Clear Cell Renal Carcinoma: MicroRNAs With Efficacy in Preclinical In Vivo Models

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Abstract. In order to identify new targets and treatment modalities for clear cell renal carcinoma, we surveyed the literature with respect to microRNAs involved in this disease. In this review, we have focused on up- and down-regulated miRs which mediate efficacy in preclinical clear-cell renal carcinoma-related in vivo models. We have identified 10 up-regulated and 33 down-regulated micro-RNAs according to this criterion. As proof-of-concept, micro-RNAs interfering with VEGF (miR-205p) and mTOR (mir-99a) pathways, which are modulated by approved drugs for this disease, have been identified. miRs targeting hypoxia induced factor-2α (HIF-2α) (miR-145), E3 ubiquitinylases speckle-type POZ protein (SPOP) (miR 520/372/373) and casitas B-lineage lymphoma (CBL) (miR-200a-3p), interfere with druggable targets. Further identified miRs interfere with cell-cycle dependent kinases, such as CDK2 (miR-200c), CDK4, 6 (miR-1) and CDK4, 9 (206c). Transmembrane receptor Ral interacting protein of 76 kD (RLIP76), targeted by mir-137, has emerged as another important target for ccRCC. Additional miRs and their targets meriting further preclinical validation are discussed.

In the US in 2020, 74,000 new cases of patients with renal cancer have been diagnosed and 15,000 patients have died (1). Renal cell carcinoma (RCC) develops in the lining of the tubules and can be classified into clear cell renal carcinoma (ccRCC), or of papillary or chromophobe subtype (2). In this review we focus on ccRCC, the most common subtype with a prevalence between 80 and 90% of all RCCs (3). Localized primary tumors can be cured by surgical resection, however, the majority of renal cancers have already metastasized to the lungs, liver, bones and brain via the bloodstream or the lymphatics at the time of diagnosis (4). An early event in the pathogenesis of ccRCC is the mutational inactivation of von Hippel-Lindau tumor suppressor (VHL), the substrate recognition component of an E3 ligase complex that ubiquitinylates hypoxia inducible factors (HIF)-1α and (HIF)-2α for proteasome-mediated degradation (5). These transcription factors accumulate due to inactivation of VHL and induce vascular endothelial growth factor (VEGF). Therefore, ccRCCs are highly vascularized and respond to anti-angiogenic therapy. In addition, loss of chromosome 3p, driver gene mutations in the mechanistic target of rapamycin (mTOR) pathway and genes involved in epigenetic modification and chromatin remodeling such as AT-rich interactive domain-containing protein 1A (ARID-1A), BRCA associated protein 1 (BAP1), lysine-specific demethylase 5C (KDM5C), protein polybromo 1 (PBRM1) and histone methyltransferase SET domain containing 2 (SETD2) have been observed (6). A further characteristic of ccRCC is profound heterogeneity. An individual tumor can contain several evolving subclones with different driver mutations (2). Several anti-angiogenic agents such as the anti-VEGF monoclonal antibody (mAb) bevacizumab and tyrosine kinase inhibitors such as sorafenib, sunitinib, pazopanib, axitinib, levatinib as well as mTOR inhibitors such as temsirolimus and everolimus have been approved for treatment of metastatic disease (7). Immune-checkpoint inhibitors such as nivolumab [anti-programmed cell death protein1 (PD1)] and a combination between nivolumab and ipilimumab [anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4)] were approved for treatment of advanced disease (8). However, the therapeutic benefit is hampered by
development of resistance to the corresponding therapies. Therefore, identification of new targets and treatment modalities for ccRCC is an important issue. In this review we focus on up- and down-regulated microRNAs (miRs) and corresponding targets with in vivo efficacy in preclinical ccRCC-related systems.

**microRNA and Cancer**

miRs are transcribed from approximately 1,000 genes in the human genome by RNA polymerase II as precursors, transported into the cytoplasm and processed (9, 10). One strand of a 22 nucleotide (nt) duplex is maintained (guide strand), while the other strand (passenger strand) is degraded (9, 10). Binding of the guide strand to the 3'-untranslated region (3'-UTR) of the corresponding mRNA leads to degradation or translational repression of the target mRNA (9, 10). A single miR can interfere with several mRNAs and a single mRNA can be targeted by several miRs (11). Therefore, miRs can potentially modulate pathways at different levels and interfere with several pathways simultaneously and have the potential to rewire oncogenic pathways; however, collateral effects due to the modulation of non-oncogenic cellular pathways is a critical issue (12). miRs can exert tumor-suppressive and oncogenic functions and their ability to modulate different genes can be context-dependent. In addition, non-canonical functions of miRs such as agonizing of toll-like receptors 7 and 8 (TLR7, 8) have been described (13). This interaction can lead to promotion of tumor growth and metastasis by induction of inflammatory responses (13). miRs play a role during all stages of tumor formation, interaction of the tumor with the tumor micro-environment (TME) and metastasis (14). We recently summarized the role of miRs in metastasis (15-21). Aberrant expression of miRs in cancer can be due to methylation of the promoters of the corresponding genes or due to dysregulation of the processing of their precursor RNAs (22). The tumor-suppressor function of miRs has been revealed by the demonstration that miR-16-1 and -15a prevent B–cell chronic lymphatic leukemia (B-CLL) in mice due to cleavage of anti-apoptotic protein B-cell lymphoma-2 (BCL-2) (23). The oncogenic role of miRs was shown by induction of hepatocellular carcinoma in transgenic mice by liver-specific expression of miR-221 (24).

