Axl and Its Mediated Signaling Axis in Cancer

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J. Haybaeck (ed.), *Mechanisms of Molecular Carcinogenesis – Volume 1*,
DOI 10.1007/978-3-319-53659-0_3

Abstract

AXL is a receptor tyrosine kinase activated by growth arrest-specific 6 (GAS6) or by ligand-independent homophilic and/or heterophilic interactions that regulate cancer cell proliferation, survival, migration, invasion, distant metastasis, the epithelial to mesenchymal transition (EMT), angiogenesis, and drug resistance. Axl belongs to the Tyro-3, AXL, and Mer (TAM) family of receptor molecules, known to be expressed in a number of organs and cell lines with a few exceptions such as lymphocytes and granulocytes. However, inappropriate Axl upregulation leads to uncontrolled cell growth, and its abundant expression is detected in a number of cancers such as colorectal and breast tumors. The transcriptional regulation of Axl is epigenetically inhibited by CpG hyper-methylation. Furthermore, the zinc finger transcriptional factor family members Sp1 and Sp3 are the constitutive regulators of Axl. Under oncogenic conditions, AP-1 family members mainly enhance its expression. Moreover, an overexpression of MZF1 induces Axl expression and mediates the migratory and invasive behavior of cells. Axl is also posttranscriptionally regulated by the small noncoding tumor suppressor microRNAs (miRNAs) miR-34 and miR-199. A malfunction of these different regulatory mechanisms in controlling Axl expression can induce Axl expression in cancer phenotypes. In addition to aspects of its regulation, this chapter will cover details of Axl structure, its expression in diverse cancer entities, and its signaling axis in the mediation of functions related to cancer phenotypes, including cell proliferation, antiapoptotic effects, EMT, cancer metastasis, angiogenesis, and drug resistance.

3.1 Axl Structure, Stimuli, and Expression

3.1.1 Discovery and Structure of Axl

Axl (also called UFO, ARK, and Tyro7) is a receptor tyrosine kinase which belongs to the TAM subfamily. This group comprises the three receptor tyrosine kinases Axl, Mer, and Tyro-3, which share a common structure. Axl was originally identified as a transforming gene in human leukemia and subsequently cloned and named as Axl from the Greek word “anexelekto,” meaning uncontrolled [1, 2]. The *Axl* gene is evolutionarily conserved between vertebrate species. The amino acid sequence of Axl revealed it to be a novel type I transmembrane protein with an intracellular tyrosine kinase domain. This gene is ubiquitously expressed, being detectable in a wide variety of organs and cell lines of epithelial, mesenchymal, and hematopoietic origin, as well as non-transformed cells, although it is absent in lymphocytes and granulocytes [2]. *Axl* is located on chromosome 19q13.1-q13.2 and yields two forms of transcripts derived through alternative splicing. Variant 1 encodes the full-length isoform (5014 bases), and variant 2 (4987 bases) lacks exon 10. The protein that results from variant 2 lacks an internal 9-amino acid sequence; apart from this, no functional differences between these isoforms have yet been reported. The full-length 894-amino acid protein has

a mass of 140 kDa and exhibits a roughly equal distribution of amino acids on either side of the plasma membrane. The structure of the extracellular domain of Axl makes it unique among receptor tyrosine kinases because it consists of a juxtaposition of two immunoglobulin-like repeats and two fibronectin type III repeats [2]. This pattern of structural elements is reminiscent of many cell adhesion molecules, and Axl has been implicated in cell–cell interactions [3]. Like other receptor tyrosine kinase molecules, the intracellular signaling functions of Axl cytoplasmic moieties (tyrosine kinase domain) have been elucidated through the cloning of receptor targets (CORT) method. Tyrosine residues at 779, 821, and 866 are active and function as docking sites for a range of intracellular tyrosine molecules including phosphoinositide phospholipase Cγ (PLCγ), growth factor receptor-bound protein 2 (GRB2), c-Src, and lymphocyte-specific protein tyrosine kinase (Lck) [4].

3.1.2 Activation of Axl Signaling

In 1995, an Axl stimulatory factor was purified from a conditioned medium of the Wi38 cell line and identified by N-terminal sequencing as growth arrest-specific gene 6 (GAS6) [5]. The 678-amino acid Gas6 protein is the latest addition to the vitamin K-dependent family of proteins. Gas6 shows a 43% amino acid sequence identity with protein S, an abundant serum protein [6]. Gas6 has the same domain organization as protein S: an N-terminal region containing 11 c-carboxyglutamic acid residues (Gla), a loop region, four EGF-like repeats, and a C-terminal sex hormone-binding globulin (SHBG)-like structure that is composed of two globular laminin G-like (LG) domains [7]. Several studies utilizing either site-specific blocking antibodies or partial protein constructs have established the SHBG region of both Gas6 and protein S as being the receptor-binding site. More detailed molecular studies revealed that the first LG domain in the Gas6 SHBG region is necessary for Axl binding [8]. The existence of a minimal Gas6–Axl complex, derived from its crystal structure, has provided a detailed view of the regions involved in their interactions [9]. In this complex, the two Ig-like domains of an Axl monomer are cross-linked by the first LG domain of a Gas6 molecule in a first high-affinity interaction. The lateral diffusion of such 1:1 complexes leads to dimerization and the formation of a circular 2:2 assembly. Two distinct sites of Gas6–Axl contact were revealed, one major and one minor; the minor site is conserved only within the Axl subfamily. No direct Axl–Axl or Gas6–Gas6 contacts were apparent in the complex (Fig. 3.1). In the major contact site, several charged residues in both Axl and Gas6 were identified to form part of polar β-sheet surfaces which interact with each other. It is interesting that protein S does not possess a distribution of charged residues similar to that found in Gas6, which may explain its inability to bind to Axl. Gas6 not only binds and activates the Axl signaling axis; it behaves the same way toward the other TAM family member receptors, with nanomolar binding affinities (0.4, 2.7, and 29 nM) in the order Axl → Tyro-3 → Mer [10].

