

microRNAs and Corresponding Targets Involved in Metastasis of Colorectal Cancer in Preclinical *In Vivo* Models

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Abstract. *The high death toll of colorectal cancer patients is due to metastatic disease which is difficult to treat. The liver is the preferred site of metastasis, followed by the lungs and peritoneum. In order to identify new targets and new modalities of intervention we surveyed the literature for microRNAs (miRs) which modulate metastasis of colorectal cancer in preclinical in vivo models. We identified 12 up-regulated and 19 down-regulated miRs corresponding to the latter criterium. The vast majority (n=16) of identified miRs are involved in modulation of epithelial-mesenchymal transition (EMT). Other categories of metastasis-related miRs exhibit tumor- and metastasis-suppressing functions, modulation of signaling pathways, transmembrane receptors and a class of miRs, which interfere with targets which do not fit into these categories. Finally, we discuss the principles of miR inhibition and reconstitution of function, prospective clinical evaluation of with miR-related agents in the context of clinical evaluation in metastasis relevant settings.*

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide with an annual incidence of 1.4 million new cases and 700,000 deaths worldwide (1). Although about half of the subjects with CRC can be cured by surgery and multimodal treatment, therapy options are limited for patients with metastatic disease (2), despite many

of the molecular alterations associated with progression from polyps, carcinoma and metastatic disease have been resolved (3, 4). Less than 5% of CRCs are inherited syndromes, such as the Lynch syndrome, formerly known as hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) (5). Lynch syndrome is caused by inherited mutations in mismatch repair genes and FAP is due to inactivating mutations in adenomatous polyposis coli (APC), a negative regulator of wingless and Int-1 (WNT) signaling (5). CRC is a heterogeneous disease at the intertumoral and intratumoral level with molecularly defined subgroups that differ in their prognosis and response to treatment (6). Subtypes characterised by chromosomal instability (CIN), microsatellite instability (MSI) or CpG island methylator phenotype (CIMP) have been identified (6). The CIN phenotype is characterized by aneuploidy and chromosomal gains and losses, the MSI phenotype exhibits the presence of frequent insertion and deletion mutations in repetitive DNA sequences and the CIMP phenotype shows patterns of aberrant gene methylation (6). The challenge is to improve treatment of CRC patients with stage III and IV disease. Presently, folfox (5-FU, leucovorin, oxaliplatin, capecitabine (capecitabin, oxaliplatin), folfoxir (leucovorin, 5-FU, irinotecan) or folfoxiri (leucovorin, 5-fu, oxaliplatin, irinotecan) are used as standard therapy with limited success in advanced disease (7). Also, combinations of chemotherapy with angiogenesis-targeting agents, such as bevacizumab, ramucirumab and aflibercept or epidermal growth factor receptor (EGFR)-targeting monoclonal antibodies (mABs) such as cetuximab and panitumab have been approved (7). Immunotherapy with antibodies disrupting the programmed cell death protein 1 (PD1) and PD-1 ligand (PD-1L) immune checkpoint, such as nivolumab and pembrolizumab, looks promising, especially in patients with the MSI subtype, however, the results have to be verified in large randomized studies (8, 9).

In order to define new targets and treatment modalities for patients with metastatic CRC we have summarized miRs

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and corresponding targets, which modulate metastasis in *in vivo* models.

Metastasis of CRC

Metastasis of CRC is a multi-step process involving local invasion, intravasation, activation of mechanisms allowing survival in the circulation, arrest at distant organ sites, extravasation, formation of micro-metastases and finally their outgrowth and colonization of distant organs (10). At the time of diagnosis, 20% of CRC patients have already developed metastatic disease (11). CRCs most commonly metastasize to the liver, lungs and peritoneum, but also various other sites such as bone, spleen, brain and distant lymph nodes have been described (12-15). More than 50% of patients with CRC will develop metastases to the liver over the course of their life, which ultimately results in death of more than two thirds of these patients (16). CRC preferentially metastasizes to the liver. This can be explained by a trapping mechanism of CRC cells in the mesenteric circulation in the liver (17, 18). However, other mechanisms may play a role such as preferred outgrowth of CRC cells in the liver due to premetastatic niche formation based on the “seed and soil hypothesis” (19, 20). This hypothesis postulates that a target organ is susceptible due to circulating signaling factors or factors packed into exosomes (19, 20). It has been found that liver metastases are formed before lung metastases are established (21). Recent sequencing of primary colorectal tumors and matching liver metastases revealed heterogeneity of metastases and metastasis-specific mutations leading to activation of phosphoinositide 3-kinase (PI3K)-AKT signaling, cell adhesion and extracellular matrix as well as induction of hepatic stellate activation genes (22).

miRs and Their Role in Oncology

miRs are transcribed in the nucleus by RNA polymerase II as primary miR transcripts (pri-miRs) which are subsequently cleaved by the microprocessor complex to produce 30 bp comprising short hairpin RNAs (shRNAs) referred to as pre-miRs (23, 24). Subsequently, exportin 5 binds to the pre-miRs and transports them to the cytoplasm (23, 24). After cleavage of the pre-miRs by DICER, an RNA-induced silencing complex (RISC)-loading complex is formed, a guide strand (antisense) is selected and the passenger strand (sense) is discarded (25). RISC can regulate mRNA expression by pairing with the 3'-untranslated region (3'-UTR) of the target mRNA and inducing cleavage of the mRNA and/or inhibition of its translation (25). miRs have many targets and are partially complementary to the 3'-UTR of their target mRNAs in contrast to siRNAs which are fully complementary to the coding region of their mRNA targets (26). miRs can interfere with several pathways because they

can target hundreds of mRNAs and therefore have the potential to rewire the oncogenic and metastatic state (27). Tumor-suppressive functions have been demonstrated first for miRs-15a and miR-16-1 in the context of pathogenesis of B-cell chronic lymphatic leukemia (B-CLL) (28-30). Deletion of the locus harboring miRs 15a and -16-1 in mice recapitulated the B-CLL phenotype (28-30). These miRs mediate the cleavage of B-cell lymphoma 2 (BCL2), which functions as an anti-apoptotic protein (28-30). We have recently summarized the functional contribution of miRs to metastasis of ovarian, breast, prostate and lung cancer (31-34). In this review, we focus on miRs involved in metastasis of CRC with documented *in vivo* activity in metastasis-related models.