**Up-regulated microRNAs**

*miRNAs targeting transmembrane receptors*

Up-regulation of adhesion molecules can inhibit tumor growth and metastasis of ccRCC cells and increased expression of corresponding miRs can counteract these functions.

**miR-146a (CADM2)**, miR-146a (Figure 1A) is induced by hypoxia and high expression correlates with low survival rate in ccRCC patients (25). Over-expression of miR-146a promotes ACHN RCC cell proliferation and tumor growth in vivo in nude mice, whereas its decrease inhibits proliferation and invasion of 786-O RCC cells (25). As a target, the cell adhesion molecule M2 (CADM2) has been identified (25). Transfection of ACHN cells with miR-146a cells promotes growth (25). miR-146a also induces epithelial mesenchymal transition (EMT) of RCC cells (25). CADM2 is a member of the synaptic cell adhesion family of transmembrane receptors, has three Ig-like domains, promotes cell aggregation by homo- and heterophilic interactions with other nectin-like family members and organizes the function of synapses through heterophilic interactions (26). CADM2 increases the level of E-cadherin and decreases the levels of vimentin (VM) (25). CADM2 exhibits tumor suppressor functions. Aberrant methylation and loss of expression of CADM2 has been observed during ccRCC tumor progression (27).

**miR-720 (E-Cadherin).** miR-720 (Figure 1A) is highly expressed in ccRCC and is a potential marker for diagnosis and prediction of survival in ccRCC patients (28). Depletion of miR-720 impairs migration, invasion, EMT and causes apoptosis in 786-O and A498 RCC cells (28). Intratumoral delivery of anti-miR-720 suppresses tumor growth of 786-O and A498 ccRCC xenografts (28). αE-catenin and E-cadherin have been identified as direct targets of miR-720 (28). Negative regulation of E-cadherin and αE-cadherin causes EMT and metastasis (28). Reduced expression of E-cadherin facilitates ccRCC progression by activation of WNT/β-catenin signaling (29). Low expression of E-cadherin correlates with poor prognosis in patients with ccRCC (30, 31).

**miRNAs targeting signalling-related proteins**

miRs targeting tumor suppressors or inhibitors of signaling pathways can increase proliferation, migration and invasion of ccRCC cells.

**miR-21 (PTEN, PTENP1).** miR-21 (Figure 1A) targets the phosphatase and tensin homolog (PTEN) and PTENP1, a pseudogene of PTEN (32, 33). In vitro, PTENP1 suppresses migration and invasion of ACHN and SN12PM6 RCC cells (32). miR-21 promotes tumor growth and metastasis of ACHN cells in vivo. In line with this, PTENP1 and PTEN inhibit tumor growth and metastasis in nude mice (32). PTENP1 RNA competes for regulation of PTEN expression by miR-21 (32). PTEN also attenuates phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling through its lipid phosphatase activity (34). PTENP1 is deleted in melanoma and non-small cell lung carcinoma (NSCLC) (35, 36) while
low PTEN is significantly associated with unfavorable outcome in ccRCC patients (37).

**miR-106-5p (WNT signaling).** High expression of miR-106-5p (Figure 1A) predicts poor survival of ccRCC patients (38). miR-106-5p induces markers of stemness in Caki-1 RCC cells (38). This miRNA targets the leucine zipper transcription factor like 1 (LZTFL1), secreted frizzled-related protein 2 (DKK2), all inhibitors of WNT signaling (38). miR-106 promotes growth of Caki-1 cells after implantation into the renal capsule and lung metastasis after tail vein injection into nude mice (38). In line with this, WNT signaling has been shown to promote proliferation, migration and invasion in ccRCC (39, 40).

**miR-122 (FOXO3).** High expression of miR-122 (Figure 1B) correlates with reduced metastasis-free survival in ccRCC patients (41). This miRNA targets transcription factor forhead box O3 (FOXO3) and promotes proliferation,
invasion and EMT of 786-0 and SN12-PM6 RCC cells (41). FOXO3 has been shown to inhibit proliferation, tumorigenic potential and invasiveness of cancer cells (42, 43). In ccRCC, FOXO3 promotes tumor metastasis and is associated with metastasis-free survival in patients (44).

**miR-144-3p (ARID-1A).** miR-144-3p (Figure 1B) promotes proliferation, invasion and clonogenicity of 786-0 and SN12-PM6 RCC cells (45). An AT-rich interaction domain-containing protein 1A (ARID-1A) has been identified as a direct target of miR-144-3p (45). The latter promotes tumor formation of SN12PM6 RCC cells in vivo (45). ARID-1A is a key member of the switch/sucrose non-fermentable (SWI/SNF) chromatin-modeling complex which functions as a negative regulator in cell cycle, apoptosis and tumorigenicity (46, 47). The target protein also triggers EMT of renal cells (48). Decreased ARID-1A expression correlates with poor prognosis of ccRCC (49) while mutations in this gene have been noted in up to 12% of ccRCCs and protein loss has been observed in 50% of ccRCCs (50, 51). In ccRCC with mutated ARID-1A, dramatically lower levels of CD8+ T-cell infiltrates have been observed, compared with those without ARID-1A mutations. This suggests that the ARID-1A mutation status can be a predictive biomarker for immune-checkpoint therapy of ccRCC (52).

