CANCER GENOMICS & PROTEOMICS *16*: 1-19 (2019)
doi:10.21873/cgp.20108

Review

The Functional Role of Prostate Cancer Metastasis-related Micro-RNAs

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Abstract. *The mortality of patients with hormone-resistant prostate cancer can be ascribed to a large degree to metastasis to distant organs, predominantly to the bones. In this review, we discuss the contribution of micro-RNAs (miRs) to the metastatic process of prostate cancer. The criteria for selection of miRs for this review were the availability of preclinical in vivo metastasis-related data in conjunction with prognostic clinical data. Depending on their function in the metastatic process, the corresponding miRs are up- or down-regulated in prostate cancer tissues when compared to matching normal tissues. Up-regulated miRs preferentially target suppressors of cytokine signaling or tumor suppressor-related genes and metastasis-inhibitory transcription factors. Down-regulated miRs promote epithelial–mesenchymal transition or mesenchymal–epithelial transition and diverse pro-metastatic signaling pathways. Some of the discussed miRs exert their function by simultaneously targeting epigenetic pathways as well as cell-cycle-related, anti-apoptotic and signaling-promoting targets. Finally, we discuss potential therapeutic options for the treatment of prostate cancer-related metastases by substitution or inhibition of miRs.*

In 2017, 160,000 new cases of prostate cancer (PC) were diagnosed in the US and an equal number in the EU with 27,000 patients dying due to metastatic PC in the US and the EU (1). Localized disease can be potentially cured by therapies such as surgery and radiation, but in some patients, the disease progresses despite these therapies (2). Androgen-deprivation therapy leads to long-lasting responses between 2 and 3 years, however the disease inevitably progresses to castration-resistant prostate cancer (CRPC) that is associated with metastatic disease and poor prognosis. Over the past 5 years, five drugs have been approved for treatment of CRPC (2). These are cabazitaxel, a taxane-based cytostatic; radium 223, an α-emitter; enzalutamide, a small-molecule androgen receptor (AR) antagonist; abiraterone, a blocker of *de novo* synthesis of androgens; and Sipuleucel, a dendritic cell-based immunotherapy (3).

The recent genetic classification of PC has revealed seven subtypes and new molecular targets for molecular intervention such as v-ets erythroblastosis virus E26 homolog (*ETS*) family transcription factor-based fusion proteins as well as mutations in speckle-type POZ protein (*SPOP*), forkhead protein A1 (*FOXA1*) and isocitrate dehydrogenase 1 (*IDH1*) (4). The serine protease inhibitor Kazal-type 1 (*SPINK*), which is up-regulated in *ETS*-rearrangement-negative tumors, is also being pursued as a new target for treatment of CRPC (5). Nucleic acid-based therapy is another approach for treatment of CRPC. Custirsen, a second-generation phosphorothioate antisense oligonucleotide targeting clusterin mRNA is presently undergoing phase III clinical studies in combination with chemotherapy in patients with CRPC (6, 7). Clusterin is up-regulated in patients with CRPC and functions as an ATP-independent molecular chaperone with anti-apoptotic function through inhibition of pro-apoptotic B-cell lymphoma virus-associated X (BAX) by altering its conformation (6, 7). In this review, we focus on micro-RNAs (miRs), another class of targets for nucleic acid-based therapy, their role in PC metastasis, and their potential function as therapeutic targets. We exclusively discuss miRs

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Key Words: In vivo metastasis models, interference with signaling networks, miR substitution and inhibition, prognostic aspects, pro-metastatic pathways, target validation, review.

validated by *in vitro* and *in vivo* preclinical experiments and exclude those which are in earlier stages of preclinical development.

Process of PC Metastasis

PC metastasis can be dissected into several functional steps (8-12). The metastatic process starts with cancer cells at the tumor edge undergoing epithelial–mesenchymal transition (EMT) associated with acquiring invasive properties, intravasation as single cells or multicellular aggregates, activation of survival programs, protection against attack by immune cells and finally extravasation and colonization of distant organs with a high preference for bone (12). Stromal-derived factor 1/C-X-C receptor 4 (SDF1/CXCR4) interactions are a prerequisite for bone tropism of PC cells and for access to metastatic niches in the bone marrow (13). The disseminated tumor cells undergo bi-directional interactions with bone-forming cells (osteoblasts), bone-degrading cells (osteoclasts) and other cells in the tumor microenvironment. The metastatic niche is populated by androgen-independent and chemotherapy-resistant cancer stem cells (CSCs), which are supported by mesenchymal cells with respect to growth and survival, while CSCs contribute to recruitment of cancer-associated fibroblasts (14). Furthermore, angiogenesis is essential for the outgrowth of bone metastases. Osteoblastic, osteoclastic and mixed lesions have been identified as PC-related metastases due to the imbalance of osteoblast-mediated bone formation and osteoclast-mediated bone resorption (9, 10). Very often, bone metastases display both osteoblastic and osteolytic elements.

An essential component of bone destruction and osteolytic metastasis is the axis formed by the receptor activator of nuclear factor-κB (NFκB)/receptor activator of NFκB (RANK)/RANK ligand (RANKL)/osteoprotegerin OPG) and members of the tumor necrosis factor (TNF)/tumor necrosis factor receptor (TNFR) superfamily (9, 11).Tumor cell-secreted parathyroid hormone, parathyroid hormone-related protein, interleukin1 (IL-1), IL-6 and RANKL mediate activation of osteoclasts and thus facilitate bone destruction (9, 11). RANK/RANKL interactions result in the activation of NFκB signaling and stimulate final effectors of osteolysis such as carboanhydrase II, H+ ATPase and cathepsin K. A vicious cycle is initiated during which osteolysis factors such as transforming growth factor β (TGFβ), insulin-like growth factor 1 (IGF1) and Ca^{2+} are released, which promote tumor cell proliferation and production of parathyroid hormone-related protein. Osteoblastic metastases are stimulated by factors, which promote osteoblast activity such as fibroblast growth factor (FGF), RANKL, platelet-derived growth factor, IGF1, and endothelin 1 (9, 11). TGFβ is activated from latent TGFβ; IGF1 can be released from inhibitory IGF binding proteins; and the osteolytic factor parathyroid hormone-related protein can be inactivated by proteases such as urokinase (9). Bone metastasis is associated with skeleton-related events such as intractable pain, bone fractions, spinal cord suppression, neurological deficits and paralysis (15).

Micro RNAs

miRs are small noncoding RNAs with a length between 18 and 25 nucleotides. They are transcribed in the nucleus as primary pre-miRs that are capped, spliced and polyadenylated. Thirty percent of miRs are processed from introns of protein-coding genes, the others are encoded by dedicated loci (16-18). Primary pre-miRs are cleaved by a complex called the microprocessor to 60- to 70-nucleotide hair-pin looped pre-miRs, which are exported to the cytoplasm by exportin 5 and subsequently are processed by multi-protein complex DICER to produce mature miRs. One strand of the mature miR (guide strand) is loaded into the miR-induced silencing complex to target mRNA by sequence complementarity. This interaction results in gene suppression by targeted mRNA degradation or translational repression in processing bodies (18). More than 1,000 miR genes have been identified and a single miR can target hundreds to thousands of mRNAs, while a single gene can be targeted by multiple miRs (19). Therefore, miRs can function as regulators of complex signaling networks.

The importance of miRs in cancer was demonstrated by correlating the deletion of *miR15/miR16* with chronic lymphocytic leukemia and its induction in a mouse model by deletion of *miR15/miR16* (20). Furthermore, miRs are involved in pro-oncogenic and tumor-suppressive pathways in a context-dependant manner (21). The crucial role of miRs in metastasis has been demonstrated for breast cancer (22, 23), ovarian cancer (24), colorectal cancer (25), and hepatocellular carcinoma (26). Here, we focus on the role of specific miRs involved in the metastatic process of PC.

Prostate Cancer Cell Lines and *In Vivo* Models

In this review, we describe PC metastasis related miRs which have been validated in at least one *in vivo* model. We excluded miRs with metastasis-related prognostic impact, but lacking *in vivo* validation in a PC-related metastasis model.

