Axl RTK and microRNAs in urogenital cancers

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Axl RTK and microRNAs in urogenital cancers

Helena Fritz

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Title and subtitle
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This thesis is based on four projects focused on the Axl receptor tyrosine kinase (RTK) and microRNAs in clear cell renal cell carcinoma (ccRCC) and in prostate cancer (PCa). Paper I focuses on differentially expressed microRNAs in ccRCC. We used bioinformatics methods to identify candidate microRNAs in a publically available cohort of ccRCC, which were measured in a cohort of 198 RCC tumors. Importantly, we identified a 2-microRNA ratio, miR(21/10b), which is an independent prognostic factor in metastasis-free ccRCC patients. These microRNAs are both linked to chronic kidney disease, a risk factor for ccRCC, and could potentially be involved in the progression from kidney disease to renal cancer. In Paper II, our aim was to elucidate if members of the miR-34 family, which is a family of microRNA tumor suppressors, could regulate Axl in ccRCC, and also to determine miR-34a/b/c expression in ccRCC tumors, as reports have been conflicting. Axl has previously been shown to be a target of miR-34a in solid cancers, and high Axl expression correlates with worsened prognosis in RCC. We showed that both miR-34a and miR-34c are direct regulators of Axl *in vitro*, however miR-34a expression is increased in ccRCC and does not correlate with Axl mRNA or protein in ccRCC tumors, and has no correlation with survival in ccRCC. Paper III was aimed at elucidating whether any of the miR-34 family members could regulate Axl expression PCa, as both miR-34a and miR-34c are downregulated in PCa, and Axl expression is increased in PCa and correlates with disease severity. In addition, we sought to elucidate the role of decreased Axl expression in miR-34a/c-mediated tumor suppression. Although we could show direct regulation of Axl by miR-34a and miR-34c, our results did not support regulation of Axl as the main function in miR-34a/c tumor suppression in PCa. The main functional outcome of miR-34a/c-mediated loss of Axl seemed to be in reduced proliferation in response to Gas6. Finally, paper IV has the aim of investigating the role of Gas6/Axl signaling in Sunitinib treatment in ccRCC. Axl has been linked to resistance to targeted therapies in cancer. Sunitinib is an angiogenesis-inhibiting drug used in treatment of advanced ccRCC, however disease progression eventually occurs in many patients. We show that Sunitinib does not inhibit Axl activation by Gas6; instead Axl phosphorylation was enhanced in the presence of Gas6 and Sunitinib, both in ccRCC cells and in endothelial cells. Moreover, we observed activation of Akt pathway in Sunitinib-treated cells, which was enhanced by the addition of Gas6. In addition Sunitinib activated the epidermal and hepatocyte growth factor receptors, an effect that was augmented by Gas6. Interestingly, Gas6 stimulation was associated with secretion of Osteopontin, associated with tumor angiogenesis, an effect that was increased by Sunitinib.

Key words

Axl, Gas6, microRNA, miR-34, miR-21, miR-10b, renal cell carcinoma, prostate cancer, clear cell renal cell carcinoma, Sunitinib

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Fritz HK, Lindgren D, Ljungberg B, Axelson H, and Dahlbäck B. The miR(21/10b) ratio as a prognostic marker in clear cell renal cell carcinoma. Eur J Cancer. 2014 jul; 50:1758-65.

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Paper II

Fritz HK, Gustafsson A, Ljungberg B, Ceder Y, Axelson H, and Dahlbäck B. Axl-regulating tumor suppressor miR-34a increased in ccRCC but not correlating with Axl mRNA or Axl protein levels. Submitted manuscript.

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Abbreviations

Ago2 Argonaute 2

APCs antigen-presenting cells

BPH benign prostate hyperplasia

ccRCC clear cell renal cell carcinoma

chRCC chromophobe renal cell carcinoma

CKD chronic kidney disease

CRPC castration-resistant prostate cancer

DCs dendritic cells

dsRBP double-stranded RNA binding proteins

ECD extracellular domain

ECs endothelial cells

EGFR epidermal growth factor receptor

EMT epithelial-to-mesenchymal transition

ERK extracellular signal-regulated kinases

FCM flow cytomtery

GLA γ-carboxyglutamic domain

HER2/3 human epidermal growth factor receptor 2/3

HIF hypoxia-inducible factor

IL interleukin

IFNα interferon alpha

LG laminin G-like domain

MAPK mitogen-activated protein kinases

NK cells natural killer cells

NSCLC non-small cell lung cancer

PCa prostate cancer

PCR polymerase chain reaction

PDGFR platelet-derived growth factor

PI3K phosphoinositide 3-kinase

pRCC papillary renal cell carcinoma

PSA prostate-specific antigen

RCC renal cell carcinoma

RISC RNA-induced silencing complex

RLC RISC-loading complex

RP radical prostectomy

RTK receptor tyrosine kinase

RT-qPCR reverse transcriptase quantitative polymerase chain reaction

sAxl soluble Axl

siRNA short interfering RNA

SLE systemic lupus erythematosus

SNP single nucleotide polymorphism

SOCS suppressor of cytokine signaling

TCGA the Cancer Genome Atlas

TGFβ transforming growth factor beta

TLR toll-like receptor

TNFα tumor necrosis factor alpha

TNM tumor-node-metastasis

TRBP TAR RNA binding protein

UTR untranslated region

VEGF vascular endothelial growth factor

VEGFR vascular endothelial growth factor receptor

VHL von Hippel-Lindau

VSMCs vascular smooth muscle cells

WB western blot

Preface

A common feature of cancers is the dysregulation of receptor tyrosine kinases (RTKs), caused for example by mutations or chromosomal amplification, or upstream dysregulation of factors that take part in regulation of the expression of RTKs, such as microRNAs. This thesis will provide an introduction to the Axl RTK and microRNAs, and their roles in renal cell carcinoma (RCC) and prostate cancer (PCa). Furthermore, the results and findings of my PhD student projects will be summarized and discussed.

The Axl receptor tyrosine kinase

Receptor tyrosine kinases

Signaling through cell surface-bound receptors is an important means for individual cells to receive external signals and to respond appropriately to stimuli from the extracellular environment. These cell surface receptors are classified into different families, primarily based on their mechanism of signal transduction, the main receptor families being G protein coupled receptors, receptor tyrosine kinases (RTKs), integrins, ion channel receptors, and cytokine receptors (Uings, 2000).

The RTKs are so-called enzyme-linked receptors, relying on extracellular binding of specific ligands and subsequent activation of an intracellular enzymatic domain to convey extracellular signals (Alberts, 2002). The most common mechanism of RTK activation is that upon binding of a protein ligand, most commonly a growth factor, the RTK dimerizes, enabling auto-phosphorylation of tyrosine residues in the intracellular kinase domain, which causes conformational changes and thereby enables the kinase domain to recruit and interact with downstream signaling mediators (Gschwind, 2004).

Approximately 10 to 15% of all protein kinase genes in metazoan organisms are tyrosine kinases, and in humans 58 of the 90 tyrosine kinase genes are RTKs (Hunter, 2009). The RTKs are regulators of important cellular processes, including survival, proliferation, differentiation and migration (Lemmon, 2010), and many RTKs have been shown to be key players in development; for example, knockdown of the epidermal growth factor receptor (EGFR) is associated with embryonic lethality and severe dysfunctions in the epithelial development of the skin, the lungs and the gastrointestinal tract (Gschwind, 2004). In the healthy resting cell, RTK activity is under tight control, however dysregulation or mutation of RTKs can cause abnormal receptor activation, associated with potent oncogenic activities which have been shown to play key roles in the development and progression of a large number of human cancers (Gschwind, 2004). For example, human epidermal growth factor receptor 2 (HER2) has been shown to be overexpressed in 30% of invasive breast cancers in humans, and the development of targeted therapies using humanized monoclonal antibodies to HER2 has improved the overall outcome for patients with HER2-amplified metastatic breast cancer tumors (Gschwind, 2004).

The TAM family of RTKs

The TAM family of RTKs comprises the three receptors Tyro3, Axl and MerTK, which have similar domain structures, with two Ig-like domains and two fibronectin type III domains, as well as a conserved kinase domain (Graham, 2014). The genes coding for the TAM receptors were initially discovered in 1991, in a study using a polymerase chain reaction (PCR)-based approach to identify tyrosine kinase genes expressed in the developing rat brain (Lai, 1991). The same year, Axl was identified as an oncogene in two separate reports (Janssen, 1991, O'Bryan, 1991). Different aspects of Axl such as expression, regulation and function will be further discussed in the Axl RTK section.

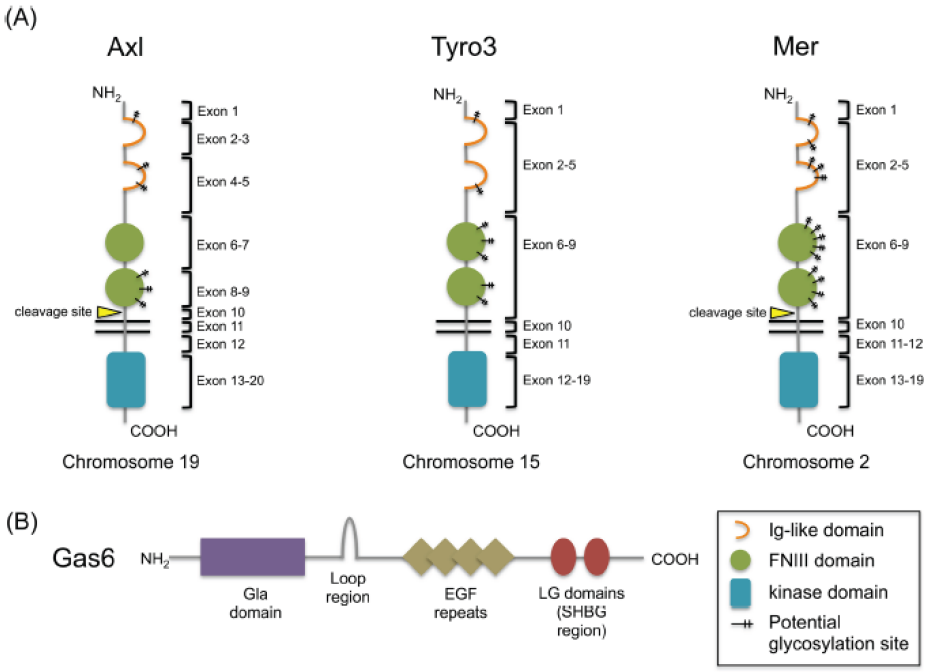

Figure 1
The structure of the TAM (Tyro3, Axl, and MerTK) receptor tyrosine kinases. Image from Axelrod et. al., 2014. Reprinted with the permission of Oncotarget/Impact Journals.

MerTK was originally identified as a viral oncogene in an acute oncogenic avian retrovirus, and was named v-ryk (Jia, 1992) and its human equivalent MerTK was later identified in neoplastic B- and T-cells, and was named Mer due to its expression in monocytes, epithelial tissues, and reproductive tissues (Graham, 1994). In addition to being expressed in reproductive tissues such as testes, prostate and ovaries, MerTK is also expressed in kidneys and lungs, and to a lesser extent in the liver, small intestine, thymus, spleen, colon and in the placenta (van der Meer, 2014). MerTK is, together with its ligands Protein S and Gas6, involved in the phagocytosis of apoptotic cells (Lemke, 2013), and MerTK knockout mice become blind shortly after birth due to insufficient phagocytosis of shed rod outer membranes by retinal epithelial cells, eventually causing photoreceptor cell death (Duncan, 2003). Furthermore, MerTK knockout mice develop systemic lupus erythematosus (SLE)-like autoimmune disease, possibly due to accumulation of apoptotic materials and decreased anti-inflammatory signaling by MerTK (Shao, 2011). Activation of MerTK by apoptotic cells has been shown to inhibit NF-κB activation and subsequent secretion of pro-inflammatory cytokines such as interleukin (IL)-12, IL-6 and tumor necrosis factor α (TNFα) (Graham, 2014, Sen, 2007). The extracellular part of MerTK can be shed from the membrane in a metalloproteinase-dependent process, yielding a soluble MerTK (sMer) protein which can function as a negative regulator of MerTK or TAM receptor activation by acting as a decoy receptor for the TAM ligands (Sather, 2007). The shed sMer is capable of inhibiting engulfment of apoptotic cells (Sather, 2007) and has been found to be increased in the serum of SLE patients, especially in those experiencing SLE flares or lupus nephritis (Wu, 2011). MerTK has been found to be overexpressed in a number of human cancers, such as melanoma, prostate cancer (PCa), breast cancer and gastric cancer (Verma, 2011). Expression of MerTK in a non-neoplastic breast epithelial cell line has been shown to stimulate efferocytosis and increase motility and chemoresistance, and it has been proposed that activation of MerTK by apoptotic cells could be a mechanism for immune suppression in cancer (Nguyen, 2014). Reduced thrombus formation and reduced platelet aggregate stabilization have been observed in MerTK knockout mice, indicating a role for MerTK in coagulation (van der Meer, 2014). In addition to Protein S and Gas6, three novel MerTK ligands involved in phagocytosis have recently been identified: Tubby, Tubby-like protein 1 and Galectin-3 (Cummings, 2013).

Human Tyro3 was first cloned and identified as an Axl-like RTK from human brain RNA, and was initially named Rse (Mark, 1994). Tyro3 is predominantly expressed in the central nervous system (Lai, 1994) but is also expressed in kidney, lungs, ovaries, testes, breast, retina, platelets, osteoclasts and monocytes and macrophages (Pierce, 2014). Like MerTK, Tyro3 is involved in phagocytosis of apoptotic cells, and knockdown of Tyro3 is associated with reduced phagocytic capability, especially in dendritic cells (DCs) but also in macrophages (Rothlin, 2015). Tyro3 has been shown

to be overexpressed in a number of human cancers, including lung cancer, PCa, multiple myeloma, and melanoma (Graham, 2014) and in PCa the Tyro3/Axl expression axis has been proposed to play a role in regulating dormancy of disseminated tumor cells in the bone marrow (Taichman, 2013).

Gas6 and Protein S

Upon their discovery, the TAM family receptors were orphan receptors with regard to the fact that their ligands remained unknown. In 1995, the Vitamin K-dependent proteins Protein S and Gas6 were identified as ligands for Tyro3 and Axl, respectively (Stitt, 1995, Varnum, 1995). Gas6 has been found to be able to bind to and stimulate all of the TAM receptors, whereas Protein S has yet only been confirmed to bind Tyro3 and MerTK (van der Meer, 2014).