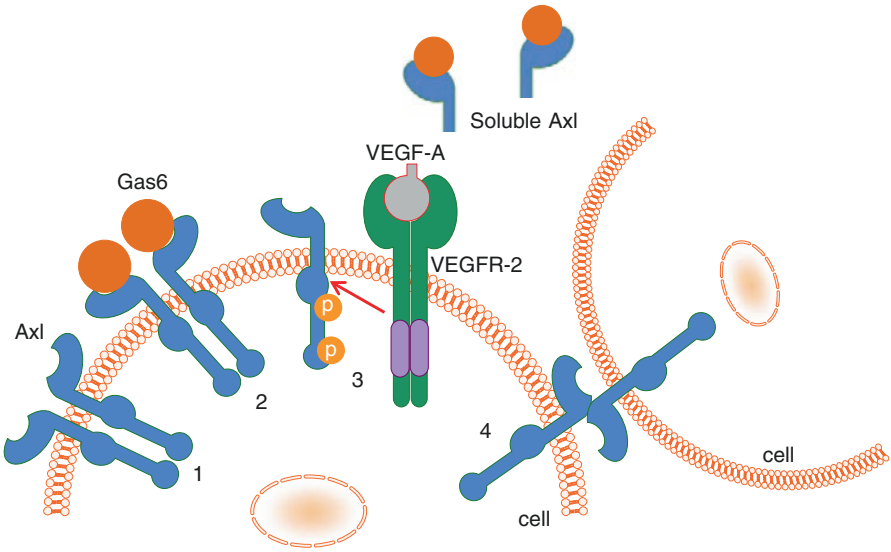

Fig. 3.1 Models for Axl signaling axis activation: (*1* and *4*) Direct, ligand-independent homophilic or heterophilic interaction between two Axl/Tyro-3 monomers in a single cell or between neighboring cells. (*2*) Ligand-induced dimerization of Axl monomers from two 1:1 (ligand–receptor) complexes to one 2:2 (2 ligand–receptor) complex. (*3*) Activation of VEGFR activates the Axl intracellular tyrosine kinase. (*4*) Hypothetical model for interaction between two Axl monomers on neighboring cells

Apart from the previously described conventional activation of the Axl signaling axis, other experimental models and a hypothetical model were proposed for Axl signaling. The first one is a ligand-independent homophilic interaction between two Axl monomers under overexpressed conditions in the same cell [11, 12]. Similar phenomena may also occur between two neighboring Axl expressing cells. The ligand-independent homophilic interactions were well described for Tyro-3, a TAM family member [13]. Axl also harbors a similar structure as Tyro-3; one could speculate that similar interactions might be possible between Axl and Tyro-3 [14]. The second way of signaling is new in its kind, which is a heterophilic interaction between two receptor signaling cascades. Binding of vascular endothelial growth factor A (VEGF-A) activates vascular endothelial growth factor receptor 2 (VEGFR2), which subsequently activates the T cell-specific adaptor protein (TSAd) and Src family kinases (SFKs), which engage Axl at its juxtamembrane domain and activates signaling [15] (Fig. 3.2). Yet hypotheses about heterodimeric interactions between TAM family members are based purely on structural similarities and have yet to be demonstrated experimentally (Fig. 3.1).

The extracellular regions of several transmembrane proteins such as adhesion molecules and growth factors and cytokine receptors have been found in circulating forms in human plasma [16]. These soluble ectodomains are shed from the full-length protein and thereby may limit the accessibility of the cell-bound receptor to the ligand.

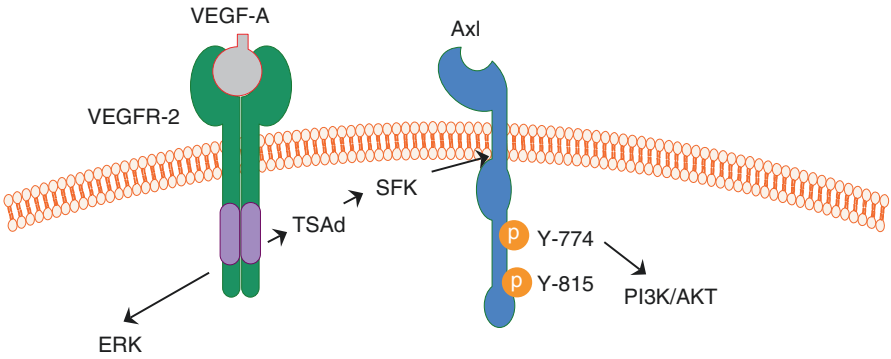

Fig. 3.2 Vascular endothelial growth factor (VEGF) signaling axis activates Axl pathway. VEGF-A activates several intracellular events that includes VEGFR2. This indeed activates TSAd, further SFK, which engages Axl and activates the Axl-mediated PI3K/AKT pathway (without Axl ligand binding or homophilic intracations). *TSAd* T cell-specific adaptor protein, *SFK* Src family kinase

Therefore they may represent an important posttranslational mechanism for controlling ligand efficacy under certain clinical conditions. The soluble Axl ectodomain is released as a result of proteolytic cleavage in conditioned media of various cell lines [17, 18]. A mouse Axl ectodomain was detected in tumor cell and dendritic cell medium and in serum. It has been suggested that proteolytic cleavage through the disintegrin-like metalloproteinase ADAM 10 is involved in its generation [19]. Furthermore, a significant amount of soluble Axl, but not Tyro-3 or Mer, was found to be in complex with Gas6 in mouse serum [19]. These observations indicate the potential value of investigating the presence of soluble Axl ectodomain in human plasma. The detection and quantitation of plasma Axl might reflect an altered regulation of Gas6–Axl system components under various clinical conditions and may therefore be of diagnostic value. In conclusion, the soluble form of Axl could reduce Axl signaling-mediated cancer phenotypes by depleting the availability of Gas6 (Fig. 3.1).