Several subtypes of PC such as adenocarcinoma, transitional cell, squamous cell and small cell carcinoma (neuroendocrine) have been described (27). The most abundant subtype is prostate adenocarcinoma (≥90%) and consequently most PC cell lines used for *in vitro* and *in vivo* studies correspond to this phenotype. The most commonly used PC cell lines are DU-145, PC3, LNCaP, C42B, LAPC4, VCaP and 22Rv as well as BPH1, PIN, RWE-1,2 as

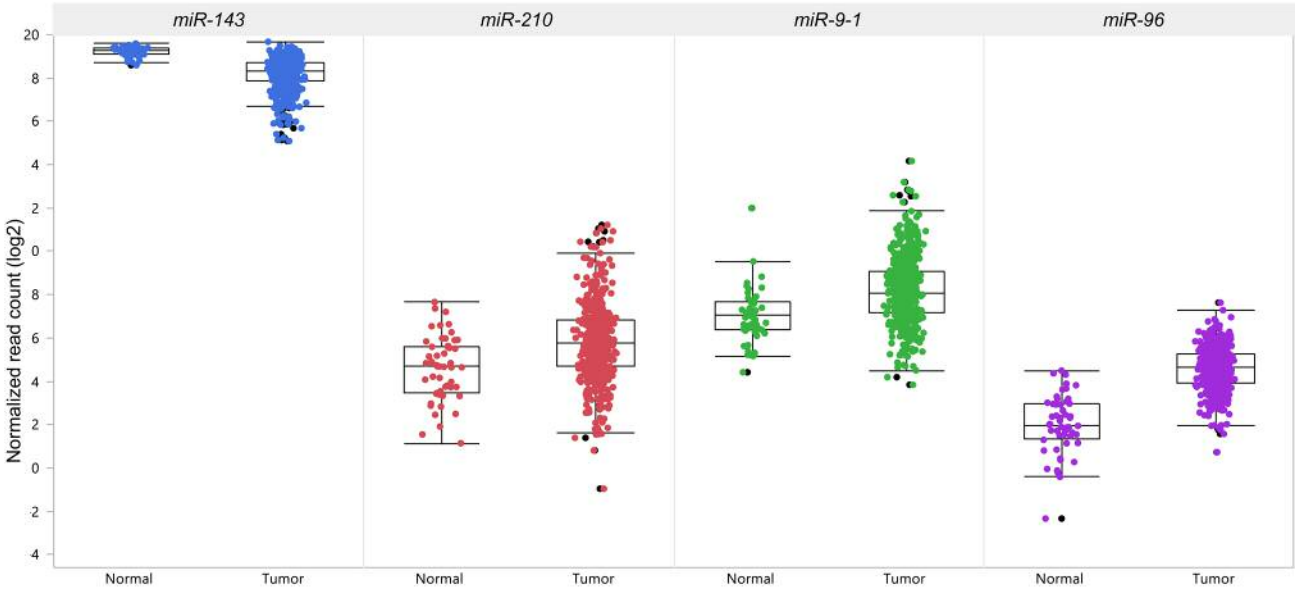

Figure 1. *Steady-state levels of miR-143, miR-210, miR-9 and miR-96 in prostate cancer and corresponding normal tissues. A total of 52 matched normal prostate and 495 prostate tumor samples were investigated. Expression as measured by RNA sequencing in The Cancer Genome Atlas prostate adenocarcinoma cohort are shown as log_2 Normalized Read Count values as provided by Broad's FIREHOSE service. Expression data are shown as box plots where the black line represents the median value and the black rectangles indicate the upper and lower 25% quartiles. Therefore, 50% of all data points are included in the black rectangle. All other data points, except for outliers, are located within the upper and lower whiskers.*

immortalized prostate cells. The generation and functional characterization of these cell lines is described in detail in (28). For functional *in vivo* metastasis-related studies, several standard *in vivo* models are used (28-30). Hematogenous metastasis-mimicking models are based on either the injection of PC cells into the tail vein of mice and monitoring the development of lung metastases, or on intracardiac tumor cell inoculation and following-up of their metastasis to distant organs. Spontaneous metastasis can be assessed by subcutaneous implantation or by orthotopic prostatic implantation. Injection of PC cells into the bones mimics the growth of PC cells in the bone microenvironment, but is not a model of metastasis *per se* (28, 29). Vossicle models are based on implantation of vertebrae from transgenic mice together with PC cells into athymic mice to study the effects of genetic alterations in the tumor microenvironment on tumor development and metastasis (28, 29). The transgenic adenocarcinoma of the mouse prostate (TRAMP) model was developed as the first *in vivo* transgenic model with spontaneous PC metastasis (30, 31). In this model, SV40 large T-antigen is expressed in prostate cells under the control of the rat probasin promoter. Metastasis to the lymph nodes, the lungs and occasionally to the kidney, adrenal glands and bones has been observed in this model.

miRs Promoting PC Metastasis

SOCS-targeting miRs (miR-9, miR-194, miR-210). miR-9 is up-regulated in 75% of patients with PC in comparison to corresponding normal tissues (33) and Figure 1. *miR-9* promoted the growth and invasion of the PC cell line M12. Subcutaneously injected M12 cells expressing a *miR-9* sponge showed reduced tumor growth. Intraprostatic injection of *miR-9* sponge expressing M12 cells revealed no metastatic sites in contrast to the control cell line (33). Cadherin 1 (CDH1) and SOCS5 were identified as direct targets of *miR-9* (33). Cleavage of CDH1 leads to accumulation of β-catenin in the nucleus, driving transcription of survival factors such as c-MYC and cyclin D1 (34). c-MYC induces additional *miR-9*. The second *miR-9* target, SOCS5, prevents phosphorylation of Janus kinase and signal transducer and activator of transcription 3 (STAT3) (35) (Figure 2A).

Serum levels of *miR-194* are higher in men with CRPC than those in men with localized disease (36). *miR-194* is induced by transcription factor GATA binding protein 2 (37, 38) and PC3 cells ectopically expressing *miR-194* showed significantly enhanced cell invasion in the chorionic allantoic membrane assay and exhibited spontaneous metastasis to visceral organs such as kidney, lung, liver and spleen, whereas control PC3 cells remained contained in the prostate (36). SOCS2, a

ubiquitin ligase, was identified as a direct target of *miR-194* (36). Inhibition of SOCS2 induced expression of pro-metastatic genes such as Fms-like tyrosine kinase 3 (*FLT3*) and *JAK1* through activation of extracellular regulated kinase (ERK)-and STAT3 signaling (39, 40) (Figure 2A).

miR-210-3p is elevated in PC, particularly in bone-metastatic PC, and promoted EMT after transfection into VCaP and C42B PC cells (41) and Figure 1. Silencing of *miR-210p* in PC3 cells reduced tumor burden and metastatic sites in bone after intracardial injection (41). *miR-210*-3p activated NF-κB signaling and the expression of metastasis-related genes such as twist family bHLH transcription factor 1 (*TWIST1*), matrix metalloproteinase 13 (*MMP13*) and *IL11* (41). The molecular basis for activation of NFκB signaling is the direct inactivation of SOCS1 and TNFα-induced protein 3 interacting protein 1 (TNIP1), both inhibitors of NFκB signaling (42-44). SOCS1 suppresses NFκB signaling by binding to DNA-bound p65 and induces its degradation (45). TNIP1 suppresses NFκB signaling through deubiquitinylation of NFκB essential modulator (44) (Figure 2B).

miRs targeting tumor-suppressor genes (miR-96, miR-154, miR-379 and miR-409). Several observations have demonstrated the involvement of *miR-96* in PC invasion and metastasis. miR-96 is transcriptionally regulated through nuclear epidermal growth factor (EGFR) and directly targets tumor-suppressor transcription factor ETS variant 6 (ETV6) (46-49). In several hematological malignancies, *ETV6* is inactivated by a mutation (50). ETV6 is inversely correlated with EGFR–*miR-96* signaling in clinical samples. A reduction of metastasis to the brain and bone has been observed after cardiac injection of RasB1 PC cells ectopically expressing ETV6 (46). Furthermore, *miR-96* directly targets forkhead box O1 (FOXO1), a tumor-suppressive transcription factor (50). FOXO1 enhances apoptosis, reduces proliferation and inhibits androgen-dependent and -independent activity of AR (51-54). In addition, it has been shown that TGFβ induces *miR-96* through SMAD family-dependent transcription and inactivation of AKT serine/threonine kinase 1 substrate 1 (*AKT1S1*) mRNA, a negative regulator of *miR-96* (55). *miR-96* was shown to promote bone metastasis in a PC model (55) (Figure 2B). In addition, dose-dependent regulation of hypoxia-induced autophagy by *miR-96* has been documented (56). miR-96 was found to be up-regulated in PC samples in comparison to matched normal prostate tissues (Figure 1).