Protein S was originally identified in 1977 (Di Scipio, 1977) and is primarily known as an anticoagulant protease regulator, acting as a cofactor to activated Protein C which regulates the activity of the procoagulant proteins Factor V and Factor VIII in the blood coagulation cascade (Dahlback, 2000). In addition, Protein S has been shown to take part in the phagocytosis of apoptotic cells (Anderson, 2003, Prasad, 2006) as well as regulation of cell survival and proliferation (Benzakour, 1995, Liu, 2003, Tomobe, 1996), and regulation of the inflammatory response (Lemke, 2008, Suleiman, 2013).

Gas6, or growth-arrest-specific 6, was first identified in 1988 as a gene expressed in growth-arrested cells (Schneider, 1988) and was later found to be a Vitamin K-dependent protein with homology to Protein S (Manfioletti, 1993). Gas6 shares 43% sequence homology to Protein S, and the two proteins have the same structure, with an N-terminal γ-carboxyglutamic acid (GLA) domain, followed by a loop region, four EGF-like repeats, and a sex hormone-binding globulin-like structure which contains two laminin G (LG)-like domains (Hafizi, 2006). The GLA domain requires post-transcriptional γ-carboxylation of glutamate residues, which is a Vitamin K-dependent process, to form complexes with calcium ions that enable the GLA domain to interact with negatively charged phospholipid membranes (Hafizi, 2006). Proper γ-carboxylation of the Gas6 GLA domain has been indicated to be essential for Axl binding and activation (Hasanbasic, 2005), although the GLA domain does not interact directly with the TAM receptors upon binding and activation (Bellido-Martin, 2008). While most of the Vitamin K-dependent proteins are synthesized in the liver, Gas6 is in contrast expressed to a greater extent in heart, kidney, and lungs, and in endothelial cells, vascular smooth muscle cells, and in the bone marrow (van der Meer, 2014).

Gas6 has been indicated to be involved in platelet activation and thrombus stabilization (Cosemans, 2010), and Gas6 knockout mice are protected from venous and arterial thrombosis (Angelillo-Scherrer, 2001). Gas6 is present in the α-granules of mouse platelets and is released upon platelet activation, however human platelets do not contain Gas6 (Balogh, 2005) although Gas6 mRNA transcripts have been found in human platelets (Angelillo-Scherrer, 2001). Although Gas6 is undetectable in human platelets, Gas6 secreted from other sources may take part in coagulation, and indeed the plasma levels of Gas6 are increased in patients with venous thrombosis (Blostein, 2011). While Protein S is an abundant serum protein, of which approximately 70% circulates in complex with the C4-binding protein (Dahlback, 2000, Hafizi, 2006), Gas6 is present at much lower concentrations, approximately 0.2 nM (Balogh, 2005), and is complexed to soluble Axl (sAxl) in circulation (Ekman, 2010).

Axl RTK

Axl, named after the Greek word anexelekto, meaning uncontrolled, was originally discovered as a transforming gene in patients with chronic myeloproliferative disorder and chronic myeloid leukemia (Janssen, 1991, O'Bryan, 1991). Since its discovery, Axl has been shown to be overexpressed in a large number of human cancers, including lung cancer, breast cancer melanoma, renal cell carcinoma, prostate cancer, osteosarcoma, and pancreatic cancer (Graham, 2014).

Structure, expression and regulation of Axl

The Axl gene is located on position q13.2 on chromosome 19, and exists in two isoforms due to alternative splicing of exon 10 (O'Bryan, 1991). Axl can be expressed in either a partially or a fully glycosylated form, with a molecular weight of approximately 120 kDa and 140 kDa, respectively (O'Bryan, 1991).

The extracellular domain (ECD) of Axl contains two Ig-like domains, which primarily take part in binding to Gas6 *via* the LG domain (Sasaki, 2006), and two FNIII domains, which have been attributed adhesive properties as these domains can be found in cell adhesion molecules (Axelrod, 2014). However, the adhesive properties of Axl seems to be dependent on context, as reports have indicated that Axl can mediate cell-to-cell adhesion in some contexts, such as in aggregation of lung cancer and myeloid cell lines as well as in schwannoma (Ammoun, 2014, McCloskey, 1997, Wimmel, 2001), while loss of Axl expression in contrast seems to increase cell-to-cell adhesion in cutaneous squamous cell carcinoma cell lines (Cichon, 2014).

A soluble form of Axl, sAxl, can be formed by extracellular proteolytic cleavage of Axl immediately outside of the transmembrane region (O'Bryan, 1995). The sAxl fragment contains the full ECD of Axl, while the transmembrane region and the intracellular domain remain in the cell membrane, and cleavage of Axl to sAxl has been proposed to be a mechanism for down-regulation of the kinase activity of Axl (O'Bryan, 1995). Furthermore, the sAxl fragment itself has been shown to be able to inhibit Gas6 signaling through Axl (Costa, 1996, O'Bryan, 1995), and sAxl correlates inversely with Gas6 in multiple sclerosis lesions (Weinger, 2009). In addition, an Axl-ECD construct resembling sAxl has been shown to inhibit the cell-to-cell adhesion of myeloid cells (McCloskey, 1997). In this context, it is noteworthy that all of the Gas6 in circulation is complexed to sAxl, indicating that although Gas6 is present in circulation, it is not available for TAM receptor stimulation (Ekman, 2010).

The kinase domain of Axl is highly conserved and homologous to several other RTK kinase domains, although it contains an unusual KWIAIES sequence (O'Bryan, 1991), which also can be found in MerTK, while the isoleucine residues are substituted with leucines in Tyro3 (Graham, 1994).

Axl is ubiquitously expressed and can be found in several human tissues, including hematopoietic cells such as platelets, DCs and macrophages as well as hematopoietic progenitor cells, and also in the kidneys, heart, endothelium, testis, skeletal muscle, endometrium, lungs, gall bladder and in cells of the digestive tract (Angelillo-Scherrer, 2001, Manfioletti, 1993, Neubauer, 1994, O'Bryan, 1991, Park, 2009, Scutera, 2009, Stenhoff, 2004, Uhlen, 2015, Yanagita, 1999, Zahuczky, 2011). In a murine study, Axl was found to be broadly expressed in late-stage embryogenesis with an onset at day 12.5 after fertilization (Faust, 1992) although Axl expression does not seem to be essential for normal embryonic development as homozygous Axl knockout mice are viable (Lu, 1999).

The promoter region of Axl is GC-rich and contains two major transcriptional start sites with recognition sites for transcriptional activators such as Sp1, AP-1, myeloid zinc finger 1 (MZF1) and the cyclic AMP response-element binding protein (Mudduluru, 2008, Mudduluru, 2010, Mudduluru, 2010, Schulz, 1993). Five different Sp-binding sites, Sp a/b/c/d/e, have been identified in the Axl promoter, and the Sp a and Sp b sites are essential for promoter activation by Sp1 and Sp3 (Mudduluru, 2008). Furthermore, Axl expression can be epigenetically regulated by CpG methylation within and around the Sp1-binding motifs in the promoter region (Liu, 2010, Mudduluru, 2008). Sp1 and Sp3 are transcription factors that are expressed in all mammalian cells, and are involved in the regulation of a vast array of cellular processes (Li, 2010). More than 12,000 Sp1/3 binding sites have been found in the human genome, and increased Sp1 and/or Sp3 expression has been observed in several human cancers (Li, 2010). Previous reports have revealed that Axl is overexpressed in renal cell carcinoma cells deficient in the tumor suppressor Von

Hippel-Lindau (VHL) protein, and reconstitution of VHL expression decreases Axl expression in a post-transcriptional manner (Gustafsson, 2009). Similarly, the hypoxia inducible factor (HIF)-1 transcription factor, which is negatively regulated by VHL, can induce Axl expression in the hypoxia response of vascular endothelial cells (Manalo, 2005) and both HIF-1 and HIF-2 have been shown to bind to hypoxia-responsive elements in the promoter region of Axl (Rankin, 2014). Expression of Axl has also been shown to be post-transcriptionally regulated by microRNAs (Mackiewicz, 2011, Mudduluru, 2011), which will be further discussed in the microRNAs section of this thesis.

Downregulation of Axl can be mediated by the E3 ubiquitin ligase Cbl-b, which tags substrate proteins with ubiquitin for subsequent degradation in the proteasome (Paolino, 2014). In addition, Gas6-mediated stimulation of Axl has been shown to result in downregulation of Axl (Gustafsson, 2009, Mishra, 2012).

Axl activation and signaling

The primary activation mechanism of Axl is by binding the LG domains of Gas6, resulting in homodimerization, which enables autophosphorylation of the kinase domains and subsequent phosphorylation of downstream mediators (Mark, 1996, Sasaki, 2002, Tanabe, 1997). Although recombinant truncated Gas6, containing the LG domains only, has been shown to be sufficient for binding to Axl (Mark, 1996, Sasaki, 2002), γ-carboxylation of the GLA domains seems to be necessary for activation of Axl by Gas6 (Hasanbasic, 2005, Tanabe, 1997). Furthermore, binding of Gas6 to Axl is facilitated by the LG domains of Gas6 forming a V-shaped structure which is stabilized by inter-domain binding of a calcium ion (Sasaki, 2002). Gas6 binds to Axl with a 2:2 stoichiometry where each Gas6 interacts with Axl at two sites which are necessary for Axl activation; the major contact between the LG1 domain of Gas6 and the IG1 domain of Axl, and the minor contact where LG1 of Gas6 interacts with the IG2 domain of Axl (Sasaki, 2006).

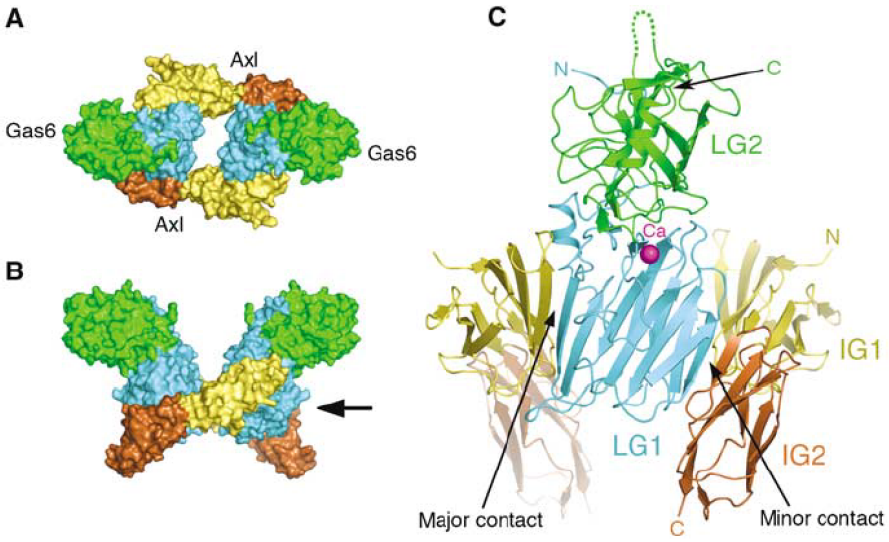

Figure 2
The Gas6-Axl interaction. Image from Sasaki et. al., 2005. Reprinted with the permission of John Wiley and Sons/The EMBO Journal.

In addition to Gas6-mediated Axl homodimerization and activation, Axl has been indicated to be capable to form heterodimers with other RTKs such as the type I interferon receptor (IFNAR), EGFR, MET, and FLT3 (Meyer, 2013, Park, 2013, Rothlin, 2007, Salian-Mehta, 2013). Moreover, co-immunoprecipitation of Axl and Tyro3 has been demonstrated in a rodent cell line, indicating that Gas6-mediated signaling could be altered by TAM receptor expression on the cell surface (Brown, 2012). Furthermore, interaction or cross-talk between Axl and other RTKs such as HER2, human EGFR-related 3 (HER3), platelet-derived growth factor receptor β (PDGFRβ), and c-Kit has been indicated (Meyer, 2013, Park, 2009).

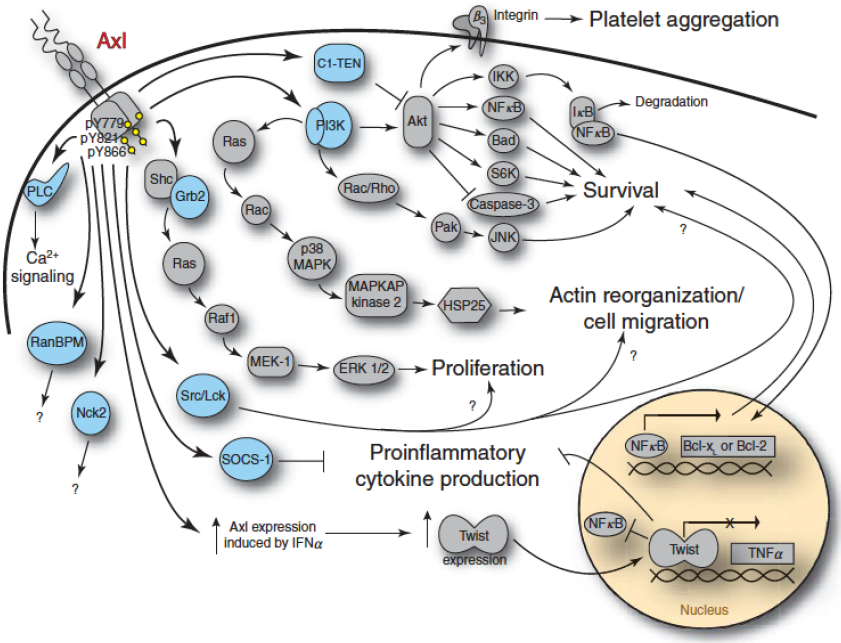

Figure 3
Axl signaling. Image from Linger et. al., 2008. Reprinted with the permission of Elsevier.

The kinase domain of Axl has been shown to activate the Ras/extracellular signal-regulated kinases (ERK) pathway *via* Grb2 and Shc (Fridell, 1996). Moreover, Axl kinase has been shown to bind to both p85 subunits of the phosphatidylinositol 3'-kinase (PI3K), the phospholipase Cγ1 (PLCγ1), c-*src* and *lck* (Braunger, 1997, Goruppi, 1997). The Axl kinase binding partners are recruited to three tyrosine residues within the Axl kinase domain; tyrosine 779 which interacts with the PI3K p85 subunits, tyrosine 821 which binds p85 subunits of PI3K, PLCγ1, Grb2, c-*src* and *lck,* and tyrosine 866 which interacts with PLCγ1 (Braunger, 1997, Weinger, 2008). Furthermore, Axl has been shown to bind to C1-TEN, the PI3K p55γ subunit, suppressor of cytokine signaling 1 (SOCS-1), RanBPM, and Nck1 (Hafizi, 2002, Hafizi, 2005). The multiple intracellular binding partners enables Axl signaling diversity through activation of signaling pathways such as PI3K, Akt, ERK, mitogen-activated protein kinases (MAPK), NF-κB, and mammalian target of rapamycin (mTOR), regulating important cellular processes such as growth and proliferation, survival, cell cycle progression, invasion and migration, and oncogenic transformation, and the functional outcomes of Axl signaling seems to be context-dependent (Axelrod, 2014, Graham, 2014).

