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Emerging Roles of Angiopoietin-like 4 in Human Cancer

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ABSTRACT

Angiopoietin-like 4 (ANGPTL4) is best known for its role as an adipokine involved in the regulation of lipid and glucose metabolism. The characterization of ANGPTL4 as an adipokine is largely due to our limited understanding of the interaction partners of ANGPTL4 and how ANGPTL4 initiates intracellular signaling. Recent findings have revealed a critical role for ANGPTL4 in cancer growth and progression, anoikis resistance, altered redox regulation, angiogenesis and metastasis. Emerging evidence suggests that ANGPTL4 function may be drastically altered depending on the proteolytic processing and post-translational modifications of ANGPTL4, which may clarify several conflicting roles of ANGPTL4 in different cancers. While the N-terminal coiled-coil region of ANGPTL4 has been largely responsible for the endocrine regulatory role in lipid metabolism, insulin sensitivity and glucose homeostasis, it has now emerged that the C-terminal fibrinogen-like domain of ANGPTL4 may be a key regulator in the multi-faceted signaling during cancer development. New insights into the mechanistic action of this functional domain have opened a new chapter into the possible clinical application of ANGPTL4 as a promising candidate for clinical intervention in the fight against cancer. This review summaries our current understanding of ANGPTL4 in cancer and highlights areas that warrant further investigation. A better understanding of the underlying cellular and molecular mechanisms of ANGPTL4 will reveal novel insights into other aspects of tumorigenesis and the potential therapeutic value of ANGPTL4.
INTRODUCTION

Cancer is a disease in which a group of cells attain the ability to proliferate uncontrollably, invade surrounding tissues, and ultimately spread to distal tissues and organs through the circulatory system to form metastatic tumors. Based on our current knowledge, the process of cancer development is somewhat perplexing and certainly multifactorial. Genetic instability fueled by DNA damage and errors generated by DNA replication machinery drive the initiation of tumorigenesis (1,2). Extensive exposure to carcinogens or genotoxic chemicals is a prime factor that causes mutations and increases the risk of cancer. In addition, by-products of normal cellular metabolism, such as reactive oxygen species (ROS), also create a constant threat to DNA integrity (1). Genetic mutations or alterations of critical regulatory genes, such as tumor-suppressor genes and proto-oncogenes cause uncontrolled proliferation and the deregulation of apoptosis further mediating tumor progression.

Tumors consist of heterogeneous cell types such as cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, immune inflammatory cells, and proliferating cancerous cells (3). This heterogeneity and interdependency sustains tumor growth and progression. CAFs form the majority of the tumor stromal population and are capable of influencing cancer cell behavior via paracrine regulation. Endothelial cells that form the tumor vasculature act as a transportation route for nutrients not only to support tumor growth but also to facilitate cancer metastasis. The pericytes provide paracrine support signals to stabilize tumor vascular integrity and its function. Immune inflammatory cells secrete pro-angiogenic effectors like angiogenic growth factor, vascular endothelial
growth factor (VEGF), and pro-invasive matrix metalloproteinases (MMPs) to promote tumor progression (3).

Reciprocal cell-cell and cell-matrix communications dictate nearly every aspect of tumor development, and they can drastically affect the efficacy of anti-tumor therapies. Such communication encourages the tumor cells of many human cancers to acquire several essential capabilities to sustain proliferative signals, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, activate invasion and metastasis, reprogram their energy metabolism, and evade immune destruction (3). Research to identify critical mediators of these hallmarks will offer novel insights into the network of molecular mechanisms that are central to cancer development.

Ongoing cancer research focusing on understanding the interaction of cancer cells with their microenvironment has intensified over recent years, and a new class of proteins known as matricellular proteins have since been implicated (4-6). Matricellular proteins are a group of non-structural, extracellular matrix (ECM)-associated glycoproteins secreted by cancer cells and neighboring stromal cells into the extracellular environment (4,5). This large group of structurally and functionally diverse proteins modulates cell-matrix interactions and cell functions by interacting with membrane receptors, proteases, hormones, and other bioeffector molecules as well as with structural matrix proteins such as collagen and vitronectin (7,8). Recent studies have implicated several members of matricellular proteins such as osteopontin (OPN), secreted protein acidic and rich in cysteine (SPARC), and angiopoietin-like 4
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(ANGPTL4) as important factors in tumor progression (4,9-11). Of particular interest, ANGPTL4 is a novel matricellular protein that was reported to interact with specific ECM proteins and integrins to facilitate cell migration during wound healing (7,8).

ANGPTL4 is a novel, glycosylated adipokine that belongs to a family of 7 ANGPTL proteins (12,13; Fig. 1). The seven proteins, designated angiopoietin-like 1-7 (ANGPTL1-7), were identified in systemic circulation and share amino acid sequence similarity with the angiopoietin family (ANG). Despite being structurally similar to ANGs, these ANGPTLs do not bind to the ANG receptors Tie 1 and Tie 2 to mediate their biological functions. The cognate receptors for ANGPTLs remain unknown; thus, ANGPTLs are considered orphan ligands (12,14). ANGPTL3, 4 and 6 play critical roles in lipid and glucose metabolism (14,15). Studies have also revealed that ANGPTL2, 3, 5 and 7 regulate hematopoietic stem cell activity (12). Interestingly, ANGPTL2 was recently reported to be a critical factor for carcinogenesis (16). ANGPTL2-associated chronic inflammation was also found to increased cancer susceptibility (16). ANGPTL2 was identified as a causative mediator of chronic inflammation, and this was strongly correlated to the frequency of carcinogenesis in vivo using a chemically-induced skin squamous cell carcinoma (SCC) mouse model. Furthermore, tumor-derived ANGPTL2 was shown to enhance metastasis and tumor angiogenesis (16). This finding suggests that other ANGPTL family proteins may have similar roles in cancer development.