Delta-like1 homolog deiodinase iodothyronine 3 (DLK-DIO3) cluster-related miRs *miR-409-3p, miR-5p, miR-154** and *miR-379* are activated in embryonic stem cells (57). *miR409-3p* and *miR-409-5p* were found to be activated in PC with high Gleason scores and expression levels of *miR-409*-3p correlated with disease-free survival (57, 58). *miR-154** and *miR-379* were also found to be highly expressed in PC and *miR-379* expression correlated with progression-free survival in patients. miRs of this cluster share a couple of common functional features with subtle differences which are not discussed in detail here. Functional studies have identified them as mediators of EMT-related changes of PC cells towards spindle-shaped cells, reduced expression of E-cadherin and increased expression of vimentin (57, 58). Ectopic expression of *miR-409*-3p/5p in the prostate gland transformed normal prostate epithelial cells, and promoted tumorigenicity, EMT and stemness *in vivo* (58). Mice inoculated intracardially with metastatic ARCaP cells had 100% incidence of bone metastasis, whereas in mice bearing cells transfected with a *miR-409*-3p/5p inhibitor did not develop metastases within 15 weeks. Similarly, inhibitors of *miR-154** reduced bone and soft-tissue metastases in the ARCaP model (58). Several tumor-suppressor genes are inhibited by miRs of this cluster: stromal antigen 2 (*STAG2*) (59, 60), ras suppressor protein 1 (*RSU1*) (61), retinoblastoma like 2 (*RBL2*) (62), nitrogen permease regulator like 2 (*NPRL2*) (63), polyhomeotic homolog 3 (*PHC3*), a polycomb group protein (64), von Hippel-Lindau tumor suppressor, an E3 ubiquitin ligase (65) and *SMAD7*, a signal transducer of TGFβ signaling (66). Several pro-metastatic pathways are activated: hypoxia-inducible factor-1α (HIF1α) through inhibition of its tumor suppressor E3 ubiquitin ligase function (65), inhibition of NPRL2 activates RAS and AKT signaling (63), RBL2 degradation induces the E2F pathway (62), deactivation of STAG2 promotes aneuploidy (59, 60) and several miRs of the cluster promote activation of polycomb proteins and osteoblastic pathways (57). The mode of action of *miR-154, miR-379, miR-409*-3p and *miR-409*-5p are summarized in Figure 3.

Targeting of intercellular adhesion molecule-1 (ICAM1) by miR-296-3p. Clinical tissue microarrays have revealed frequent up-regulation of *miR-296*-3p in PC patients and concomitant down-regulation of *ICAM1* (67). Knock-down of *miR-296*-3p in M12 PC cells reduced pulmonary metastasis in a model of experimental metastasis in nude mice (22). *miR-296*-3p inhibits ICAM1 by directly targeting its 3'UTR and is pivotal for the metastasis of PC9 cells to the lungs, probably by enhancing survival of circulating tumor cells by mediating resistance to natural killer (NK) cells (67). The described *in vivo* effects were abolished by a natural killer cell-eliminating anti-sialo GM1 antibody (67). ICAM1 has been identified as a co-stimulator of natural killer cell-mediated cytotoxicity (68, 69).

AR as a target of miR-301a. miR-301a is a predictor of PC metastasis, which is induced in tumor cells by adipocytes (70). It was shown that PC recruits more adipocytes than normal prostate tissues and *in vitro* enhanced pre-adipocyte recruitment increases PC cell invasion (70). Overexpression of *miR-301a* enhanced lung metastasis of LNCaP and PC3 cells after subcutaneous implantation into nude mice (70).

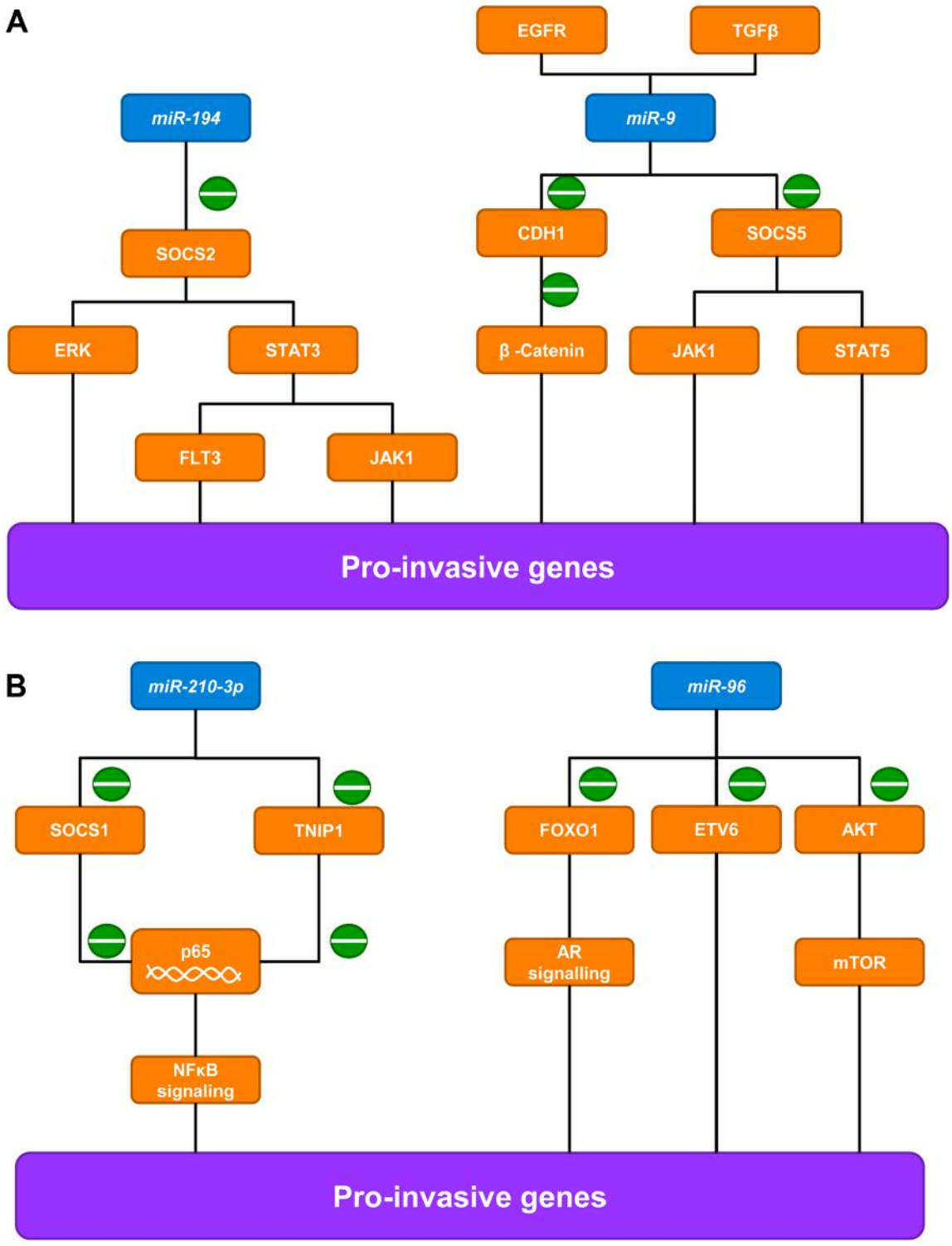

Figure 2. *miRs up-regulated in metastatic prostate cancer: A: miR-194 and miR-9; B: miR-210-3p and miR-96. Suppressors of cytokine signaling (SOCS) 1, 2, 5 are direct targets of miR-210-3p, miR-194 and miR-96. Other direct targets are cadherin 1 (CDH1), TNFα-induced protein 3-interacting protein (TNIP1), forkhead box 1 (FOXO1), ETS variant 6 (ETV6) and AKT8 viral oncogene cellular homolog (AKT). Inhibition of these targets by the corresponding miRs results in activation of pro-invasive genes. AR: Androgen receptor; EGFR: epidermal growth factor receptor; ERK: extracellular-regulated kinase; FLT3: FMS-like tyrosine kinase 3; JAK1: Janus kinase 1; mTOR: mechanistic target of rapamycin; NFκB: nuclear factor κB; p65: protein 65; STAT: signal transducer and activator of transcription factor; TGFβ: transforming growth factor β.*