Axl in the immune system

The TAM receptors are expressed in human myeloid immune cells, such as DCs and macrophages, and they play a role in regulating inflammation by limiting the immune response, and by taking part in phagocytic events (Rothlin, 2015). Although the effects are more profound in triple and double TAM receptor knockout mouse models, Axl knockout mice exhibit phenotypes that are linked to dysfunctional regulation of inflammation such as hyperactivation of antigen-presenting cells (APCs) and production of autoantibodies (Lu, 2001).

Axl, as well as Tyro3 and MerTK, is essential for late-stage maturation of natural killer (NK) cells, and NK cells lacking Axl show severely impaired cytotoxic activity (Caraux, 2006). Furthermore, blocking Gas6/Axl signaling decreases the IL-15-mediated differentiation of hematopoietic progenitor cells to NK cells (Park, 2009). Axl is upregulated upon interferon α (IFNα)-mediated differentiation of monocytes to DCs, and Gas6/Axl signaling protects DCs from growth factor deprivation-induced apoptosis and induces chemotaxis towards Gas6 in a dose-dependent manner (Scutera, 2009). The Axl-upregulating effect of IFNα-mediated differentiation is inhibited by activation of pro-inflammatory toll-like receptors (TLRs) by increasing the expression of metalloproteinases that take part in cleaving Axl to sAxl, thereby decreasing Gas6/Axl signaling (Scutera, 2009). In mature DCs, knockdown of Axl is associated with increased interleukin-6 (IL-6) and TNFα secretion upon activation of TLRs, and conversely, Gas6-mediated activation of TAM receptors inhibits the TLR-induced cytokine production by activation of SOCS1 and SOCS3 *via* the transcription factor STAT1 (Rothlin, 2007). Interestingly, the proinflammatory IFNα receptor, IFNAR, colocalizes with Axl in DCs and is essential for the Gas6/Axl-mediated activation of STAT1 (Rothlin, 2007). In macrophages, Gas6 signaling through IFNα-induced Axl induces expression of the transcription factors Twist1 and Twist2, which inhibit proinflammatory TNFα secretion (Sharif, 2006). Axl takes part in phagocytic events in both macrophages and DCs, most likely in concert with MerTK and Tyro3, and the TAM receptors seem to take part in immune homeostasis by acting as negative regulators of APC activation upon inflammatory stimuli (Lemke, 2003, Seitz, 2007). In the brain, Gas6/Axl signaling has been shown to protect oligodendrocytes from TNFα-mediated apoptosis (Shankar, 2006). The levels of sAxl are increased in human multiple sclerosis (MS) lesions, and in a mouse model of MS with Axl knockdown, autoimmune encephalomyelitis is more severe, fewer microglia and macrophages are activated, and the clearance of myelin debris is impaired (Weinger, 2009, Weinger, 2011).

Axl in the vascular system

Gas6 and Axl are expressed in endothelial cells (ECs), and have been indicated to take part in regulation of the immune response by inhibiting the adhesion of granulocytes to activated ECs when high levels of Gas6 are present (Avanzi, 1998, Manfioletti, 1993). In contrast, Gas6 has been reported to promote inflammation by enhancing the interactions between ECs, leukocytes, and platelets, and by promoting extravasation of leukocytes, as well as thrombosis (Tjwa, 2008). Gas6 has a well-established role in thrombus stabilization by increasing platelet aggregation and degranulation, which is dependent on TAM receptor expression (Angelillo-Scherrer, 2005, Cosemans, 2010, Gould, 2005).

In addition, Gas6/Axl signaling is involved in promoting survival in ECs and vascular smooth muscle cells (VSMCs) (D'Arcangelo, 2006, Healy, 2001, Melaragno, 2004, Nakano, 1996). In ECs, Axl-mediated activation of PI3K results in subsequent phosphorylation and activation of the Akt pathway which promotes cellular survival *via* the transcription factor NF-κB, increased expression of the anti-apoptotic protein Bcl-2 as well as inhibition of pro-apoptotic Caspase-3 activation (Hasanbasic, 2004). Similarly, survival in VSMCs is mediated through the PI3K/Akt pathway (Melaragno, 2004). In a mouse model of vascular injury, Gas6/Axl signaling was shown to be increased in vascular neointima, and Axl expression was induced by thrombin and angiotensin II (Melaragno, 1998). In a later study, the role of Axl was studied in oxidative stress-mediated vascular remodeling, and Axl was shown to be an important mediator of intima proliferation by activating the Akt pathway (Konishi, 2004). Similarly, in a model of flow-induced vascular remodeling, Axl-deficient mice were shown to have thinner intima and media, increased apoptosis, and fewer VSMCs, macrophages, and neutrophils (Korshunov, 2006). In the context of vascular remodeling, Axl seems to protect VSMCs from apoptosis and promotes intima and media thickening, and it also plays a role in the increased secretion of proinflammatory cytokines and chemokines (Gerloff, 2012).

The Gas6/Axl system has also been shown to play a role in atherosclerosis (Lutgens, 2008, Ming Cao, 2001). Gas6/Axl signaling in VSMCs induces expression of the class A scavenger receptor (SRA) that mediates the uptake of lipids and thereby enables the formation of foam cells and atherosclerotic plaques (Ming Cao, 2001). Moreover, Gas6 is expressed in ECs, VSMCs, and macrophages of atherosclerotic plaques, and Gas6 deficiency is associated with increased plaque stability, and fewer plaque-associated macrophages as well as decreased inflammation (Lutgens, 2008).

Gas6/Axl signaling has been reported to inhibit vascular endothelial growth factor (VEGF) receptor 2-mediated angiogenesis as well as chemotaxis stimulated by VEGF-A (Gallicchio, 2005). In contrast, Axl has been reported to be an essential mediator of VEGF-A-dependent activation of the PI3K/Akt pathway, which induces EC

migration, vascular permeability and angiogenesis (Ruan, 2012). Moreover, knockdown of Axl in primary human umbilical vein ECs (HUVECs) caused a significant decrease in endothelial tube formation, indicating a role for Axl in EC morphogenesis (Li, 2009). In addition, lactate has been shown to induce ligand-dependent stimulation of Axl, VEGF receptor 2 as well as Tie2, resulting in activation of the PI3K/Akt pathway, tube formation and vessel sprouting (Ruan, 2013).

Axl in cancer

Axl was originally identified as a transforming gene, and its expression has since been confirmed in a large number of human cancers (Graham, 2014, O'Bryan, 1991). For example, overexpression of Axl has been reported in squamous cell carcinoma, non-small cell lung cancer (NSCLC), myeloid leukemia, prostate cancer, osteosarcoma, and ocular melanoma (Brand, 2015, Linger, 2013, Neubauer, 1994, Paccez, 2013, Tian, 2014, van Ginkel, 2004). Moreover, increased Axl expression has been associated with worsened prognosis in several cancer types, such as RCC, squamous cell carcinoma, and ocular melanoma (Brand, 2015, Gustafsson, 2009, van Ginkel, 2004). In cutaneous squamous cell carcinoma, Axl expression seems to be associated to epithelial-to-mesenchymal transition (EMT), a process involved in the initiation of metastasis, and resistance to chemotherapy (Cichon, 2014). In a xenograft study of NSCLC, Axl knockdown was associated with reduced tumor growth and improved response to chemotherapy (Linger, 2013). High Axl expression has also been reported to be associated with decreased survival in breast cancer (Gjerdrum, 2010). Chromosomal amplifications and translocations as well as point mutations of Axl have been reported, however genetic aberrations of Axl are rather uncommon and have not been extensively studied (Graham, 2014).

The role of Axl in cancer metastasis seems to be linked to its role in EMT and cellular migration and invasion. In breast cancer, Axl is induced upon EMT by Vimentin, a protein associated to mesenchymal phenotype, and knockdown of Axl inhibited metastasis in a xenograft model, and in addition, Vimentin-induced Axl confers a migratory phenotype in non-malignant mammary epithelial cells (Gjerdrum, 2010, Vuoriluoto, 2011). Moreover, Axl expression seems to be able to induce expression of EMT markers in breast epithelial cells (Asiedu, 2014). Similarly, Axl expression is associated with migration and invasion in hepatocellular carcinoma *in vitro*, and Gas6/Axl signaling promotes expression of Slug, a protein involved in EMT which seems to mediate Axl-dependent invasion(Lee, 2014). In addition, Gas6 signaling through Axl has been linked to dormancy in the bone marrow, and resistance to chemotherapy-induced apoptosis in PCa (Shiozawa, 2010, Taichman, 2013).

As previously mentioned, Axl expression seems to be supported by hypoxic conditions. In clear cell RCC (ccRCC) cell lines, Axl expression is associated with loss of the VHL tumor suppressor, and the HIF transcription factors, which are negatively regulated by VHL, directly activate Axl transcription (Gustafsson, 2009, Rankin, 2014). Similarly, Gas6-mediated downregulation of Axl has been shown to be inhibited in PCa cells under hypoxic conditions (Mishra, 2012). In acute myeloid leukemia (AML), AML cells have been shown to induce expression of Gas6 by bone marrow-derived stromal cells, and the stroma-derived Gas6 stimulates AML cell proliferation, survival, and chemoresistance *via* Axl (Ben-Batalla, 2013). Axl has also been indicated to be involved in regulating the secretion of pro-inflammatory mediators from tumor-associated macrophages in a model of breast cancer (Ye, 2010).

In addition to its roles in tumor growth, apoptosis resistance and metastasis, Axl seems to be involved in acquired resistance to targeted therapies in cancer. In NSCLC, Axl expression was shown to be increased in cells resistant to Gefitinib and Erlotinib treatment, inhibitors of EGFR, and similarly, Axl expression was increased in NSCLC and head and neck squamous cell carcinoma with acquired resistance to another EGFR inhibitor, Cetuximab (Bae, 2015, Brand, 2014, Zhang, 2012). Moreover, Axl expression seems to predict response to EGFR-targeted therapies (Byers, 2013, Meyer, 2013).

The increasing evidence supporting a role for Axl in various human cancers has lead to the development of different Axl inhibitors, for experimental use, and also for potential use in the clinic. Moreover, recent findings identifying Axl to be involved in acquired resistance to targeted therapies in several cancer types, as well as its role in mediating responsiveness to chemotherapy, indicate a potential use of targeted Axl therapies as a means to overcome drug resistance, and also as a complement to chemotherapies (Linger, 2010, Wu, 2014). For example, a monoclonal Axl-targeting antibody was shown to reduce tumor growth in xenograft models of breast cancer and NSCLC, and in addition the antibody was shown to reduce secretion of inflammatory molecules and to improve the effect of anti-VEGF therapy, EGFR inhibitor therapy and chemotherapy (Ye, 2010). Similarly, a small molecule inhibitor of Axl has been shown to reduce metastasis, angiogenesis, and proinflammatory cytokine secretion, and to improve survival in xenograft models of metastatic breast cancer (Holland, 2010). An inhibitor of Axl is currently going through clinical trials (Sheridan, 2013).

microRNAs

MicroRNAs are short, approximately 23 nucleotides (nt) long, non-coding RNA strands, that can act as post-transcriptional regulators of mRNA transcripts (Bartel, 2009). The first microRNA was identified in 1993 in a study aimed at determining the role of the lin-4 gene in regulation of LIN-14 in *C. elegans,* where the lin-4 gene was found to produce two short non-coding RNA transcripts with sequence complementarity to the 3' untranslated region (UTR) of the LIN-14 mRNA transcript (Lee, 1993). A few years later, lin-4 was found to also regulate the expression of another protein, LIN-28, again by sequence complementarity in the 3' UTR of the LIN-28 mRNA transcript (Moss, 1997). After its discovery, the lin-4 microRNA remained a unique finding for several years, and it was not until a second microRNA, let-7, was discovered in 2000 that microRNAs were recognized as an important non-coding part of the genome (Bartel, 2004, Reinhart, 2000). Since then, microRNAs have been found to be expressed in plants, viruses and animals, including mammals, and they have been shown to take part in many cellular processes such as proliferation, apoptosis and differentiation, in the organism development and in the pathogenesis of many diseases (Eulalio, 2008). For example, microRNA has been shown to play a role in inflammatory bowel disease, myocardial infarction, amyotrophic lateral sclerosis (ALS), atherosclerosis, and in a large number of human cancers (Boon, 2015, Chapman, 2015, Hata, 2015, Hayes, 2014, Hosin, 2014, Volonte, 2015).

Expression and regulation of microRNAs

Many microRNAs are evolutionally conserved, and they are expressed in distinct patterns during development and play a role in regulation of basic cellular processes as well as specialized processes that enable cellular tissue specificity (Lee, 2007). For example, the lin-4 microRNA plays a key role in regulating LIN-28 expression in specific stages in the development of *C. elegans*, and removal of the lin-4 complementary sequence in the 3' UTR of LIN-28 causes a retarded phenotype (Moss, 1997). Similarly, let-7 has been shown to regulate the expression of the LIN-41 protein in *C. elegans*, which plays a key role in regulating the developmental

timing in order to onset the expression of adult specialization transcription factors in the cell (Slack, 2000). In a study using transgenic microRNA reporter zebra fish, certain microRNAs were shown to be expressed in specific tissues during embryonic development, with overlapping expression of predicted target mRNAs, and with a general pattern of decreased target mRNA expression in microRNA-expressing cells, although expression of some mRNA targets was found to be increased (Shkumatava, 2009). It is noteworthy that the appearance of the microRNA pathway seems to be simultaneous with the appearance of multicellular organisms in the evolution, and it has been proposed that microRNAs could enable diverse cell specialization, as the diversity of microRNA expression correlates with cell specitation (Lee, 2007).

MicroRNAs are encoded in the genome and are expressed in a stepwise process that is subject to regulation on multiple levels (Hata, 2015). First, a primary transcript (pri-miRNA) is transcribed from the microRNA-encoding gene by RNA polymerase II, which is then processed in the nucleus by a complex of Drosha, a ribonuclease III type enzyme, and its cofactor, DGCR8, into a hairpin precursor microRNA (pre-miRNA) (Hata, 2015). The pre-miRNA is dependent on the Exportin-5/RanGTP complex for transport out of the nucleus into the cytoplasm, where GTP is hydrolyzed, the complex is disassembled, and the pre-miRNA is released into the cytosol (Ha, 2014). Finally, in the last step of microRNA maturation, another ribonuclease, Dicer, cleaves the pre-miRNA into a single-stranded, mature microRNA.