Emerging evidence has identified a novel role for ANGPTL4 in cancer progression, while the other ANGPTL members have been mostly characterized as factors involved in angiogenesis and metabolism. The involvement of other ANGPTL
family members in cancer remains unclear and warrants further investigation. In this review, we discuss the potentially diverse roles of ANGPTL4 in tumor development and discuss certain discrepancies regarding ANGPTL4 that require further clarification.

FUNCTIONAL DOMAINS OF ANGPTL4

ANGPTL4 is characterized by the presence of a highly hydrophobic signal peptide, an N-terminal coiled-coil domain, and a large angiopoietin/fibrinogen-like C-terminal domain, which is well conserved in the ANG and ANGPTL families (17; Fig. 2). The native full-length ANGPTL4 (flANGPTL4) exists in the form of dimeric or tetrameric complexes that can undergo proteolytic processing to generate the N-terminal coiled-coil fragment (nANGPTL4) and the C-terminal fibrinogen-like domain (cANGPTL4; 17). Although ANGPTL4 are known to be proteolytically processed, the mechanism of cleavage, the importance of the processing and the roles of the various ANGPTL4 fragments are only beginning to be elucidated.

The proteolytic cleavage of ANGPTL4 is mediated by proprotein convertases (PCs; 17; Fig. 2). Because ANGPTL4 is proteolytically processed in the liver, the hepatocellular carcinoma cell line Huh7 has been used in studies investigating ANGPTL4 cleavage. Using Huh7 cells transfected with expression vectors encoding for the various convertases, Lei et al showed that in vitro several PCs, including furin, PC5/6, paired basic amino acid-cleaving enzyme 4, and PC7, are able to cleave human ANGPTL4 at the -RRXR- consensus cleavage site (17). Although this study showed that ANGPTL4 is cleaved by PCs, the correlation between the expression of PCs and
the truncated forms of ANGPTL4 remains unknown. The PCs have been shown to be enzymatically activated after passing through the late Golgi. Therefore, how ANGPTL4 is proteolytic processed remains to be determined (18). Importantly, the expression of these PCs in tumors and whether they are indeed responsible for the \textit{in vivo} processing of flANGPTL4 is unknown.

The nANGPTL4 is responsible for the oligomeric assembly of ANGPTL4 and binds to lipoprotein lipases (LPL) to inhibit their activities (19). Disulfide bond formation facilitates the oligomeric formation of nANGPTL4 and flANGPTL4, which enhances its inhibitory effects on LPL activity. Mutations that prevent oligomerization severely compromise the capacity of ANGPTL4 to inhibit LPL. The flANGPTL4 can also bind to heparin sulfate proteoglycans to participate in the inhibition of endothelial cell migration and tubule formation (20). However, it remains unknown whether flANGPTL4 and nANGPTL4 can directly stimulate intracellular signaling because neither a receptor nor a cell-surface interaction partners has been identified for ANGPTL4. The cANGPTL4 binds to and activates integrins $\beta_1$ and $\beta_5$ to regulate cell migration via the FAK/PAK signaling cascade (7). Recently, cANGPTL4 was found to trigger vascular disruption by binding to and dispersing vascular-endothelial cadherin (VE-Cad) and claudin-5 at endothelial junctions via the activation of integrin $\alpha_5\beta_1$ (21). Furthermore, cANGPTL4 can also associate with specific matrix proteins and delay their proteolytic degradation by metalloproteinases. This interaction does not appear to interfere with integrin-matrix protein recognition (7,8). Taken together, these studies
suggest that different ANGPTL4 fragments exhibit tissue-dependent functions and that the different fragments of ANGPTL4 may have distinct roles in human cancers.

**ANGPTL4 EXPRESSION AND ITS TRANSCRIPTIONAL REGULATION IN HUMAN CANCERS**

The expression of ANGPTL4 is regulated by the nuclear hormone receptors of the peroxisome proliferator-activated receptors (PPARs) family as well as by hypoxia and fasting (15,22,23). PPARs act as lipid biosensors and are crucial for lipid metabolism and homeostasis (15). Recently, PPARs have been implicated in the development of tumors (24). Studies have shown that ANGPTL4 is one of the PPAR target genes and contains several putative PPARs response element (PPRE) consensus sequences within its promoter (25). Reports have proposed that ANGPTL4 may be regulated by all three PPARs isoforms (PPARα, PPARβ/δ, and PPARγ), suggesting that ANGPTL4 may exert tissue-dependent effects governed by the differential expression of the PPAR isoforms (15). Furthermore, despite conflicting results, PPARs are known to be involved in tumorigenesis. This further implicates ANGPTL4 in cancer progression, although evidence that PPARs upregulate ANGPTL4 directly in tumor cells is still lacking. Girrol *et al* (2008) suggested that PPARβ/δ modulates ANGPTL4 expression during tumorigenesis in human breast cancer and melanoma cell lines, while Zhu *et al* (2011) reported no apparent correlation between the expression of PPARs and ANGPTL4 in squamous cell carcinoma (11,26). A recent study suggested that prostaglandin E2 (PGE₂) may transactivate PPARβ/δ to regulate the expression of
ANGPTL4 under hypoxic conditions (25). Due to the aberrant growth of tumor cells and poor vascularization, the tumor microenvironment tends to become hypoxic. Several new studies have reported that hypoxia can induce the expression of ANGPTL4 and that the upregulation of ANGPTL4 is induced by the transcription factor hypoxia-inducible factor 1α (HIF-1α) (11,25,27). Indeed, Zhang et al (2011) identified a functional HIF-1α binding site located 1.6 kb upstream of the human ANGPTL4 gene (28). This was further confirmed by another independent group using the hepatocellular carcinoma cell lines MHCC-97L and SMMC-7721 (27).

