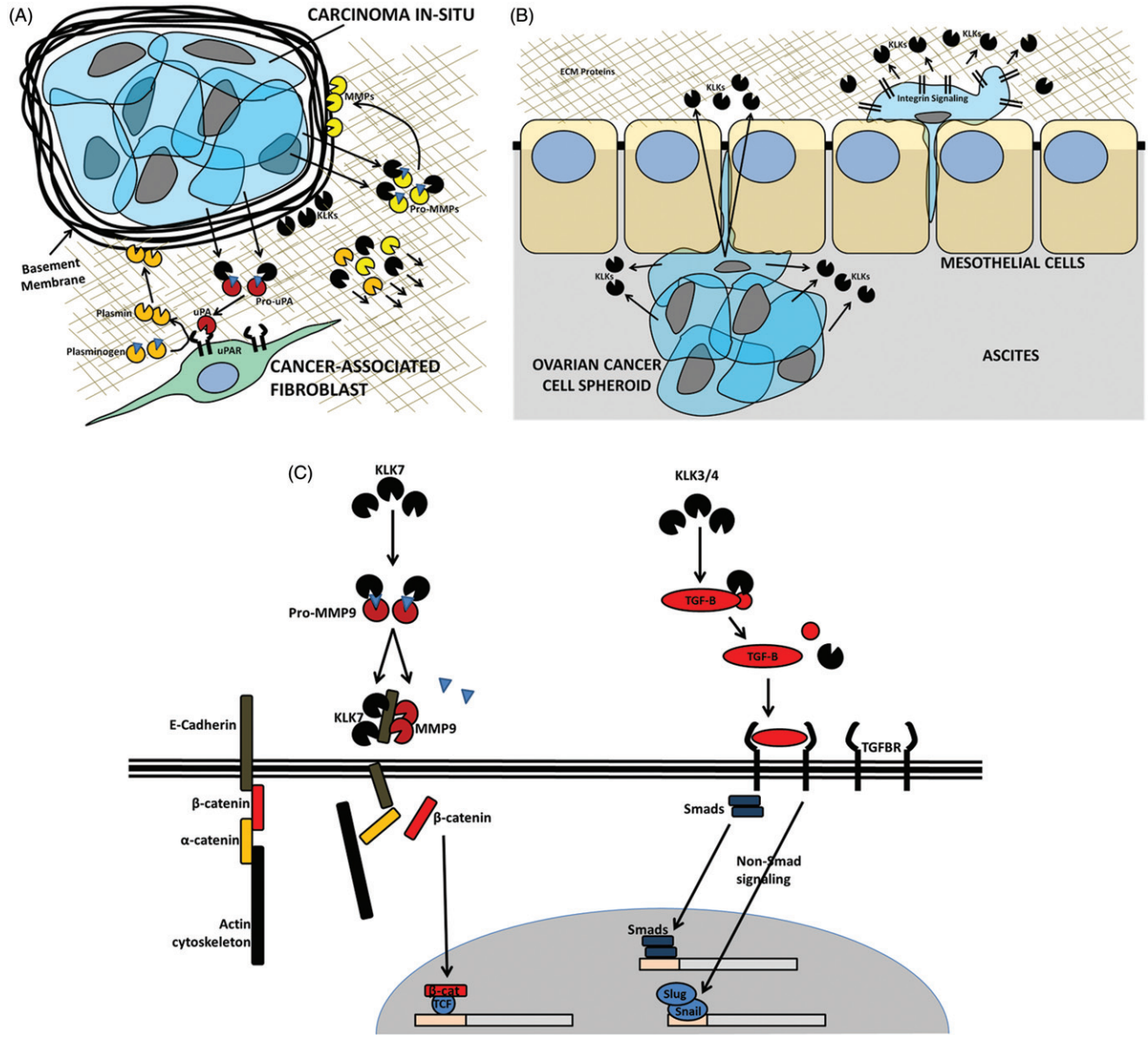

Figure 3. Kallikrein-mediated regulation of cancer cell invasion and metastasis. (A) Proteolytic relationships involving KLKs in the tumor microenvironment. Note that KLKs and the related proteases cleave not only the extracellular matrix proteins, but the basement membrane as well. (B) KLK-mediated cell–cell and cell–ECM adhesion and (C) regulation of epithelial-to-mesenchymal transition by KLKs. See abbreviation list and text for more details. Molecules are not drawn to scale.

in zymography and can efficiently degrade high-molecular-weight ECM proteins such as FN, laminin, VN and collagen[78]. As such, all these data indicate that KLKs may support altered tumor cell–ECM adhesion machinery in the cancer microenvironment.