In the cytosolic maturation process, Dicer complexes with a double strand RNA-binding protein (dsRBD), the TAR RNA-binding protein (TRBP), which seems to modulate the efficiency by which the pre-miRNAs are processed into mature microRNAs as well as tuning of the length of mature microRNAs (Ha, 2014). Dicer has also been shown to bind a dsRBD cofactor, PACT, whose role in microRNA processing remains unknown (Ha, 2014). In *Drosophila,* the TRBP homologs seem to be essential for maturation of most microRNAs; in contrast, purified mammalian Dicer function has been shown to be comparable to that of Dicer-dsRBD complexes (Lee, 2006). Dicer also interacts with the Argonaute 2 (Ago2) protein, and TRBP to form the RISC-loading complex (RLC) which is essential for efficient transfer of matured microRNA strands to Ago2 (Wang, 2009). In the RLC complex, TRBP has been shown to be essential for the transfer of microRNA strands in complex with Dicer to Ago2, and the subsequent assembly of the RNA-induced silencing complex (RISC) which enables gene regulation by the mature microRNA (Chendrimada, 2005).

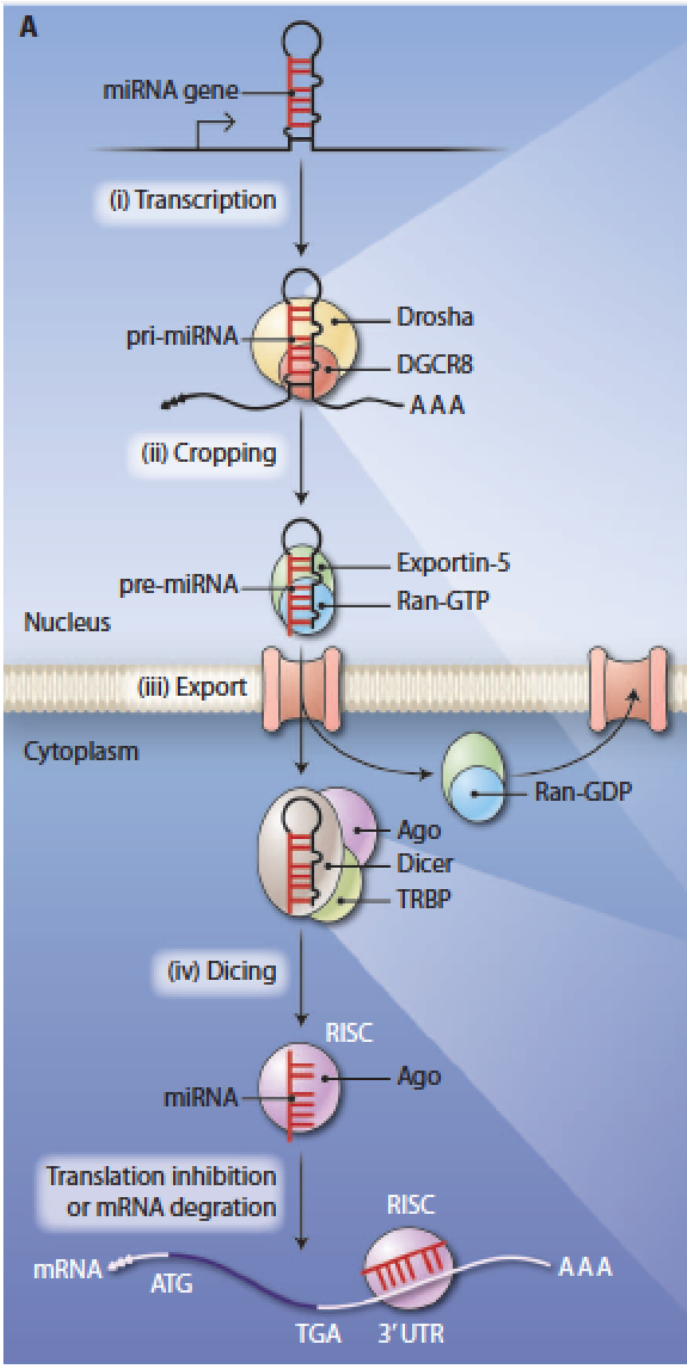

Figure 4
MicroRNA biogenesis. Image from Hata et. al., 2015. Reprinted with the permission of AAAS.

Although the exact structure of RISC remains to be elucidated, it is clear that the main catalytic activity of the complex resides in the Argonaute proteins, as a complex, called the minimal RISC, containing purified Ago2 and a siRNA strand is sufficient to cleave target RNAs (Rivas, 2005). Argonaute proteins have numerous binding partner proteins and seem to form a variety of complexes, which is supported by the variety in size of isolated RISC complexes, ranging from relatively small complexes at around 150 kDa, to molecular complexes which can be as large as 8 MDa (Pratt, 2009). The mechanisms by which microRNAs bind to and regulate target mRNA strands will be further discussed below.

Regulation of microRNAs occurs at several levels. For example, cell fate-determining signaling pathways, such as estrogen receptor signaling, transforming growth factor β (TGFβ) signaling, the MAPK pathway, and the p53-mediated DNA damage response, affect the overall and specific biogenesis of microRNAs (Olive, 2015). In addition, expression of microRNAs can be directly induced by transcription factors, as with the miR-34 family which is directly transactivated by the tumor suppressor p53, and the expression of these microRNAs has been found to be epigenetically inactivated by CpG methylation in the promoter region of the microRNA gene in cancer (Vogt, 2011).

Processing of nuclear pri-miRNAs is subject to regulation by RNA-binding proteins such as DDX5 and DDX17, which bind to Drosha-DGCR8 to function as restricted promoting factors which are important for the processing of specific pri-microRNA subsets (Shen, 2015). For example, in p53-mediated damage response and in TGFβ-mediated responses, DDX5 binds to the Drosha-DGCR8 complex and enhances the processing of specific microRNA clusters or a specific microRNA, respectively (Shen, 2015). In fact, each step in the microRNA processing machinery is subject to post-translational regulation and/or modulating protein interactions (Hata, 2015). In addition, the microRNA molecule itself carries intrinsic regulatory elements such as nucleotide sequences and structures that can affect both the biogenesis and the stability of the microRNA (Ha, 2014). Examples of such elements are single nucleotide polymorphisms (SNPs) which can affect both microRNA biogenesis and target repression activity, RNA editing by adenosine deaminases which can disturb the interaction between the microRNA and Drosha, RNA methylation, and regulation of RNA stability by nucleases that cleave pre-miRNAs or RNA strands that bind to and destabilize specific microRNAs (Ha, 2014).

MicroRNA – mRNA interactions and post-transcriptional regulation

In humans, more than 400 different microRNAs, and more than 45,000 predicted microRNA target sites in human mRNA 3' UTRs, have been identified, and furthermore, each microRNA can potentially regulate a large number of mRNA targets (Bartel, 2009, Friedman, 2009, Lim, 2005). Most commonly, microRNAs bind to target mRNA transcripts *via* 5' canonical seed sequence sites, known as the 7mer-A1 site, the 7mer-m8 site and the 8mer site (Bartel, 2009). These canonical sites rely on complementarity between nucleotides 2-7 in the microRNA and the seed match region in the target mRNA, along with additional flanking nucleotide paring (Bartel, 2009). Furthermore, another type of seed sequence matching, the marginal sites, is characterized by binding to the target mRNA by 6mer-matching of nucleotides 2-7 or 3-8 in the microRNA, which generally is less efficient than 7mer or 8mer sites (Bartel, 2009). Less commonly, microRNAs can bind to mRNAs in so-called atypical sites, with 7mer, or 7mer with a central mismatch, primary seed match sites and a microRNA 3' supplementary or compensatory site, respectively, which increases the efficiency of the functional site (Bartel, 2009). In addition to matching of the microRNA-mRNA *via* seed sequences, the context of the 3' UTR of the target mRNA seems to have impact on the efficacy of regulation by microRNAs, as mRNAs containing identical 3' UTR seed sequences can be differently regulated (Bartel, 2009).

Other features that boost site efficiency and thereby translational regulation are AU-rich elements situated close to the seed sequence, positioning at least 15 nt from the 3' UTR stop codon, off-center positioning in long 3' UTRs, proximity to seed sequences of coexpressed microRNAs, and additional pairing to nucleotides 13-16 in the microRNA (Grimson, 2007). In addition to the most commonly occurring targeting of 3' UTRs, microRNAs have also been shown to be able to efficiently repress expression of mRNAs by binding to target sites introduced in the 5' UTR, and targeting can also occur in the open reading frames (ORFs) of mRNA transcripts (Baek, 2008, Lewis, 2005, Lytle, 2007). In addition, interaction of microRNAs with gene promoters has been demonstrated (Zardo, 2012).

The mechanisms by which microRNAs regulate gene expression are diverse; target recognition can result in translational repression by inhibition of translation elongation, by promoting premature ribosome dissociation, by preventing initiation of translation, and by deadenylation of mRNA transcripts (Eulalio, 2008). In addition, microRNAs can act in the opposite manner and activate translation (Lee, 2013). For example, the mouse miR-34a and miR-34b have been shown to be able to increase the translational efficiency of an alternatively adenylated β-actin mRNA

transcript, and mutation or blocking of the 3' UTR seed sequence markedly reduces protein expression (Ghosh, 2008). However, microRNAs are thought to most commonly act to repress translation, primarily through destabilization of mRNA transcripts (Baek, 2008, Eichhorn, 2014, Guo, 2010).

Apart from the impacting factors discussed above, the functional outcome of microRNA-mediated gene regulation is highly dependent on the cellular context. For example, miR-34a, which is primarily known as a tumor suppressor microRNA, exerts tumor suppressive functions in neuroblastoma, but in contrast seems to support proliferation of rat renal proximal tubule cells and is overexpressed in several cancer types (Dutta, 2007). In line with this finding, it was recently reported that microRNA-mediated regulation is affected by the abundance of target mRNA transcripts (Arvey, 2010). Moreover, in a study analyzing single cells, target repression varied dramatically between individual cells and microRNAs were found to establish mRNA threshold levels at which protein production can be readily repressed, enabling microRNAs to act both as a fine-tuner as well as a switch of gene expression (Mukherji, 2011).

It is noteworthy that some microRNA families exhibit sequential redundancy, especially microRNA families involved in development, and revealing the functional implications of these microRNA families requires removal of the redundancy, at least in part (Olive, 2015).

MicroRNAs in cancer

Dysregulation of microRNAs has been reported in a broad range of human cancers. While some microRNAs act as tumor suppressors, other microRNAs are highly expressed in cancers and exert pro-oncogenic functions, hence they are called oncomiRs (Esquela-Kerscher, 2006, Ma, 2008). Because of their roles as tumor suppressor and oncogenes, microRNAs have been identified as potential tools for diagnosis and prognosis of human cancers, and also as potential targets for therapeutic intervention (Metias, 2009). Some, but not all, microRNAs are released into bodily fluids, such as urine and in the bloodstream, and therefore present a potential non-invasive method of cancer diagnosis and prognosis (Khoury, 2015). For example, in a recent report, three different microRNA panels were identified as diagnostic and prognostic markers in the serum of human PCa patients and patients with benign prostatic hyperplasia (BPH) (Haldrup, 2014). MicroRNAs found in tumor tissue and cells have also been found to be useful as prognostic and diagnostic tools, for example in PCa where a four-microRNA ratio, the miQ, was shown to be a powerful prognostic marker as well as a diagnostic tool to predict tumor aggressiveness, metastasis and overall survival (Larne, 2013). Similarly, the expression of a single microRNA, miR-21, has been correlated to decreased survival and lymph node metastasis, and is an independent prognostic factor in human primary breast cancer (Yan, 2008). In addition, tumor microRNAs have been shown to be powerful tools to classify cancer tumors (Lu, 2005).

The miR-34 family

The miR-34 family comprises three similar microRNAs, miR-34a, miR-34b, and miR-34c, which are located at two different sites in the genome; miR-34a is found on chromosome 1p36, whereas miR-34b and miR-34c are located on chromosome 11q23 (Hermeking, 2010). Studies in mice have revealed that miR-34a is ubiquitously expressed, whereas miR-34b and miR-34c are primarily expressed in lungs, which is the only tissue where miR-34b/c expression exceeds miR-34a expression (Misso, 2014).

The members of the miR-34 family are directly regulated by p53, and function as tumor suppressors by regulating the expression of genes that control apoptosis, cell cycle and proliferation (Bommer, 2007, Chang, 2007, Corney, 2007). The p53 transcription factor is activated upon cellular stress, such as DNA damage or hypoxia, and initiates expression of a wide array of genes (Sax, 2014). Dysregulation of the p53 network is frequently found in human cancers, such as mutations that prevent p53 from binding to specific target DNA sequences, but also aberrations of p53-regulating

factors, mislocalization of p53 into the cytoplasm, and viral infections where viral oncogenes interfere with p53 (Vogelstein, 2000). Moreover, the miR-34 family has been reported to be independently inactivated *via* methylation of CpG islands in the promoter region, rendering these microRNAs irresponsive to transcriptional activation by p53 (Lodygin, 2008, Vogt, 2011). Interestingly, the epigenetic inactivation of miR-34 family members seems to be mutually exclusive with p53 mutations, indicating that inactivation of miR-34a/b/c expression could be an alternative route to loss of p53-mediated regulation of cellular processes (Vogt, 2011). The miR-34 family members have been shown to negatively regulate target genes that are anti-apoptotic, such as BCL2 and SIRT1, genes that promote proliferation and growth, including E2F3, Notch2 and CREB, genes that are involved in migration and invasion, such as MET and SNAIL, as well as stemness-promoting genes like NANOG and SOX2 (Agostini, 2014). Moreover, miR-34a has been found to target CD24 and Scr, proteins involved in tumorigenesis (Muppala, 2013). Decreased expression of miR-34 family members has been observed in several types of cancer, for example in pancreatic cancer, NSCLC, bladder cancer, and in PCa (Bommer, 2007, Catto, 2011, Chang, 2007, Hagman, 2010).

The implications of the microRNA-34 family in cancer has indicated that miR-34 mimics potentially could be used in treating human cancers, and in fact, a miR-34 analog is the first microRNA-based therapeutic to enter phase I clinical trials for liver cancer (Agostini, 2014).

Interestingly, miR-34a has been shown to be a negative regulator of Axl expression by direct binding to the 3' UTR of the Axl mRNA transcript in NSCLC, colorectal cancer and breast cancer (Mackiewicz, 2011, Mudduluru, 2011). Moreover, Axl expression was shown to correlate inversely with miR-34a expression in NSCLC, colorectal cancer and breast cancer cell lines, and in breast cancer tumor tissue (Mackiewicz, 2011, Mudduluru, 2011). In addition, miR-34a has recently been reported to directly regulate Axl in B-cell chronic lymphocytic leukemia (Boysen, 2014).