The tumor microenvironment is constantly under chronic inflammatory pressure (29). Recently, the potent regulator of inflammation transforming growth factor β (TGF-β) was found to regulate the expression of ANGPTL4 via a Smad3 signaling pathway (30). The upregulation of ANGPTL4 in cancer cells when they extravasate into the circulatory system could explain their inclination towards colonizing lung tissue. The rationale is based on the ability of ANGPTL4 to disrupt the integrity of vascular tight junctions thereby increasing the permeability of the capillaries in the lung to promote the intravasation into the lung tissue (30). Whether TGF-β can serve as a good therapeutic target still remains unclear due to its dual role in inhibiting and promoting tumor cell growth. However, this demonstrates the capacity of the primary tumor microenvironment to promote the progression of cancer via the production of cytokines (29).

Although early expression profiling studies have detected ANGPTL4 in a variety of organs or tissues such as the skin, intestines, kidneys, adipose tissues and the
liver (14, 15, 22), little is known about the relative expression of the various ANGPTL4 fragments in these tissues. Recently, it was shown that adipocytes express the post-translationally modified fANGPTL4 (~65 kDa), whereas the liver produces both cANGPTL4 (~47 kDa) and nANGPTL4 (~26 kDa) (17, 31, 32; Fig. 2). The mechanism for such tissue-dependent expression of ANGPTL4 remains unclear.

While information regarding the relative expression of the different ANGPTL4 fragments in normal tissues is emerging, much less is known about the expression of various ANGPTL4 fragments in various tumors (Table 1). To date, four studies have reported the elevated expression of cANGPTL4 in various tumors. Using an in vitro model where they overexpressed ANGPTL4 cDNA in B16F0 mouse melanoma cells, Galaup et al (2006) showed the presence of a small ANGPTL4 fragment whose molecular weight corresponded to the cANGPTL4 fragment (10). Using a monoclonal antibody against cANGPTL4 (mAb11F6C4), Zhu et al (2011) showed that cANGPTL4, but not nANGPTL4, is highly expressed in major epithelial tumors such as SCC (11). Similarly, Huang et al (2011) detected elevated cANGPTL4 from fine needle aspirates of breast tumors, basal cell carcinomas, melanomas, and in several cancer cell lines derived from breast, lung and liver cancers (21). Consistent with the above findings, Kim et al (2011) showed that colorectal cancer cells only secrete cANGPTL4 proteins (25). A high ANGPTL4 expression in oral cancer correlates to an enhanced rate of tumorigenesis and a poor prognosis based on the tumor clinicopathology (33, 34; Table 2). An elevated ANGPTL4 in colorectal cancers is also correlated with shorter disease-free survival rates (25). Furthermore, the downregulation of ANGPTL4 impairs tumor
growth and metastasis (11,35,36). In contrast, studies have also demonstrated that increased ANGPTL4 expression inhibits melanoma, lung and colorectal tumor growth, metastasis and angiogenesis (10,37). High ANGPTL4 level in mouse tumors also impaired tumor cell migration and invasiveness, thus inhibiting metastasis (10). The reason for this discrepancy is unclear. These results suggest that the expression and roles of ANGPTL4 may be context- and tumor type-dependent. The expression of ANGPTL4 fragments in lymphatic and hematopoietic cancers such as lymphoma and leukemia also remains unclear. Despite the increasing emphasis on the differential roles of fANGPTL4, cANGPTL4 and nANGPTL4, most studies have not vigorously addressed the biological significance of this phenomenon in cancer. Emerging studies have also revealed that the post-translational modification of ANGPTL4 can affect its biological functions, which suggests another level of complexity in understanding the role of ANGPTL4 in cancer. Thus, further investigation is necessary to better understand how tumor microenvironment influences the transcriptional regulation of ANGPTL4 in cancer.

**ANGPTL4 AND TUMOR GROWTH**

There are increasing lines of evidence that clearly support a tumor-promoting role of inflammation in the development and progression of cancer (38). The tumor microenvironment strongly resembles the wound environment, which contains numerous growth factors and pro-inflammatory lipid mediators such as prostanoids. However, instead of signaling for the removal of the abnormal cells, these pro-
inflammatory mediators have been found to be co-opted by the cancer cells to promote their growth, invasion and metastasis (38).

Cyclooxygenase-2 (COX-2) is an inducible enzyme that converts free arachidonic acid into prostanoids including prostaglandins (PGs) and thromboxanes (25). Under normal conditions, COX-2 expression is undetectable in most cells. During inflammation, COX-2 expression is upregulated in activated macrophages to increase the production of prostanoids that modulate the actions of the various immune cells (39). More recently, COX-2 has been shown to be upregulated in various carcinomas and appears to play a fundamental role in tumorigenesis. The expression of COX-2 is elevated in approximately 50% of colorectal adenomas and in up to 85% of adenocarcinomas; it is associated with poor survival among colorectal cancer (CRC) patients (25). Importantly, the tumor-promoting function of COX-2 is strongly linked to prostaglandin E2 (PGE2), which is also the most abundant prostaglandin found in CRCs (25). A recent study showed a synergistic effect between PGE2 and hypoxia in promoting colorectal cancer cell proliferation, and the authors ascribe tumor-promoting effects to ANGPTL4 (25). Specifically, hypoxia induced the expression of EP1, a PGE2 receptor, in CRC. The elevated level of PGE2 stimulated EP1 receptor signaling, which enhanced ANGPTL4 expression and cANGPTL4 secretion (Fig. 3). More importantly, the authors of this study went on to show that cANGPTL4, and not fANGPTL4 or nANGPTL4, was responsible for the increased colorectal carcinoma cell proliferation and tumor growth in vitro and in vivo (25). Notably, the study further showed that cANGPTL4 regulates cancer cell proliferation and that STAT1 induction is dependent
on a NADPH oxidase-mediated production of superoxide (O$_2^-$), as well as the Src and MAPK pathways. This is consistent with the findings by Zhu et al (2011) who showed that cANGPTL4 stimulates a redox-based mechanism to enhance tumor cell survival via the alteration of the O$_2^-$:H$_2$O$_2$ ratio leading to the activation of Src and ERK (11).