miRs Up-regulated in Metastatic Colon Cancer Cells

miRs targeting tumor and metastasis suppressor mRNAs. miR-15b increases invasion and migration of CRC cell line HCT-116 and intrasplenic injection of HCT-116 cells transfected with anti-miR-15b exhibits decreased number of metastatic nodules in the liver of nude mice (35). Metastasis-suppressor 1 (MTSS1) and klotho were identified as direct targets of miR-15b (Figure 1). MTSS1 is a multi-functional protein, which functions as a tumor suppressor (TS) and inhibitor of metastasis (36). Klotho is a transmembrane protein and TS which acts as a co-receptor for fibroblast growth factor (FGF) receptors, modulates WNT/ β -catenin signaling and suppresses metastasis of renal cancer xenografts in mice (37-39). High plasma levels of miR-15b in CRC patients are associated with local recurrence, distant metastases and poor prognosis (35).

miR-103/107 (Figure 1) increases motility and invasiveness of HCT-116 CRC cells and overexpression of miR103/107 in HT-29 and HCT-116 cells promotes cell matrix adhesion and inhibits cell-cell adhesion (40). HCT-116 cell transfected with miR103/107 expression vectors exhibit significantly increased size and number of metastatic nodules in the liver after implantation into the caecum of nude mice (40). Death-associated protein kinase (DAPK) and krüppel-like factor 4 (KLF4) were identified as direct targets of miR-103/107 (40). DAPK acts as a TS and inhibits cell-matrix adhesion by inactivation of integrin β 1 and interferes with cell migration (41). DAPK also has been identified as a suppressor of CRC metastasis (42, 43). The other target, KLF4, is a zinc finger transcription factor, which suppresses tumor formation in genetic and pharmacological mouse models of colonic tumorigenesis (44). From a clinical point of view, a signature of miR-103/107 high, DAPK low and KLF4 low, correlates with metastasis and poor prognosis in CRC patients (40).

miR-182 (Figure 1) accelerates growth, invasion and migration of CRC cell line SW480 *in vitro* (45). SW480 cell transfected with miR-182 and subcutaneously implanted into

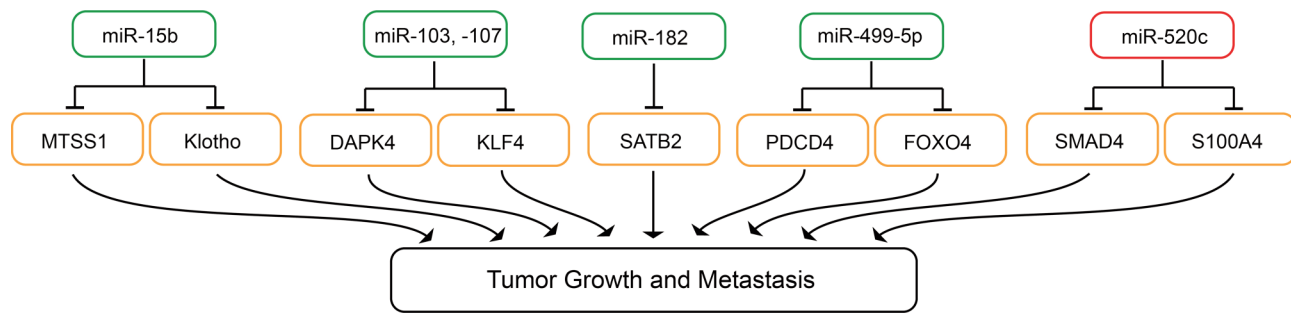


Figure 1. *miRs modulating tumor- or metastasis-suppressor genes with efficacy in metastasis-related colorectal cancer in vivo models. miRs up-regulated in CRC biopsies are boxed in green, downregulated miRs are boxed in red. The corresponding targets are boxed in yellow. DAPK: Death associated protein kinase; FOXO4: forkhead bOX protein 04; KLF4: krüppel-like factor 4; Klotho: transmembrane protein klotho; MTSS1: metastasis suppressor 1; PDCD4: programmed cell death protein 4; S100A4: S100 binding protein 4; SATB2: special AT-rich sequence binding protein; SMAD4: mothers against decaplegic homolog 4.*

nude mice exhibit increased metastasis to the abdominal cavity, liver, lymph nodes and lungs (45). Special AT-rich sequence binding protein 2 (SATB2), a member of the nuclear matrix attachment proteins that recognize AT-rich sequences at the base of looped-out chromatin, was identified as a target of miR-182 (45, 46). SATB2 is mainly expressed in epithelial cells of colon and rectum and in the nuclei of neurons in the brain (47). SATB2 is down-regulated in CRC patients with metastasis and poor prognosis (48). miR-182 is highly expressed in CRC patients with metastasis compared to patients without metastases (45).

miR-499-5p (Figure 1) transfectants of the SW480 CRC cell line show increased migration and woundhealing in comparison to control cells (49). After tail vein injection of miR-499-5p transfectants of SW480 cells into nude mice, number and size of lung and liver metastatic nodules were increased in comparison to the untransfected cell line (49). As direct targets, forkhead box protein O4 (FOXO4) and programmed cell death protein 4 (PDCD4) have been identified (49). FOXO4 is a transcription factor which suppresses functions downstream of PI3K/AKT (50, 51). The other target, PDCD4, has been identified as a TS, which inhibits proliferation, cell-cycle progression and induces apoptosis (52). In colon cancer cells, down-regulation of PDCD4 promotes invasion and activates β -catenin/transcription factor seven 7 (TCF7) and activator protein 1 (AP1)-dependent transcription in colon carcinoma cells (53). In CRC, PDCD4 reduces zinc-finger protein SNAIL, up-regulates E-cadherin, down-regulates transcription factor c-MYC and urokinase plasminogen activator receptor (uPAR), inhibits mTOR complex 2 (mTORC2) and decreases interleukin 6 (IL6), tumor necrosis factor α (TNF α) and nuclear factor κ B (NF κ B) (54). miR-499-5p expression in CRC is associated with clinical stage and lymph node metastasis (49). miRs interfering with signaling are shown in Figure 2.