The role of the miR-34 family members in PCa and RCC will be further discussed in the respective sections below.

miR-21

The miR-21 is one of the most well-known oncomiRs, and overexpression of miR-21 has been reported in a large number of human cancers, including lung cancer, glioblastoma, breast cancer, PCa, and B-cell lymphoma (Selcuklu, 2009, Zhu, 2014). The gene for miR-21 is located within the TMEM49 gene, and is independently transcribed from two promoter regions with conserved elements such as binding sites

for AP-1 and STAT3 (Kumarswamy, 2011). Expression of miR-21 has been reported to be induced by IL-6 *via* the STAT3 transcription factor, and targets the tumor suppressor PTEN and in addition seems to be involved in activating an epigenetic switch to activate a positive feedback loop to maintain a transformed state in a transformed breast epithelial cell line (Iliopoulos, 2010). Interestingly, miR-21 expression is triggered by inflammatory stimuli, primarily in bone-marrow derived cells, where miR-21 seems to be involved in resolution of the inflammatory response, as LPS-induced miR-21 expression in macrophages has been associated with decreased pro-inflammatory signaling and resolved inflammation (Sheedy, 2015). The complexity of miR-21 function in the regulation of inflammation is further illustrated by the fact that miR-21 overexpression has been reported in diseases associated with impaired immune responses, such as asthma, psoriasis and chronic viral and bacterial infections, as well as with diseases characterized by chronic inflammation such as atherosclerosis, SLE, and colitis (Sheedy, 2015). In the cardiovascular system, miR-21 is highly expressed, and dysregulated miR-21 expression has been reported in several cardiovascular diseases such as myocardial infarction, and moreover, miR-21 expression in VSMCs is associated with increased proliferation and survival (Cheng, 2010).

High expression of miR-21 is associated with poor prognosis in human cancers including NSCLC, breast cancer and RCC (Faragalla, 2012, Markou, 2008, Yan, 2008). For example, miR-21 is frequently overexpressed in breast cancer tissues and experimental inhibition suppresses growth of breast cancer cell lines *in vitro* as well as in tumor xenografts *in vivo* (Si, 2007). Moreover, reexpression of miR-21 in a breast cancer cell line was associated with increased expression of EMT markers, increased expression of cancer stem cell surface markers, and increased sphere formation ability, which is associated with stem cell characteristics (Han, 2012).

In cancer, miR-21 has been shown to target tumor suppressor genes, for example PTEN in hepatocellular carcinoma and RCC, and the programmed cell death 4 (PDCD4) gene in breast cancer and colorectal cancer (Asangani, 2008, Dey, 2012, Lu, 2008, Meng, 2007). In breast and colon cancer, miR-21 expression has been shown to be indirectly induced by the adhesion molecule CD24, an effect that can be inhibited by miR-34a-mediated downregulation of CD24 and its mediator Src (Muppala, 2013). Moreover, miR-21 seems to be involved metastasis, as demonstrated by decreased lung metastasis and intravasation of colorectal cancer cells in a chicken embryo metastasis assay where miR-21 had been inhibited (Asangani, 2008). A role for miR-21 in tumor angiogenesis has also been indicated, as miR-21 expression in PCa cells has been shown to be associated with increased expression of VEGF and HIF1α and increased tumor angiogenesis (Liu, 2011). Further discussion of miR-21 and its role in RCC can be found in the section discussing the kidneys and RCC.

miR-10b

The miR-10b is most commonly recognized as an oncomiR, and has been found to be overexpressed in glioblastoma, NSCLC, breast cancer, and hepatocellular carcinoma (Gabriely, 2011, Lu, 2014). The miR-10b gene is located within the HOXD cluster on chromosome 2, in-between the HOXD4 and HOXD8 genes (Biagioni, 2013).

Increased expression of miR-10b has been reported in metastatic breast cancer cell lines, and overexpression of miR-10b in non-invasive breast cancer cell lines promoted invasion and metastasis (Ma, 2007). Furthermore, expression of miR-10b correlated with clinical progression in breast cancer patients (Ma, 2007). In contrast, another study reported that miR-10b was downregulated in breast cancer (Iorio, 2005). In addition, downregulation of miR-10b has been shown in primary and metastatic colorectal carcinoma (CRC), and in ccRCC (Pizzini, 2013, Wotschofsky, 2012). Moreover, miR-10b has been indicated to be downregulated by CpG methylation in gastric cancer, and miR-10b overexpression was associated with reduced migration, invasion and proliferation, and induction of apoptosis, and miR-10b has been proposed to function as a tumor suppressor in gastric cancer (Kim, 2014, Li, 2015). Moreover, retinoic acid-induced differentiation of neuroblastoma cell lines, resulting in reduced proliferation and less aggressive cell behavior, was associated with increased miR-10b expression and furthermore, miR-10b was shown to be involved in mediation of the differentiated phenotype (Meseguer, 2011).

Increased expression of miR-10b has also been associated with congenital heart defects, and miR-10b has been shown to negatively regulate the TBX5 transcription factor which is involved in cardiac development in the embryo (Wang, 2014).

Most mRNAs that have been validated as targets of miR-10b are tumor suppressor genes, such as KLF4 in esophageal squamous cell carcinoma, HOXD10 in breast cancer, and E-cadherin in NSCLC (Ma, 2007, Tian, 2010, Zhang, 2015). In breast cancer, miR-10b expression has been reported to be induced by TGFβ, and miR-10b seems to take part in mediating EMT induced by TGFβ (Han, 2014). The effects of miR-10b expression seem to be cell-type dependent, as miR-10b expression promotes invasion and metastasis in breast cancer, while miR-10b-rexpressing glioblastoma cells are characterized by increased proliferation and resistance to apoptosis (Gabriely, 2011). The role of miR-10b in RCC will be further discussed in the respective section below.

The kidneys and renal cell carcinoma

The kidneys

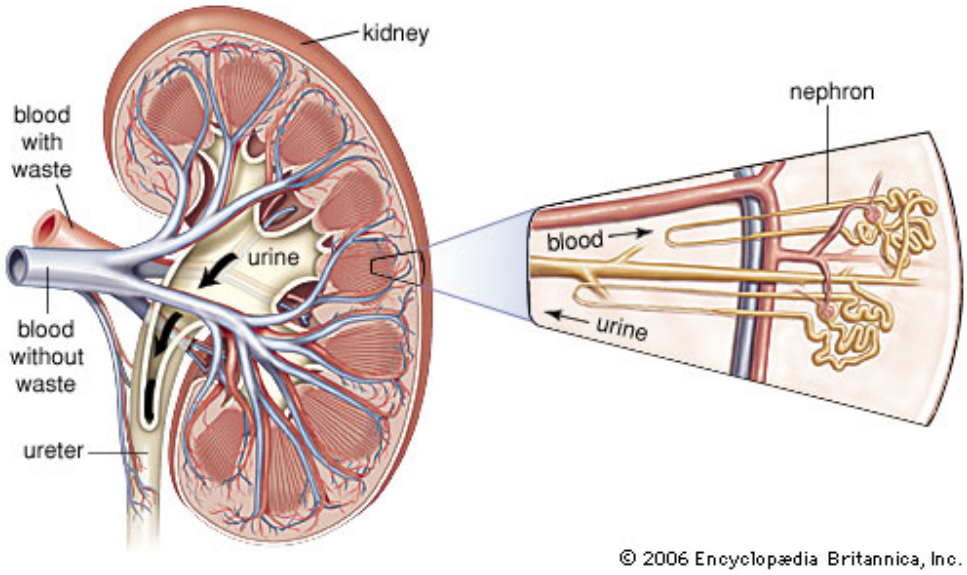

Figure 5
The human kidney. Reprinted with the permission Encyclopaedia Britannica.

The main functions of the kidneys are removal of waste and foreign materials from the blood stream into the urine, to regulate the acid-base balance, electrolyte concentration, and blood pressure, and to reabsorb glucose and amino acids from the primary urine as well as producing hormones, such as erythropoietin (Epo), and Vitamin D production (Cotran, 2005, Widmaier, 2006). The kidneys take up approximately 20% of the cardiac blood output and receive blood through the renal arteries which branch out form the abdominal aorta (Boron, 2004).

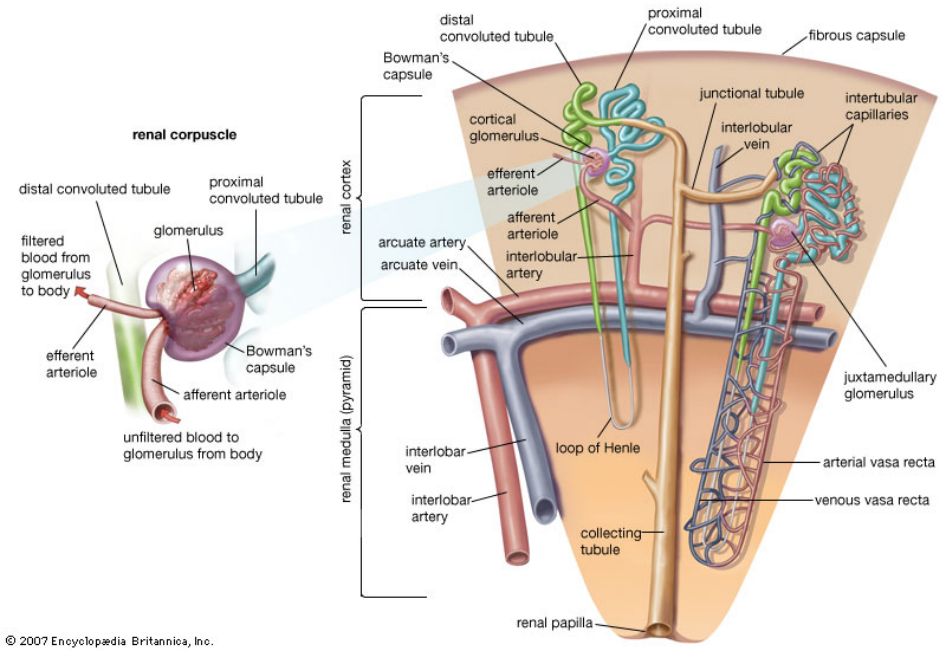

Figure 6
The human renal corpsule and the nephron. Reprinted with the permission Encyclopaedia Britannica.

The kidneys are protected by the fibrous renal capsule, which in turn is surrounded by perirenal fat, forming an adipose capsule, the renal fascia, which encapsulates the kidneys and the adrenal glands in connective tissue, and on the outside of the capsule, paranephric fat. On the inside, the kidney is divided into two major structures, the renal cortex, and the renal medulla. The renal cortex is organized into pyramid-shaped structures, where the renal corpsule, in which blood ultrafiltration occurs, can be found. The renal tubules extend down through the renal medulla, where they are connected to the collecting ducts, which lead the urine into the ureter and subsequently into the urine bladder. The cortex- and medulla-spanning tubules, known as nephrons, form a distinct structure capable of efficient blood filtration, removal of waste and reabsorption of nutrients. Blood filtration begins in the glomeruli, situated in the Bowman's capsule inside the renal cortex, where approximately 20% of the water in the blood plasma passing the glomeruli is filtrated out along with solutes present. The filtrate then passes through the proximal convoluted tubule, where the brush border, which is lined with epithelial cells specialized in resorption, can be found. Subsequently, the filtrate passes through Henle's loop which helps concentrating the urine using an urea concentration gradient which draws out water from the urine filtrate (Widmaier, 2006). Some nephrons, known as juxtamedullar nephrons, span deeper into the renal medulla and

have collecting blood capillaries, vasa recta, that are intertwined with Henle's loop and are supplied with reabsorbed substances from the nephron. Finally, the filtrate passes the distal convoluted tubule, which take part in regulation of pH and electrolyte concentration.

Renal cell carcinoma

RCC is the most common type of cancer in the kidney, and it accounts for approximately 2-3% of all cancer cases in adults (Escudier, 2012). In Europe, more than 88,000 individuals are diagnosed with RCC each year, and almost 40,000 patients die from RCC yearly (Ljungberg, 2010). The prevalence of RCC is slightly higher in men than in women, about 60% off RCC cases are in men, and the peak age of diagnosis is between 60 and 70 years (Ljungberg, 2010). The incidence of RCC in the world has been increasing with approximately 2% yearly until recently (Ljungberg, 2010). A small portion, around 2-3%, of all RCC cases are hereditary and are caused by inherited mutations in VHL, c-Met, fumarate hydratase, or in the folliculin gene (Weikert, 2010).

RCC is further classified into subtypes, of which clear cell RCC (ccRCC), papillary RCC (pRCC), and chromophobe RCC (chRCC) are the most commonly occurring (Lopez-Beltran, 2009). Of the RCC subtypes, ccRCC is by far the most common, accounting for around 75% of all RCC cases (Cohen, 2005, Lopez-Beltran, 2009). Papillary RCC is much more common in men than in women, and has a 5-year survival of approximately 90%, however the prognosis of patients with metastatic pRCC is poor (Cohen, 2005). Around 75% of all sporadic pRCC cases are characterized by duplications of chromosome 7 (Cohen, 2005). Chromophobe RCC is thought to arise from a cell type in the collecting ducts called type B intercalated cells (Cohen, 2005). Clear cell RCC has overall worse prognosis than pRCC and chRCC, and is often characterized by mutations in the tumor suppressor VHL gene (Lopez-Beltran, 2009). VHL mutations are found in approximately 60% of ccRCC cases, and loss of VHL allows hypoxia-inducible genes to be overexpressed, favoring epithelial cell proliferation (Cohen, 2005).

As of today, surgery is the only curative treatment for RCC, in localized RCC most commonly by nephron-sparing procedures aimed at removing the tumor while allowing the kidney to remain functional, at least in part (Ljungberg, 2010). Patients with metastatic RCC are treated with systemic therapeutics, for example immunotherapy with IFNα and IL-2, which have a response rate of around 6-15% and 7-27%, respectively (Ljungberg, 2010). In addition, targeted therapies are used, including tyrosine kinase inhibitors such as Sorafenib and Sunitinib, as well as anti-

VEGF therapy with Bevacizumab, and mTOR inhibitors such as Temsirolimus (Abe, 2013).

The disease-specific 5-year survival of patients with localized RCC is approximately 90%, with decreasing survival for patients with more advanced disease (Novara, 2010). Although targeted therapies have improved the survival of RCC patients, the prognosis of patients with metastatic RCC remains poor with a 5-year survival of less than 10% (Abe, 2013, Ljungberg, 2010). Approximately 25% of all patients do not respond when treated with targeted therapies, and in addition some patients who initially respond eventually develop resistance (Bielecka, 2014).

The main prognostic tool for RCC is the Tumor-Node-Metastasis (TNM) staging system (Ljungberg, 2010, Wittekind, 2002). In addition, factors such as histological grade, tumor size and presence of necrosis have been shown to be useful in assessing the prognosis, and prognostic systems and nomograms that combine different independent prognostic factors have been developed, such as the SSIGN score which is based on TNM stage, tumor size, grade and necrosis (Frank, 2002, Fuhrman, 1982, Leibovich, 2003).