Given the distinct functions of the different ANGPTL4 fragments, this information provides a focal point for future ANGPTL4 research highlighting the need to demarcate the different biological functions of cANGPTL4 and nANGPTL4 in cancer progression. More extensive studies will certainly be necessary to elucidate the underlying mechanisms by which ANGPTL4 modulates cancer proliferation. One important discovery will be the identification of the receptors that mediate the downstream effects of ANGPTL4.

**ANGPTL4 AND ANOIKIS RESISTANCE**

The abnormal growth pattern of tumor cells suggests that the tumor cells grow under a myriad of physiological stresses that should trigger apoptosis and halt cancer progression. However, tumor cells evade apoptosis using multiple dysregulated mechanisms, and anoikis resistance is a hallmark of cancer (3).

An early study showed that ANGPTL4 acts as a negative regulator of apoptosis, protecting endothelial cells from serum deprivation-induced apoptosis. Although the mechanism was unknown, it was postulated that ANGPTL4 could bind to a receptor to mediate its anti-apoptotic effect. This suggests that cancer cells may employ a similar mechanism to resist stress-induced apoptosis (23). Indeed, ANGPTL4 has been shown
to contribute to anoikis resistance in hepatoma cells grown in a detached state. These hepatoma cells tend to form a synokis-like multicellular aggregate that was resistant to apoptosis inducers (40). The suppression of ANGPTL4 by RNA interference enhanced detachment-induced apoptosis and sensitized the tumor cells to anti-tumor drug treatment (40). Recently, the anoikis-protective effect of ANGPTL4 was attributed to cANGPTL4. In this study, tumor-derived cANGPTL4 activates a redox-mediated survival mechanism that is used by tumor cells to facilitate cancer progression through the modulation of ROS (11). Tumour-derived cANGPTL4 interacts with integrins β1 and β5 to modulate O$_2^-$ levels using the NADPH oxidase-dependent machinery to maintain an elevated oncogenic O$_2^-$:H$_2$O$_2$ ratio in tumors. This cANGPTL4-integrin interaction activates Src kinase by oxidation and consequently stimulates the pro-survival PI3K/PKBα and mitogenic ERK pathways to sustain attachment-independent survival (Fig. 3). Importantly, the suppression of ANGPTL4 via RNAi or immunosuppression by monoclonal antibody (mAb11F6C4) modulates intracellular ROS generation to attenuate tumor growth that is associated with enhanced apoptosis in vitro and in vivo (11). mAb11F6C4 binds to the human cANGPTL4 protein and has been shown to block the interactions between cANGPTL4 with integrin, VE-cadherin and claudin 5 to attenuate tumor growth and metastasis (11,21). Similar observations were also recently made in CRC showing that cANGPTL4-mediated activation of ERK and Src kinase enhanced colorectal cancer cell proliferation under hypoxic conditions, further confirming the unique functions of cANGPTL4 in cancer (25). Despite the loss of cell-matrix attachment, the hijacking of integrin-mediated signaling by tumor-derived
cANGPTL4 provides an alternative survival mechanism, which is important for cancer cells during metastasis (11). This finding suggests that anticancer strategies focusing on anoikis or redox-based apoptosis in tumors are viable. However, more studies will be required to validate the role of cANGPTL4 in anoikis. Of particular interest would be studies on the effect of cANGPTL4 on other pro-survival pathways during anoikis and whether other ANGPTL4 fragments influence tumor survival.

ANGPTL4 AND ANGIOGENESIS

The oxygen consumption of rapidly growing tumor cells may outweigh an insufficient oxygen supply leading to a pathological condition known as hypoxia (41). This hypoxic environment triggers the onset of pathological angiogenesis, which forms new blood vessels to supply oxygen and nutrients and remove metabolic waste products supporting tumor survival (41). Angiogenesis is a multistep process that is regulated by angiogenic factors like VEGF, ANG and more recently, ANGPTL4 (42). An in vivo hypoxic signature includes ANGPTL4 as a robust hypoxia-related gene. Previous studies have indicated that ANGPTL4 mRNA and protein levels become elevated in endothelial cells in response to hypoxia and exert a VEGF-independent pro-angiogenic effect (42). An increased expression of ANGPTL4 was also observed in the majority of human cancers with the expression of cANGPTL4 highly correlated to the expression of hypoxia-inducible factor 1α (HIF-1α) (See ANGPTL4 expression and its transcriptional regulation in human cancers). These observations indicate that ANGPTL4 could be a key modulator in tumor angiogenesis in a hypoxic tumor microenvironment.
Recent studies highlighted a prominent role for ANGPTL4 in tumor angiogenesis and vascular permeability. Using a transgenic mouse model expressing a viral G-protein coupled receptor (vGPCR), ANGPTL4 expression was up-regulated by vGPCR in Kaposi’s sarcoma (43). This increase in ANGPTL4 expression enhanced endothelial cell migration and differentiation, both of which are important processes in angiogenesis, resulting in markedly enhanced neovascularization. The inhibition of ANGPTL4 using siRNA prevented the neovascularization process and reduced vascular permeability as well as vGPCR-induced tumorigenesis in vivo (43). In subsequent studies, Huang et al (2011) revealed that ANGPTL4-knockdown allograft tumors express consistently lower levels of endothelial cell marker CD31 compared to wild type allograft tumors, which is indicative of impaired angiogenesis further confirming the pro-angiogenic role of ANGPTL4 in tumors (21). While these findings suggest a pro-angiogenic role for ANGPTL4 in tumors, an anti-angiogenic role has also been reported for ANGPTL4. Ito et al (2003) found that ANGPTL4 acts as an anti-angiogenic factor inhibiting VEGF-induced endothelial cell proliferation, chemotaxis and tubule formation, which are important processes for vascularization (37). An in vivo corneal neovascularization assay revealed that ANGPTL4 strongly impairs VEGF-induced neovascularization and that ANGPTL4 alone does not influence neovascularization. These results seem to suggest that in the context of VEGF-induced angiogenesis, ANGPTL4 exerts anti-angiogenic effects. Furthermore, a study investigating transgenic mice that overexpress ANGPTL4 in the basal epidermis, the authors showed that elevated levels of ANGPTL4 impaired neovascularization resulting
in a decrease amount of invading capillary vessels into the xenograft tumors effectively limiting tumor growth (37). Interestingly, the same group also reported a pro-angiogenic effect for ANGPTL4 further highlighting the controversial roles of ANGPTL4 (10,20,42).