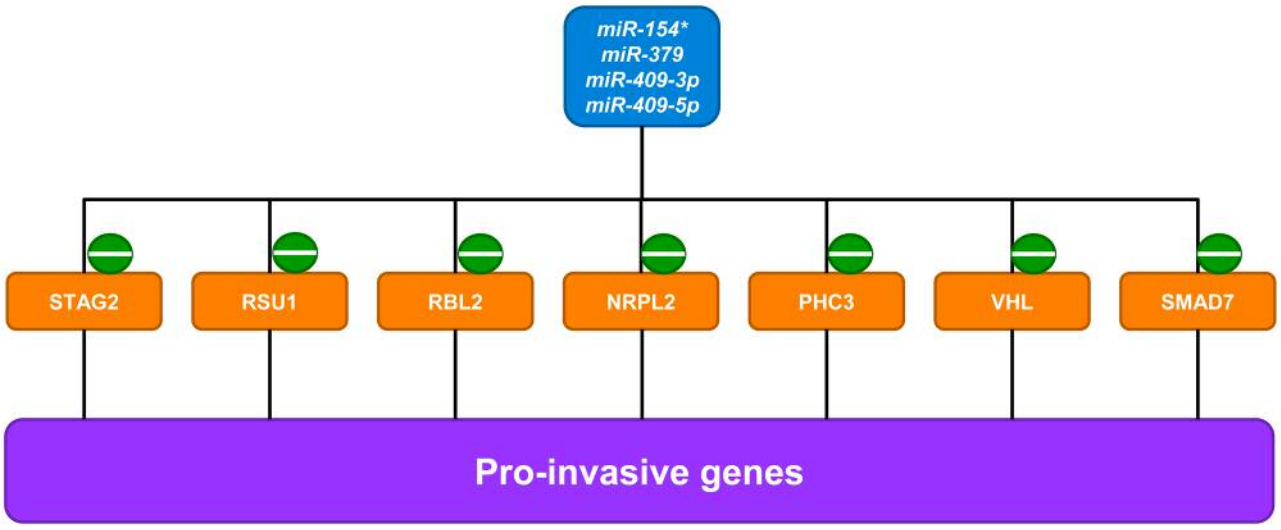

Figure 3. *miR-154*, miR-379, miR-409-3p and miR-405-5p target tumor suppressor genes. These miRs are up-regulated in metastatic prostate cancer and inactivation of tumor suppressor-related mRNAs results in the activation of pro-invasive genes. NRPL2: Nitrogen permease regulator-like 2; PHC3: polyhomeotic homolog 3; RBL2: retinoblastoma-like 2; RSU1: RAS suppressor protein 1; STAG2: stromal antigen 2, VHL: von Hippel Lindau gene.*

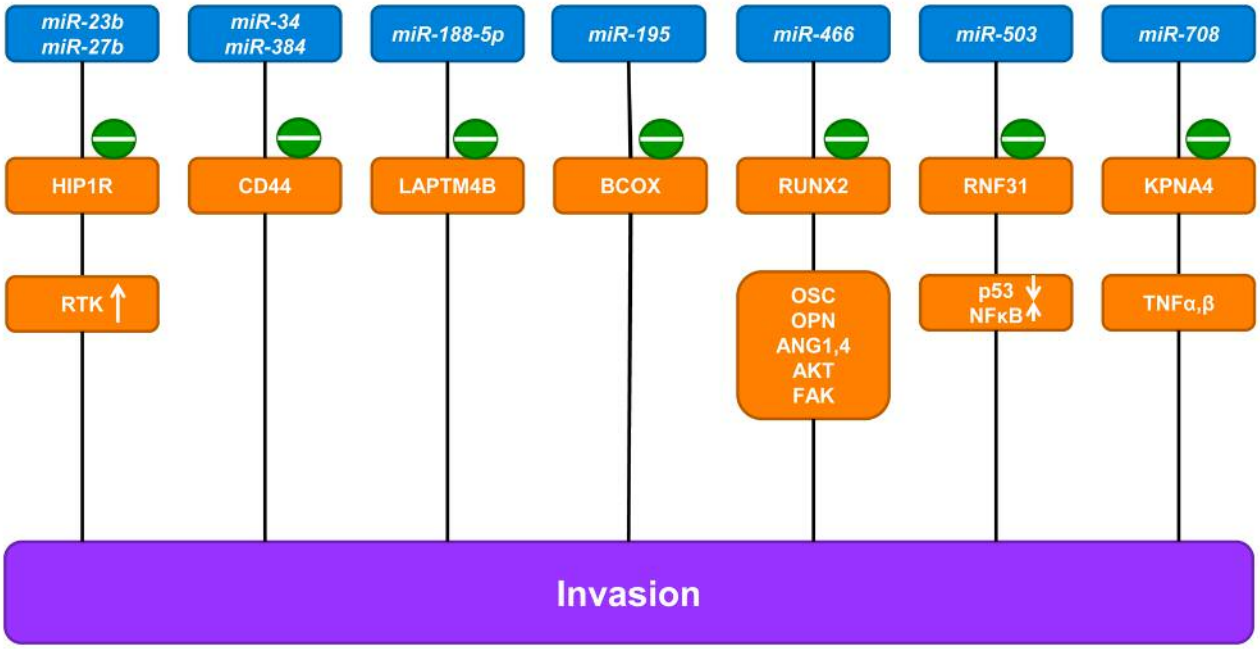

Figure 4. *Overview of miRs down-regulated in metastatic prostate cancer targeting mRNAs exerting different functions. These miRs include miR-23b, miR-27b, miR-34, miR-188-5p, miR-195, miR-384, miR-466,miR-503 and miR-708. AKT: AKT8 viral oncogene cellular homolog; ANG: angiopoietin; BCOX (KIAA0100): breast cancer overexpressed gene 1; CD44: cluster of differentiation 44; FAK: focal adhesion kinase; HIP1R: Huntington-interacting protein 1-related protein; KPNA4: karyopherin 4; LAPTM4B: lysosome transmembrane protein 4; NFκB: nuclear factor κB; OPN: osteopontin; OSC: osteocalcin; p53: protein 53; RNF-31: ring finger protein 31; RTK: receptor tyrosine kinase; RUNX2: RUNT-related transcription factor; TNF: tumor necrosis factor.*