3.1.3 Axl Is Upregulated in Cancer

After the identification of Axl and structural and function determinations establishing it as a receptor tyrosine kinase and transforming gene, a number of screens have been carried out to detect its presence in a number of normal and cancer cell lines and resected patient tumor specimens. These studies have demonstrated that Axl is overexpressed in several cancer entities (Table 3.1). Furthermore, it has been shown that Axl is overexpressed in most cancer cell lines such as non-small cell lung cancer (NSCLC), breast cancer (BRC), and colorectal cancer (CRC), and its expression positively correlates with cell adhesion or invasive potential [18, 36, 37]. Increased Axl expression in cancer entities positively correlated with the cancer progression and poor prognosis and has also been identified as a potential druggable target in patient treatment.

Table 3.1 Axl expression is increased in different human cancers when compared to respective healthy specimens

Tumor entity	Sample size	Method	Correlations with clinical parameters	References
Colon	17 and 223	WB, IHC, RT-PCR	Potential marker for cancer progression and an oncotarget	[20–22]
Gastric	96	IHC	Poor prognosis	[23]
Breast	569	RT-PCR	Lymphovascular invasion	[24]
Lung	88	RT-PCR, IHC	Worse clinicopathological features and prognosis	[25]
Thyroid	27	IHC	Cancer progression	[26]
Liver	137	IHC	Lymph node metastasis	[27]
Prostate	96	RT-PCR	Cancer progression	[28]
Osteosarcoma	62	IHC	Poor prognosis	[29]
Renal Cell	221	ELISA	Cancer progression	[30]
Glioma	121	RT-PCR, IHC	Poor prognosis	[31]
Leukemia				
Myeloid	112	RT-PCR, WB	Poor prognosis	[32]
Chronic Lymphocytic	22	WB	Unique target molecule for treatment	[33]
Ovarian	72	RT-PCR, IHC	Poor prognosis	[34]
Esophageal	92	IHC	Cancer progression	[35]

IHC immunohistochemistry, *IFC* immunofluorescence, *RT-PCR* real-time polymerase chain reaction, *WB* Western blot, *ELISA* enzyme-linked immunosorbent assay, *TMA* tissue microarrays

3.2 Axl-Mediated Hallmarks of Cancer

3.2.1 Axl Signaling in Cell Proliferation

Cell proliferation is a normal physiological process which occurs in all types of cells and organs. Under normal physiological circumstances, cell proliferation is tightly controlled. Changes at cellular, genetic, and/or epigenetic levels lead to a reprogramming in which cell proliferation occurs and may result in uncontrolled cell division. Janssen et al. were the first to report that Axl is a transforming and cell-proliferating gene under induced expression conditions [1]. After this initial report, studies in more cancer entities buttressed a functional role for Axl in inducing cell proliferation. Varnum et al. identified Gas6 as a stimulus of an Axl signaling axis which mediates mouse fibroblast cell transformation and increased proliferation [5]. Before the identification of Gas6 as an Axl ligand, a study with interleukin-3-dependent 32D cells using a chimeric receptor containing the recombinant epidermal growth factor (EGF) receptor extracellular and transmembrane domains and the Axl kinase domain showed that PI3K is dispensable for Ras/ERK-mediated

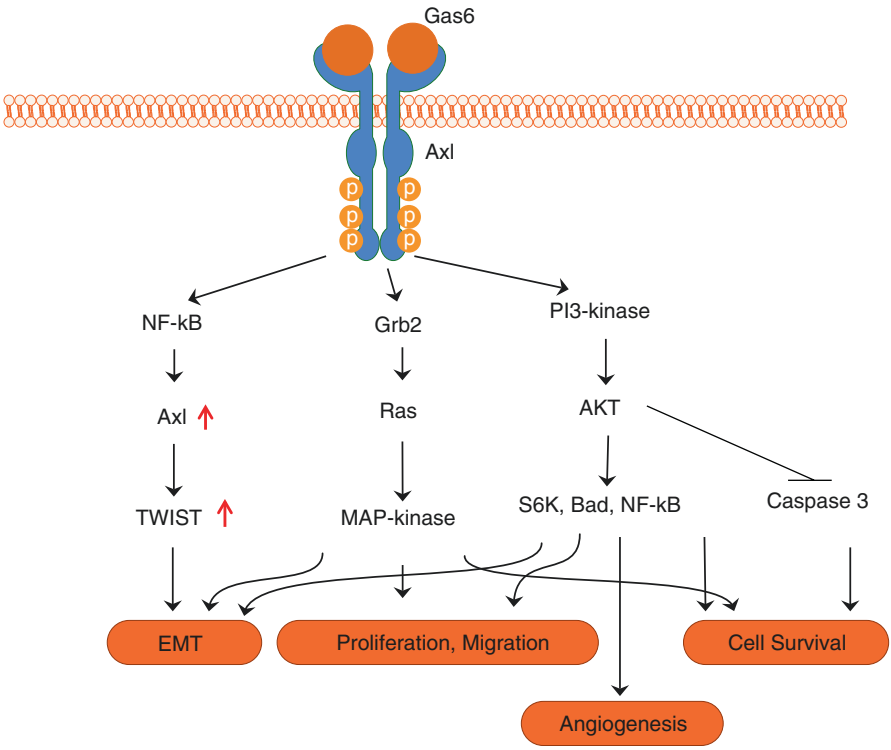

Fig. 3.3 Axl signaling axis: Activation of different intracellular molecules, mediating cell proliferation, survival, migration, invasion, metastasis, EMT, and angiogenesis (*red upward arrow*: Gas6/Axl signaling activates the transcriptional regulation of the gene) (color figure online)

cell proliferation and also that different threshold levels are needed for Ras/ERK activation. This study gives a demonstration that different extracellular domains dramatically alter the intracellular responses of receptor kinases to different stimuli, and this is especially true for Axl [38]. Later studies with different cell types revealed that the Ras/ERK pathway contributes to Axl-mediated cell survival [39]. In addition to mechanisms involving Gas6 activation, the induction of Axl expression can stimulate progression through the cell cycle and the division of NIH3T3 cells through the mitogen-activated protein kinase (MAPK) pathway [40]. Depending on the availability of Gas6 ligand and Axl protein in various cell types, this means that the MAPK/ERK pathway might be important in Gas6/Axl-mediated cell signaling in some contexts [41].