Axl and Gas6 in the kidney and RCC

Gas6 and Axl are expressed in the kidney, and increased expression of Gas6 and/or Axl seems to be associated with several inflammatory renal diseases (Fiebeler, 2004). In mesangial cells, specialized kidney VSMCs that often have a proliferative phenotype in glomerular disease, Gas6 stimulation of Axl results in Axl phosphorylation and cellular proliferation, and this effect seems to be dependent of proper γ-carboxylation of Gas6 (Yanagita, 1999). Moreover, in a model of induced glomerulonephritis, both Axl and Gas6 were upregulated and were associated with the progression of mesangial cell proliferation, an effect that could be inhibited by administration of Warfarin, which inhibits Vitamin K-dependent γ-carboxylation, or by administration of Axl-Fc (Yanagita, 2001). Later, the same group reported that the effects of Gas6 stimulation on mesangial cell proliferation were mediated by the STAT3 transcription factor (Yanagita, 2001). In addition, in a mouse model of nephrotoxic nephritis, mortality as well as the progression of glomerular disease were decreased in Gas6-deficient mice (Yanagita, 2002). Similarly, increased Gas6 and Axl expression were observed in the glomeruli of rats with induced diabetic nephropathy, but not in Gas6-deficient rats, and Warfarin administration could inhibit mesangial and glomerular hypertrophy (Nagai, 2003). Later, it was reported that Gas6 activates AKT and mTOR signaling in glomerular hypertrophy, and that high glucose treatment could induce Gas6/Axl expression in mesangial cells, resulting in mesangial hypertrophy in a Gas6-dependent manner (Nagai, 2005). Increased expression of

Gas6 has also been observed in a rat model of chronic renal rejection after kidney transplantation, however this was accompanied by high expression of Tyro3 rather than Axl (Yin, 2002). In a later study of human specimens of chronic renal transplant rejection, Gas6 expression was found to be increased in acute tubular necrosis as well as in acute transplant reject, and increased expression of Axl was observed in acute tubular necrosis whereas differential expression of the other TAM receptors could not be detected (Yin, 2003). Increased Gas6 expression has also been observed in humans with chronic kidney disease (CKD), however in a mouse model of CKD, Gas6/Axl signaling was reported to a protective mechanism, associated with slower progression to renal failure (Hyde, 2014, Lee, 2012).

In the light of the reported differential expression of Gas6 and Axl in kidney disease, it is noteworthy that there is a well-established connection between renal disease and the risk of developing kidney cancer (Russo, 2012).

Axl is expressed in RCC tumors, and high expression of Axl, or low expression of Gas6, correlates with poor prognosis in RCC (Chung, 2003, Gustafsson, 2009). Moreover, Axl mRNA expression levels in the tumor have been shown to be an independent prognostic factor in RCC (Gustafsson, 2009). In VHL-deficient ccRCC cell lines, Axl expression is high and seems to be post-transcriptionally regulated by VHL, as reconstitution of VHL results in decreased expression of Axl protein, but not Axl mRNA (Gustafsson, 2009). Similarly, Axl could be linked to direct transcriptional regulation by VHL in a study using proteomic and transcriptional profiling approaches to compare VHL-positive and -negative ccRCC cells (Boysen, 2012). Moreover, HIF1 and HIF2, which are negatively regulated by VHL, have been shown to directly bind to the hypoxia-responsive element in the Axl promoter, and inactivation of Axl in metastatic ccRCC cells reversed the metastatic phenotype *in vivo* and reduced invasion *in vitro* (Rankin, 2014). In ccRCC tumors, Axl is expressed both in tumor cells and in endothelial cells, and epithelial Axl expression correlated with poor prognosis, whereas high endothelial expression of Axl correlated with high nuclear differentiation grade (Boysen, 2012). Interestingly, short-term stimulation of Axl by Gas6 in ccRCC cell lines is associated with initial Axl phosphorylation, followed by downregulation of Axl protein, and Gas6-stimulated ccRCC cells exhibit decreased cell viability, and decreased migratory capacity which seemed to be dependent on Axl (Gustafsson, 2009). Similarly, siRNA-mediated knockdown of Axl been associated with G0/G1 cell cycle arrest, however the effect of Gas6 was not studied in this context (Chung, 2003).

The miR-34 family in RCC

As previously mentioned, the miR-34 family members are primarily considered to be tumor suppressor microRNAs. However, the reports on miR-34, in particular miR-34a, expression in RCC are contrasting. In several studies aimed at identifying differentially expressed microRNAs in ccRCC, miR-34a was found to be upregulated in tumor specimens (Juan, 2010, Jung, 2009, Liu, 2010, Osanto, 2012, White, 2011, Wu, 2012). In contrast, epigenetic inactivation of both miR-34a and miR-34b/c has been reported in RCC, and decreased expression of miR-34a has also been reported in RCC tumors in a study using RT-qPCR (Vogt, 2011, Zhang, 2014). Low miR-34a expression has also been reported in RCC cell lines, and miR-34a was shown to regulate CD44 *in vitro*, a cell surface adhesion molecule often upregulated in RCC (Yu, 2014). In addition, miR-34a has been indicated to regulate Notch1 and c-Myc, c-Met, and Bcl2 in RCC *in vitro*, however, direct regulation of Notch1 by miR-34a was not confirmed by Luciferase assay (Yamamura, 2012, Zhang, 2014). On the other hand, high miR-34a expression was observed in the renal tubules of rats in an experimental model of oxidative stress-induced renal carcinogenesis, and siRNA-mediated knockdown reduced proliferation renal carcinoma cells, thus the authors proposed that effects of miR-34a could be cell type-dependent (Dutta, 2007).

It is possible that the contrasting reports on miR-34a expression could be explained by differences in sample size, sample preparation and experimental procedures. However, most of the reports conducted with human tumor samples seem to indicate that miR-34a expression indeed is increased in ccRCC tumors, similar to what we have observed (Paper II). In addition, although Luciferase assays could be used to efficiently prove miRNA-mRNA interactions, these assays are usually performed under relatively synthetic conditions with high concentrations of microRNA and Luciferase-mRNA transcripts which may be rather unrepresentative of physiological conditions. Thus, proving a potential regulation of a mRNA transcript by a microRNA, even if performed in a tissue-specific cell lines, does not automatically imply that this interaction is the primary mode of action by the microRNA in that cell type. Similarly, experiments performed with microRNA mimics or microRNA blockers are usually also conducted at relatively high molecular concentrations, and in addition, the fact that each microRNA usually has numerous targets further aggravates attempts to elucidate microRNA function in a physiological context.

miR-21 in RCC

High expression of miR-21 in ccRCC has been reported by numerous research groups, and miR-21 expression was correlated to worse outcome in the Cancer Genome Atlas study (Cancer Genome Atlas Research, 2013, Chow, 2010, Huang, 2009, Juan, 2010, Jung, 2009, Osanto, 2012, Wotschofsky, 2012, Wu, 2012). In RCC, miR-21 has been shown to directly regulate TIMP3, a metalloproteinase inhibitor, and Fas ligand, an apoptosis-inducing protein, affecting survival and proliferation (Zhang, 2011). Moreover, miR-21 is a direct negative regulator of the transcription factor TCF21 which drives expression of a member of the metastasis suppressor family, KISS1 (Zhang, 2012). Interestingly, increased levels of miR-21 has been observed in the kidneys and serum of patients with renal fibrosis, which is the end stage for many chronic kidney diseases (Glowacki, 2013, Zarjou, 2011). Expression of miR-21 can be induced by TGFβ, which is involved in RCC pathogenesis and metastasis (Bostrom, 2013, Glowacki, 2013, Zarjou, 2011). In the context of renal fibrosis, it is noteworthy that miR-21 has been indicated to be involved in an epigenetic switch linking inflammation to cancer and maintenance of a transformed state, as has previously been mentioned (Iliopoulos, 2010).

miR-10b in RCC

Unlike miR-21, the reports on miR-10b expression in various human cancers are inconsistent. For example, upregulation of miR-10b has been reported in more than 10 human cancers, while downregulation of miR-10b has been reported in melanoma and colon cancer, and in RCCs (Lu, 2014). Similar to our findings (Paper I), decreased expression of miR-10b in ccRCC has been observed by others (Cancer Genome Atlas Research, 2013, Juan, 2010, Osanto, 2012). Furthermore, decreased expression of miR-10b seems to be associated to metastatic disease, early relapse, and poor prognosis in ccRCC (Heinzelmann, 2011, Khella, 2012, Slaby, 2012, Wotschofsky, 2012, Wu, 2012).

As previously mentioned, miR-10b has been validated to target tumor suppressor genes, and the effects of miR-10b expression are potentially cell-type dependent (Gabriely, 2011, Ma, 2007, Tian, 2010). To date, little is known about the role of miR-10b in ccRCC, and how decreased expression of miR-10b could contribute to the cancer phenotype, however in a recent report, significant downregulation of miR-10b was reported in renal allograft rejection and inhibition of miR-10b in human glomerular cells mimicked the features of allograft rejection (Liu, 2015).

The prostate and prostate cancer

The prostate

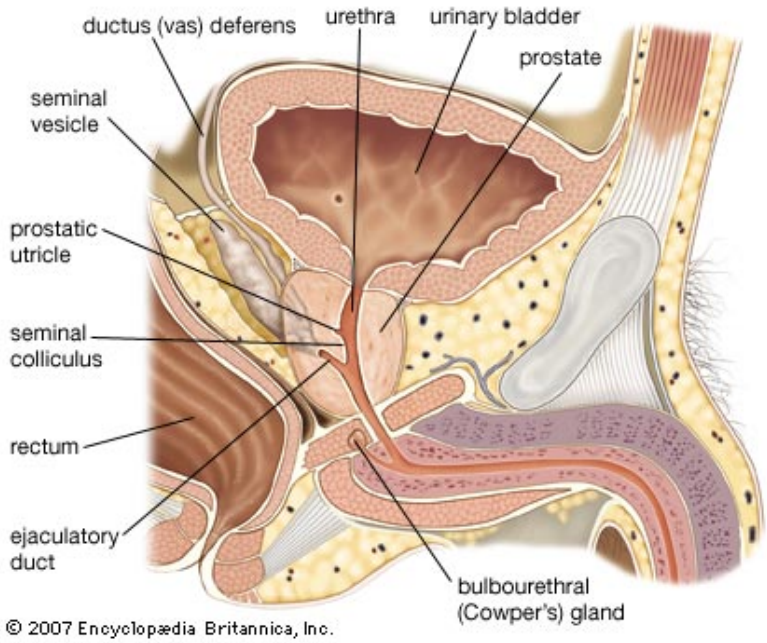

Figure 7
The human prostate. Reprinted with the permission Encyclopaedia Britannica.

The prostate is an exocrine gland in the male reproductive system, whose main function is to secrete an alkaline fluid, that together with spermatozoa and seminal vesicle fluid make up the semen (Neil, 2005). The size of the prostate is just larger than a walnut, and it sits just below the urinary bladder, with the urethra and the ejaculatory ducts passing through. The prostate gland can be divided into zones, the peripheral zone, the central zone, which surrounds the ejaculatory ducts, and the transitional zone, lateral to the urethra (Myers, 2000).

Prostate cancer

Prostate cancer is the most common neoplasm in men, and has an incidence rate of around 214 cases per 1000 men in Europe (Heidenreich, 2014, Jemal, 2008). The risk of developing PCa increases over age, which is reflected by the fact that PCa is more common in developed countries, as compared to developing countries (Ferlay, 2010). The 5-year standardized survival of PCa patients is approximately 83%, and patients diagnosed with early-stage PCa have even better survival, close to 100% over a 5-year period (De Angelis, 2014). However, PCa imposes a great economic burden to the healthcare, costing almost 5.5 billion EUR yearly in Europe (Luengo-Fernandez, 2013). In addition, even though the overall survival of PCa patients is high, the 4% of PCa patients that develop metastatic disease face a 5-year survival rate of approximately 30% (Fong, 2012).

PCa can be diagnosed using digital rectal examination, transrectal ultrasound-guided biopsy, and analysis of prostate-specific antigen (PSA) concentration in the serum (Heidenreich, 2014). In general, high PSA levels are associated with increased likelihood of PCa, however not all PCa patients exhibit increased serum PSA (Heidenreich, 2014). The PSA has been debated because of the potential false positive and negative results, and in a systematic review of two large PCa screening studies, no conclusive evidence supporting population-based screening for PCa could be found (Ilic, 2007). Increased PSA levels can also occur in BPH (Chang, 2012). In a randomized study with more than 160,000 European men, the authors concluded that PSA screening does reduce the number of deaths from PCa with 20%, however there is a risk of overdiagnosis associated with the PSA test (Schroder, 2009). To completely diagnose PCa, histological examination of biopsy samples is needed (Heidenreich, 2014). The Gleason scoring system is based on the histological appearance of the prostate tissue, where a low score represents well-differentiated tumors which still have structure similar to healthy prostate, whereas patients with high Gleason score have tumors which in large have lost their glandular structure and are poorly differentiated (Cotran, 2005). The Gleason score also has prognostic significance (Heidenreich, 2014, Rusthoven, 2014). In addition to the Gleason score, TNM staging is used for prognosis and treatment selection, and is based on tumor histology, spread, lymph node involvement, and metastasis status (Cheng, 2012).

For patients with low-risk PCa, therapy may not be needed; instead, active surveillance is recommended for patients with a localized disease, few biopsy samples with confirmed cancer and a Gleason score of 6 or lower (Heidenreich, 2014). Surgical removal of the whole prostate, radical prostectomy (RP), has been found to especially beneficial for patients who are 65 years old or younger, and those who have intermediate or high risk PCa (Heidenreich, 2014). In addition, radiotherapy is used to treat low-risk PCa tumors (Heidenreich, 2014). Treatments for intermediate and

high risk PCa tumors include RP as well as different types of radiotherapy (Heidenreich, 2014).

More than 90% of PCa metastases are found in the bone marrow, and cause great pain and high risk of skeletal complication (Heidenreich, 2014). For the treatment of metastatic PCa tumors, therapeutic androgen deprivation strategies are most commonly used. Androgen deprivation can for example be achieved using agonists of the luteinizing hormone-releasing hormone, which causes testosterone levels to decrease (Heidenreich, 2014). Relapsing PCa after hormone treatment is often referred to as castration-resistant PCa (CRPC), and while some of these will respond to second line therapy targeting the androgen receptor, others are truly hormone-resistant and are resistant to all hormone-targeting therapies (Heidenreich, 2014). Chemotherapeutic agents, such as Docetaxel, are also used in second line treatment of CPRC (Heidenreich, 2014).