Apart from their proteolytic action, KLKs have been demonstrated to have non-proteolytic functions during cancer metastasis[66,83]. For instance, during ovarian cancer progression, it has been shown that KLK7 enhances the expression and function of integrin adhesion receptors and both forms of the produced serine protease (KLK7 and the non-proteolytic form) play a role in ovarian cancer peritoneal invasion[82] (Figure 3B). Moreover, over-expression of *KLK10* in ES-2 ovarian cancer cells reduced their anchorage-independent growth *in vitro* and caused smaller tumor burden *in vivo*[84]. Adding recombinant KLK10 protein that lacked catalytic activity into cell cultures confirmed these findings, indicating a non-proteolytic function during disease progression.

Kallikrein-mediated regulation of epithelial-to-mesenchymal transition (EMT)

EMT is necessary for cancer cells to develop an invasive phenotype. The cadherin switch (i.e. decreased E-cadherin and parallel increased N-cadherin) and increased vimentin expression are prominent features associated with EMT[85]. Several studies suggest that KLKs play a role in modulating EMT-related mechanisms. For example, prostate cancer cell lines that were transfected with *KLK3* and *KLK4* demonstrated an altered morphology and increased motility, which were a result of a decreased expression of E-cadherin and increased expression of vimentin[86].

Table 3. KLKs associated with immune evasion, energy metabolism and angiogenesis.

Cancer hallmark	Involved KLK	Cancer promoting or inhibiting function	Implicated pathway	Reference
Immune evasion	KLK3	Promotion	Activates TGFbeta	93
	KLK5, 7	Promotion	Cleaves LL-37	95,96
	KLK14	Promotion	Cleaves C3	97
	KLK3, 4	Inhibition	Induces cytotoxic T cell responses	98–102
Energy metabolism	KLK7	Promotion	Insulin degradation	108
	KLK5	Inhibition	Modulates mevalonate pathway	109
Angiogenesis	KLK1	Promotion	Activates type IV collagenases	47
	KLK2, 4, 12	Promotion	Activates uPA–uPAR system	48,49
	KLK12	Promotion	Cleaves members of the CCN family	51
			Release of PDGF-beta	54
	KLK3, 5, 6, 13	Inhibition	Generates angiostatin-like fragments	9,56
	KLK1, 2, 12	Promotion	Kinin signalling pathway	35,49,63,65

Moreover, KLK6 and KLK7 can also cleave E-cadherin either indirectly via another metalloproteinase[87] or directly[88], thus generating high levels of shed fragments in metastatic ovarian cancer and ascites. Interestingly, MMP9, which is also a substrate of KLK7, may cause the same loss of E-cadherin[89], which directly leads to EMT via the β-catenin/TCF-dependent pathway (Figure 3C).

Certain KLK substrates may also induce EMT. For instance, TGF-beta has a number of recognised effects on prostate cancer cells, including activation of cytoskeletal modulators, such as the GTPase, RhoA[90], which results in the rearrangement of the actin cytoskeleton and induction of cell migration. Furthermore, TGF-beta can also down-regulate E-cadherin expression by promoting hypermethylation of the E-cadherin gene via TGF-beta/Smad signalling cascade activation, leading to a loss of the epithelial phenotype and EMT[91]. The latent (pro-) form of TGF-beta is known to be activated by KLK3 and KLK4[12] (Figure 3C).

On the other hand, opposing reports on the protective role of KLK6 against breast cancer progression may likely be mediated by an inhibition of EMT, which is characterized by a down-regulation of vimentin and concomitant up-regulation of epithelial markers[92]. Therefore, a clear consensus of whether KLKs act as EMT-promoters or EMT-suppressors has yet to be elucidated, though the available literature supports a context-dependent function, as in the case of most cancer hallmarks described in this review.

Evading immune destruction

The natural response of the immune system during cancer represents a significant barrier to tumor formation and progression. However, the majority of tumors deploy molecular strategies to avoid immune-mediated destruction. Escape from immunosurveillance can be achieved through the secretion of immunosuppressive cytokines and deficient expression of immunomodulatory molecules and major histocompatibility complex (MHC) class I antigens[10]. Strategies that cancer cells use to evade immune destruction include the elimination of induced effector cells, the disruption of critical signalling pathways, resistance of tumor cells to cytotoxicity and tumour cell evasion of immune reactivity[10]. As such, the association of KLKs with mechanisms of immune evasion have only recently started to emerge, but they appear to be robustly established in the field of cancer immunology (Table 3)[93].