Apart from the MAPK/ERK pathway, many Axl-mediated downstream pathways have been linked to PI3K/AKT and the ribosomal protein S6 kinase (S6K)-mediated signaling. Goruppi et al. used specific PI3K and S6K inhibitors to show that these two kinases are essential mediators in Gas6/Axl-mediated cell proliferation (Fig. 3.3). Studies involved pretreatments of serum-started NIH3T3 cells with the specific PI3K inhibitor Wortmannin and S6K inhibitor rapamycin before

stimulation with Gas6. Wortmannin treatment leads to a significant inhibition of the Gas 6-induced S-phase entry of cells. The previously described Gas6/Axl-mediated activation of MAPK was also abolished with this treatment. Moreover, rapamycin treatment abrogated Gas6-induced S6K activation, leading to an inhibition of cell proliferation. While Axl does not appear to directly bind Src, Gas6 activation nonetheless induced the phosphorylation of Src in serum-starved NIH3T3 cells [42]. These studies clearly demonstrated a role for the Gas6/Axl-mediated signaling axis as an inducer of cell proliferation through distinct intermediary molecules including PI3K, Ras/ERK, and Src.

3.2.2 Antiapoptotic Functions of Axl Signaling

Under normal physiological conditions, most cells generally undergo programmed cell death events (apoptosis) to control cell growth or remove damaged cells from multicellular organisms. Studies with the interleukin-3-dependent 32D cell line first demonstrated that Gas6/Axl signaling reduced the induction of apoptosis [38]. Even in the absence of a stimulus, the overexpression of Axl alone increased cell proliferation and protected NIH3T3 cells from cell death under serum-starved conditions [40]. A treatment of primary chondrocytes that overexpressed Axl with Gas6 resulted in increased survival in colony formation assays [43]. The binding of PI3K led to Axl activation and triggered multiple downstream pathways and increased cell survival [42]. Bellosta et al. used fibroblasts from Axl knockout mice in a further demonstration of the antiapoptotic activities of Axl. Serum-starved Axl knockout cells showed increased levels of apoptosis which could not even be rescued by the addition of Gas6. In addition, Axl protected these fibroblast cells from apoptosis even after treatment with TNF alpha or an overexpression of c-Myc [39]. These initial reports revealed that Gas6/Axl has antiapoptotic signaling functions both in vitro and in vivo.

The expression of Ark, the mouse homologue of Axl, was detected in Gn10 GnRH cells derived from migrating tumors in olfactory cells, but not in GT1-7 cells derived from the post-migratory tumor in the forebrain. Comparatively in these lines, Gn10 are more resistant to serum-stimulated apoptosis. Gas6/Axl signaling activated the PI3K and ERK pathways in Gn10 cells, and the effects were blocked by the ERK-specific inhibitor PD98059 and PI3K inhibitor Wortmannin [41]. Gas6/Axl signaling similarly induced antiapoptotic effects in human umbilical vein endothelial cells and human pulmonary artery endothelial cells [44, 45]. Further its shown that Gas6/Axl mediated antiapoptotic signaling is mediated through AKT activation [46]. These studies reveal a clear and general role for antiapoptotic processes mediated by Gas6/Axl in different cells under conditions of Axl overexpression.

Nuclear factor kappa B (NF-κB) is known to translocate into the nucleus upon activation of the PI3K/AKT cell survival pathway. Under normal conditions, NF-κB is found in the cytoplasm as a homodimer or a heterodimer including members of a family of structurally related proteins. Five members of the family have been identified: RelA (p65), RelB, cRel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100). All are

inactive in association with inhibitory proteins of the IκB family (IκBα, β, ε, and Bcl3) or as precursors of NF-κB1 (p105) and NF-κB2 (p100), whose nuclear localization signals are masked until they are further processed. Upon stimulation, the inhibitors IκB and p105 are activated, a process which triggers their proteolytic degradation via the ubiquitin-proteasome pathway. This leaves activated forms of NF-κB that can translocate to the nucleus and induce the transcriptional regulation of antiapoptotic genes like BCl-X_L and BCL2 [47, 48]. Demarchi et al. showed NF-κB as one of the key downstream molecules in mediating the Gas6/Axl signaling antiapoptotic property. As described above, Gas6 stimulation increased the nuclear translocation of active NF-κB and its binding to BCl-X_L promoter and induced the BCl-X_L expression in serum-starved NIH3T3 cells [49]. These authors also observed glycogen synthase kinase 3 (GSK3) activation, which is known to induce cell proliferation and antiapoptotic functions [50]. A similar functional role of NF-κB was shown under the Gas6/Axl signaling axis in endothelial cells. Gas6 treatment led to the activation of AKT and NF-κB and increased the expression of the antiapoptotic gene BCL2—one mechanism by which Axl-stimulated pathways protect cells from apoptosis. The treatment had the supportive effect of decreasing the expression of the caspase 3 activation products p12 and p20 [51], which are proapoptotic. In vascular smooth muscle cells, Son et al. showed that Gas6/Axl signaling inhibited the function of the proapoptotic molecules BAD and caspase 3 [52]. An shRNA-mediated Axl knockdown in NSCLC cell lines showed increases of apoptosis over controls [53]. In OE33 and OE19 esophageal cancer cells, Axl overexpression attenuated cellular and molecular markers of apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). In support of this finding, Axl knockdown FLO-1 cells were sensitive to TRAIL-induced apoptosis. However, another mechanism seemed to be involved: Axl expression did not alter DR4 or DR5 expression, but associations of Axl with DR5 were detected. This blocked the recruitment of caspase 8 to the death-inducing signaling complex. This confirms that Axl mediates TRAIL-induced apoptosis by mitigating the death-inducing signaling complex [54].