miRs inducing EMT. miRs-135b and -210 (Figure 3B) both separately and in combination induce migration and invasion of RKO and HCT-116 CRC cells *in vitro* (55). RKO and HCT-116 cells transfected with miR-135 or miR-210 exhibit enhanced metastasis to lungs and liver after injection into chicken embryos (55). Seven in absentia homolog1 (SIAH1), set domain containing 2 (SETD2) and forkhead box protein N 3 (FOXN3) were identified as targets for both, miR-135b and miR-210 (55). SIAH1 functions as an E3 ubiquitin ligase, which induces proteasome-mediated degradation of specific proteins (56). SETD2 is a histone methyltransferase specific for lysine-36 of histone H3 which is involved in transcriptional regulation, chromatin regulation, DNA repair and splicing (57). FOXN3 suppresses N-Cadherin, a transmembrane receptor involved in EMT (58). miR-135 and -210 and their targets are reciprocally expressed in CRC tissues (55).

miR-181a (Figure 2) stimulates growth, invasion, migration and induces EMT of HT-29 CRC cells *in vitro* (59). HT-29 cells transfected with miR-181a give rise to increased liver metastasis after implantation into the spleen of nude mice (59). WNT inhibitory factor-1 (WIF-1) has been identified as a target of miR-181a (59). WIF-1 is a lipid binding protein, which binds to WNT proteins and prevents them from triggering signaling (60). WIF-1 was shown to be down-regulated in prostate, breast, lung and bladder cancer and restauration of expression of WIF-1 reversed EMT, invasion and tumor growth (61, 62). Elevated expression of miR-181a in CRC samples correlates with advanced clinical stage, distant metastasis and serves as an independent prognosis marker for poor survival (60).

miR-196a-5p (Figure 3B) promotes proliferation of SW480, SW620 and HCT-116 CRC cells (63). After implantation into the spleen, SW480 cells transfected with miR-196a-5p exhibit more liver metastases than the control cell line, whereas its

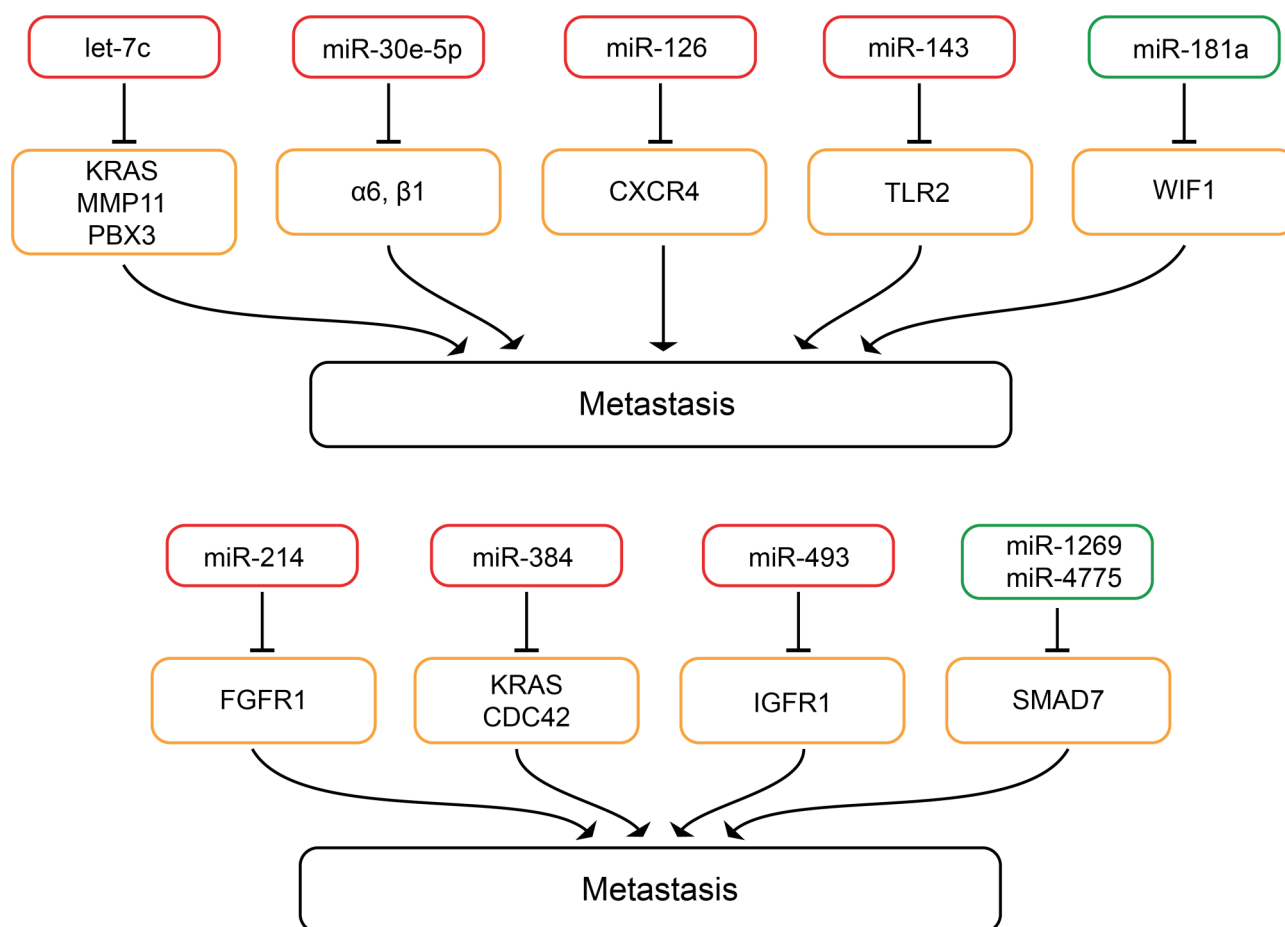


Figure 2. miRs modulating signaling pathways with efficacy in metastasis related colorectal cancer in vivo models. miRs up-regulated in CRC biopsies are boxed in green, downregulated miRs are boxed in red. The corresponding targets are boxed in yellow. $\alpha 6, \beta 1$: Integrin $\alpha 6, \beta 1$; CDC42: cell division control protein 42 homolog; CXCR4: C-X-C chemokine receptor 1; FGFR1: fibroblast growth factor receptor 1; IGFR1: Insulin-like growth factor receptor 1; KRAS: kirsten ras; MMP11: matrix metalloproteinase 11; PBX3: pre-B-cell leukemia transcription factor X3; SMAD7: mother against decaplegic homolog 7; TLR2: toll like receptor 2; WIF1: WNT-Inhibitory factor 1.