Kallikrein-mediated modulation of the immune-tumor microenvironment

One of the mechanisms by which tumors mediate immunosuppression is through the release of soluble immunosuppressive factors. It has been well established that tumor cells produce TGF-beta and other immunosuppressive cytokines (i.e. IL-10) that inhibit normal anti-tumor function of immune effector cells[94] (Figure 4A). Interestingly, human kallikreins lead to the activation of TGF-beta, through KLK3 or plasmin[93], thereby demonstrating their indirect role in the regulation of tumorigenicity (Figure 4A).

Another prominent kallikrein-mediated manipulation of the tumor microenvironment may be the fact that KLKs can cleave and process powerful chemo-attractant molecules of the innate immune defense, like LL-37, which stimulate immune responses in neutrophils, monocytes and T-cells[90]. KLK5 has been shown to control the activation of LL-37, whereas both KLK5 and -7 are capable of further digesting LL-37 into smaller peptides[95,96].

Kallikrein involvement in tumor cell resistance to anticancer immunity

The complement network is an essential component of the innate immune system, which exerts important functions in immune surveillance and homeostasis. C3 is one of the major signalling effectors that exert a broad spectrum of biological effects in immune cell activation, thereby contributing to innate and adaptive immune functions. As such, proteolytic activation of C3 constitutes another mechanism that may contribute to tumorigenicity. Interestingly, the activation and/or degradation of C3 have been associated with different proteases[97]. Given the wide distribution of KLKs in tissues and biological fluids where complement components are also expressed, it was suggested that via C3 processing, tissue-localized KLKs can play an extrinsic complement-related role during activation of the innate immune response[97] (Figure 4B). More specifically, KLKs, such as KLK14, were efficiently able to cleave C3, the point of convergence of the complement cascade, indicating a potential modulation of C3-mediated functions[97].