All of these studies provide clear evidence for the importance of Gas6/Axl signaling or Axl hemophilic activation through an increased activity of downstream signaling pathways (PI3K/AKT, MAPK, GSK3, TRAIL) and specific molecules (NF-κB, BAD, BCl-X_L, BCL2) that are involved in cell survival/antiapoptosis in a range of cell types.

3.2.3 Axl Signaling Induces Angiogenesis

Angiogenesis is important in sustaining tumor growth and survival. A tumor mass stimulates the formation of new blood vessels to permit tumor expansion, local invasion, and metastasis and mainly to deliver oxygen, nutrients, and survival and growth factors. Several factors are known to be important for angiogenesis, including the vascular endothelial growth factor receptor (VEGFR) and corresponding receptor-associated signaling that promotes endothelial proliferation and migration.

Other angiogenesis regulators include EGF-like domain-containing protein 7 (EGFL7) and alpha-5 beta-1 (α5β1) integrin, which contribute to the formation of new blood vessels. Angiogenesis processes have been further extrapolated through studies of interactions between endothelial cell interactions and the extracellular matrix [55–58].

A functional role for Axl in vasculature was first demonstrated by O'Donnell et al. [45]. Axl expression was detectable in capillary endothelium, in vascular smooth muscle cells of arterioles and veins, and in a subset of synovial cells in the synovial tissue of a patient suffering from rheumatoid arthritis (RA), a disease whose key features are abnormalities in angiogenesis and synovial cell hyperplasia. In vitro studies showed that Gas6/Axl signaling protected human umbilical vein endothelial cells (HUVECs) from tumor necrosis factor alpha (TNFα)-mediated cytotoxicity [45]. The hint that Axl might be involved in angiogenesis came from observing its high expression in vascular cells.

This role was confirmed by Axl gain- or loss-of-function experiments in vitro and in vivo. Axl antisense screening reduced the proliferation of HUVECs and their directed migration to vitronectin haptotaxis. Furthermore, in co-culture branching morphogenesis/tube formation VGFR-dependent assays, HUVEC/primary pulmonary artery smooth muscle cells showed a functional role for Axl in angiogenesis. Knockdowns of Axl mitigated tube formation, fiber length, and branching, which are main functional events in endothelial tube morphogenesis. Similar results were pronounced in an SCID mouse angiogenesis model. shRNA-Axl- and shRNA-VEGFR2-silenced HUVECs were mixed with Matrigel, seeded into poly-L-lactic acid scaffolds and subsequently implanted into SCID mice. After 14 days, a significant reduction in human Tie2 expression levels was observed in both shRNA-Axl and shRNA-VEGFR2 cells, which indicates a role for Axl in neovascularization [59]. Similarly, Axl-siRNA-silencing reduced the expression of Dickkopf-homologue 3 (DKK3) and Tie2, which play a role in tube formation. However, silencing these two genes had no effect on Axl expression in HUVECs. Anti-VEGF treatment attenuated the reduction of tube formation introduced by siRNA-Axl or siRNA-DKK3 [60]. Implanting cells that stably expressed Axl-dominant negative constructs in nude mice using the dorsal skinfold chamber model demonstrated that Axl-dominant negative-expressing cells reduced the density and diameter of tumor vessels to a degree comparable to that of Axl wild-type-expressing cells [12].

Angiogenesis is promoted by high levels of lactate, which are also essential for wound healing and tumorigenesis. Lactate activates the PI3K/AKT pathway in primary human endothelial cells and is essential for their organization into tubes, as well as for vessel formation in mouse aortic explants. This activation is mediated by the ligand-mediated activation of the three receptor tyrosine kinases Axl, Tie2, and VEGF receptor 2 and has been confirmed through pharmacological inhibitions of their kinase activity or by suppressing their expression [61]. VEGF-A generally activates a series of intracellular events through the activation of VEGFR2 and subsequent downstream molecules such as Src family kinase (SFK) in a T cell-specific adaptor protein (TSAd)-dependent manner. It is also known that VEGFR2 activates

the PI3K/AKT pathway in endothelial cells. An interesting and novel aspect of these findings is that activated SFKs engage Axl via its JM domain and thereby promote autophosphorylation at Y773 and Y815 in the absence of any external mediated activation of Axl. The activated tyrosine residues within the optimal motif bind to the SH2 domains of p85, and this further activates PI3K, which produces lipids that are essential for the activation of AKT [15]. This is one of the most important aspects of the Axl signaling, responding for other receptor signaling through intracellular kinase domain activation, without its receptor or ligand (Figs. 3.2 and 3.3). These studies clearly demonstrate that Axl has a signaling function in angiogenesis and by consequence a role in the angiogenic mechanisms related to metastases.

3.2.4 Axl Signaling Induces Epithelial–Mesenchymal Transition

Epithelial–mesenchymal transition (EMT) is a process whereby epithelial cells lose their cell polarity and cell–cell adhesion and gain migratory and invasive properties to become mesenchymal stem cells. As cells undergo EMT, they gain increased resistance to apoptosis and alter their production of extracellular matrix (ECM) components and ECM-degrading enzymes. The switch that occurs in EMT initiation is also accompanied by changes in a number of key molecules including the expression and activity of specific transcription factors and specific cell-surface proteins, a reorganization and expression of cytoskeletal proteins, and changes in microRNA expression patterns [62]. Kalluri et al. provided a summary of many genes that function as EMT markers, including key transcriptional regulators such as Snail, Slug, and Twist, in a recent review [62].