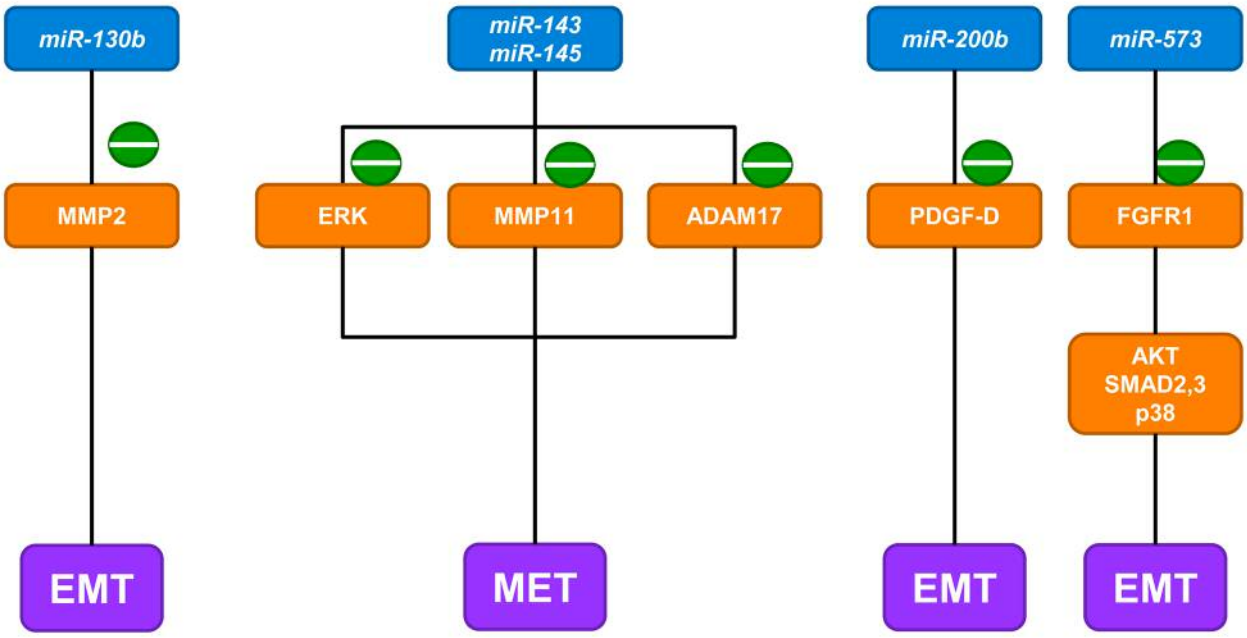

Figure 5. *miR-130b, miR-143, miR-145, miR-200b and miR-573 mediate epithelial–mesenchymal transition or mesenchymal–epithelial transition. These miRs are down-regulated in metastatic prostate cancer and cause epithelial–mesenchymal transition (EMT) or mesenchymal–epithelial transition (MET). AKT: AKT8 viral oncogene cellular homolog; ADAM 17: a disintegrin and metalloproteinase 17; platelet-derived growth factor D; ERK: extracellular-regulated kinase; FGFR1: fibroblast growth factor receptor-1; MMP: matrix metalloproteinase; p38: p38 mitogen-activated protein kinase.*

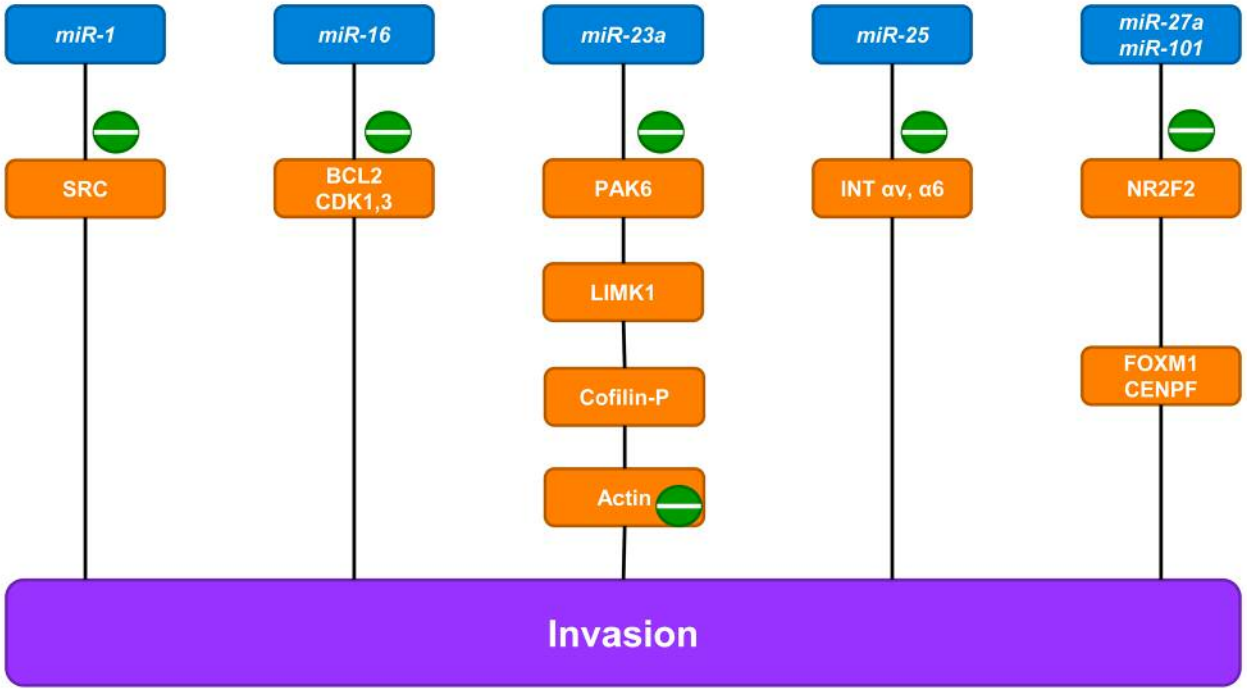

Figure 6. *miR-1, miR-16, miR-23a, miR-25, miR-27a and miR-101 interfere with cellular signaling mechanisms. These miRs are down-regulated in metastatic prostate cancer and target mRNAs which promote invasion. BCL2: B-Cell lymphoma 2, CDK: cyclin-dependent kinase; CENPF: centromer protein F precursor; Cofilin-P: phosphorylated cofilin; NR2F2: nuclear receptor subfamily 2, group F, member 2; FOXM1: forkhead box M1; INT: integrin; LIMK1: LIM kinase 1; PAK6: p21-activated kinase 6; SRC: tyrosine kinase SRC.*

Orthotopically engrafted PC CWR22Rv-1 cells gave rise to increased metastatic foci when co-implanted with immortalized pre-adipocytes. Mechanistically, the observed effects were due to the inhibition of AR by *miR-301* in tumor cells (70). AR has been identified as a negative regulator of TGFβ/SMAD/MMP9 signaling (71-75).

miRs Inhibiting PC Metastasis

miR-34a and miR-383 target CD44. It has been shown that PC stem cells express CD44 and confer tumorigenic properties on these cells (76, 77). CD44 has been identified as a direct target of *miR-34a* and *miR-383* in PC cells (78, 79) (Figure 4). In addition, *miR-373, miR-520c* and *miR-708* were shown to target CD44 and modulate *in vitro*-invasion of PC cells (80, 81). *miR-34a* inhibited clonal and clonogenic properties of PC cells (78). Tail vein injection of *miR-34a* into nude mice with orthotopic LAPC9 tumors inhibited lung metastasis and extended the survival of the animals without affecting tumor growth (78). Ectopic expression of *miR-383* suppressed the tumorigenicity of DU145, PC3 and LNCaP PC cell lines, while silencing of *miR-383* induced tumorigenicity of normal prostate epithelial cells (78). *miR-383* inhibited the capacity of $CD44^+$ PC cells to initiate tumors, and its ectopic expression in PC3-3M cells tagged with luciferase suppressed growth of xenografts *in vivo* (78). Inoculation of these cells expressing *miR-383* ectopically into the left ventricles of nude mice reduced metastasis (78). Thus, CD44 is a crucial mediator of metastasis (82). Low expression of *miR-383* is associated with poor survival outcome in patients with PC and an inverse correlation of expression of *miR-383* in PC tissues and lymph node metastasis has been found (78).

miR-573 targets FGF receptor R1 and attenuates EMT. miR-573 is down-regulated in PC and its expression levels inversely correlate with survival (83). *miR-573* is induced by transcription factor GATA-binding protein 3, a key factor in preventing PC progression through antagonizing AKT signaling (84). *miR-573* suppressed migration and invasion of VCaP and PC3 PC cell lines (83). VCaP transfected with *miR-573* gave rise to significantly fewer lung metastases compared to control cells after implantation into the dorsal flank of immuno-deficient mice (83). FGFR1 was identified as a direct target of *miR-573* (83). FGFR1 is a mediator of EMT and an activator of downstream signaling pathways such as AKT, SMAD2, SMAD3 and p38 mitogen-activated protein kinase (85) (Figure 5). An inverse correlation between expression levels of *miR-573* and FGFR1 has been found in prostate tumors (83).