knock-down in HCT-116 cells attenuates liver metastasis (63). miR-196a-5p promotes EMT transition in CRC cells accompanied by down-regulation of E-cadherin and up-regulation of N-cadherin and fibronectin (63). Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α ($\text{I}\kappa\text{B}\alpha$), a negative regulator of NF κB signaling was identified as a target of miR-196a-5p (63). $\text{I}\kappa\text{B}\alpha$ blocks the ability of NF κB transcription factors to bind to DNA (64). Data addressing correlations of expression of miR-196a-5p and clinical parameters of patients with CRC are not available yet.

miR-544a (Figure 3B) mediates invasion of HCT-116 and SW480 CRC cells (65). Inhibition of miR-544a transfected HCT-116 cells leads to reduction of lung metastasis of subcutaneously implanted cells in comparison to control cell lines (65). The mRNA of homeobox gene HOXA10 has been identified as a target of miR-544a (65). Overexpression of

HOXA10 leads to up-regulation of E-cadherin, a validated inhibitor of EMT (65-67). Expression of miR-544a in CRC tissues correlates with metastatic propensity (65).

miR-885-5p (Figure 3B) expression induces cell migration in HCT-116 and DLD-1 cells through activation of EMT (68). miR-885-5p enhances stressfiber formation through rearrangement of the cytoskeleton (68). HCT-116 cells transfected with miR-885-5p give rise to increased liver and lung metastasis after orthotopic implantation into the caecum (68). Cytoplasmic polyadenylation element binding protein 2 (CPEB2) has been identified as a target of miR-885-5p (68). CEBP2 is a negative regulator TWIST, a transcription factor involved in EMT (69-71). miR-885-5p is overexpressed in liver metastases of CRC patients (68).

miRs-1269a and -4775 (Figure 2) activate transforming growth factor β (TGF β) signaling (72, 73). Ectopic

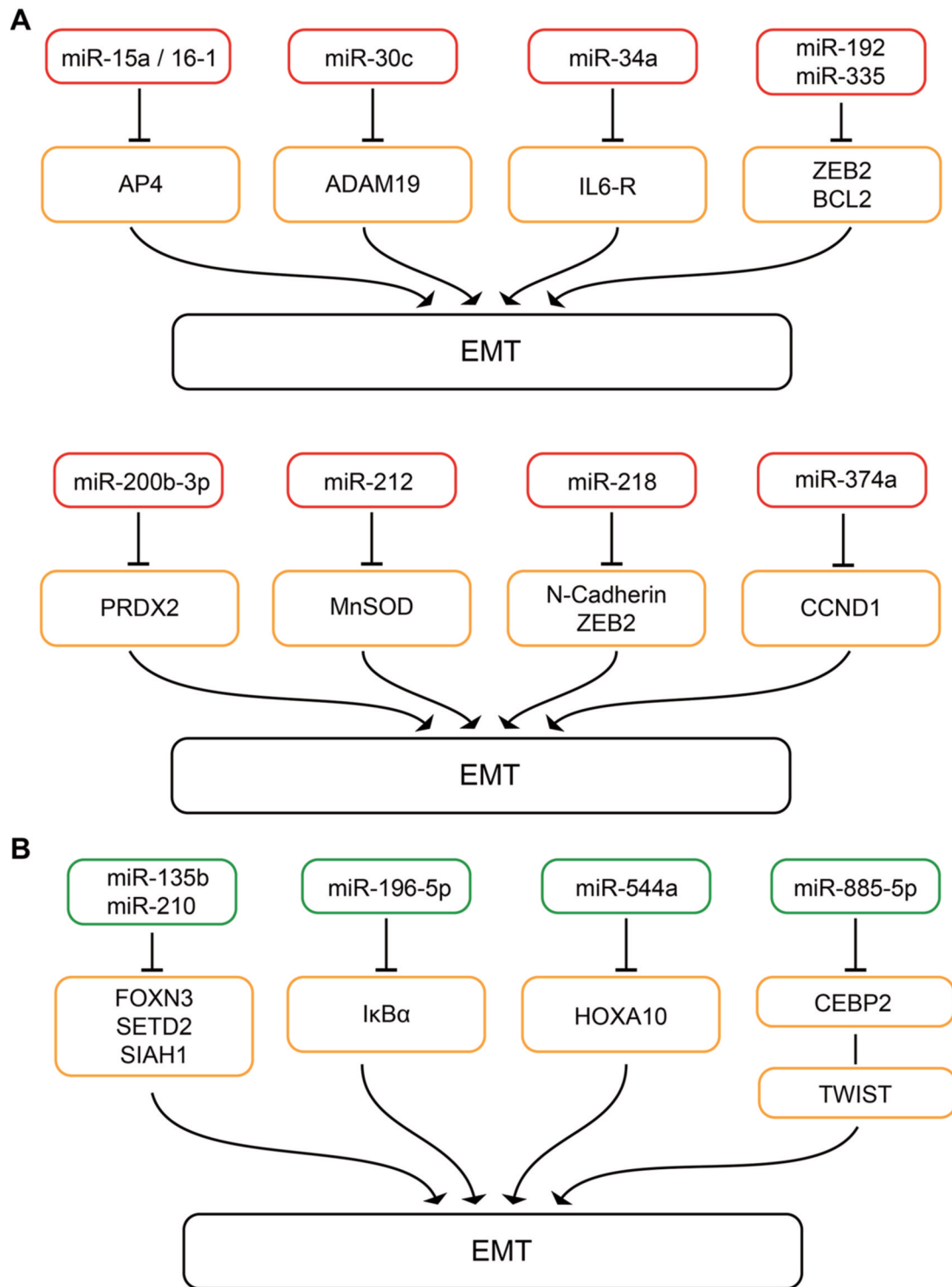


Figure 3. miRs modulating epithelial-mesenchymal transition with efficacy in metastasis-related colorectal cancer *in vivo* models. Down-regulated miRs are boxed in red, up-regulated miRs in green, targets are boxed in yellow. A: miRs 15a/16-1, -30c, -34a, -192, -335, -200b-3p, -212, -218 and -374a. B: miRs 135b, -210, -196a-5p, -544a and -885-5p. ADAM19: A disintegrin and metalloproteinase 19; AP4: activating enhancer binding protein 4; BCL2: B-cell lymphoma 2; CCND1: cyclin D1; CEBP2: cytoplasmic polyadenylation element binding protein; FOXN3: forkhead box protein N3; HOXA10: homeobox gene HOXA10; IL-6R: interleukin 6 receptor; IkBa: nuclear factor of κ light chain polypeptide gene enhancer in B cells Inhibitor α ; MnSOD: manganese superoxide dismutase; N-cadherin: neural cadherin; PRDX2: periredoxin 2; SETD2: set domain containing 2; SIAH1: seven in absentia homolog 1; TWIST: transcription factor TWIST; ZEB2: zinc finger E-box binding homeobox 1.