A role for Axl functions in EMT in pancreatic cancer was first demonstrated [63]. This work showed that shRNA-Axl silencing in MIAPaCa-2 cells led to a significant reduction in signaling in MAPK/ERK and PI3K/AKT pathways. It also revealed a significant downregulation in the major transcriptional factors that initiate the mesenchymal switch: Snail, Slug, and Twist [63]. Interestingly, the overexpression of these three factors in immortalized mammary epithelial cells enhances Axl expression and autocrine signaling loop with Gas6 ligand (Fig. 3.3). This also enhanced the expression of the mesenchymal markers N-cadherin and vimentin and reduced the expression of the epithelial markers E-cadherin and β-catenin. In parallel, shRNA-Axl silencing in highly metastatic breast cancer cells MDA-MB-231 reduced the threshold of mesenchymal-like features and mediated cancer progression events [64, 65]. Another study showed that EMT induced by Slug and H-Ras is mediated through Axl via vimentin in breast cancer cells [66]. Furthermore, Axl expression positively correlated with the vimentin expression in EGFR-mutant NSCLC tumors in vivo [67]. This evidence confirms increased Axl expression induction by mesenchymal cells and that its expression is induced by the mesenchymal transcriptional factors Slug, Snail, and Twist through vimentin.

Axl is posttranscriptionally downregulated by the tumor suppressor and EMT inhibitor miRNA miR-34a [36, 68]. miR-34a is also known to downregulate other

EMT-inducing genes including axin-like protein (AXIN2), carbonic anhydrase 9 (CA9), C-X-C motif chemokine 10 (CXCL10), FOS-like antigen 1 (FOSL1), fucosyltransferase 8 (FUT8), growth arrest-specific protein 1 (GAS1), Kruppel-like factor 6 (KLF6), and podocalyxin-like protein 1 (PODXL) [68]. As discussed above, Gas6/Axl signaling activates the AKT and NF-κB signaling cascades, which are known to induce the EMT phenotype in a range of cancers.

3.2.5 Axl Signaling in Cancer Metastasis

In 2012 the World Health Organization (WHO) registered more than 8.2 million cancer-related death cases mainly lung, liver, stomach, colorectal, breast, and esophageal cancer. More than 90% of these cancer-related deaths are due to cancer metastasis, which is induced by a loss of the normal self-control of signaling cascades [69]. Cancer metastasis is a multistep process accompanied by a number of changes in morphology and molecular functions. The way cancer cells spread from a primary site to different local or distant organs in a patient depends on the tumor type. This process begins after gaining self-sustainability at the tumor site through angiogenesis and extracellular degradation and a gain in migratory properties, followed by the steps of migration, local invasion, intravasation, and transport through the circulatory system to different organs, extravasation, and formation as micrometastasis or macrometastasis, which require local angiogenesis for establishment [69, 70].

Axl is capable of inducing all aspects of cancer metastasis events including migration, invasion, angiogenesis, and EMT in different cancer entities. An overexpression of Axl has been reported using differential display PCR methods in the highly metastasizing prostate cancer cell line DU145 [71]. However, for the first time, the role of Axl in terms of cancer metastasis, explained through adenovirus type V E1A protein (E1A), is known to reverse the transformed phenotype, to inhibit metastasis, and to induce apoptosis. Overexpression of E1A inhibited the expression of Axl and prevented the Gas6/Axl signaling axis which induces signaling cascades required for cell survival, including AKT and NF-κB; it also inactivated or downregulated apoptosis-inducing genes such as BAD and Fas-ligand, thus inhibiting cancer progression [72]. Nakano et al. derived low and highly metastatic cell lines from highly metastatic cell lines through the dilution plating method [73]. A cDNA array analysis by the authors revealed that five genes, including Axl, were significantly upregulated in highly metastatic cell lines compared to the lines with low metastatic activity. Similarly, suppression subtractive hybridization screening of lung cancer cell lines revealed an induction of Axl expression in the highly metastatic cell line PLA-801D compared to the low metastatic cell line PLA-801C [74]. Axl overexpression induced the expression of matrix metalloproteinase 9 (MMP-9) and activated the ERK pathway, enhanced the transactivation of NF-κB, and induced the translocation of brahma-related gene 1 (Brg-1) to the nucleus. Axl-mediated MMP-9 expression and the invasiveness of cancer cells were also significantly inhibited by interfering with the pathway: either through a dominant negative overexpression of ERK, IkB, or Brg-1 or a specific inhibition of ERK and NF-κB [75].

Axl staining was positive in 54% of pancreatic cancer specimens and significantly associated with lymph node metastases. Here, too, a specific inhibition of the ERK and P13K/AKT pathway showed a loss of Axl function in in vitro studies. Axl silencing decreased the amount of the GTPase proteins Rho and Rac and mirrored the migration and invasion of MIAPaCa-2 cells [63]. Even a dominant negative inhibition of just the Axl kinase domain activity had similar effects by inhibiting tumor cell invasion in fetal rat brain aggregates [12]. Similarly, inhibition of Axl expression and/or functions inhibited cell migrations, invasions, and distant metastasis to the lung in an orthotopic breast cancer model [60, 64].

Interfering with other molecules functionally associated with Axl has similar effects in multipole cancers. Yes-associated protein 1 (YAP1) plays a major role in tumorigenesis and tumor progression in multiple cancers. A shRNA-YAP1-mediated knockdown of YAP1 significantly inhibited the expression of Axl, the proliferating cell nuclear antigen (PCNA), and MMP-9 and mediated the invasive potential of LAC A549 and GAC SGC-7901 cell lines [76]. In clear cell renal cell carcinoma (ccRCC), Rankin et al. demonstrated cross talk between von Hippel–Lindau/hypoxia-inducible transcription factor (HIF) and Gas6/Axl signaling [77]. HIF1 and HIF2 transactivate Axl expression by binding the hypoxia-response element in the Axl proximal promoter. In parallel, the authors determined that Gas6/Axl signaling uses lateral activation of met proto-oncogene (MET) through SRC to maximize cellular invasion abilities [77]. Similarly, Axl activates transforming growth factor-β (TGF-β) signaling and the tumor progression events it mediates in hepatocellular carcinoma (HCC) [78, 79].