miR-143 and miR-145 induce mesenchymal–epithelial transition (MET). Down-regulation of *miR-143* and *miR-145* is associated with tumor progression and metastasis (86). *miR-143* and *miR-145* were shown to induce transdifferentiation of mesenchymal cells towards epithelial cells (86). As shown with miR-transfected PC3 cells, both *miR-143* and *miR-145* inhibited growth of tibial injected cells and reduce the size of skeletal lesions (1). *miR-143* can interfere with extracellular signal-regulated kinase (ERK) signaling, which is involved in the MET pathway (87, 88), and *miR-145* has been shown to target MMP11, and a disintegrin and metalloproteinase17 (ADAM17) (89) (Figure 5). *miR-143* is down-regulated in prostate tumors when compared with matching normal prostate tissues (Figure 1).

miR-200b attenuates EMT. miR-200 was up-regulated by AR and reversed EMT in PC3 cells (90). *miR-200* induced an increase in expression of differentiation markers of prostate epithelium such as cytokeratins CK8 and CK18, suppressed mesenchymal features such as zinc finger and homeobox transcription factor1 (ZEB1) and reduced invasion of PC3 cells *in vitro* (90). Transfection of PC3 cells with *miR-200b* suppressed growth and angiogenesis of PC3 xenografts (90). Orthotopic injection of these cells dramatically reduced spontaneous metastasis as assessed by monitoring fluoresence. miR-200b also inhibited platelet-derived growth factor-D in PC3 cells (91) (Figure 5). From a mechanistic point of view, the direct targets of *miR-200b* need to be identified in further detail.

miR-130b targets MMP2. *miR-130b* is down-regulated in clinical PC specimens (92). Stable expression of *miR-130b* in M12 PC cells suppressed migration and invasion in wound-healing assays without affecting proliferation. Intracardiac injection of luciferase-tagged PC3 cells reduced tissue metastasis, as detected by bioluminescence. MMP2 was identified as a direct target of *miR-130b* (92) and acts as a promoter of metastasis through degradation of the extracellular matrix (93) (Figure 5).

miR-449a targets prostate leucine zipper gene (PrLZ). Down-regulation of *miR-449* is related to PC clinical stage and distant metastasis (94). *miR-449a* suppressed proliferation, promoted apoptosis and restricted invasion and tube formation by LNCaP and PC3 cells (94). *miR-449* suppressed tumor formation of subcutaneously implanted transfected PC3 cells and reduced the number of animals with tumor metastases (94). PrLZ has been identified as a direct target of *miR-449*. PrLZ is a member of the tumor protein D52 (TPD52) family, which is specifically expressed in prostate tumors and is associated with progression of PC (95-97).

miRs Interfering with Signaling

miR-25 targets integrin components. *miR-25* was down-regulated in aldehyde dehydrogenase (ALDH)-positive normal and transformed PC stem cells and steadily increased upon luminal differentiation (98). ALDH is a functional marker of

CSCs and progenitors (99). *miR-25* overexpression reduced integrin αv and integrin α6 in human PC cell lines and PC stem cells (98) (Figure 6). Integrins αv and α6 have been identified as pro-invasive integrins (100, 101). In PC stem cells, *miR-25* affected morphology and reduced migration, switching them to a less invasive phenotype due to cytoskeletal reorganization (98). The impact of *miR-25* on metastasis was monitored by injecting fluorescently labeled *miR-25* expressing PC3 cells into the circulatory system of embryonic zebrafish (98, 102). It was found that *miR-25* disrupted extravasation and colonization of distant sites *in vivo* (98).

miR-1 targets v-src sarcoma viral oncogene homolog (SRC). *miR-1* is reduced in PC compared with benign tissue and its expression further decreases with metastatic progression (103-105). *miR-1* was induced by AR and inhibits *in vitro* proliferation and *in vivo* growth of RASB1 cells, a cell line derived from experimental bone metastases of DU145 PC cells expressing mutated RAS V12G37 (103, 106). SRC was identified as a direct target of *miR-1* (103) (Figure 6). Bone and brain metastases of RASB1 cells transfected with *miR-1* were evaluated after intracardiac inoculation. Developing metastases were delayed in time from 3 weeks to 10 weeks, diminished in size and mainly found in the brain. Expression of SRC in *miR-1*-expressing RASB1 cells restored metastatic ability. Furthermore, it was shown that EGFR promotes PC bone metastases by down-regulating *miR-1* and activating the transcription factor TWIST1 (107). In this context, translocation of EGFR into nuclei and increased nuclear EGFR as well as reduced *miR-1* expression correlate with worse clinical outcome (107).

miR-16 targets BCL2 and cell-cycle-related genes. *miR-16* was identified in a functional screen for miRs which inhibit proliferation of 22Rv1 PC cells (108). Expression of *miR-16* was low in the majority of PC clinical samples in comparison to matching normal tissues. *miR-16* can induce apoptosis of PC cells by targeting BCL2 by inhibiting the translation of its mRNA (20) (Figure 6). Transfection of PC with *miR-16* down-regulated the expression of several cell cycle-related genes such as cyclin D3 as well as cyclin-dependent kinases 1 and 3 (108). *In vivo* bone metastasis of luciferase-tagged PC3 cells ectopically expressing *miR-16* cells was inhibited after intracardiac injection. A *miR-16* mimetic complexed to atelocollagen administered *i.v.* into mice inhibited metastasis into the thorax, jaws and legs, suggesting that systemic delivery of *miR-16* could be a novel strategy to inhibit prostate tumor growth in the bones (108).

miR-23a targets p21-activated kinase 6 (PAK6). *miR-23a* is reduced in PC cell lines and tissues in comparison to controls and low expression is associated with aggressive and poor prognostic PC phenotype (109). *miR-23a* suppressed invasion and migration of PC3, DU-145 and C4-2B PC cell lines *in vitro* (109). Intraprostatic injection of luciferase-tagged PC3 cells, transfected with *miR-23a* gave rise to fewer metastatic lesions as revealed by bioluminescence (109). *miR-23a* was found to dissolute stress fibers, suppress the formation of actin fibers (109) and inhibit PC cell motility (110, 111). PAK6 was identified as a direct target of *miR-23a* (109) (Figure 6). PAK6 directly phosphorylates LIM kinase 1 (112) and the latter phosphorylates cofilin (113, 114), suppressing stress fibers and actin filaments required for cell motility and invasion (110-112).

miR-27b and miR101 target nuclear receptor subfamily 2 group F member 2 (NR2F2) (previously COUP-TFII). *miRs-27b* and *miR-101* directly target NR2F2, a master regulator of the metastatic gene regulatory network and member of the orphan nuclear receptor family, by modulating the expression of transcription factors ZEB1 and ZEB2 (115, 116) (Figure 6). Loss of *miR-101* correlated with increase in the NR2F2–forkhead box M1 (FOXM1)-centromer protein F precursor (CENPF) signaling activity in clinical data sets (115). FOXM1 is a transcription factor which is overexpressed in PC and contributes to proliferation, angiogenesis and metastasis (117). CENPF is a structural component of the kinetochore and a known target of FOXM1 (118). Both FOXM1 and CENPF levels are increased in metastatic PC (48). *In vivo*, lymphatic metastasis was promoted in LNCaP cells in which *miR-101* and *miR-27b* were inhibited after injection into mouse prostate (115). Clinical data revealed that loss of function of *miR-101* and *miR-27b* leads to overexpression of NR2F2, FOXM1 and CENPF, and enhancement of metastasis and drug resistance (115).

miRs Targeting Bone-related Targets

miR-466 targets Runt-related transcription factor 2 (RUNX2). *miR-466* was underexpressed in PC compared to normal tissues and inhibited invasion and migration of PC3 and DU145 PC cells (119). *In vivo*, PC3 cells constitutively expressing *miR-145* exhibited reduced growth of the primary tumor after orthotopic implantation into the posterior prostatic lobe of athymic mice (119). Intracardiac injection of these cells suppressed spontaneous metastases compared to controls. *miR-466* directly targets bone-related transcription factor RUNX2, which is involved in expression of several genes related to tumor growth and metastasis, such as osteopontin, osteocalcin, angiopoietins 1 and 4, MMP11, vimentin, focal adhesion kinase (FAK) and AKT (120, 121) (Figure 4).