**miR-193a, miR-224 (ST3Gal IV).** Over-expression of miR-193a and miR-224 (Figure 1B) increases RCC proliferation and migration both in vitro and in vivo (53). α2,3 sialyltransferase IV (ST3GalIV) enzymatic activity has been identified as a direct target of these miRs (53). Down-regulation of ST3GalIV correlates with induction of PI3K/AKT signaling (53). ST3GalIV is highly expressed in adjacent normal tissues (53). Sialyltransferases add sialic acid to nascent oligosaccharides and each sialyltransferase is specific for a particular sugar substrate (54, 55). Sialylation is involved in cell fate decisions and cancer progression and aberrant glycosylation is a new hallmark of cancer (56). ST3GalIV enzymatic activity has been found in several types of cancer (57, 58). However, how down-regulation of ST3GalIV mediates ccRCC pathogenesis still remains to be resolved.

**miR-137 (RLIP76).** miR-137 (Figure 2) is significantly down-regulated in ccRCC tissues in comparison to corresponding non-cancerous tissues (63). Ectopic expression decreases proliferation, invasion and induces apoptosis in RCC cells (63). This miRNA inhibits pulmonary metastasis of ccRCC cells after tail vein injection into nude mice (63). Ral interacting protein of 76 kD (RLIP76) has been identified as a direct target of miR-137 (63). RLIP76 is a multifactorial protein with transport and signaling functions. It contains a surface domain (171 to 186 amino acids) but its membrane topology is not yet resolved (64). Transport of glutathione-conjugates is a documented function of RLIP76 (64).

**miR-141 (EPH2).** miR-141 (Figure 2) is decreased in ccRCC tissues in comparison to corresponding normal tissues (68). Overexpression of miR-141 attenuates proliferation and motility of 786-O and SN12-PM6 RCC cell lines (68). In an orthotopic RCC xenograft model, miR-141 suppressed tumorigenesis and metastasis to the liver, lungs, lymph nodes and peritoneum (68). Erythropoietin-producing human hepatocellular receptor A2 (EPHA2) was identified as a direct target of this miRNA (68). EPHA2 is frequently up-regulated...
in ccRCC and activates focal adhesion kinase (FAK) and AKT signaling (68) while its expression in tumor tissues predicts locally aggressive behaviour and poor outcome in patients with ccRCC (69). Accumulation of EPHA2 modulates cytoskeleton dynamics, loss of cell contact, proliferation, oncogenic signaling and metastasis (70, 71). Multiple drugs targeting EPHA2 are under preclinical and clinical development in several types of cancer (72).

miR-182-5p (FLOT-1). miR-182-5p (Figure 2) is down-regulated in ccRCC and inhibits proliferation and tumorigenicity of 786-O and Caki-1 RCC cells in vitro and in vivo (73). Upregulation of miR-182 leads to G1 phase arrest, inhibition of AKT signaling and induction of transcription factor FOXO3 (11). Flotillin 1 (FLOT-1) was identified as a target of miR-182-5p (73). FLOT-1 is a caveolae-associated integral membrane protein which tethers membrane receptors and acts as a signaling molecule (74, 75). Flotilin-1 oligomer-based microdomain scaffolds are involved in molecular sorting, endocytic pathways, phagosomal trafficking and coordinate a variety of signaling processes (76). FLOT-1 plays a role in the progression of several types of carcinomas (76).

miR-193b (IGF1R). Expression of miR-193b (Figure 2) is decreased in ccRCC tissues in comparison to matched normal tissues (77). miR-193b inhibits proliferation, invasion and migration of Caki-1 RCC cells in vitro and tumor growth in vivo in nude mice (77). Insulin-like growth factor receptor 1 (IGFR1) has been identified as a target of miR-193b (77). IGFR1 promotes malignant transformation, induces proliferation, but inhibits apoptosis in ccRCC (78, 79). Several IGFR1 inhibitors are undergoing clinical trials in several types of carcinomas (80).

miR-28-5p (RAP1B). miR-28-5p (Figure 3A) is down-regulated in ccRCC in comparison to corresponding normal tissues (81). miR-28-5p suppresses proliferation of A498 and ACHN RCC cells in vitro and tumor growth in vivo in nude mice through targeting ras-related protein 1B (RAP1B) (81). miR-28-5p represses mitogen activated protein kinase (MAPK) signaling by inhibiting phosphorylation of p38 mitogen-activated kinase (p38) and ERK1/2 (81). As a physiological function, RAP1B GTPase is involved in platelet activation/adhesiveness after injury (82). In cancer, RAP1B activates multiple signaling pathways associated with tumor cell proliferation, invasion, cell adhesion and angiogenesis (83, 84).

miR-99a (mTOR). miR-99a (Figure 3A) is down-regulated in ccRCC and correlates with overall survival (85). In 786-O and OS-RC-2 RCC cells, miR-99a inhibits proliferation, induces G1 phase cell-cycle arrest and inhibits migration and invasion (85). In vivo, miR-99a suppresses tumor growth of 786-O xenografts in nude mice (85). mTOR has been identified as a target of miR-99a (85). mTOR knock-down
miR-106a-5p (PAK5). miR-106a-5p (Figure 3A) is down-regulated in ccRCC in comparison to corresponding normal tissues (91). In 786-O and ACHN RCC cells, miR-106-5p inhibits migration and invasion by targeting p21 activated protein kinase (PAK5) (91). In an experimental metastasis model, miR-106a-5p suppresses metastasis to the lungs in nude mice (91). PAK5 is a member of a family of six isoform versions which play a role in cytoskeletal dynamics, cell survival and proliferation. They are overexpressed, hyperactivated or amplified in several types of cancer and function as signal transducers in pathways such as RAS, RAF, nuclear factor xB (NFκB), AKT and protein 53 (p53) (92). PAK5 can act as an oncogene as well as an effector of GTPases ras-related C3 botulinum toxin substrate (RAC) and cell division control protein 42 homolog (CDC42) and is presently under validation as a target for cancer therapy (93, 94).