These findings suggest a role for ANGPTL4 in tumor vascularization, although the underlying mechanism by which ANGPTL4 modulates angiogenesis remains unresolved. The discrepancies between the findings suggest a context- and tissue-dependent effect of ANGPTL4, which is further complicated by post-translational modification of ANGPTL4 where N-glycosylated cANGPTL4 was found to suppress the Raf/MEK/ERK signaling cascade in endothelial cells impairing bFGF and VEGF-induced angiogenesis (32). To resolve these disagreements, future investigations should focus on understanding the roles of post-translation modifications on ANGPTL4 and on the mechanisms that govern the pro- and anti-angiogenic effect of fANGPTL4 and its truncated fragments.

ANGPTL4 IN TUMOR INVASION AND METASTASIS

Metastasis is the spread of malignant cells to distant organs and is frequently the final event in tumor progression (3). Metastasis is largely responsible for the majority of cancer deaths as the metastatic cancer cells are typically highly aggressive and resistant to anticancer drugs. However, metastasis remains the most poorly understood aspect of cancer (3). Metastasis is a complex, multi-step process where tumor cells locally invade the surrounding tissue and then migrate across the vascular endothelium of the
lymphatic and vascular systems (intravasation). While in circulation, tumor cells must evade immunodetection and resist anoikis before seeding at a distal site. These tumor cells leave the circulation through extravasation to establish a new secondary tumor (44). One of the fundamental mechanisms required during metastasis is the disruption of the vascular integrity, which facilitates the transmigration of the tumor cells across the endothelium (44).

Early studies have suggested that flANGPTL4 inhibits VEGF-induced angiogenesis and vascular permeability (37). In a different study, ANGPTL4 was shown to inhibit vascular leakiness and this impaired the intravasation and extravasation of tumor cells in vivo (10). Notably, only a single form of ANGPTL4 was detected in sera in vivo. Because the molecular weight of ANGPTL4 fragment size was not provided in the study, it was not possible to know whether the detected protein band corresponded to flANGPTL4, nANGTL4 or cANGPTL4. Galaup et al (2006) went further by using the overexpression of ANGPTL4 in melanoma to show that the expression of ANGPTL4 impairs the migration and invasion of melanoma cells (10). Interestingly, flANGPTL4 in the presence of a smaller ANGPTL4 fragment whose molecular weight corresponds to the cANGPTL4 was found to be produced by the melanoma cells (10). However, it is unclear which ANGPTL4 fragment was actually responsible for the inhibition of cell migration and invasion.

However, recent studies have demonstrated that ANGPTL4 is highly expressed in metastatic cancers, suggesting that it may play a role in the metastatic processes. Furthermore, these studies have pinpointed ANGPTL4 as a critical mediator in the
transmigration process (11,21,45). ANGPTL4 enhances vascular permeability and promotes the metastasis of breast tumor cells to the lung and the metastasis of melanoma cells to the brain (21,28,30,46). TGF-β upregulates ANGPTL4 in breast tumor cells and disrupts the endothelial cell-cell junctions leading to enhanced vascular leakiness and transendothelial migration of the tumor cells. Furthermore, clinical studies have correlated ANGPTL4 expression with venous and lymphatic invasion in human gastric and colorectal carcinomas, which further emphasizes the role of ANGPTL4 in tumor metastasis (47-49). ANGPTL4 was found to promote transendothelial migration and metastasis of hepatocellular carcinoma cells through the upregulation of vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (EC) and the activation of the VCAM-1/integrin β1 signaling axis (27). In this mechanism, the increased VCAM-1 expression on endothelial cells facilitates the attachment of the metastatic cancer cells in circulation and the subsequent trans-endothelial extravasation to establish a metastatic tumor. ANGPTL4 also disrupts EC-EC interactions leading to enhanced vascular leakiness and promotes the metastasis of cancer cells to the lung (21). Another study pinpointed the vascular disruptive effect of ANGPTL4 to the cANGPTL4 fragment. In this study, the authors showed that cANGPTL4 interacts with integrin α5β1 to activate the Rac/PAK signaling axis weakening the EC-EC contacts and facilitating cANGPTL4 interaction with VE-Cadherin and claudin-5 (21; Fig. 3). This results in the internalization and declustering of the EC tight junction and adherens junction proteins leading to the disruption of the vascular barrier. Clearly, more investigations are necessary to clarify the pro-metastatic role of ANGPTL4 and its underlying mechanism.
of action. Taken together, these results suggest that ANGPTL4 may have both homotypic and heterotypic influences in the tumor microenvironment (Fig. 3).