expression of miR-1269a and -4775 increases migration of SW480 and SW620 CRC cells (72, 73). SW480 cells transfected with miR-1269a and implanted into the caecum give rise to increased metastases to the liver. After tail vein injection of the transfectants, metastases to the lungs and the liver are increased (72). Overexpression or knocking-down of miR-4775 in SW480 or SW620 cells increases or decreases lung and liver metastasis after injection into the tail vein of nude mice (73). Both of them are inducers of EMT and target mothers against decapentaplegic homolog 7 (SMAD7). miR-1269a in addition targets the mRNA of homeobox gene HOXD10 (72, 73). SMAD7 inhibits TGF β signaling which is induced by interaction of heterodimeric transmembrane serine/threonine kinase complexes, including transforming growth factor β receptor I and II (TGF β RI and TGF β RII) with cytokines (74, 75). SMAD7 blocks receptor-regulated SMAD (R-SMAD) phosphorylation by occupying the catalytic domain of TGF β RI. Inhibition of SMAD7 leads to activation of R-SMADs through TGF β RI. Activated R-SMADS (p-SMAD2 and p-SMAD3) form a complex with SMAD4 which translocates into the nucleus and regulates transcription of specific target genes (75, 76). The role of TGF β signaling in the pathogenesis of CRC is well documented (77). Elevated TGF β production is associated with the risk of CRC relapse and metastases (77). Both, miR-1269a and -4775 are up-regulated in CRC tissues, correlate with CRC progression and are indicators of poor survival in CRC patients (72, 73).

miRs Down-regulated in Metastatic Colon Cancer Cells

miRs targeting KRAS. miRs-let-7c and -384 (Figure 2) target KRAS (78, 79) (Figure 3). KRAS is a member of the small GTPase super-family and is constitutively activated by mutation of a single amino acid and is one of the early oncogenic drivers in CRC with a prevalence of 40% in this type of tumor (80, 81). miR-let-7c in addition targets matrix metalloproteinase 11 (MMP11) and pre-B-cell leukemia transcription factor 3 (PBX3) (80). Expression of MMP11, also known as stromelysin 3, correlates with the aggressiveness of CRC (82). PBX3 is a homeobox gene which is part of the EMT regulatory network and indicates poor outcome in patients with CRC (83). In addition to KRAS, miR-384 directly targets cell division control protein 42 homolog (CDC42), a member of the RHO family of GTPases. miR-384 is involved in cell-cycle progression, actin filopodia formation and migration (84). miRs-let-7c and -384 inhibit migration of LoVo and SW620 cells *in vitro* (78, 79). Over-expression of let-7c in LoVo cells inhibits growth and metastasis in the chorioallantoic model (CAM) (78). Inhibition of miR-384 in LoVo and SW620 results in more hepatic metastases after intrasplenic injection (79). Decreased expression of miR-let-7c is associated with metastasis in CRC

patients (78). miR-384 is down-regulated in CRC biopsies in comparison to adjacent normal tissue and is closely associated with aggressive characteristics of CRC (79).

miRs targeting transmembrane receptors. miR-30e-5p (Figure 2) overexpression reduces cell adhesion and migration of CRC cells RKO and HCT-116 *in vitro* (85). In RKO and HCT-116 cells miR-30e-5p overexpression decreases tumor weight and metastasis in the CAM assay (85). Integrins α 6 and β 1 (INT α 6 and INT β 1) have been identified as direct targets of miR-30e-5p (85). miR-30e-5p suppresses the adhesion of INT α 6 in CRC cells to laminin (85). miR-30e-5p induces p21 and p27 inhibitors of cell-cycle progression (85). The anti-metastatic effects of INT α 6 and INT β 1 are due to inhibition of adhesion to ECM, migration, invasion and proliferation (85, 86, 87). miR-30e-5p is down-regulated in tumors of CRC patients in comparison to corresponding normal tissues (85).

miR-126 (Figure 2) inhibits subcutaneous growth of HCT-116 and SW480 CRC cells and decreases lung metastasis of these cells after tail vein injection (88). miR-126 targets C-X-C chemokine receptor type 4 (CXCR4) and Ras homolog gene family, member A (RHO A) signaling pathway components in human CRC cells (88). CXCR4-G13 RHO signaling has been shown to drive transendothelial migration of breast cancer cells (89). Overexpression of CXCR4 and its ligand stromal-derived factor 1 (SDF-1) correlates with dissemination and reduced overall survival in patients with gastrointestinal tumors (90, 91).

miR-143 (Figure 2) reverses the invasive and migratory phenotype of CRC cells SW620 and HCT-116 (92). miR-143 overexpression in SW620 CRC cells significantly decreases lung colonization after tail vein injection in nude mice (92). Toll-like receptor 2 (TLR2) has been identified as a direct target of miR-143, indicating a novel pathway that controls CRC cell invasion and migration (92). Also, TLR2-dependent inflammation plays an important role in metastatic progression (93). TLRs play a critical role in innate immunity and subsequent induction of the adaptive immune response (94, 95). Low levels of miR-143 are associated with advanced pathology stages of CRC and lymph node metastasis (92).

miR-214 (Figure 2) transfected HCT-116 and LoVo cells exhibit significantly slower growth rate and markedly reduced migration and invasion (96). Ectopic expression of miR-214 in HCT-116 cells mediates significant reduction of metastatic nodules in the liver after intrasplenic implantation (96). Fibroblast growth factor receptor 1 (FGFR1) has been identified as a direct target of miR-214 (96). FGFR1 is a member of the FGFR kinase family that contains four transmembrane tyrosine kinases (97). FGFR1 inhibition prevents liver metastasis of colon xenografts by modulating the pre-metastatic niche. Expression of FGFR1 in CRC patients is associated with metastasis (98, 99).

miR-493 (Figure 2) inhibits liver metastasis of HCT-116 cells after injection into the spleen of nude mice (99). miR-493 expression induces cell death of HCT-116 cells in colonized liver parenchyma (99). Insulin-like growth factor receptor1 (IGFR1) was identified as a direct target of miR-493 (99). IGFR1 promotes survival and metastasis of several types of cancer including CRC (100, 101).