Myeloid zinc finger 1 (MZF1) is a transcription factor known to induce cell proliferation, antiapoptotic properties, and transformation of cells. siRNA-MZF1 silencing reduced the number of tumors and prolonged the time it took them to form [80–82]. The overexpression of MZF1 induced migration and invasion by inducing Axl expression in colorectal and cervical cancer cell lines. Furthermore, an in vivo chicken embryo metastasis assay showed that the overexpression of MZF1 induced tumor growth and the formation of distant metastases in an Axl-dependent manner. A positive correlation was found between Axl and MZF1 expression in colorectal cancer tumor specimens [22].

Axl is transcriptionally induced by AP-1 family members through the MAPK pathway, under oncogenically stimulated conditions [83]. As described above, the MAPK/ERK pathways are one of the key downstream pathways activated by different oncogenic stimuli-like phorbol esters, which activates the AP-1 family. Interestingly, the invasive capacity of a panel of NSCLC, breast cancer, and CRC cells correlated positively with Axl mRNA and protein expression [36]. Less invasive cells, on the other hand, exhibited low levels of Axl expression due to epigenetic regulation [84]. Additionally, the Axl posttranscriptional regulators miR-34 and miR-199 significantly reduced Axl-mediated migration, invasion, and distant metastasis in NSCLC, BRC, and CRC cell lines. miR-34 and miR-199 expression negatively correlated with Axl expression in NSCLC patient tumor specimens as compared with levels in normal specimens [36].

Further evidence that Axl is an important cancer-inducing receptor tyrosine kinase comes from loss- or gain-of-function studies of key molecules in the

pathway, including Axl and its transcriptional or posttranscriptional regulators. Studies based on a range of drugs that specifically inhibited the functions of Axl, or are in combination with other relevant molecules such as VEGFR- and EGFR-specific inhibitors, have supported this finding [85–90].

3.2.6 Axl in Cancer Stemness

Cancer stem cells (CSC) can be divided into types that divide or differentiate rapidly; others develop into mature cells without any further divisions. Two explanations have been offered. The first (cancer stem cell theory) is a more systematic model proposing that cells divide and feed tumor growth with self-renewal ability. These cells divide certain number of times and then differentiate as specialized mature tumor cells. The second (stochastic) model proposes that each cancer cell possesses the same potential to self-renewal and differentiate [91–97]. Till today, a number of cancer stem cell markers were reported generally as well as cancer entity specific [98].

Ahtiainen et al. reported Axl CSC functional properties in breast cancer cell lines. The authors checked the innate immunity to oncolytic adenovirus Ad5/3-Delta24 in conventional treatment-resistant, non-cancer-initiating cells (CIC) with $CD44^{+}/CD24^{-/low}$ population and normal breast tissue $CD44^{+}/CD24^{-/low}$ stem cells. Under these conditions, the authors observed that normal breast tissue cells have intact type I INF signaling compared to the breast cancer CIC $CD44^{+}/CD24^{-/low}$ population, which showed dysregulated innate immune response due to the dysfunctional virus recognition caused by impaired trafficking of Toll-like receptors (TLR) 9 and cofactor MyD88 and the absence of TLR2, having a deleterious impact on TLR pattern recognition receptor signaling. Further, they increased inhibitory signaling via the suppression of cytokine signaling of Axl/Tyro-3 and Mer. The presence of these CIC stem cells has been put forward as an explanation for the resistance of relapsed or metastatic cancers to treatments [99]. CD44 and ALDH1 are known and well characterized CSC markers for many cancer entities. shRNA-Axl silencing significantly altered the expression of intercellular junction molecules increasing cell–cell adhesion with downregulation of Wnt and TGFβR signaling and negatively correlated with CD44 and ALDH1 expression in squamous cell carcinoma (SCC) cell lines [100]. Specific inhibition of Axl with amuvatinib inhibited the breast cancer stem cell self-renewal and restored chemosensitivity for the drug [101]. Further studies showed that the overexpression of Axl and $CD44^{+}$ positively correlated with drug resistance against metformin and imatinib [102, 103].

3.3 Axl as Regulator of Drug Resistance in Cancer

Resistance to chemo and molecular targeted therapies is a major problem in present cancer treatments, which has stimulated research into the mechanisms that cause resistance and strategies to overcome them. Drug resistance can be divided into two

types. One is intrinsic (an inbuilt system that arises in a cancer mass or cells due to the molecules that are expressed or genetic modifications that it has experienced). The second is acquired resistance, which is a change in the molecular profiles of cells or the tumor mass that has been stimulated by the treatment itself [104]. A number of studies have reported that Axl-induced expression increases drug resistance in different cancer entities.

Macleod et al. developed an ovarian carcinoma cell line that was resistant to 20-fold dosages of cisplatin ($PE01^{CDDP}$) by exposing the parental cell line PE01 to increasing concentrations of cisplatin. Under these conditions, Axl is overexpressed 2.9 times in the resistant cell line compared to the original parental cell line PE01 [105]. Cisplatin is used as a first-line treatment for esophageal adenocarcinoma (EAC), known for a high rate of chemotherapy resistance and poor outcome. Inducing Axl expression increased the IC^{50} value twofold and increased cell survival threefold. The inhibition of Axl expression reduced the cell survival twofold when treated with cisplatin. Cisplatin is known to activate endogenous p73β and increase expression of p-c-ABL(Y412) and p-p73β(Y99). In general, this molecular mechanism plays a role in inducing apoptosis, which is blocked by Axl expression [106]. Kurokawa et al. reported that acquired resistance to cisplatin is due to the EMT-like changes that Axl induces in NSCLC cells [107]. Bladder cancer cell line and patient derived xenografts after treatment with PI3K/mTOR (PF-04691502) and MEK (PD-0325901)-specific inhibitors reduced tumor growth and decreased the secretion of the vascular endothelial growth factor. However, this increased Axl expression [108]. Another study based on NSCLC cells confirmed Axl's role in acquired resistance under treatment with drugs like cisplatin [109].