The role of ANGPTL4 in vascular permeability and metastasis is controversial. The apparent discrepancy that was observed in previous studies appears to derive from the use of different experimental approaches and models in each study. Additionally, the functions of ANGPTL4 may be affected by the tumor microenvironment suggesting a context- and tissue-specific activity. Presently, the lack of structural information on ANGPTL4 limits our ability to fully explain this phenomenon. Unfortunately, numerous studies that have attempted to decipher the role of ANGPTL4 in cancer have failed to address the post-translational modifications and proteolytic processing of ANGPTL4, which clearly have a significant influence on the functions of ANGPTL4 (10,30,37,47-49). For example, the expression of either the nANGPTL4 or the cANGPTL4 truncation in transgenic mice remains unknown (43). The differential processing of ANGPTL4 has a major effect on the different biological functions of ANGPTL4 under various contexts and may have contributed to the observed discrepancies (11,21,50). Indeed, only cANGPTL4 can interact with integrin and stimulates intracellular signaling to promote tumor cell survival (7,8,11). In addition, it appears that the processing of ANGPTL4 in humans is tissue-dependent as different organs such as the liver and kidney have been found to utilize different forms of ANGPTL4 (50). One recent finding has implicated the glycosylated form of ANGPTL4 in augmenting the leakiness of the kidney glomerular epithelium suggesting that this type of post-translational modification of
ANGPTL4 may direct its function in this case (50). Further *in vitro* and *in vivo* experiments are necessary to clarify the role of ANGPTL4 in metastasis.

**CLINICAL RELEVANCE OF ANGPTL4**

The endocrine regulatory role of ANGPTL4 in lipid metabolism, insulin sensitivity and glucose homeostasis has been extensively studied (14,15,22). As a critical mediator in energy homeostasis, it is not surprising that ANGPTL4 has been suggested as a potential therapeutic target for metabolic and cardiovascular diseases. Antibodies against nANGPTL4 could potentially lower the levels of triglycerides and cholesterol in heart disease patients (http://www.lexparma.com). The targeted silencing of ANGPTL4 offers another potential therapeutic strategy in the treatment of atherosclerosis, dyslipidemia and diabetic wounds (8,14,51,52). These reports emphasize the clinical relevance of ANGPTL4 as an attractive therapeutic candidate.

ANGPTL4 as an anti-tumor and anti-metastatic target remains debatable because the multi-faceted roles of ANGPTL4 in tumorigenesis need further clarification. However several research groups have shown promising results that suggesting that ANGPTL4 could be a potential interventional therapeutic target in the battle against cancer (11, 21, 25, 30).

On one hand, it was shown that the immunosuppression of cANGPTL4 by a neutralizing antibody results in tumor regression, which was attributed to reduced cell proliferation and enhanced redox-based cell apoptosis of the regressed tumor (11). The suppression of cANGPTL4 by either a neutralizing antibody or RNA interference also
reduces vascular disruption and thus metastasis (21). In addition, the inhibition of cANGPTL4 may confer chemosensitivity to cancer cells (25). On the other hand, an increased level of ANGPTL4 has been found to inhibit tumor angiogenesis, vascular permeability and invasiveness of cancer cells (10,20,37,42). Although discrepant in their results, these studies have utilized different cancer cell models suggesting that potential inhibitory therapy may have a restricted therapeutic range or may be restricted to certain cancer types.

ANGPTL4 could also be a potential diagnostic biomarker in cancer. ANGPTL4 expression was observed to be elevated in numerous cancers at both the mRNA and protein level (Table 2). Given the implications that ANGPTL4 may have on cancer progression, a recent report suggested that ANGPTL4 mRNA could be used as a diagnostic marker to distinguish primary and metastatic clear cell renal-cell carcinoma (53). Several other studies have also delineated the ANGPTL4 expression level in primary and invasive tumors, showing that high ANGPTL4 mRNA and protein expression in human tumor biopsies often correlates with a poor prognosis and a poor disease outcome (25,33,34). As a secreted protein, ANGPTL4 protein levels could also be detected in the serum (23). Zhu et al (2011) have also shown that increased cANGPTL4 protein levels could be detected in the serum of tumor-bearing mice (11). cANGPTL4 was also found to be secreted by hypoxic cancer cells, lending further credence to the potential use of ANGPTL4 as a biomarker (25).
CONCLUSION AND FUTURE DIRECTION

Recent work has suggested that ANGPTL4 has increasingly important roles in cancer development and progression. Therefore, many researchers have postulated that diverse and sometimes conflicting roles for ANGPTL4 exist in cancer. Based on the collective data, ANGPTL4 exerts a pro-angiogenic effect by promoting vascularization under hypoxic conditions in tumors (42). Moreover, ANGPTL4 promotes invasion and metastasis by enabling vascular permeability and conferring anoikis resistance to the tumor cells (11,21,28,46). However, anti-tumorigenic roles of ANGPTL4 have also been established particularly in the inhibition of tumor angiogenesis and metastasis (10,37). The role of ANGPTL4 in human cancer could certainly be context-dependent or tissue-dependent. These seemingly controversial results underscore the need to address the function of flANGPTL4 and its truncated fragments in cancer and the post-translational modifications that may clarify the context-dependent roles of the different fragments of ANGPTL4 in cancer.

The role of ANGPTL4 in systemic lipid and glucose metabolism has been established recently (15). However, it would be interesting to examine the impact that ANGPTL4 may have on tumor cell metabolism. Evidence has shown that AKT/PKB can regulate glucose consumption and glycolysis during the metabolic reprogramming that occurs in cancer cells. Coupled with the ability of ANGPTL4 to regulate the function of AKT/PKB in conferring tumor cell resistance against anoikis, it remains to be observed if ANGPTL4 plays a role in the alteration of cancer cell metabolism as well (11,54). Another area that remains to be elucidated is the biological function and the
inherent signaling pathway of the different fragments of ANGPTL4 (Fig. 2). It is interesting to note that a ‘receptor’ for the full-length and the N-terminal ANGPTL4 has yet to be identified (See functional domains of ANGPTL4 ). The cANGPTL4 has been reported to bind to several interacting partners, resulting in the activation of different pathways in a context- and tissue-dependent manner (11,21,25). However, it is unknown whether the C-terminal domain of the flANGPTL4 activates similar signaling pathways and biological functions.