miRs targeting EMT-related mRNAs. The miR-15a/16-1 (Figure 3A) cluster is hosted by the DLEU2 gene which is frequently deleted or down-regulated in human tumors (102). EMT-inducing transcription factor activating enhancer binding protein 4 (AP4) is the target of the p53-inducible miRs-15a/16-1 (102, 103). AP4 has similar functions as other EMT mediators such as zinc finger proteins SNAIL and SLUG, zinc finger E-box-binding homeobox 1,2 (ZEB1, ZEB2), TWIST and MYC (104, 105). AP4 forms a double-negative feedback loop with miR-15a/16-1 (106). SW620 CRC cells transfected with miR-15a/16-1 exhibit decreased lung colonization after tail vein injection (106). An inverse correlation between expression of AP4 and miR-15a/16-1 has been noted in primary CRC (106).

miR-30c (Figure 3A) inhibits proliferation, invasion, migration and EMT of CRC cells (HCT-116, LoVo and SW620) (107). HCT-116 cells transfected with miR-30c show a decrease in tumor size after subcutaneous implantation into nude mice and inhibition of lung colonization after tail vein injection (100). ADAM19 was identified as a direct target of miR-30c (107). A disintegrin and metalloproteinase 19 (ADAM19) is involved in promotion of invasiveness of tumors by degradation of components of the ECM and has been shown to be up-regulated in renal carcinoma and brain tumors (108-110).

IL6 induces EMT and invasion of CRC cells, such as DLD-1. This phenomenon is mediated by repression of miR-34a (Figure 3A) by signal transducer and activator of transcription 3 (STAT3) (111). DLD-1 cells treated with IL6 *ex vivo* exhibit lung colonisation after tail vein injection into nude mice (111). IL-6R has been identified as a direct target of miR-34a (111). Down-regulation of STAT3 or IL-6 receptor (IL-6R) by small interfering RNA (siRNA) in SW620 CRC cells suppresses lung metastasis in nude mice after tail vein injection (111). An IL-6R/STAT3/miR-34a feedback loop is manifest in carcinoma cell lines with mesenchymal characteristics and activation of this feedback loop is associated with metastasis of CRC (111). In a mouse model of colitis-associated CRC cancer, induced by azomethane and dextran sulfate (112), knock-out of miR-34a results in invasive tumors that penetrate through the muscularis mucosa and may ultimately result in metastasis (111). Activation of IL6/IL6R pathway can lead to metastasis and inhibition of the IL6/IL6R/STAT3 pathway has been shown to inhibit CRC growth and metastasis (113, 114).

miRs targeting ZEB2. miRs-192, -218 and -335 (Figure 3A) target ZEB2, miR-218 also targets N-cadherin and miR-335 in addition to ZEB2 targets BCL2 directly and vascular endothelial growth factor-A (VEGF-A) indirectly (55, 115, 116). In an orthotopic mouse model, HCT-116 cells transfected with miR-192 exhibited sixfold reduction of incidence of liver metastasis, whereas growth of primary tumors after subcutaneous implantation was only modestly affected (115). Nude mice injected with SW620 cells expressing miR-218 into the tail vein live significantly longer than animals injected with control cells (55). miR-218 transfected SW620 cells also exhibit reduced ability to metastasize into the lungs and liver of chicken embryos (55). SW620 cells transfected with miR-335 show a decrease of lung and liver metastases after tail vein injection (116). ZEB2 is a member of the homeodomain transcription factors with 9 zinc fingers and one homeodomain (117). ZEB-2 binds to regulatory gene sequences at E-boxes and functions as a transcriptional activator and repressor by inhibition of epithelial genes and activation of mesenchymal genes (117). In CRC, induction of EMT functions as a culprit of metastases (118). EMT-related functions of ZEB-2 are mediated by its interaction with R-SMAD, an effector of TGF β signaling (119). It has been shown previously that silencing of ZEB-2 by siRNA decreases migration and invasion of CRC cell lines (120). EMT enables CRC cells to migrate through the ECM and colonize lymph/blood vessels, thereby initiating the first step of the metastatic cascade (121). For miR-192 an inverse relationship between miR-192 expression and ZEB-2, BCL2 and VEGF-A in CRC biopsies has been detected (115). Reduced expression of miR-218 has been noted in CRC biopsies in comparison to corresponding normal tissues (56). Low expression of miR-335 is associated with shorter survival in CRC patients (116).

miRs targeting redox-related miRs. miR-200b-3p (Figure 3A) inhibits growth of LoVo CRC cells transfected with miR-200b-3p after subcutaneous implantation and metastasis to the liver in an orthotopic model with this cell line (122). miR-200b-3p inhibits EMT and is inhibited by c-MYC (122). Peroxiredoxin 2 (PRDX2), an antioxidant enzyme which reduces peroxides, has been identified as a target of miR-200b-3p (122, 123). It has been shown that PRDX2 silencing contributes to metastasis due to decreased c-MYC expression by down-regulating WNT/ β -catenin signaling (124). Furthermore, miR-200b-3p disrupts c-MYC protein stability through inhibition of the AKT2/ glycogen synthase kinase 3 β (GSK3 β) pathway (122). miR-200b-3p is down-regulated in metastatic CRC cell lines and CRC biopsies compared to their normal counterparts (124).

Ectopic expression of miR-212 (Figure 3A) inhibits EMT transition in HCT-116 and SW480 cells (125). Liver metastasis of these cells is inhibited after injection into the

spleen of nude mice (125). Manganese superoxide dismutase (MnSOD) has been identified as a direct target of miR-212 (125). MnSOD is a mitochondrial anti-oxidant enzyme which dismutates superoxide radicals into either oxygen or hydrogen peroxide and protects CRC cells from tumor necrosis factor related apoptosis inducing ligand (TRAIL)-induced apoptosis. Furthermore, SOD promotes anoikis resistance and tumor metastasis (126, 127). It was hypothesized that overexpression of MnSOD in CRC cells leads to a greater hydrogen peroxide flux which leads to accumulation of hypoxia-inducible factor-1 α (HIF-1 α) which in turn confers EMT traits to CRC cells (128). HIF-1 α can induce aggressive malignant traits in tumor cells independent of its canonical transcription factor function (129). Low level of miR-212 expression is associated with an aggressive and poor prognostic phenotype in CRC patients (56).