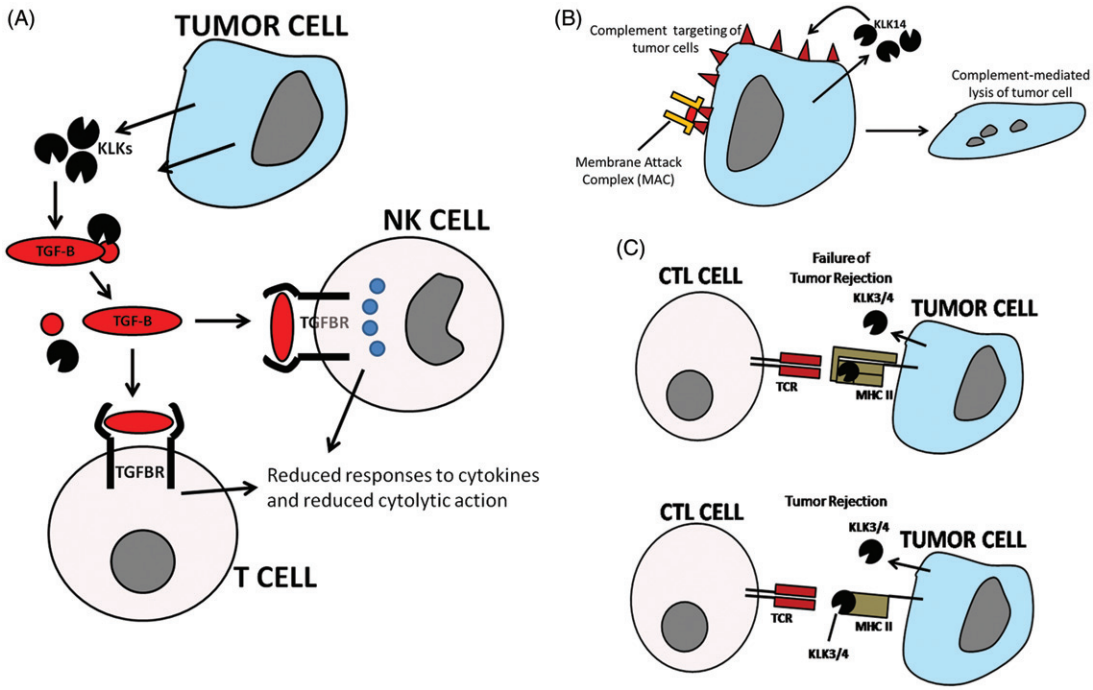

Figure 4. Kallikrein-mediated regulation of tumor cell resistance to anticancer immunity. (A) Modulation of the immune-tumor microenvironment via TGF by KLKs, (B) kallikrein-mediated modulation of tumor cell resistance mechanisms via proteolytic activation of C3 and (C) kallikrein-mediated anti-tumor immunity. See abbreviation list and text for more details. Molecules are not drawn to scale.

Kallikrein-mediated anti-tumor immunity

In addition to directly eliminating immune cells and suppressing their function by release of inhibitory factors that directly or indirectly suppress immune function, tumors also evolve mechanisms to evade immune cell recognition. The detection of tumor cells by effector T cells is based upon a complex pathway of processing and presentation of endogenous tumor antigens in the context of MHC Class II molecules. The critical role of $CD4^+$ and $CD8^+$ T cells for both the establishment and the maintenance of the anti-tumor response has been widely demonstrated. Interestingly, two prostate-specific expressed kallikreins, KLK3 and KLK4, are able to promote immune and anti-tumor responses[98–100] (Figure 4C). KLK4 represents an immunogenic molecule capable of inducing cytotoxic T cell (CTL) responses, as $CD4^+$ T-cells recognize peptide sequences specific for KLK4[100]. $CD4^+$ T helper responses are considered crucial in maintaining effective CTL responses and a number of MHC Class II-restricted epitopes within KLK4 have been identified[101] (Figure 4C). Additionally, the generation of KLK3-specific $CD8^+$ T cells[102], through a recombinant adenovirus vector expressing the antigen-PSA, actively engaged in the destruction of tumor cells expressing the cognate antigen, thus leading to anti-tumor responses[102]. Mechanisms by which prostate cancer cells may evade cytotoxic responses that are driven by the presentation of KLK3 and KLK4 on their surface MHC II molecules remains to be established.

Deregulated cellular energetics

Two major constituents of the tumor microenvironment may contribute to the reprogramming of cancer cell metabolism and energetics. First, cancer cells may regulate glucose uptake and metabolism to favor glycolysis, even under aerobic conditions. Second, cancer cells may regulate and manipulate oxygen homeostasis to their own favor, to facilitate malignant progression[10].

Oxygenation in tumors fluctuates due to the instability and disorganization of tumor vasculature. In response to hypoxic stress, epigenetic modifications, such as histone methylation and acetylation may be dynamically altered. Interestingly, *KLK* gene expression is often regulated via such mechanisms. For instance, histone acetylation in the *KLK3* enhancer region may facilitate gene transcription under hypoxic conditions[103]. This hypoxic microenvironment also increases the expression of AR-targeted genes, like *KLK2* and *-3* through specific Jumonji C domain-containing histone demethylases (JMJDs)[103]. Perhaps the most well-established pathway involved in gene transcription during hypoxia is the *HIF-1* pathway[104]. Among the wide range of factors analyzed for hypoxia-related mediation by HIF-1, KLK9 was also found[104]. Moreover, hypoxia is also implicated with the activation of oncogenes (i.e. *RAS, MYC*) in many tumors[105,106], which in turn may enhance *KLK3* expression[103] through androgen-regulated pathways.

Numerous studies suggested that the kallikrein–kinin system may participate in the regulation of glucose delivery and metabolism by skeletal muscle[107]. Evidence also suggests that kinins mediate the increase in insulin sensitivity after administration of enzyme inhibitors[108]. Human insulin has been identified as a substrate of KLK7, whereas co-expression of vaspin (a serpin inhibitor) inhibited insulin degradation by KLK7 in the circulation[108]. Therefore, increased glucose metabolism is based on the insulin-stabilizing effect by inhibiting KLK7-mediated insulin degradation (Table 3)[103]. Whether a possible KLK7/insulin/glucose axis exists in the cancer setting has yet to be determined. Interestingly, it was recently shown that KLK5 could alter

the expression of genes encoding enzymes involved in cholesterol/isoprenoid metabolism[109]. Finally, the up-regulation of lipogenesis by androgens is one of the most characteristic metabolic features of prostate cancer cells[110]. Given that KLK3 may transactivate the *AR*[111], it is tempting to speculate that KLK3 may also serve as a master regulator of lipid homeostasis in advanced prostate cancer.