miR-143, -216b (KRAS). miR-143 and -216b target Kirsten rat sarcoma viral oncogene homolog (KRAS). miR-143 (Figure 3A) is down-regulated in ccRCC tissues, compared to corresponding normal tissues (94). A synthetic version miR-143 inhibits growth of Caki-1 ccRCC cells in vitro and tumor growth in vivo after systemic polyion complex-based delivery in nude mice (94). miR-143 perturbs cancer specific energy metabolism and induces autophagy (94). A partial metabolic shift from glycolysis to oxidative phosphorylation has been observed. miR-143 down-regulates glucose transporter 1 (GLUT1) and also suppresses PI3K/AKT and MAPK/ERK signaling. miR-216b (Figure 3A) is down-regulated in ccRCC specimens, in comparison to corresponding normal tissues. It suppresses proliferation and invasion of 786-O and ACHN RCC cell lines and inhibits tumor growth of ACHN ccRCC-based xenografts in vivo (95). It also interferes with AKT and ERK pathways (95).

KRAS functions as a small GTPase and transduces signals from cell surface receptors to the cytoplasm through specific effector pathways such as MAPK/ERK and PI3K/AKT, while regulating diverse cellular responses (96, 97). KRAS is involved in ERK1/2-based phosphorylation and nuclear translocation of pyruvate kinase muscle isoform 2 (PKM2) promoting the Warburg effect (98). Due to its de-regulation in many types of cancer, KRAS is an important target, however its druggability is a critical issue (99, 100). Recently significant progress has been achieved in the treatment of KRAS mutated tumors (101). In ccRCC, KRAS mutations rarely occur, however, de-regulation of KRAS signaling is a frequent event (102).

miR-148a (AKT2). miR-148a (Figure 3A) is down-regulated in ccRCC cell lines and tissues and its down-regulation is associated with lymph node metastasis (103). miR-148a inhibits proliferation, colony formation and migration of 786-O ccRCC cells via suppression of AKT2 (103). Tumor growth in nude mice from 786-O cells transfected with miR-148a is attenuated (103). AKT2 is an isoform of the AKT2 family which functions as an oncogene by enhancing survival, migration and invasion of cancer cells. This gene is a member of the PI3K/AKT pathway and phosphorylates downstream targets including mTOR (104, 105). Independently it was shown that AKT2 is de-regulated in ccRCC (106). mTOR inhibitors everolimus and temsirolimus are approved for treatment of ccRCC (107).

miR-205-5p (VEGF). Expression of miR-205-5p (Figure 3B) is down-regulated in ccRCC and correlates with poor prognosis of patients (108). miR-205-5p inhibits proliferation, migration, EMT and induces apoptosis of 786-O and ACHN ccRCC cells (108). VEGF-A was identified as a direct target of miR-205-5p (108). This miRNA inactivates the PI3K/AKT/mTOR signaling pathway (108). 786-O xenografts in nude mice expressing miR-205-5p exhibit decreased tumor growth (108). The VEGF pathway plays an important role in ccRCC (109). The VEGF inhibitors sorafenib, sunitinib, bevacizumab, pazopanib, cabozaclinib and axitinib are approved for treatment of ccRCC (110,111).

miR-30a-3p, -30c-2-3p, -145 (HIF-2α). Reduced expression of miR-30a-3p and miR-30c-2-3p (Figure 3B) was significantly associated with poor prognosis in patients with ccRCC (112). Both target hypoxia-inducible factor 2α (HIF-2α) directly (112). In vivo, tumor growth of UMRC2 xenografts is inhibited by both miRs (112). miR-30a-3p and miR-30c-2-3p repressor expression enhance HIF-2α expression, a mechanism whereby the tumor-suppressive effect of constitutive HIF-1α expression is attenuated in HIF1α/HIF2α tumors. Under high oxygen conditions, HIF1α and HIF2α are hydroxylated by prolylhydroxylase enzymes, resulting in VHL recognition, polyubiquitinylation and subsequent proteasomal degradation (113). Under low oxygen conditions, HIF-2α subunits are stabilized and activate numerous genes involved in metabolism, angiogenesis, motility/invasion and extracellular matrix (ECM) remodeling (114). HIF-1α acts as a TIS, whereas HIF-2α promotes tumorigenesis (115,116). Restricted expression of miR-30a-3p and miR-30c-2-3p in ccRCC enhances HIF-2α activity.
Androgen receptor (AR) down-regulates miR-145 (Figure 3B) via promoter interaction in ccRCC cell lines (117). miR-145 inhibits proliferation and invasion of OS-RCC (VHL wild-type) and SW-839 (VHL inactivating mutant) RCC cells (117). miR-145 targets HIF-2α and suppresses HIF2α/VEGF/matrix metalloproteinase 9 (MMP9) / cyclin D1 (CCND1) signals in OS-RCC and SW-839 ccRCC cell lines (117). In an orthotopic kidney capsule model, miR-145 suppresses tumor growth and metastasis of OS-RCC cells (117). ccRCC patients with higher AR expression had lower overall survival rates, linking AR to poor prognosis (118). AR in ccRCC cells increases proliferation and AR inhibitors enzalutamide and abiraterone inhibit TG of Caki-1 xenografts in nude mice (119). Intracrine androgen biosynthesis has been observed in ccRCC and AR increases hematogenous metastasis of ccRCC (120).