R428 is a selective, small molecule inhibitor of Axl that blocks its catalytic and precancerous activities. R428 treatment reduced Axl-induced AKT phosphorylation, cancer cell invasion, angiogenesis, and the production of pro-inflammatory cytokines. It also reduced the expression of the cytokine granulocyte macrophage colony-stimulating factor and Snail in a dosage-dependent manner. Interestingly, using R428 to inhibit Axl-mediated cellular and molecular functions during cisplatin treatments achieved an enhanced suppression of liver metastases [110]. Axl knockdowns in RAC cell lines reduced migration, invasion, and in vivo engraftment, accompanied by a downregulation in the activity of the Ral GTPase proteins (RalA and RalB). Similar effects were obtained using an A428 inhibitor. Blocking Axl functions also abrogated the phosphorylation of ERBB2 (Her-2/neu) at the Tyr877 residue, which reveals the cross-functional effects of R428 on different receptor signaling axes [35].

Induced EGFR expression has been associated with the development of head and neck cancer (HNC) and a poor prognosis for patients. Clinical trials based on the EGFR inhibitor erlotinib were not successful in HNC patients. Glies et al. developed a cell line, which is resistant to erlotinib. Compared to the parental cell line, the HN5-erlotinib resistant cell line exhibited an EMT phenotype and affected migrations. Surprisingly, Axl exhibited a higher degree of phosphorylation in this line [68]. Similarly, cases of NSCLC with activating mutations of EGFR respond to EGFR-targeted tyrosine kinase inhibitors such as erlotinib, but the sensitivity is

short and these cells acquire resistance to the drug. These resistant cells are characterized by an increased activation of Axl and EMT [67]. Specifically inhibiting Axl with R428 induced the cells to become sensitive to erlotinib treatment [68], suggesting that the expression or activation of Axl might be the cause of resistance in the patient's samples. Another study demonstrated that inhibiting Axl activity increased the sensitivity of head and neck squamous cell carcinoma to chemotherapy, cetuximab, and radiation [111]. Axl is upregulated in metformin resistant prostate cancer cell lines and inhibits Axl with R428 sensitized the cells to metformin treatment [102]. In support of these findings, it has been shown that Axl expression is increased in myeloid leukemia cell lines and also Gas6/Axl signaling for chemotherapy. The cells also exhibited increased Bcl-2 and Twist expression [112]. More evidence of Axl's role in cancer stemness comes from the different responses of metastasized lesions to small molecule inhibitors. R428 effectively inhibited liver and lung metastasic lesions more effectively than when applied to peritoneal metastasis-derived cells [113].

An overexpression of Axl was also found in HER2-positive and estrogen receptor (ER)-positive lapatinib-resistant breast cancer clones derived from lapatinib-sensitive BT474 cells by chronic exposure to lapatinib. The authors found that Axl overexpression is the cause for this drug resistance; treatment with foretinib (a multikinase inhibitor of Axl, MET, and VEGFR) restores the sensitivity of these cells to lapatinib [114]. Metastatic renal cell carcinoma (RCC) patients show high rates of resistance to antiangiogenic therapy. To understand this, Zhou et al. generated RCC cell lines with resistance to sunitinib (an antiangiogenic small molecule). Gene profiling assays revealed an upregulation and activation of Axl, MET, and EMT genes. Angiogenesis was also enhanced by co-culturing RCC with human umbilical vein endothelial cells. Further, the authors stained tissues from the RCC patients that had been treated with sunitinib, which revealed that Axl and MET are mediators of sunitinib resistance. Pretreatments with specific inhibitors of Axl and MET or a suppression of these genes inhibited the metastatic behavior of RCC cell lines and rescued the acquired resistance to sunitinib in a xenograft model [115]. Similarly, Axl knockdown in glioblastoma cell lines led to higher sunitinib sensitivity, reduced migration, and increased apoptosis [116]. Many other studies have confirmed the importance of Axl expression and its activated signaling in drug resistance. All of this evidence clearly demonstrates that Axl pays an important role in drug resistance and acquired resistance in different treatment strategies.

Conclusions

Axl is known to be overexpressed in a number of cancer entities and induces cell survival, proliferation, antiapoptosis, colony formation, migration, invasion, EMT, and distant metastasis formation. These hallmarks of cancer can be mediated either through the overexpression of Axl or Gas6 stimulation, through their different effects on cancer-associated signaling cascades. Moreover, Axl overexpression is associated with poor patient survival in several cancer entities. Drug resistance is a major problem in cancer therapies, and Axl is known for inducing drug resistance and cancer stemness. A number of gain- or loss-

of-function experiments have demonstrated that Axl has potential as a druggable target both in vitro and in vivo. The expression status of Axl and availability of its ligand Gas6 play major roles in Axl-mediated signaling. As discussed above, under normal conditions, the regulation of Axl at transcriptional and posttranscriptional stages is tightly controlled. However, during cancer progression, Axl expression is induced in cancer cell lines and tissues through a loss of regulation. The inhibition of Axl expression—either through inhibitors at the transcriptional level or miRs at the posttranscriptional level—would control its expression. A second way to inhibit Axl-mediated cancer progression and metastasis would be to block Axl signaling either at its Gas6 binding site and/or through active intracellular motifs and specific inhibitors. This would inhibit cross talk signaling between Axl and enhance other signaling cascades such as VEGFR2 receptor tyrosine molecules. Recent advances in technology and research into cancer genotypes have thus produced an enormous amount of data on Axl and its effects and are encouraging signs that a personalized approach based on Axl might be an effective way to treat patients. All of this evidences the importance of Axl as a potential therapeutic target for mitigating many of the hallmarks of cancer.

Acknowledgments This work was supported by the German Cancer Consortium (DKTK). I thank Prof. Ulrike Stein for her suggestions, critical reading and encouragement in writing this chapter and Harikrishna Raddhakrishna, Steffen Fuchs, and Kathrina Ilm for their critical reading and discussion of this chapter.

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