miRs targeting cell-cycle related proteins. miR-374a (Figure 3A) inhibits proliferation and invasion of miR-374a transfected HCT-116 cells (130). Subcutaneously implanted SW620 cells transfected with miR-374a give rise to markedly smaller tumors and fewer metastasis after intrahepatic injection (130). miR-374a decreases EMT-markers such as N-Cadherin, ZEB1, vimentin, SLUG and TRAIL (130). Cyclin D1 (CCND1) was identified as a direct target of miR-374a (130). CCND1 has been shown to suppress the PI3K/AKT pathway (130). PI3K/AKT pathway can promote EMT transition (131, 132). CCND1 forms a complex and functions as a regulatory subunit of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) which are required for cell-cycle G1/S transition (131, 132). High miR-374a with a low level of CCND1 expression correlates with a good prognosis in CRC patients (130).

miRs targeting other mRNAs. miR-22 (Figure 4) suppresses cell proliferation, colony formation, migration and invasion of CRC cell lines SW620 and LoVo (133). Subcutaneously implanted SW620 cells transfected with miR-22 exhibit decreased cell growth in comparison to the control cell line. Tail vein injection of these cells give rise to reduced numbers and size of lung nodules (133). Specificity protein 1 (Sp1) has been identified as a direct target of miR-22 (133). Sp1 is a member of the transcription factor family, promotes growth, migration and invasion of CRC cells and plays an important role in CRC development and progression (134, 135). miR-22 suppresses the phosphatase and tensin homolog (PTEN)/AKT pathway by targeting Sp1 expression (133). PTEN is a key tumor suppressor that antagonizes the PI3K/AKT pathway, which is highly involved in CRC cancer progression (136-138). From a mechanistic point of view, Sp1 can inhibit PTEN expression by binding to the PTEN promoter region (139). Down-regulation of miR-22 is associated with disease progression and metastasis in CRC patients (133).

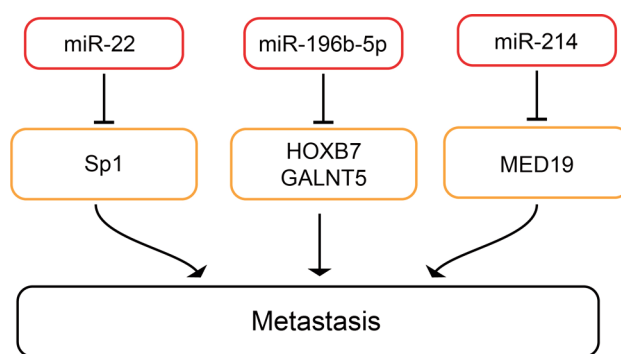


Figure 4. miRs modulating other targets with efficacy in metastasis-related colorectal cancer in vivo models. Down-regulated miRs are boxed in red, targets are boxed in yellow. GALNT5: Polypeptide N-acetylgalactosaminyltransferase 5; HOXB7: homeobox protein B7; MED19: mediator 19; Sp1: specificity protein 1.

miR-196b-5p (Figure 4) inhibits proliferation and invasion of a panel of 8 CRC cell lines and its inhibition in HCT-116 CRC cell line inhibits intra-abdominal metastasis (140). Homeobox protein B7 (HOX B7) and N-acetylgalactosaminyltransferase 5 (GALNT5) have been identified as targets of miR-196b-5p (140). Transcription factor HOX B7 promotes proliferation and tumorigenic growth of human CRC cells both *in vitro* and *in vivo* (141). GALNTs are involved in modulating ECM composition and affecting cell adhesion and numerous pathways in tumor formation and cancer progression (142). Low levels of miR-196b-5p in CRC biopsies correlate with bad prognosis (140).

miR-214 (Figure 4) inhibits proliferation and invasion of six CRC cell lines and its expression is up-regulated by forkhead box P3 (FOXP3) (143). *In vivo*, SW480 cells transfected with miR-214 give rise to fewer and smaller lung metastatic nodules after tail vein injection in comparison to the control cell line (143). miR-214 directly targets mediator 19 (MED19), a component of the mediator complex (143). Mediator is a multiprotein complex, which transmits signals from transcription factors to RNA polymerase II and directly interacts with these components (144, 145). The role of mediator components MED1, MED12, MED28, cyclin C and cyclin-dependent kinase 8 (CDK8) in malignancy are well documented (145). MED19 binds to gene-specific regulatory factors and provides support for the basal RNA polymerase II activity and its inhibition decreases growth of hepatocellular carcinoma, prostate cancer and osteosarcoma cell lines (146-149). With respect to clinical correlations of miR-214, it was found to be down-regulated in CRC biopsies and to be correlated with lymphatic metastasis, probably by promoter hypermethylation (143).

miR-520c (Figure 1) significantly inhibits migration and invasion of HCT-116 and SW620 cells and targets S100

calcium-binding protein A4 (S100A4) (150). Intraspinal injection of HCT-116 cells transfected with miR-520c results in reduction of TG and liver metastases in comparison to the control cell line (150). S100A4 is a member of the S100 calcium binding protein family which interacts with receptor of advanced glycation end products (RAGE) and has been classified as a metastasis-related protein which is also referred to as metastasin (151-153). S100A4 is an activator of proliferation and invasion-promoting WNT signaling (154). Furthermore, S100A4 promotes metastasis by interaction with myosin and as a promoter of angiogenesis (155, 156). S100A4 has been identified as a prognostic marker for poor survival of CRC patients (157, 158). An important clinical aspect is down-regulation of miR-520c in CRC biopsies due to hypermethylation of its promoter region (150).

Therapeutic Aspects

Recent studies have identified several targets for treatment of CRC-related liver metastasis (17). Apoprotein B mRNA editing enzyme 3G (APOBEC 3G), cluster of differentiation 133 (CD133), chemokine receptor 4 (CXCR4), L1 cell adhesion molecule (L1 CAM), lipase C (lipase C), metastasis associated in colon cancer-1 (MACC1), hepatocyte growth factor (HGF) and tyrosine kinase MET (c-MET), phosphatase of regenerating liver 3 (PRL3), tumor-associated signal transducer 2 (Trop-2), S-family proteins S100A4 and S100 P and notch receptors are targets under preclinical and clinical evaluation for treatment of CRC-related liver metastasis (17). In order to extend this panel of targets, we surveyed the literature for miRs, which modulate formation of CRC-related metastasis to distant organs.