miR-32-5p (TR4). miR-32-5p (Figure 3B) is down-regulated in ccRCC tissues of patients with distant metastases compared to those from metastasis-free patients (121). miR-32-5p inhibits invasion of ACHN, SW839 and OS-RCC ccRCC cells by targeting testicular nuclear receptor 4 (TR4). After implantation into the renal capsule, miR-32-5p inhibits metastasis of OS-RCC xenografts into the lungs, spleen and liver (121). In this experimental system, sunitinib suppresses metastasis via induction of miR-32-5p and suppression of TR4 (121). miR-32-5p attenuates metastasis through interference with TR4/fibroblast growth factor (FGF)/MET signaling (121). TR4 is an orphan receptor of the family of steroid receptor transcription factors (122, 123). This receptor can alter hepatocyte growth factor (HGF)/MET signaling by binding to the TR4-response element of the
DNA-damage repair, DNA and RNA metabolism and signal transduction (139). Several CDK2 inhibitors are evaluated in dispensable for normal development (139). In addition to its involvement in cell-cycle progression, miR-200c mediates associated with several types of cancer, however, it is (138). Deregulation of CDK2 is clinical studies, however it emerged that due to pronounced induction of cell-cycle arrest and tumor growth in subcutaneous and orthotopic ccRCC xenografts (142). CDK4, CDK9 and CCND1 have been identified as direct targets of miR-206c (142). As already outlined, CDK4 is a validated target for treatment of hormone receptor positive, HER2-negative metastatic breast cancer (143). CDK9 is a serine-threonine kinase which is involved in DNA transcription by phosphorylating RNA polymerase II and several transcription factors (144). However, more selective inhibitors of CDK9 have to be generated to explore their therapeutic potential (144). CCND1 is involved in cell-cycle transition to the S-phase by activating cyclin-dependent enzymes (145, 146).

miR-1 targets CDK4, CDK6, Caprin 1 and SLUG. miR-1 (Figure 4A) down-regulation correlates with clinico-pathological characteristics and overall survival of ccRCC patients (133). miR-1 inhibits proliferation, migration and invasion of 786-O ccRCC cells (133). It targets cyclin-dependent kinases 4 and 6 (CDK4, 6), caprin 1 and metastasis related gene SLUG (SNAIL-2) (133). In vivo, miR-1 inhibits tumor growth of ACHN-derived xenografts after subcutaneous and orthotopic implantation in nude mice (133). CDK4 and CDK6 inhibitors such as palbociclib, ribociclib and abemaciclib have been approved for hormone-sensitive, human epidermal growth factor receptor 2 (HER2) negative breast cancer (134). The other target, caprin-1, is a mediator of proliferation and cell-cycle progression (135). Target SLUG (SNAIL-2) is a zinc finger-based transcriptional repressor which is involved in EMT and mediates anti-apoptotic activity (136, 137).

miR-200c targets CDK2. miR-200c (Figure 4A) is down-regulated in ccRCC specimens (138). In SN12-PM6 and 786-O cells, this miRNA suppresses proliferation and induces cell-cycle arrest (138). Cyclin-dependent kinase 2 (CDK2) has been identified as a direct target of miR-200c (138). In orthotopic xenografts, miR-200c suppresses growth of SN12-PM6 RCC cells (138). Deregulation of CDK2 is associated with several types of cancer, however, it is dispensable for normal development (139). In addition to its involvement in cell-cycle progression, miR-200c mediates DNA-damage repair, DNA and RNA metabolism and signal transduction (139). Several CDK2 inhibitors are evaluated in clinical studies, however it emerged that due to pronounced toxicity effects, more selective inhibitors need to be evaluated (140). In ccRCC, CDK2 is a strong predictor of recurrence (141).

miR-206c targets CDK4,9, and CCND1. miR-206c (Figure 4A) inhibits proliferation of ccRCC cells through induction of cell-cycle arrest and tumor growth in subcutaneous and orthotopic ccRCC xenografts (142). CDK4, CDK9 and CCND1 have been identified as direct targets of miR-206c (142). As already outlined, CDK4 is a validated target for treatment of hormone receptor positive, HER2-negative metastatic breast cancer (143). CDK9 is a serine-threonine kinase which is involved in DNA transcription by phosphorylating RNA polymerase II and several transcription factors (144). However, more selective inhibitors of CDK9 have to be generated to explore their therapeutic potential (144). CCND1 is involved in cell-cycle transition to the S-phase by activating cyclin-dependent enzymes (145, 146).

microRNAs targeting components of the extracellular matrix Components of the ECM such as laminins and collagens have a positive impact on angiogenesis and metastasis of ccRCC cells. This can be achieved by down-regulation of the corresponding miRs.

miR-200b targets LAMA4. miR-200b (Figure 4B) expression inversely correlates with survival in patients with ccRCC (147). miR-200c impedes cell spreading and migration, but not growth, in ccRCC cell lines OS-RC2 and Caki-1 (147). Laminin subunit α4 (LAMA4) has been identified as a direct target of miR-200b (147). In vivo, miR-200b has no impact on proliferation, but inhibits metastatic colonies in the lungs after tail vein injection in nude mice (147). LAMA4 is an ECM glycoprotein and is part of the laminin complex. It is secreted into the ECM, up-regulates integrin α5β1 and activates the integrin-linked kinase (ILK)/FAK/ERK pathway (148). α4 chain laminins are widely expressed in ccRCC and have a de-adhesive function (148). LAMA4 promotes angiogenesis and is enriched in blood vessels of ccRCC patients while its expression correlates with poor prognosis (149, 150). Intra-tumoral injection of miR-200b mimetics decreases lung metastasis in mice (147).