As noted, the deregulation of KLK expression is mostly viewed as a consequence of deregulated cellular energetics in cancer, rather than the cause of this hallmark. To which extent, such KLK deregulation is simply a ''passenger effect'' of the metabolic perturbation or indeed holds critical tumor-promoting or tumor-suppressive potential is a subject of debate. Given the causative relationships (as explained in the previous sections) of elevated/reduced KLK expression documented in the previously discussed cancer hallmarks, the non-passenger potential of KLKs during energy metabolism is highly possible.

Enabling characteristics of cancer

Enabling genomic instability

Genome instability, leading to gene mutations, chromosomal rearrangements and/or fusion gene development is considered a major characteristic for the acquisition of the hallmark capabilities of most human cancers[38]. Literature evidence on the contribution of KLKs to genome instability currently remains poor and elusive; nevertheless, it points to a significant unmet need and an interesting field for investigation.

KLKs may indirectly regulate the master regulator of the antioxidant response, the nuclear factor (erythroid-2)-related factor 2 (Nrf2), through a PAR2-mediated mechanism[112]. In contrast, intracellular ROS production is usually augmented by B2R activation[113], pointing to a possible contribution of KLKs to the generation of ROS. Exposure to recombinant KLK1 mimics ROS effects[114]. Moreover, the cleaved product of kininogen (HKa) by the kallikrein–kinin system significantly increases intra-cellular ROS and p38 kinase phosphorylation[115]. Specifically, HKa-induced ROS may act through p38 kinase to up-regulate p16INK4a expression, which may, in turn, regulate telomerase activity[115]. Accumulation of ROS in most cell types is associated with high expression of p16INK4a, which leads to G1 arrest cell senescence acceleration[115]. Furthermore, HKa may inhibit Akt phosphorylation and eNOS phosphorylation, leading to reduced nitric oxide (NO) production[115], thereby affecting cell cycle arrest and apoptosis. It is thus concluded that ROS induction by KLKs might represent a common avenue for genomic instability, leading to the acquisition of multiple hallmarks of cancer.

Tumor-promoting inflammation

Immune cells infiltrate tumors and induce inflammatory responses, which can paradoxically enhance tumorigenesis by providing molecular strategies for acquiring the hallmarks of cancer[10]. Inflammation contributes to multiple hallmark capabilities by supplying bioactive molecules to the tumor microenvironment (i.e. growth factors, survival factors, proangiogenic factors and other hallmark-facilitating programs). Additionally, inflammatory cells can release chemicals, notably ROS, which are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward states of heightened malignancy.

Notably, KLKs may be the cause or consequence of tumor-promoting inflammatory responses. The kallikrein–kinin system has been linked to cellular inflammatory processes in many diseases[20]. Kinin receptors might play a pivotal role in the recruitment of pro-inflammatory cells within the tumor microenvironment, including leukocyte activation, as well as in the release of pro-inflammatory mediators, such as cytokines, prostaglandins, leukotrienes and ROS[20]. Notably, B2R signalling on neutrophils is involved in their extravascular migration at sites of inflammation[20]. Moreover, signal transduction through B1R and B2R in macrophages may result in the production of pro-inflammatory mediators, such as TNF-alpha, IL-1 and PGE2[116]. Finally, immature human monocyte-derived dendritic cells were found to constitutively express B1R and B2R and as a result, BK binding increased their migratory behaviour *in vitro*[116]. Collectively, these observations propose the presence of an active KLK-B1R/B2R axis in multiple inflammatory cells of the neoplastic milieu.

In general, inflammation and immune defense largely depend on the secretion and actions of specific chemical signals, such as interleukins. Inflammatory cytokines and KLKs are now shown to regulate each other, through autocrine or paracrine signalling loops and/or cascades. For instance, KLKs can activate pro-IL-1beta *in vitro,* as indicated by KLK7 and KLK13[117]. In the epidermis, LL-37, which is a proteolytic product of KLKs, acts synergistically with IL-1beta to augment immune responses[118]. The action of LL-37 is mediated by the PI3K/AKT pathway, which regulates specific *KLK* genes[119], probably via a positive feedback loop. At the same time, IL-19, IL-20, IL-22, IL-24 and IL-26 may up-regulate the expression of KLK6, -7 and -14, thus mediating critical ECM rearrangements[117]. Despite such evidence, the exact contribution of KLK-related cascades in the acquisition of cancer hallmarks in the inflammatory tumor microenvironment remains to be explored.