Other miR-related Targets

miR-23b and miR-27b target Huntington-interacting protein 1-related protein (HIP1R). *miR-23b* and *miR-27b* are encoded by the miR cluster 23b/27b and are down-regulated in CRPC

(122). Ectopic expression of *miR-23b*, *-27b* in CRPC cell lines ALV31 and PC3 ML reduced mobility and invasion, increased E-cadherin and reduced expression of Ras-related C3 botulinum toxin substrate 1 (RAC1), even though the proliferation rate was not altered (122, 123). *In vivo*, orthotopic xenografts of aggressive PC cells transduced with *miR-23b* and *miR-27b* were not affected with respect to growth of the primary tumor, however, distant metastases were significantly reduced (123). *miR-23b* and *miR-27b* down-regulated HIP1R, a component of clathrin-coated pits and vesicles that may link the endocytic pathway to the actin cytoskeleton (123, 124) (Figure 4). HIP1R binds 3-phosphoinosites and may promote survival by stabilizing receptor tyrosine kinases following ligand-induced endocytosis (124).

miR-188-5p targets lysosome transmembrane protein 4 (LAPTM4B). Down-regulation of *miR-188*-5p is associated with metastasis and poor prognosis in PC (125). *miR-188*-5p inhibited proliferation, invasion and migration of PC3 and LNCaP PC cell lines *in vitro* (125). Ectopic expression of *miR-188*-5p inhibited tumor growth and metastasis of PC3 cells implanted into the dorsal flank of nude mice (125). LAPTM4B has been identified as a direct target of *miR-188*-5p in PC3 and LNCaP cells (125) (Figure 4). LAPTM4B is located in late endosomes and lysosomes and can promote proliferation, invasion and inhibit apoptosis and is able to initiate autophagy (126). LAPTM4B is associated with poor prognosis and metastasis in several types of tumors (127-130).

miR-195 targets breast cancer overexpressed gene 1 (KIAA0100, previously BCOX1). PC patients with low miR-195 expression were found to have a poorer prognosis (131). Restoring *miR-195* expression in PC3 and LNCaP cells inhibited proliferation, migration and invasion (131). *miR-195* down-regulates BCOX1 (Figure 4, which is associated with the recurrence and progression of triple-negative breast cancer (132, 133). BCOX1 is significantly overexpressed in patients with PC and lymph node metastasis and is associated with PC progression (131). *miR-195* reduced growth and metastasis of PC3 and LNCaP cells implanted into the dorsal flanks of immunodeficient mice (131).

miR-503 targets ring finger protein 31 (RNF31). miR-503 inhibits proliferation, migration and invasion of PC3 and LNCaP cells *in vitro* (134) and targets RNF31, also known as HOIL-1 interacting protein (135, 136) (Figure 4). RNF31 catalyzes the formation of linear peptide bonds between the amino terminus of methionine and the carboxy-terminal glycine (137). RNF31 promotes degradation of p53 (138) and facilitates NFκB signaling (139). *RNF31* knockdown in PC3 cells attenuated metastasis of these cells implanted subcutaneously into the dorsal flanks of nude mice (134). RNF31-positive staining is associated with PC progression and unfavorable outcome (134).

miR-708 targets karyopherin. *miR-708* targets karyopherin (KPNA4) (Figure 4). The expression levels of karyopherin correlate with PC progression. *miR-708* inhibited migration of PC3 cells. *KPNA4* knockdown attenuated primary tumor invasion of PC3 xenografts as well as bone metastasis (140). Central mediators of karyopherin function are TNFα and TNFβ, which enhance PC cell migration, stimulate osteoclasts and bone resorption (141,142) and can alter macrophage polarization towards the M2 subtype in the tumor microenvironment (143).

miRs with a Multitude of Identified Targets

miR-141 targets CD44, methyltransferase EZH2 and several Rho-GTPases. *miR-141* is a member of the *miR-200* family and is underexpressed in PC stem/progenitor populations in xenografts and tumors (144). Forced expression of *miR-141* in $CD44^{+}$ and bulk PC cells suppressed tumor regeneration and invasion and enforced an epithelial phenotype with loss of the mesenchymal phenotype (144). *miR-141* inhibited invasion in PC3, DU145, VCaP, LAPC4, LNCaP and C4-2 PC cells. Ectopic expression of *miR-141* in $CD44^{high}$ PC3 and DU-145 PC cells and implantation into the dorsal prostate of non-obese diabetic/severe combined immunodeficient mice resulted in smaller primary tumors and fewer lung metastases. Doxycycline-inducible expression of *miR-141* in LAPC9 xenografts reduced growth of xenografts and diminished metastasis (144). The mechanism of action of *miR-141* is based on inhibition of CSC-related molecules, cytoskeleton- and cell-cycle-related components, resulting in inhibition of tumor growth and metastasis. CSC-related targets such as CD44 and histone methyltransferase EZH2, a component of the polycomb repressive complex 2, both involved in CSC maintenance, invasion and metastasis, are direct targets of *miR-141* (144, 145). In addition, several invasion- and motility-modulating Rho GTPases, such as cell division cycle 42 (CDC42), ras-related C3 botulinum toxin substrate 1 (RAC) and actin-related protein 2/3 complex subunit 5 (ARPC5) have been identified as direct targets of *miR-141* (Figure 7). These targets are involved in formation of actin stress fibers and lamellipodia, and cytoskeletal reorganization (146-148). Furthermore, *miR-141* inhibited cell-cycle-related molecules such as CDK4 and CDK6. However, it is not yet clear, whether these are direct targets of *miR-141*.

miR-141-3p targets TNF receptor-associated factors (TRAF) 5 and 6. *miR-141-3p* is an inhibitor of bone metastasis of PC cells and is down-regulated in bone-metastatic PC tissues