Let 7d targets COL3A1. Decreased let-7d (Figure 4B) is associated with advanced tumor stages in ccRCC patients and is inversely correlated with macrophage infiltration (151). Over-expression of let-7d impedes growth and migration of 786-O and 769-P RCC cell lines in vitro (151). In vivo, in 786-O cell-derived xenografts, let-7d inhibits tumor growth (151). In patient-derived xenografts (PDX), intra-tumoral injection of let-7d mimetics inhibit tumor growth and metastatic nodules in the lungs of nude mice.
α1 chain of type III collagen (COL3A1) and chemokine (C-C motif) ligand 7 (CCL7) have been identified as direct targets of let-7d (151). Col3A1 is involved in ECM organization, cell adhesion, migration and invasion through the MAPK pathway (152). Col3A1 expression is correlated with a poor prognosis in patients with bladder cancer and ovarian carcinoma (153, 154). CCL7 promotes tumor growth by recruiting leukocytes including monocytes, macrophages and neutrophiles to the tumors facilitating TME formation, invasion and metastasis (155).

**microRNAs targeting E3-ubiquitin-ligases**

Ubiquitinylation marks proteins for degradation by the proteasome leading to deregulation of transcription factors and signaling components. This results in proliferation, migration and survival of tumor cells. Down-regulation of miRs targeting ubiquitinylation enzymes mediates pro-tumoral effects in ccRCC cells.

**miR-200a-3p targets CBL.** miR-200a-3p (Figure 4B) is down-regulated in ccRCC tissue in comparison to normal adjacent tissue (156). miR-200a-3p suppresses proliferation and migration and induces apoptosis in vitro and in vivo (152). Casitas B-lineage lymphoma (CBL) has been identified as a target for miR-200a-3p (156). CBL functions as an E3 ubiquitin-ligase and consists of an N-terminal tyrosine kinase binding domain, a ring finger domain, a proline rich domain which interacts with adaptor proteins and signaling components. This results in proliferation, migration and survival of tumor cells. Down-regulation of miRs targeting ubiquitinylation enzymes mediates pro-tumoral effects in ccRCC cells.
and a C-terminal domain which binds ubiquitin (157). CBL is involved in survival, migration and proliferation (157). However, inhibition of angiogenesis has also been reported for CBL (158). Taken together, the role of CBL in ccRCC has to be resolved in further detail.

**miR-520/372/373 target SPOP.** In vitro miRs-520/372/373 (Figure 4B) suppress proliferation and migration in A498 and ACHN ccRCC cells (159). Tumor growth of ACHN xenografts is suppressed by each of these miRs (159). Speckle-type POZ protein (SPOP) has been identified as a target of these miRs (159). SPOP functions as an E3 ubiquitin ligase which is highly expressed in ccRCC (160, 161). It is transcriptionally activated by HIF-2α and functions as a key hub in kidney cancer (162). It induces degradation of tumor suppressors such as PTEN and dual-specificity phosphatase (DUSP7), pro-apoptotic target death-associated protein 6 (DAXX6) and is part of a novel pathway in ccRCC (162). SPOP expression correlates with a bad prognosis in ccRCC (163). Small molecules have been identified which inhibit SPOP-substrate protein interaction and suppress oncogenic SPOP signaling pathways in ccRCC (164).

**microRNAs targeting apoptosis-related proteins**

Anti-apoptotic proteins are crucial for survival of tumor cells. This is achieved by down-regulation of miRs which inactivate mRNAs encoding anti-apoptotic enzymes.

**miR-337-3p targets CAPN4.** miR-337-3p (Figure 5A) is down-regulated in ccRCC cell lines (165). It inhibits proliferation, colony growth and invasion, but enhances cell adhesion (165). Knock-down of miR-337-3p exerts the opposite effects (165). miR-337-3p inhibits EMT and suppresses growth of ccRCC xenografts in nude mice (165). Calpain small regulatory subunit 4 (CAPN4) was identified as a target of miR-337-3p (165). The calpain family of Ca-dependent non-lysosomal cysteine proteases is involved in proteolysis of many substrates, cytoskeletal remodeling, cell survival, apoptosis and cellular signaling. Proteins of the NFκB, FAK pathways and c-MYC have been identified as targets of CAPN4-regulated enzymes (166, 167). The latter are also involved in metastasis (168). CAPN4 has been shown to be a poor prognostic factor in gastric carcinoma (169).

**miR-708 targets survivin.** miR-708 (Figure 5A) expression is attenuated in ccRCC (174). miR-708 induces apoptosis, inhibits migration and invasion, reduces adhesion to ECM and increases the fraction of spindle-shaped cells of A498 and Caki-2 ccRCC cells (174). Intra-tumoral delivery of this miRNA in xenografts of A498 ccRCC cells leads to regression of established tumors in nude mice (174). Survivin was identified as a direct target of miR-708 (174). Its knockdown phenocopies miR-708 re-expression in ccRCC cells (174) and is a predictor of progression and death from ccRCC (175). It is a member of the inhibitor of apoptosis family which modulates the cell cycle, microtubules dynamics and inhibits caspase activation (176). Several survivin inhibitors are under preclinical investigation (177, 178).