Axl and Gas6 in PCa

Axl is overexpressed in PCa cell lines as well as in PCa tumor tissue, where increased Axl expression seems to be associated with the tumor Gleason score (Jacob, 1999, Sainaghi, 2005, Shiozawa, 2010). Gas6 signaling through Axl has been implicated to increase the proliferation of PCa cell lines, and knockdown of Axl has been shown to decrease tumor weight and IL-6 secretion in tumor xenograft models, and decreased cellular growth and survival, migration and invasion *in vitro* (Paccez, 2013, Sainaghi, 2005). However, another study has indicated that Annexin II, which is abundantly expressed in the bone marrow, induces Axl expression and osteoblast-secreted Gas6, which inhibits growth and prevents chemotherapy-induced apoptosis in PCa cells, possibly to induce dormancy in the bone marrow niche (Shiozawa, 2010). In line with these findings, the same group later presented a report indicating that PCa cells express Axl, and that Gas6 signaling through Axl indeed inhibits the growth of a human PCa cell line (Taichman, 2013). Furthermore, immunofluorescence staining in xenografts indicated that Tyro3 is expressed in primary tumors but not in bone marrow-disseminating cells, and is upregulated upon the formation of metastatic lesions, and conversely, Axl is primarily expressed in the disseminating cells prior to metastasis *in vivo* (Taichman, 2013). Based on their findings in the xenograft imaging experiments, the authors presented the hypothesis that when Tyro3 expression is equal to or higher than Axl expression, Gas6-mediated signaling promotes cellular proliferation and that possibly, the dynamics in Tyro3/Axl expression could be a mechanism to induce dormancy in the bone marrow (Taichman, 2013). In another study by the same research group, small interfering RNA (siRNA)-mediated knockdown of Axl in PCa cell lines resulted in decreased expression of several markers of EMT (Mishra, 2012). Moreover, Gas6 stimulation of PCa cells resulted in

downregulation of Axl, similar to what has been observed in RCC cells, and in experiments mimicking hypoxic conditions, Gas6-mediated downregulation of Axl was inhibited (Mishra, 2012).

Axl imaging using radiolabeled monoclonal antibodies has also been proposed as a potential tool to identify patients that could benefit from targeted Axl therapy (Nimmagadda, 2014).

The miR-34 family in PCa

Differential expression of all of the microRNA-34 family members has been reported in PCa (Ambs, 2008, Hagman, 2010, Walter, 2013). Downregulation of miR-34c in PCa correlates with Gleason grade and low expression of miR-34c is associated with worsened prognosis (Hagman, 2010, Tsuchiyama, 2013). Furthermore, miR-34c has been shown to be a direct negative regulator of E2F3, BCL2, and MET, all of which are oncogenes involved in proliferation, survival and metastatic progression, and miR-34c expression correlated inversely with MET expression in PCa tumor tissue (Hagman, 2010, Hagman, 2013). Similar to miR-34c, decreased expression of miR-34a has also been observed in PCa tumors (Ambs, 2008, Hagman, 2010). In PCa miR-34a has been shown to be a negative regulator of SIRT1 expression *via* interaction with the SIRT1 promoter, and ectopic miR-34a expression in a PCa cell line was associated with growth inhibition, cell cycle arrest, and decreased resistance to apoptosis when the cells were treated with Camptothecin, a drug used in cancer therapy (Fujita, 2008). Later, the same group presented evidence that re-expression of miR-34a also could attenuate resistance to the chemotherapy drug Paclitaxel in a PCa cell line (Kojima, 2010). Moreover, miR-34a has been shown to target CD44, which is associated with cancer stemness and metastasis, in PCa, and in addition systematic treatment with miR-34a decreased metastasis and increased survival in a xenograft study (Liu, 2011). While differential miR-34b expression in PCa has been reported, the results are inconsistent; miR-34b has been indicated to be upregulated in one study, another study reported that miR-34b was downregulated in PCa (Ambs, 2008, Martens-Uzunova, 2012). Also in the case of miR-34a and miR-34c there has been controversing reports, as both miR-34a and miR-34c were reported to be upregulated in PCa tumors in a recent study (Walter, 2013).

The present investigation

Paper I

Introduction

Clear cell renal cell carcinoma is the most common type of cancer in the kidney, and while the prognosis of patients with organ-confined disease is relatively good, patients who develop metastasis face a poor prognosis with a 5-year survival of less than 10% (Cohen, 2005, Novara, 2010, Weikert, 2010). MicroRNAs, short non-coding RNAs, act as post-transcriptional regulators of protein expression by binding to target mRNAs, and more than 30% of the human genome is thought to be regulated by microRNAs (Bartel, 2004, Lewis, 2005). Moreover, dysregulation of microRNA expression has been linked to cancer, and in ccRCC microRNAs have been reported to be differentially expressed in tumor tissues, and microRNA expression signatures have been indicated to be useful prognostic tools (Osanto, 2012, Slaby, 2012, Wotschofsky, 2012, Zaman, 2012). Recently, the Cancer Genome Atlas (TCGA) presented a report where a large number of ccRCC tumor samples were extensively characterized using arrays for mRNA transcripts and microRNAs, as well as protein, DNA methylation, and SNP arrays (Cancer Genome Atlas Research, 2013). Our aim was to evaluate the prognostic significance of microRNAs identified from the TCGA cohort in a large RCC cohort.

Methods and results

We used biostatistical methods to identify microRNAs associated to patient outcome and disease severity in the TCGA cohort, which is publically available. Candidate microRNAs were selected based on their feasibility to be measured by RT-qPCR, and four candidate microRNAs were subsequently measured in a cohort of 198 RCC tumor samples of which 152 were ccRCC, and 50 normal kidney samples. Using SPSS-based statistics, we evaluated the expression of the candidate microRNAs in RCC subtypes and their association to survival and disease severity. Importantly, we identified a two-microRNA ratio, $miR^{21/10b}$ which correlated significantly with patient

survival, and showed that the $miR^{21/10b}$ is an independent prognostic factor for ccRCC patients without metastasis at the time of diagnosis.

Conclusions and future perspectives

In this report we presented a microRNA ratio which could provide additional diagnostic information for metastasis-free ccRCC patients. The concept of a microRNA ratio, or a microRNA index quota, is relatively novel and has several advantages as it eliminates the need for housekeeping genes in the RT-qPCR reaction, it may amplify the prognostic impact of a single microRNA, and meanwhile, combining several factors into a prognostic ratio could also increase the prognostic robustness with regard to variation in basal expression of single prognostic factors between individuals (Larne, 2013).

In our microRNA ratio, miR-21 is the numerator and seems to be upregulated in ccRCC tumor tissue. This finding is supported by previous reports by other research groups, as well as by the report presented by the TCGA on their ccRCC cohort (Cancer Genome Atlas Research, 2013, Faragalla, 2012, Juan, 2010, Zaman, 2012). Moreover, miR-21 expression has been shown to mediate survival, invasion and proliferation in ccRCC cells *in vitro* (Zhang, 2011, Zhang, 2012). In this context, it is interesting to note that increased expression of miR-21 has been reported in renal fibrosis, and in addition, miR-21 seems to be involved in an epigenetic switch induced by inflammatory signaling which enables maintenance of a transformed state in mammary cells (Glowacki, 2013, Iliopoulos, 2010, Zarjou, 2011). The role of miR-21 in renal inflammation as well as in RCC could be an indication that miR-21 is involved in malignant transformation in the kidney, as chronic kidney disease and renal fibrosis is linked to increased risk of RCC (Russo, 2012).

Although miR-10b has been reported to be upregulated in several cancer types, our finding of decreased miR-10b expression in ccRCC is supported by other reports (Cancer Genome Atlas Research, 2013, Juan, 2010, Lu, 2014, Osanto, 2012). The targets of miR-10b in the kidney are unknown, however miR-10b has been validated to target tumor suppressor genes in other cancers where miR-10b seems to be upregulated, and it is possible that effects of miR-10b expression are cell-type dependent (Gabriely, 2011, Ma, 2007, Tian, 2010). As previously mentioned, decreased expression of miR-10b in ccRCC has been linked to poor prognosis as well as metastatic disease and early relapse (Heinzelmann, 2011, Khella, 2012, Slaby, 2012, Wotschofsky, 2012, Wu, 2012). Similar to miR-21, dysregulation of miR10b expression has been linked to renal disease, as miR-10b was recently reported to be significantly downregulated in renal allograft rejection (Liu, 2015). Further elucidation of the roles of miR-10b and miR-21, either alone or together, in kidney

disease and ccRCC could potentially increase the understanding of RCC tumor formation and biology.

Paper II

Introduction

In previous reports from our group, high Axl expression has been identified in ccRCC cells *in vitro*, and furthermore, high Axl expression in RCC patients correlates with poor survival and is an independent prognostic factor (Gustafsson, 2009, Gustafsson, 2009). The miR-34 family, known as a family of tumor suppressor microRNAs mediating p53-induced cellular stress response, has been shown to be epigenetically inactivated in several solid cancers, including RCC, and moreover miR-34a has been shown to be downregulated in ccRCC cells *in vitro* (Lodygin, 2008, Vogt, 2011, Yu, 2014). However, reports are in conflict as miR-34a also has been reported to be upregulated in ccRCC tumors (Liu, 2010). Axl expression correlates inversely with miR-34a expression in breast cancer, NSCLC, and colorectal cancer, and in addition, miR-34a has been shown to directly bind to the 3' UTR of Axl to regulate Axl expression post-transcriptionally (Mackiewicz, 2011, Mudduluru, 2011).
In this report, our aim was to elucidate the role of the miR-34 family members, miR-34a, miR-34b, and miR-34c, in the regulation of Axl expression in ccRCC cells *in vitro*. Furthermore, we sought to determine if Axl expression correlated inversely with miR-34a/b/c expression in human ccRCC tumors, and whether any of the miR-34 family members could predict patient outcome in ccRCC.

Methods and results

786-O, a ccRCC cell line, was transiently transfected with miR-34 family members, and cells were analyzed for total Axl protein expression using Western Blot (WB), surface Axl protein expression using flow cytometry (FCM), as well as for Axl mRNA levels using RT-qPCR, and we observed significant downregulation of Axl protein as well as decreased Axl mRNA levels in cells transfected with miR-34a or miR-34c. To determine if miR-34a/c could directly regulate Axl mRNA by binding to the 3' UTR, we performed Luciferase reporter assay with an Axl 3' UTR construct and microRNA mimics, and showed that both miR-34a and miR-34c bind directly to Axl 3' UTR. The binding of miR-34a and miR-34c to Axl 3' UTR was seed sequence-specific as an introduced mutation in the seed sequence of the Axl 3' UTR abrogated miR-34a/c-mediated downregulation of Luciferase activity.

Moreover, expression analysis of the miR-34 family members in RCC tumor samples using RT-qPCR revealed that miR-34a is upregulated in ccRCC patients, however miR-34a does not predict outcome in ccRCC. In addition, expression of miR-34a was analyzed with regard to correlation with previously determined Axl mRNA levels in tumor, sAxl in patient serum, as well as immunohistochemistry for Axl in tumor tissue, however no correlation with Axl expression was found.

Conclusions and future perspectives

We concluded that although miR-34a/c has the capability to regulate Axl *in vitro*, the results of our study do not support the hypothesis of miR-34 family members as key regulators of Axl expression in ccRCC. In addition, our findings do not support miR-34a as a tumor suppressor in ccRCC. This finding is in agreement with other reports where miR-34a seemed to support cellular proliferation in renal carcinogenesis (Dutta, 2007). Interestingly, miR-34a has also been shown to support survival when co-expressed with Myc in B-lymphoid cells (Sotillo, 2011). Differential targeting of a microRNA in different cell types could be explained by the effects of target mRNA abundance on target regulation (Arvey, 2010). In this context, it would be of interest to determine the role of miR-34a in ccRCC pathogenesis, and to elucidate which targets are regulated by miR-34a in ccRCC.

Although our study does not support this hypothesis, it is still possible that Axl expression is regulated by miR-34a/c in RCC, although the fact that miR-34a expression is increased in ccRCC does not strengthen this hypothesis. As tumor RNA extracts do not only contain tumor cells but also tumor stroma, it is possible that Axl and miR-34a are not expressed in the same cells. This could potentially be elucidated using *in situ* hybridization or animal models utilizing reporter genes to identify exactly which cells express Axl and miR-34a, as well as localization of Axl protein or mRNA and miR-34a.

Paper III

Introduction

As previously discussed, high expression of Axl has been reported in PCa, and has been linked to metastasis and aggressive phenotype (Mishra, 2012, Paccez, 2013, Shiozawa, 2010). Similar to RCC, epigenetic inactivation of miR-34a has been reported in PCa and has been shown to regulate cellular growth and resistance to

chemotherapy (Fujita, 2008, Kojima, 2010, Lodygin, 2008). Moreover, decreased expression of miR-34c predicts worsened outcome in PCa, and miR-34c has been shown to be a direct regulator of MET and Bcl2 in PCa (Hagman, 2010). As previously mentioned, direct regulation of Axl by miR-34a has been reported, and in addition, overexpression of miR-34c is associated with decreased Axl expression in PCa (Boysen, 2014, Hagman, 2013, Mackiewicz, 2011, Mudduluru, 2011).

In this report, we sought to elucidate the role of the miR-34 family members in the regulation of Axl expression in PCa cell lines *in vitro*, as well as to determine the role of Axl in miR-34-mediated regulation of cellular functions such as growth, apoptosis and migration.

Methods and results

We performed transient transfection with miR-34 family members in PCa cell lines, and analyzed the cells for total Axl protein expression using WB, surface Axl protein expression using FCM, and in addition we used RT-qPCR to measure Axl mRNA levels. Significant downregulation of Axl protein was observed, as well as decreased Axl mRNA levels in PCa cells transfected with miR-34a or miR-34c. Moreover, we performed Luciferase reporter assay with an Axl 3' UTR construct and microRNA mimics, and both miR-34a and miR-34c were found to bind directly to the 3' UTR of Axl. We confirmed that binding of miR-34a and miR-34c to Axl 3' UTR occurred at the predicted seed sequence using an introduced mutation in the miR-34 seed sequence of the Axl 3' UTR which inhibited downregulation of Luciferase-Axl 3'UTR by miR-34a and miR-34c. In addition, we assayed PCa cell lines transiently transfected with miR-34a/b/c or Axl siRNA, and found that miR-34a/c-mediated inhibition of proliferation in the presence of Gas6 potentially could be mediated by Axl. However, transfection with Axl siRNA did not replicate miR-34/c transfection with respect to cellular growth or migration, although a similar insignificant trend to increased apoptosis in PC3 cells transfected cells with miR-34a/c or Axl siRNA.