New and promising findings have already earmarked ANGPTL4 as a diagnostic biomarker in various cancer types. Despite the reported conflicting roles of ANGPTL4, there are several exciting findings that offer a novel perspective promoting the therapeutic use of cANGTPL4 antibody as a primary clinical drug. Clearly, the diverse roles of ANGPTL4 in cancer remain to be discovered. However, given the complex nature of cancer development and the many proteins involved, the likelihood of curing cancer by targeting a single protein is unlikely to achieve the desired outcome. Indeed, multi-modal therapy is a new yet extremely important advance in cancer treatment. By building on the current mechanistic insights of ANGPTL4 function, elucidating the diversified roles of ANGPTL4 in human cancer promises to be a challenging yet provocative endeavor.
ACKNOWLEDGEMENT

We apologize to colleagues whose works are not cited due to space constraints.

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Angiopoietin-like 4 in Human Cancers

al..Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. Proc Natl Acad Sci U S A 2006;103(49):18721-18726.


### Table 1 Expression of ANGPTL4 protein in various human cancers

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Detection technique</th>
<th>Antibody used *</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Various cancer</td>
<td>Immunofluorescence, real-time PCR, and Western blot</td>
<td>nANGPTL4 and cANGPTL4</td>
<td>Zhu et al (2011) (11)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Real-time PCR and Western blot</td>
<td>Full-length ANGPTL4</td>
<td>Nakayama et al (2011) (47)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Real-time PCR and Western blot</td>
<td>Full-length ANGPTL4 and cANGPTL4</td>
<td>Kim et al (2011) (25)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Real-time PCR and Western blot</td>
<td>Full-length ANGPTL4</td>
<td>Zhang et al (2011) (28)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Real-time PCR and Western blot</td>
<td>Full-length ANGPTL4</td>
<td>Nakayama et al (2010) (48)</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>Immunohistochemistry</td>
<td>Full-length ANGPTL4</td>
<td>Shibata et al (2010) (49)</td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
<td>Real-time PCR and Western blot</td>
<td>Full-length ANGPTL4</td>
<td>Ma et al (2010) (43)</td>
</tr>
</tbody>
</table>

* Indicates antibodies raised against full-length, n-terminal, and c-terminal ANGPTL4.

# We apologize to colleagues whose work was not included in this table, particularly those using real-time PCR or microarray analysis only.
### Table 2

Expression of ANGPTL4 in various human cancers and its implicated roles in tumor growth, metastasis and angiogenesis.

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Detection technique</th>
<th>Expression in tumor</th>
<th>Disease outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain metastasizing melanoma</td>
<td>Real-time PCR</td>
<td>High expression in metastasized melanoma cells</td>
<td>Expression of ANGPTL4 correlated with metastasis of melanoma</td>
<td>Izraely <em>et al</em> (2011)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Real-time PCR and Western blot</td>
<td>High expression in colorectal carcinoma cells under synergistic effect of PGE2 and hypoxia</td>
<td>Increased cancer cell proliferation, survival, motility, and tumor-associated angiogenesis</td>
<td>Kim <em>et al</em> (2011)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Real-time PCR and Western blot</td>
<td>High expression in breast cancer cells under hypoxia</td>
<td>Increased metastasis by promoting extravasation in the lungs</td>
<td>Zhang <em>et al</em> (2011)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Real-time PCR and Western blot</td>
<td>High expression in 100% of patients with metastatic tumor</td>
<td>Increased expression facilitates venous invasion and distant metastasis</td>
<td>Nakayama <em>et al</em> (2011)</td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>Real-time PCR and bisulfite sequencing</td>
<td>Low expression in 12% of primary carcinoma</td>
<td>Methylation-silenced Angptl4 is present in mammary carcinoma</td>
<td>Hattori <em>et al</em> (2011)</td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
<td>Real-time PCR and Western blot</td>
<td>High expression in 92% of oral Kaposi’s sarcoma</td>
<td>Enhances Kaposi’s sarcomagenesis</td>
<td>Hu <em>et al</em> (2011)</td>
</tr>
<tr>
<td>Tumor Type</td>
<td>Methodology</td>
<td>Findings</td>
<td>Reference</td>
<td></td>
</tr>
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<td>--------------------------------</td>
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</tr>
<tr>
<td>Endometrial cancer</td>
<td>DNA microarray, real-time PCR, and Immunohistochemistry</td>
<td>Expressed in tumor</td>
<td>Mannelqvist et al (2011) (56)</td>
<td></td>
</tr>
<tr>
<td>Esophageal squamous cell carcinoma</td>
<td>High expression in metastatic tumor</td>
<td>Angptl4 promotes lymphovascular invasion</td>
<td>Shibata et al (2010) (49)</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Real-time PCR, and Western blot</td>
<td>High expression of Angptl4 in 36.9% of adenocarcinomas</td>
<td>Nakayama et al (2010) (48)</td>
<td></td>
</tr>
<tr>
<td>Kaposi’s sarcoma</td>
<td>Real-time PCR and Western blot</td>
<td>Upregulated by virus-encoded G-protein-coupled receptor</td>
<td>Ma et al (2010) (43)</td>
<td></td>
</tr>
<tr>
<td>Hepatoma</td>
<td>Increased expression in cancer</td>
<td>Angptl4 contributes to anoikis resistance</td>
<td>Zhang et al (2008) (40)</td>
<td></td>
</tr>
<tr>
<td>General cancer</td>
<td>Transgenic mice, corneal neovascularization, and Miles permeability assays</td>
<td>Low expression in tumor</td>
<td>Mice expressing Arp4 in the skin exhibited remarkable suppression of tumor growth</td>
<td>Ito et al (2003) (37)</td>
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<td>---------------</td>
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</tr>
<tr>
<td>Gastric cancer</td>
<td>Low expression detected in tumor</td>
<td>Angptl4 gene is silenced by methylation, contributing to cancer development</td>
<td>Kaneda et al (2002) (58)</td>
<td></td>
</tr>
</tbody>
</table>
Legends