**microRNAs targeting other proteins**

In addition to proteins outlined in the previous sections, other proteins are implicated in oncogenesis and metastasis. They can regulate cell movement, angiogenesis, autophagy, EMT and protein degradation via the proteasome. miRs can modulate their activity.

**miR-29b targets MMP2.** miR-29b (Figure 5A) inhibits the capacity of ccRCC cells to promote capillary tube formation and to invade ECM gel in vitro (179). LCM6 RCC cells transfected with dexamethasone inducible miR-29b subcutaneously or orthotopically implanted into nude mice give rise to smaller tumors with decreased microvessel density and decreased occurrence of intrahepatic metastases, indicating that miR-29 suppresses both angiogenesis and metastasis (179). Matrix metalloproteinase 2 (MMP2) has been identified as a direct target of this miRNA (179). It exerts its anti-angiogenic effect by inhibiting VEGFR2 signaling in endothelial cells (179). It is well documented that degradation and remodelling of the ECM mediates tumor growth and metastasis (180). However, inhibition of MMPs in cancer therapy did not meet the projected clinical endpoints as revealed by numerous clinical trials (181).

**miR-204 targets LC3B**

miR-204 (Figure 5A) is a VHL-regulated tumor suppressor in ccRCC (182). VHL is lost in the vast majority of ccRCC carcinomas (182). miR-204 containing lentivirus particles inhibit subcutaneously implanted 786-O RCC xenografts after intra-tumoral injection (182). miR-204 expressing 786-O cells do not infiltrate into the kidney parenchyma after implantation into the kidney capsule in contrast to cells expressing an inactive mutant (182). The miRNA is...
cytotoxic to VHL−, but not to VHL+ RCC cells (182). The autophagy protein LC3B is a direct target of miR-204 and is a mediator of its cytotoxicity (182). LC3B induces macroautophagy which is necessary for ccRCC progression (182). LC3B is also involved in phagosome biogenesis and substrate selection in ccRCC and other types of cancers (183,184). Autophagy plays a context-dependant role in different types of cancers, therefore inhibition or stimulation of this process could be helpful as therapeutic intervention (185). Nevertheless, the role of autophagy in ccRCC remains to be investigated in more detail.

miR-338 targets KIFC1. miR-338 (Figure 5B) inhibits proliferation, migration and invasion of ccRCC in vitro and in vivo by targeting kinesin family member 1 (KIFC1) through the PI3K/AKT signaling pathway (186). KIFC1 is up-regulated in ccRCC and correlates with aggressive clinicopathological parameters (186). It is a member of the kinesin protein family, which are motor proteins that deliver cargo to different cellular destinations by ATP hydrolysis and tubulin binding (187). KIFC1 associates with poor survival in ccRCC, drives tumor malignancy, prevents cancer cell death and is involved in spindle pole organization (188, 189). Several kinesin inhibitors are under preclinical and clinical evaluation in diverse types of cancer (190).

miR-490-3p targets vimentin. Orphan testicular receptor T4 (191) induces vimentin (VIM) and inhibits miR-490-3p (Figure 5B) by binding to its promoter (192). VIM was identified as a direct target of miR-490-3p (192). miR-490-
3p inhibits tumor growth and metastasis after implantation into the renal capsule of transfected Caki-1 cells in nude mice (192). VIM belongs to the intermediate filament family, is ubiquitously expressed in mesenchymal cells, represents a marker for EMT and correlates with accelerated tumor growth, invasion and prognosis in several types of cancer (193, 194).

miR-646 targets NOB1. miR-646 (Figure 5B) is a predictor of distant metastasis in patients with ccRCC (195). In vitro, miR-646 inhibits proliferation and colony formation of 786-O and ACHN ccRCC cell lines (195). In vivo, miR-646 attenuates tumor growth of 786-O and ACHN RCC after subcutaneous implantation into nude mice (196). In these cell lines, miR-646 inhibits formation of key components of the MAPK signaling pathway such as p38, ERK1/2 and c-jun N-terminal kinase (JNK) (196). Nin one binding protein (NOB1) was identified as a direct target of miR-646 (196). Down-regulation of NOB1 inhibits activation of the proteasome and stabilizes proteins which increase phosphorylation of key components of the MAPK pathway (196, 197). In addition, NOB1 acts as a RNA binding protein. NOB1 binds to 18S ribosomal RNA and is involved in its processing (198, 199). NOB1 expression is associated with poor prognosis in several types of cancers (199).