Conclusion

Hanahan and Weinberg described in 2011 the ''hallmarks of cancer'' – the eight biological capabilities acquired during the multistep development of human tumors[10]. In view of the emerging concept of the pleiotropic nature of cancer, the discovery of novel cancer biomarkers as well as targets for anti-cancer therapy is of great interest. In this review, we analyzed the role of KLKs in carcinogenesis, as it relates to these hallmarks. Since KLKs are implicated in cancer progression, their role as important modulators in the hallmarks of cancer is of importance and could provide novel insights into the molecular mechanisms of tumorigenesis.

Targeting each hallmark separately, and/or more hallmarks in parallel, is proposed as an effective strategy for therapeutic intervention in cancer. Since KLKs have emerged as novel targets for pharmacological intervention[4,120], it is worth

considering KLKs as targets for anti-cancer therapy in a ''hallmark-regulatory'' mode of action. For instance, anti-angiogenic therapy provides a novel approach for cancer management and anti-angiogenic drugs are centered on the blockade of the VEGF signalling pathway. However, other angiogenic factors (i.e. MMPs and ECM molecules) are also worth considering[121], providing the direct/indirect targeting of KLKs through these substrates. Moreover, considering the significant role of KLKs in immune response, these serine proteases might be considered as important candidates for immunotherapy. For instance, the prostate tissue-specific proteins have been identified for their utility as targets of antitumor vaccines[122]. Furthermore, research in testing the anti-metastatic potential of KLK inhibitors and increasing knowledge will enable further efforts to target KLK's action in the tumor microenvironment.

Interestingly, the activity of KLKs seems to contribute to all of the documented hallmarks. It is possible to view KLKs as members of a putative ''kallikrein balance''. For instance, this balance is biased toward antiangiogenesis when one KLK expression exceeds the expression of other KLKs. What is surprising and of considerable interest, is the contradictory effect of KLK activity for different KLKs in the same hallmark. This observation, although seems confusing, might have a better global application in anti-cancer treatment by protease inhibitors. Notably, over the last 20 years, we have been witnessing the failure of MMP inhibitors as cancer therapeutic agents in clinical trials[123]. This failure has been partially attributed to the weakness for developing specific protease inhibitors rather than inhibitors that block all proteases of a class. Similar to MMPs, KLKs also seem to have tumor-promoting and tumor-suppressive effects in a given microenvironment and as such, these overlapping effects most probably co-exist during the progression of the disease.

Perhaps the most significant conclusion and suggestion we would like to bring forward as a future perspective is that this ''hard lesson'' from the MMP inhibitors failure should be taken into serious consideration in the case of KLKs, and as such, targeted approaches should be opted instead. Given the pleiotropic roles of KLKs, both activators and inhibitors of KLK activities should be of therapeutic interest[4,120]. Emerging *in silico* and next generation high-throughput screening for protease inhibitors and/or activators[124,125], will provide the first steps towards this goal[126]. The development of such technologies for the discovery of KLK regulatory molecules is likely to advance our understanding of the complicated nature of interaction between the microenvironmental dynamics involving KLKs. Consequently, the development of specific KLK modulators will be paramount to provide better chances of therapeutic efficacy[120].

Here, we focus on different sets of KLKs that regulate one or more hallmarks, thus contributing with different strategies to their acquisition. Apparently, this is not the case in a personalized cancer setting. Our purpose here was to simply highlight various molecular pathways that are implicated in the acquisition of the sufficient and necessary signals that dictate a hallmark. To which extent (if indeed any) one or more KLKs have a causative role is, in our opinion, a context- and patient-dependent aspect.

To conclude, focusing on a ''hallmark-KLK targeting'' strategy for anti-cancer therapy, the mapping of the KLKs' cellular signalling pathways implicated in the hallmarks of cancer, is fundamental for the design of KLK modulators. The major biological programs elicited by cancer cells during cancer progression and which have been described in this review, should be viewed collectively as an entire program, in which KLKs may act as regulatory cues among a plethora of other microenvironmental factors. Therefore, in the next decade we anticipate that targeting of KLK pathways associated with each hallmark may allow development of novel anti-cancer therapies.

Declaration of interest

The authors report no declarations of interest.

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