Conclusions and future perspectives

Similar to our conclusions in Paper II, our conclusion in Paper III was that miR-34 indeed has the capability to regulate Axl *in vitro*, however it seems that miR-34a/c-mediated effects in PCa are most likely primarily mediated by regulation of other targets. Surprisingly, we could not completely reproduce previously reported effects of miR-34c with regard to proliferation, growth and apoptosis (Hagman, 2010). Possibly, this could be explained by experimental differences. Although we could show that Axl is directly regulated by miR-34a/c, this does not necessarily have to

imply that Axl is regulated by miR-34a/c in PCa *in vivo*, especially considering the complexity of microRNA targeting and how target regulation is dependent on target mRNA abundance (Arvey, 2010, Bartel, 2009). As miR-34c regulation of MET in PCa has been shown to have impact on tumorigenic processes such as invasion, it is possible that miR-34c-MET mRNA interactions, and potentially regulation of other targets by miR-34c, are favored in ectopic reexpression of miR-34c in PCa (Hagman, 2013). To further elucidate whether regulation of Axl by miR-34a/c in PCa has physiological relevance, it would be interesting to evaluate if Axl and miR-34a/c are coexpressed in PCa tumors, for example by immunohistochemistry and *in situ* hybridization in tumor tissue, or by reporter assays *in vivo*. In addition, altered experimental setups could possibly enable further knowledge regarding Axl-mediated effects of miR-34 family dysregulation in PCa.

Paper IV

Introduction

High expression of Axl has been associated with poor prognosis in several human cancers including RCC, and in addition, increasing evidence has been presented indicating a role for Axl in acquired resistance to targeted therapies in cancer (Brand, 2014, Gustafsson, 2009, Wu, 2014). Axl is involved in tumorigenesis, for example in proliferation and migration, and it also plays a role in immunology and in vascular biology (Li, 2009, Verma, 2011). Furthermore, Axl is involved in tumor angiogenesis and has been shown to crosstalk with other RTKs such as EGFR and VEGFR (Holland, 2005, Korshunov, 2012). Moreover, Axl seems to be involved in acquired resistance to targeted therapies, for example EGFR-targeted therapies in breast and lung cancer, and resistance to Imatinib, an inhibitor targeting multiple kinases including PDGFR, in gastrointestinal stromal tumors (Mahadevan, 2007, Meyer, 2013, Zhang, 2012). In addition, therapy targeting Axl has been shown to have additive effect with VEGFR-targeted therapy in EC tube formation, and targeting Axl in xenograft models improves the effect of anti-VEGF therapy in breast cancer and NSCLC, and EGFR inhibitor therapy in NSCLC (Li, 2009, Ye, 2010). In RCC, the multi-kinase inhibitor Sunitinib which targets RTKs such as VEGFR and PDGFR, is often used for therapy of advanced RCCs (Faivre, 2007). However, although targeted therapies have been shown to be beneficial, disease progression often occurs in patients with metastatic RCC even when treated with targeted therapies (Calvo, 2014).

In this report, we hypothesized that Gas6 and Axl, similar to what has been observed in other human cancers, possibly could be involved in resistance to targeted therapy in ccRCC. Thus, our aim was to elucidate the role of Gas6/Axl signaling in acquired resistance to Sunitinib treatment.

Methods and results

Subconfluent and confluent 786-O ccRCC cells were stimulated with Gas6 for up to 5 days, and Gas6-mediated activation of Axl was evaluated in total cell lysates using Axl immunoprecipitation (IP) and subsequent immunblotting for Axl phosphorylation. Interestingly, long-term Axl activation by Gas6 was only observed in 786-O cell grown to full confluence prior to stimulation, whereas Axl phosphorylation in subconfluent cells seemed to be highly induced within 15 minutes, started to decrease after 30 minutes, and was followed by reduction of total Axl expression in the cells. Moreover, IP and WB for phosphorylated Axl and WB for phosphorylated Akt revealed that both Axl and Akt are activated even after 14 and 10 days, respectively, of Gas6 stimulation in confluent 786-O cells. In addition CellTiter-Glo 2.0 and CellTox-Green assays revealed that Gas6 could not mediate increased viability and growth, or decreased apoptosis, in subconfluent cells, as was observed with confluent Gas6-stimulated cells.

Short-term stimulation of 786-O cells with or without Sunitinib pretreatment revealed that Sunitinib does not inhibit Gas6-mediated Axl activation; instead Axl phosphorylation was increased in Sunitinib and Gas6 co-treated cells and similarly, enhanced phosphorylation of Akt and Erk was observed in the presence of Sunitinib and was further enhanced by Gas6. Sunitinib-induced enhancement of Axl phosphorylation could be inhibited using the small molecule Axl inhibitor R428, and furthermore, R428 could inhibit the Sunitinib-mediated increase of Akt and Erk activation even when Gas6 was not present. Similar results were obtained in human aortic endothelial cells (HAECs) with respect to enhanced Axl and Akt activation when cells were co-treated with Gas6 and Sunitinib. Using a phospho-kinase array, we observed increased activation of a downstream target of Akt, PRAS40, which is involved in cancer progression, after treatment of 786-O cells with Gas6, and this effect was further enhanced with co-treatment of Gas6 and Sunitinib. Interestingly, we observed activation of EGFR and HGFR in 786-O cells co-treated with Gas6 and Sunitinib, but not in cells stimulated with Gas6 alone, and the Gas6/Sunitinib-mediated increase in EGFR and HGFR phosphorylation could be partially inhibited using the R428 Axl inhibitor. Co-treatment of Gas6 and Sunitinib in 786-O cells was associated with increased growth and viability compared to Gas6 or Sunitinib alone, and reduced apoptosis similar to what was observed with Gas6 alone. Moreover, Gas6-mediated activation of Axl was accompanied by increased secretion of

Osteopontin, a protein involved in tumor angiogenesis and stroma adaptation, and this effect was further increased in the presence of Sunitinib.

Conclusions and future perspectives

The results presented in this preliminary report suggests that in the presence of Sunitinib, Gas6/Axl signaling is enhanced and is acting in concert with other RTKs. As previously mentioned, Gas6 is expressed in the kidney, and in ccRCC tumors (Gustafsson, 2009, Manfioletti, 1993). In RCC, Sunitinib is thought to act primarily on ECs (Huang, 2010). The observed enhancement of Akt activation in Gas6 and Sunitinib co-treated cells could imply a role for Axl and Gas6 in the resistance to Sunitinib treatment. In NSCLC, increased Akt signaling in ECs is associated with increased tumor angiogenesis characterized by leaky vessels, causing hypoxia, and moreover, hypoxia seems to stabilize Gas6/Axl signaling in PCa (Graves, 2010, Mishra, 2012). Furthermore, the observed Gas6-inducible Osteopontin secretion, which was shown to be enhanced in the presence of Sunitinib, could indicate a role for Gas6/Axl in activating alternative pathways to angiogenesis, thus providing a mechanism to restored tumor angiogenesis, as Osteopontin seems to be involved in tumor angiogenesis (Anborgh, 2010).

As this is a project that is still undergoing, future perspectives include repeating experiments as well as confirming observations from the protein array experiments. In addition, we are currently analyzing the effects of Gas6 and Sunitinib co-treatment on 786-O cell cycling, and in addition, we are setting up experiments for assaying effects on migration and invasion in this context. Furthermore, we would like to evaluate functional effects of Gas6/Sunitinib co-treatment in ECs using migration and tube formation assays. Moreover, we aim to repeat selected experiments in primary RCC tumor cells isolated from patients.

Populärvetenskaplig sammanfattning

I vårt DNA finns det ett stort antal gener, som beskriver för cellen hur den skall tillverka de proteiner som behövs för att cellen ska kunna fungera normalt. Olika celler uttrycker olika proteiner, och det är viktigt att tillverkningen av proteiner, och även mängden av olika proteiner i cellen, är under kontroll och i balans. När en cell uttrycker för mycket eller för lite av ett protein, eller om proteinet har förändrats, t.ex. genom mutationer i genen, kan olika sjukdomar, t.ex. cancer, uppstå. Det finns många sätt för cellerna att kontrollera proteinmängden i sig själva, dels genom kontroll av själva processen där generna används för att tillverka proteiner, men också genom att påverka processer som styr proteinernas aktivitet eller som gör att proteiner bryts ned. Ett sätt att kontrollera tillverkningen av proteiner är med så kallade mikro-RNA. Mikro-RNA är väldigt korta bitar av RNA, som också finns i vårt DNA, men de beskriver inte hur något protein ska tillverkas; istället kan de reglera proteintillverkningsprocessen genom att interagera med de meddelanden som bär generna beskrivning av proteinerna. På så sätt kan en mikro-RNA påverka tillverkningen av ett visst protein, oftast genom att hindra tillverkningen. I min avhandling har jag arbetat med olika mikro-RNA, samt med ett protein som heter Axl, som har visats sig finnas i stora mängder i många olika typer av cancerceller. Axl-proteinet är en receptor som kan ta emot signaler från cellens utsida, och Axl har funktioner som kan bidra till egenskaper som ofta kopplas till cancer, som till exempel cancercellers förmåga att motstå programmerad celldöd, en viktig process för kroppen att ta död på celler som inte beter sig normalt, eller egenskaper som gör att cellerna växer snabbare och kan förflytta sig i kroppen.

I mitt första projekt mätte vi olika mikro-RNA i prover från njurcancertumörer, och vi kunde visa att två mikro-RNA, miR-21 och miR-10b, uttrycks olika mycket i njurcancertumörer jämfört med i frisk njurvävnad. Dessutom kunde vi visa att det förändrade uttrycket av dessa mikro-RNA kan användas för att skilja på patienter som har god prognos och de som har sämre prognos, hos patienter som inte har någon metastas när de får sin diagnos. Alla funktioner av dessa mikro-RNA är inte helt utredda, men man vet att miR-21 ofta uttrycks i större mängd i tumörceller från olika typer av cancer, och när det finns mycket miR-21 så verkar cancercellerna vara mer aggressiva, t.ex. så är de mer motståndskraftiga mot programmerad celldöd. Uttrycket av båda dessa mikro-RNA verkar också förändras i njursjukdomar, på samma sätt som

vi sett i njurcancer, vilket är väldigt intressant eftersom det finns en tydlig koppling mellan njursjukdomar och ökad risk för njurcancer.

I det andra projektet undersökte vi om en familj av mikro-RNA, miR-34-familjen, kan reglera uttrycket av Axl-proteinet, samt hur dessa mikro-RNA uttrycks i njurcancer. Bakgrunden till detta var att vi tidigare kunnat visa att Axl uttrycks i njurcancertumörer, och att njurcancerpatienter som har mycket Axl i tumören har sämre prognos än de som inte har så mycket Axl. Dessutom har det sedan tidigare varit känt att en av de mikro-RNA som ingår i miR-34-familjen, miR-34a, kan reglera uttrycket av Axl i andra cancersjukdomar, och man har också kunnat visa att ju mer miR-34a man har i tumören, desto mindre Axl finns det. Med hjälp av experiment i njurcancerceller kunde vi visa att både miR-34a och miR-34c, en annan medlem i miR-34-familjen, kunde reglera mängden Axl i njurceller, men när vi undersökte uttrycket av miR-34-familjemedlemmarna i patientprover så visade det sig att mängden miR-34a var högre i tumörprover än i prover från frisk njure, något som andra också har sett. Eftersom miR-34-familjen oftast brukar vara uttryckt i lägre mängd i tumörer än i frisk vävnad, och dessutom verkar kunna motverka egenskaper som är viktiga för tumörceller, brukar man beskriva dessa mikro-RNA som en del av cellens mekanism för att förhindra att cellen utvecklar tumörliknande egenskaper. I njurcancer verkar dock miR-34a ha en omvänd roll. Andra forskargrupper har tidigare kunnat vissa att just miR-34a verkar kunna ha olika effekt i olika celltyper, och man har också visat att miR-34a kan göra så att celler från njuren växer snabbare.

I det tredje projektet fortsatte vi vår undersökning av miR-34-familjen, denna gång i prostatacancer. Även här ville vi undersöka om miR-34-familjen kan reglera uttrycket av Axl-proteinet. I detta projekt var bakgrunden att Axl uttrycks i prostatacancertumörer, och uttrycket verkar vara högre ju allvarligare sjukdomen är. Det är också känt sedan tidigare att uttrycket av både miR-34a och miR-34c är lägre i prostatacancer, och att uttrycket av miR-34c kan användas för att bedöma prognosen i prostatacancer. Även i detta projekt gjorde vi experiment i celler, denna gång prostatacancerceller, och vi kunde visa att både miR-34a och miR-34c, en annan medlem i miR-34-familjen, kunde reglera mängden Axl. Vidare gjorde vi experiment för att utröna vilken betydelse regleringen av Axl av dessa mikro-RNA kunde ha, och vi undersökte bland annat celltillväxt, rörlighet och programmerad celldöd. Vi kunde visa att nedreglering av Axl verkade vara kopplat till minskad celltillväxt, så kallad proliferation, när man stimulerar cellerna med Gas6, ett protein som stimulerar Axl. I övrigt verkade inte Axl ha så stor inverkan på de egenskaper vi undersökte, och vi konstaterade att de effekter som uppstår när uttrycket av miR-34a och/eller miR-34c förloras i prostatacancer troligtvis beror i första hand på andra proteiner, som också regleras av dessa mikro-RNA.

I det fjärde och sista projektet, som vi fortfarande arbetar med i vårt labb, har vi fokuserat på Axl, och Gas6, proteinet som binder till Axl på cellens utsida och på så

sätt kan aktivera Axl, samt vilken roll de har i behandling av njurcancer med ett läkemedel som heter Sunitinib. Sunitinib är ett läkemedel som verkar genom att förhindra aktiveringen av receptor-proteiner på blodkärlsceller, och på så sätt förhindrar nybildning av blodkärl i tumören, en process som ofta sker i cancertumörer. Sunitinib har effekt i njurcancer, men tyvärr är det många patienter som efter ett tag återfår tumörer. Anledningen tros vara så kallad resistens, det vill säga att tumören hittar vägar att fortsätta växa, t.ex. genom att få igång nybildningen av blodkärl igen, och i andra cancersjukdomar har man sett att Axl verkar delta i den här processen. Genom experiment med både njurcancerceller och blodkärlsceller har vi kunnat visa att när man behandlar njurcancerceller med Sunitinib, så verkar de Axl-aktiverande effekterna av proteinet Gas6 öka. Dessutom aktiveras andra receptorer, som kan delta i processer som bidrar till cancer, när man behandlar cellerna med Sunitinib, och denna effekt blir starkare när man också har med Gas6 i behandlingen. Gas6 verkar också kunna stimulera utsöndringen av ett protein som kan bidra till nybildningen av blodkärl, och även denna effekt förstärks när man dessutom behandlar med Sunitinib. Detta är väldigt intressant eftersom det sedan tidigare är känt att Gas6 uttrycks i njuren, och Axl uttrycks som bekant i njurcancerceller, och även i blodkärlsceller. Därför tror vi att Axl och Gas6 deltar i utvecklingen av resistans mot Sunitinib-behandling, och vi kommer att utföra ytterligare experiment för att få mer information om hur detta skulle kunna gå till.

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