Therapeutic Aspects

We have identified 10 up-regulated and 33 down-regulated miRs with efficacy in ccRCC-related in preclinical in vivo models. Up-regulated miRs can be inhibited by single-stranded RNAs such as locked nucleic acids (LNA), 12-25 nucleotides complementary to the corresponding mRNA (200). An alternative type of miR inhibitors are miR sponges which contain multiple miR binding sites that can compete with the corresponding mRNA for binding of the corresponding miR (201). Inhibition of miRs can also be achieved with small molecules which interfere with their transcription or their secondary structure, but specificity issues of the identified compounds are a major issue (200, 201). The corresponding targets are candidates for reconstitution with small molecules or gene therapy. The identified up-regulated miRs are shown in Figure 1. They cover adhesion molecules CADM2 and E-cadherin (miRs-146a and -720), tumor suppressors such as PTEN and PTENP1 (miR-21), WNT-signaling inhibitors (miR-106-5p), transcription factors such as FOXO3 and enzymes such as DICER (miR-122) and ST3GalV (miR-193a, -224). Noteworthy, our survey revealed only one hit addressing an epigenetic modifier (miR-144-3p, ARID-1A). This is surprising since several epigenetic modifiers such as BAP-1, PBRM1, SET2 and KDM5C play an important role in the pathogenesis of ccRCC (6).

Down-regulated miRs (Figures 2, 3 and 4) are candidates for miR reconstitution therapy. Their targets are moieties for inhibition by small molecules or mAb-derived entities. Double stranded RNAs functioning as miR-mimetics can be used for restoration of the function of miRs (202). Another option for functional reconstitution of miRs is to express them with appropriate vectors in recipient cells (202).

Among the down-regulated miRs identified, miR-99a (mTOR) and miR-205-5p (VEGF) are directed against targets with drugs approved for treatment of ccRCC as outlined in the previous sections. Several approved drugs for ccRCC target VEGF or vascular endothelial growth factor receptors 1-3 (VEGFR1-3), often jointly (7). Two approved drugs for ccRCC target mTOR (107). Three of the identified down-regulated miRs (miR-30a-3p, -30c-2-3p and -145) target HIF-2α, which after dimerization with HIF-1α, induces transcription of genes involved in the pathogenesis of ccRCC such as VEGF, platelet-derived growth factor B (PDGFR-B), transforming growth factor α (TGFα), erythropoietin (EPO), SPOP and ECM proteins (115, 116). NOB1, an E3 ubiquitin ligase, is inactivated in 95% of sporadic ccRCC with the consequence that its substrates, HIF-2α and HIF-1α, are not degraded (115, 116). PTP 2977 (MK-6482), an orally active and selective HIF-2α inhibitor showed promising efficacy and tolerability and is presently in Phase III clinical studies in patients with inactivating VHL mutations (203-205).

Our search has identified SPOP (targeted by miR-520,372/373) as a tractable target and miR-based agent for treatment of ccRCC. SPOP is induced under hypoxic conditions and functions as an E3 ubiquitin ligase which mediates degradation of PTEN and other tumor suppressors and promotes tumorigenesis by acting as a key regulatory hub in ccRCC (160, 161). Another E3 ubiquitin-ligase, CBP (targeted by miR-200a-3p) as a potential therapeutic target, has been revealed by our search. However, more target validation experiments are necessary to substantiate its role as a therapeutic target in ccRCC.

Our survey has identified CDKs and corresponding miRs as targets and therapeutic tools for treatment of ccRCC such as miR-1 (CDK4, CDK6), miR-200c (CDK2) and miR-206c (CDK4, CDK9). CDK4 and CDK6 inhibitors have been approved for treatment of hormone-dependent, HER2-negative breast cancer (134). PD-0332991, a potent and selective inhibitor of CDK4 and 6 inhibits proliferation of RCC cells at nanomolar concentrations (206). It remains to be explored whether a therapeutic window exists for CDK inhibitors in patients with ccRCC. Furthermore, RLIPT76 (targeted by miR-137) was identified as a druggable transmembrane receptor which induces apoptosis in ccRCC cells both in vitro and in vivo.

In addition to these high priority targets as outlined above, other druggable targets and corresponding miRs have been
noticed such as CAPN4 (miR-337-3p), c-FLIP/SURVIVIN (miR-708), VHL regulated LC3B (miR-204), kinesin KIF1C (miR-338), AKT2 (miR-148a), PAK5 (miR-106-5p), EphA2 (miR-141) and IGFR1 (miR-193b). However, more target validation experiments are necessary for these targets and their associated miRs to explore their potential for treatment of ccRCC.

Other targets with pending druggability and preliminary target validation have been identified such as: Let-7d (COL3A), miR-28-5p (RAS-1B), miR-32-5p (TR4), miRs-143 and -216 (KRAS), miR-200b (LAMA4), miR-490-3p (VIM) and miR-646 (NOB1).

Of note, miR-122 targets DICER (38) and FOXO3 (17) (Figure 1). This may be due to selection of different in vitro and in vivo systems for target identification. The same may be true for miR-780 which targets c-FLIP and Survivin (Figure 3A) (170, 174).

miR-based therapy has experienced serious drawbacks in clinical trials due to lack of efficacy or serious adverse reactions (207). Clinical studies evaluating a miR-17 inhibitor in patients with polycystic kidney disease and treatment of patients with hepatitis C virus infection were put on hold due to toxicity issues (207). A Phase I study of MRX34, a synthetic mimic of miR-34, in patients with multiple types of tumors was shut down due to immune-related side effects (207). Next generation of miR-related therapeutics with an expected profile of better tolerability and efficacy have been generated (207). Also progress in delivery issues has been achieved (207). COBOMARSEN (MRG-106), an antagonist of miR-155, which is presently evaluated in clinical trials in haematological malignancies is well tolerated and looks promising with respect to efficacy (207). The next couple of years will give us a more realistic estimation of the potential of miRs for the therapy of cancer.

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Weidle and Nopora: Target Identification With MicroRNAs in Clear Cell Renal Carcinoma (Review)
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