We have identified 12 up-regulated and 19 down-regulated miRs conferring *in vivo* efficacy in CRC-related models. The vast majority of identified miRs (n=16) contributes to modulation of EMT. ZEB2 as a single target or in combination with BCL2 or N-Cadherin is targeted by miRs -192, -218 and -335 and is the most prevalent target in the EMT-related category. ADAM19, IκBα, FOXN3, HOXA10, AP4, CEBP2, IL6R, CCND1, PRDX2, MnSOD, SETD2 and SIAH1 were identified as further EMT-modulating targets. Noteworthy, PRDX2 and MnSOD are proteins with anti-oxidative function. Inhibition of proteins, which negatively affect signaling pathway by miRs is another theme emerging from survey of the literature. Examples of EMT-related targets are WIF-1 (miR-181a), an inhibitor of WNT/β-catenin signaling and SMAD7 (miR-1269a and -4775), an inhibitor of TGFβ signaling. A further category of identified miRs affects tumor- or metastasis suppressing mRNAs such as MTSS1 and Klotho (miR-15b), DAPK4 (miRs-103, -107), SATB2 (miR-182), PDCD4 (miR-499-5p) and S100A4 (miR-520c). Another subclass of identified miRs promotes or interferes with signaling pathways such as KRAS which is

mutated in 40% of CRC. KRAS is inhibited by miRs let-7c and -384. Another subclass of identified miRs target transmembrane tyrosine kinase receptors such as FGFR1 (miR-214) and IGFR1 (miR-493). Further signal-transducing receptors identified by our survey are integrins α6 and β1 (miR-30e-5p), CXCR4 (miR-126) and TLR2 (mi-143). Other identified targets do not match with the target categories as described above. These are Sp1 (miR-22), HOXB7 and GALNT5 (miR-196b-5p) and MED19 (miR-214). Depending on the context, miR-196-5p can mediate EMT by targeting IκBα (Figure 3B) or metastasis by targeting HOXB7 and GALNT5 (Figure 4).

With respect to therapeutic intervention, up-regulated miRs are candidates for inhibition of function and down-regulated miRs are candidates for reconstitution of function. miR-inhibitors are referred to as antagomirs and are single-stranded RNA molecules in the range of 12-25 nucleotides complementary to the target mRNA or miR sponges that are constructs with multiple reiterated miR binding sites which compete with the natural mRNA target for binding to the corresponding miR (23-25). The alternative therapeutic option, functional reconstitution of corresponding miRs, can be performed with miR mimetics, small double-stranded RNAs designed to mimic endogenous mature miRs (23-25). Alternatively, miR function can be reconstituted through expression of the corresponding miR by appropriate vectors or small double-stranded RNA mimetics (23-25). Since the approaches for identification of miRs involved in metastasis of CRC rely on very similar methods and neither inhibition or reconstitution of identified miRs has been translated into clinical studies, a prioritization of their therapeutic potential is presently not possible. The same holds true for their cognate targets. However, there are many critical issues associated with treatment of metastatic disease with miR-related agents which are not discussed in this review and are summarized in (32-34). For therapeutic intervention there are two concepts: preventive treatment before the tumor has colonized the parenchyma of distant organs such as the liver and treatment of established metastases. Preventive treatment is a critical issue due to potential toxicity issues associated with long-term treatment. Treatment of distant metastases with miR-related agents should result in a cytotoxic effect on metastatic disease, alone or in combination with other agents. Many problems are associated with the treatment of distant metastases such as their heterogeneity, their tortuous vasculature, elevated hydrostatic pressure causing poor drug penetration and resistance against drugs, which have an effect on the primary tumor (159-161). Proof-of-concept for treatment of metastatic disease has been shown with alpha particles emitting radionuclides alone or linked to monoclonal antibodies and immuno-therapy with antibodies directed against immune checkpoint targets such as PD-1, PD-1L and CTLA4 (159-162). In preclinical models, development and

growth of metastases can be monitored by non-invasive imaging methods such as bioluminescence or MRI (159). Metastasis modulating capability of other miRs are evaluated in the experimental metastasis model (tail vein injection) (n=11: 15a/16-1, 22, 34a, 126, 143, 214, 218, 335, 499-5p, 1269a and 4775), spontaneous metastasis after subcutaneous implantation (n=3: 30c, 182, 544a), intrasplenic injection and dissemination to the liver and peritoneum (n=8: 15b, 181a, 196a-5p, 212, 214, 384, 493, 520c), orthotopic implantation into the caecum (n=5: 103/107, 192, 200b-3p, 885-5p, 1269a), intrahepatic metastasis assay (n=1, 374a) and the CAM assay in chicken embryos (n=6: let-7c, 30e-5p, 135b, 182, 210, 218). Of note: miRs -218 and -1269a have been evaluated each in two *in vivo* models. A sub-category of identified miRs affects tumor growth in addition to modulating metastasis (n=9: let-7c, 30c, 30e-5p, 103/107, 126, 200b-3p, 214, 374a and 499-5p). The steps of the metastatic cascade affected by the corresponding miRs are not defined and a reduction of metastatic nodules in distant organs could be due modulation of intra- and extravasation or colonization of distant organs. An impact of corresponding miRs on the size of the nodules might be an indicator of modulation of growth of distant metastases, but modulation of intra- and extravasation, or colonization can not be excluded. Evaluation of CRC-related *in vivo* models with established liver metastasis and treatment with miR-related agents is not addressed in the *in vivo* models described in this review and should be investigated in future *in vivo* models (163). Noteworthy, 17 of the described miRs are associated with prognosis in patients with CRC (let-7c, miRs- 15b, 103/107, 143, 181, 182, 196a-5p, 212, 214, 335, 374a, 384, 499-5p, 520c, 544a, 1269a, 4775).

In the near past, miR-based therapeutic agents have witnessed serious drawbacks in clinical studies addressing kidney disease, hepatitis C infection and cancer, mostly due to serious side-effects (164). Clinical evaluation of second-generation miRs with an improved side-effect profile is underway. In August 2018, the FDA has approved an RNAi drug, Onpatro (Alynham Pharmaceuticals), acting on the liver for treatment of patients with transthyretin amyloidosis and polyneuropathy (25). Multiple RNAi-based drug candidates are in Phase I, II or III clinical studies for renal, liver and ocular indications. The next upcoming years will show whether miR-based agents will play a game changing role in oncology and metastatic disease.

Conflicts of Interest

UB and SA are and UHW was an employee by Roche.

Authors' Contributions

UHW, UB and SA jointly prepared the manuscript and the figures.

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