Figure 1. Phylogenetic trees for the members of the angiopoietin (ANG) and angiopoietin-like protein (ANGPTL) families discovered in humans. There are 7 members in the ANGPTL family and 5 in the ANG family. Both families of proteins share structural similarities. However, ANGPTLs do not bind to ANG receptors Tie 1 and Tie 2 to mediate their biological functions ANGPTLs are commonly considered to be orphan ligands.

Figure 2. Structure and biological functions of ANGPTL4. Full-length ANGPTL4 (flANGPTL4; ~65 kDa) comprises a highly hydrophobic signal peptide (SP), an N-terminal coiled-coil domain (CCD) and a C-terminal fibrinogen-like domain (FLD). ANGPTL4 protein oligomerizes prior to secretion and cleavage. The N- and C-terminal domains are separated by a short linker that after being secreted from the cell can be cleaved into the nANGPTL4 (~26 kDa) and cANGPTL4 (~47 kDa) fragments. Cleavage is catalysed by proprotein convertases (PC) at a conserved basic sequence (-RRKR-). The nANGPTL4 remains oligomeric, whereas cANGPTL4 will dissociate into monomers. Genetic variation in ANGPTL4 provides insights into protein processing and function. Oligomerized flANGPTL4 is able to bind to the extracellular matrix via the heparan sulfate proteoglycans and inhibit endothelial cell adhesion, migration, and tubule formation. Both flANGPTL4 and nANGPTL4 are involved in the modulation of triglycerides disposition via their inhibitory effect on lipoprotein lipase (LPL). However this inhibitory effect on LPL appears to be weaker in the case of flANGPTL4 inhibition
Angiopoietin-like 4 in Human Cancers

(dotted arrow). In contrast, cANGPTL4 binds to β1-integrin and activates two downstream processes. One process involves the stimulation of NADPH oxidase leading to the production of $O_2^-$. A high ratio of $O_2^-:H_2O_2$ has been found to activate the Src/ERK/PKBα prosurvival pathway thereby conferring anoikis resistance and promoting tumor growth. Alternatively, cANGPTL4-bound β1-integrin can activate the FAK/Src/PAK1 cascade to trigger the PKC-mediated signaling pathways that are crucial for effective wound healing. It is also interesting to note that cANGPTL4 has been found to be able to inhibit angiogenesis by suppressing the Raf/MEK/ERK signaling cascade.

**Figure 3. Diverse roles of ANGPTL4 in human cancer.** The roles of ANGPTL4 in human cancer are controversial. The fANGPTL4 (depicted by a rectangle and an oval), and cANGPTL4 (depicted by an oval) have been implicated in the various aspects of cancer. The tumor microenvironment is inflammatory and hypoxic in nature. In the event of promoting tumor growth, the expression of ANGPTL4 appears to be upregulated by pro-inflammatory TGF-β via the SMAD pathway as well as by COX2-induced PGE2 signaling in carcinomas. ANGPTL4 subsequently activates NADPH oxidase (NOX) to promote tumor cell proliferation via the Src/ERK/STAT1 pathway. As the aberrant growth of tumor cells progresses, a mechanism to evade apoptosis via anoikis resistance ensures the survival of the tumor cells through ANGPTL4-mediated inhibition of proapoptotic Bcl-2 associated death promoter (BAD; see text). In addition, fANGPTL4 was shown to upregulate the expression of VCAM-1 on endothelial cells through an unknown mechanism facilitating the attachment of circulating metastatic
cancer cells to endothelial cells promoting their transendothelial extravasation and metastatic tumor formation. In contrast, anti-angiogenic effects of flANGPTL4 have also been reported whereby it inhibits endothelial cell proliferation, migration and tubule formation via an unknown mechanism. In addition, N-glycosylated cANGPTL4 has been shown to impair bFGF and VEGF-induced angiogenesis by suppressing the Raf/MEK/ERK pathway, suggesting that other post-translational modifications may also play a role in the modulation of the functions of ANGPTL4. One study has also demonstrated the inhibitory effect that flANGPTL4 has on VEGF-induced vascular permeability; however, the mechanism remains unknown. Despite the establishment of an anti-angiogenic effect, whether flANGPTL4 or either of its truncated fragments binds to a receptor to modulate these effects remains to be determined.
Tan et al, Figure 1
Tan et al, Figure 2

PC cleavage site

fANGPTL4 N

SP CCD

25 143

nRKR

FLD

C ~65 kDa

Oligomerisation

Secretion and cleavage

26 kDa

nANGPTL4 (oligomer)

C CD

~26 kDa

Oligomerisation

Inhibition of lipoprotein lipase (LPL)

Interacts with heparan sulphate proteoglycans

Inhibition of endothelial cell adhesion, migration, and tubule formation

NADPH oxidase

O2 production

Increased O2 : H2O2 ratio

SIP/ERK/PKB

Increased anoikis resistance

Tumour cell survival

NADPH oxidase

FAK/Src/PAK1

PKC

Increased keratinocyte migration

Increased wound closure efficiency

STAT1

Tumour cell proliferation

Suppression of Raf/MEK/ERK

Inhibition of angiogenesis
Tan et al, Figure 3

Pro-Tumorigenesis

Anti-Tumorigenesis

Anchorage

Metastasis

Angiogenesis

Intervasation

Secondary tumor