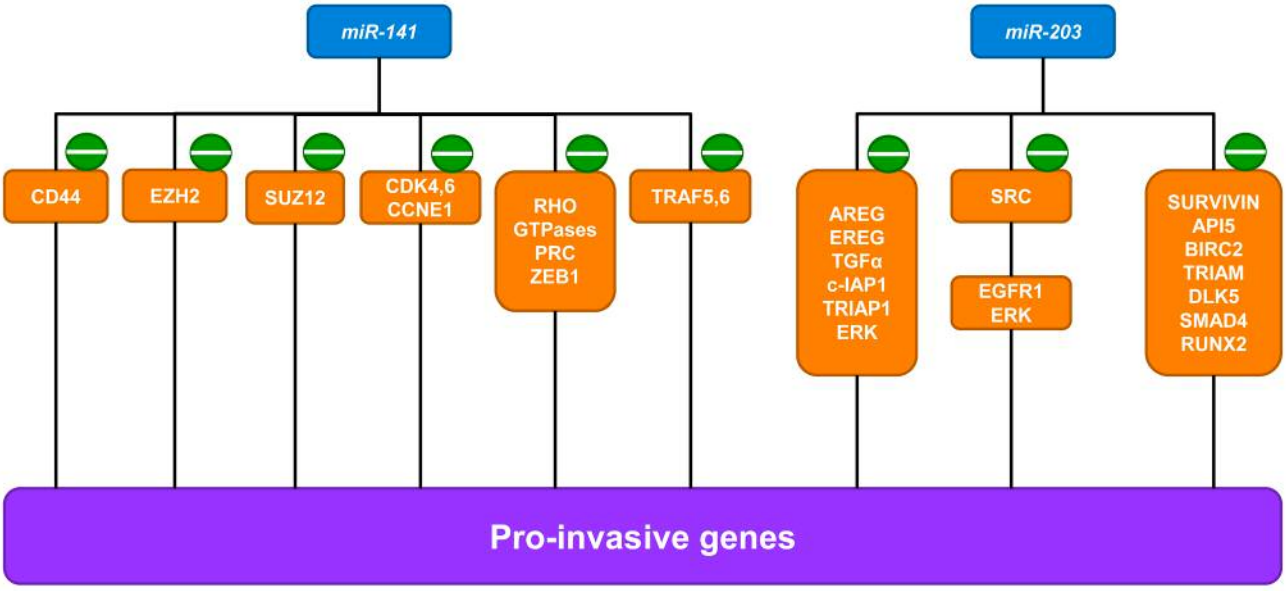

Figure 7. *miRs down-regulated in metastatic prostate cancer and targeting several mRNAs simultaneously. The mechanism of action of miR-141 and miR-203 are outlined. API5: Apoptosis-inhibitor protein 5; AREG: amphiregulin; cIAP1: cellular inhibitor of apoptosis 1; CCNE1: cyclin E; CD44: cluster of differentiation 44; CDK: cyclin-dependent kinase; DLX5: distal-less homeobox 5; EGFR: epidermal growth factor receptor; EREG: epiregulin; ERK: extracellular-regulated kinase; EZH2: histone methyltransferase EZH2; PRC: polycomb repressor complex; RHO GTPases: RHO guanosine triphosphatase; RAP1: small GTPase RAP1; RUNX2: Runt-related transcription factor 2; SMAD4: transcription factor SMAD4; SRC: tyrosine kinase SRC; SUZ12: core protein of EZH2; TGFα: transforming growth factor α; TRAF: TNF receptor-associated factor; TRIAP1: TP53-regulated inhibitor of apoptosis 1; ZEB1: zinc finger E-box binding homeobox 1.*

(149). Intracardiac injection of PC3 cells overexpressing *miR-141*-3p reduced metastasis to the bones in comparison to controls with fewer metastatic nodules and smaller osteolytic areas of metastatic tumors (149). TRAF5 and TRAF6 were identified as direct targets of *miR-141*-3p (149). TRAF5 and TRAF6 modulate TNF-induced activation of NFκB signaling through binding to TNF receptor cytoplasmic domains (150) (Figure 7).

miR-203 inhibits EGFR signaling and anti-apoptotic proteins. *miR-203* levels are reduced during progression of PC. Ectopic expression of *miR-203* inhibited proliferation, invasion, migration and adhesion of PC cell lines such as DU145, RASB1 and PC3 (151-153). miR-203 induced MET in PC cells with concomitant expression of E-cadherin (153). RASB1 cells ectopically expressing *miR-203* exhibited a significant decrease in brain and bone metastases after intracardiac injection (151). In a second *in vivo* model, *miR-203* was shown to inhibit growth of DU-145 cells after subcutaneous injection (152). Several *miR-203*-related targets have been identified, depending on the system under investigation. The first system is based on DU145/RASB1 bone metastasis-derived clones whose parental cell line was transformed with RAS G37 (151). In these cell lines, the mechanism of action of *miR-203* is based on activation of EGFR signaling as well as inhibition of anti-apoptotic proteins (151). EGFR ligands amphiregulin (AREG), epiregulin (EREG) and transforming growth factor α (TGFα) as well as apoptosis-inhibiting proteins apoptosis inhibitor 5 (API5), cellular inhibitor of apoptosis protein (c-IAP1) (BIRC2) and p53-regulated inhibitor of apoptosis 1 (TRIAP1) have been identified as targets of *miR-203*, which result in activation of metastasis-promoting SRC and ERK signaling (151). In a second system, RAP1, a member of the RAS family of GTPases which are involved in cell–cell and cell–extracellular matrix interactions (154, 155) was identified as a direct target of *miR-203* in DU145 cells (152). In a third system using PC3 cells, survivin, ZEB2, RUNX2, distal-less homeobox 5 (DLX5) and SMAD4 were identified as direct targets of *miR-203* (156-158). In PC3 cells, *miR-203* reduced expression of osteopontin and osteocalcin genes, which are key osteoblastic genes, indicating that *miR-203* may regulate the osteomimetic properties of PC cells (152). In LNCaP cells, *miR-203* was identified as a suppressor of LIM and SH3 protein 1 (LASP1), which impaired proliferation and migration of PC cells (159-161). The mechanism of action of *miR-203* is summarized in Figure 7.

Therapeutic Aspects

We have summarized PC-metastasis related miRs based on preclinical validation by *in vitro* and *in vivo* systems, as well as clinical correlations. Some of them are up-regulated in PC tissues in comparison to corresponding normal tissues, but the majority are down-regulated in PC tissues. Reconstitution or inhibition of their functions are potential therapeutic options. Therapeutic strategies and current pitfalls are summarized in several excellent reviews (162-167) and therefore these issues are not discussed in detail in this review.

Options for reconstitution are ectopic expression by expression vectors for the corresponding miRs, double-stranded synthetic RNAs that mimic endogenous miRs (165), or non-specific induction of corresponding miRs with small molecules (165). To inhibit the functionality of miRs, short nucleotides with chemically enhanced stabilities and nuclease resistance are administered. Types of miR-interfering drugs include antisense oligonucleotides and single-strand DNA-like molecules such as locked nucleic acids. In the same way, small double-stranded interfering RNAs (siRNA) and antagomirs, which are RNAs of 23 nucleotides modified with cholesterol at the 3'-end, ultimately lead to RNA silencing by RNA interference. miR sponges, another approach to inhibiting unwanted actions of miRs, contain multiple binding sites for a miR of interest, thus interfering with their functionality (163-167). Considerable progress has been achieved by optimizing the pharmacokinetic/pharmacodynamic properties of these agents by chemical modification significantly improving the binding affinity, stability and target modulation (164-167).

Issues which have to be addressed are: hybridization-associated off-target effects, hepatotoxicity, design of efficient delivery systems, rapid clearance by the reticulo-endothelial system, and enhancement of circulation time when injected into the bloodstream (165-167).

By increasing the molecular weight of RNAs through conjugation to polyethylene glycol a fast renal clearance can be circumvented. The serum half-life can be further prolonged by coupling of the interfering nucleotides to albumins or cholesterol which bind to circulating lipoproteins, thus shielding the nucleotides from renal clearance (168-170). Apart from this, complexing the nucleotides with nanoparticles also reduces the risk of side-effects, *e.g.* immunogenicity and cytokine release syndrome, while the increased molecular weight is expanding the retention time (171).

Efficient delivery systems are currently being explored to allow hydrophilic RNAs to cross the cell membrane and to minimize off-target effects. To facilitate the entry of nucleotides into the endosomes, lipophilic polymers such as lipid nanoparticles, dynamic polyconjugates or oligonucleotide nanoparticles, which mask hydrophilic RNAs, are used (172-179). Targeting ligands, aptamers and antibodies coupled to nucleotides not only shield miR-interfering drugs from fast renal clearance, but also allow targeting of specific cell types. Thus, hapten-binding bi-specific antibodies were shown to facilitate the knockdown of the target gene by delivering haptenylated siRNA complexed with dynamic polyconjugates (180-190). Upon internalization, release of RNA can be facilitated by conjugates coupled *via* acid-sensitive or protease cleavable linkers within the acidic endosome (191, 192). Furthermore, complexation with cell-penetrating viral proteins, *e.g.* trans-activating transcriptional activator-derived protein from human immunodeficiency virus 1, Drosophila-derived penetratin or chimeric peptides overcome the endosomal barrier and accomplish endosomal escape (193-195). However, potential toxicity and immunogenicity favor the use of human-derived cell-penetrating peptides such as neurturin as they display reduced toxicity and enable the passage of nucleotides through the endosomal membrane to the cytosol (195-197). In this way, cell-specific delivery packages can be assembled by formulating nucleotides with lipophilic polymers and targeting entities for delivery of RNAs (180-184, 186).

As a corollary of the many different factors that influence the delivery of nucleotides, such as tissue-specific targeting, cell penetration and endosomal escape, further studies – especially in *in vivo* mouse models – should explore and address the current delivery bottleneck to resolve the limitations of the delivery system.

The extent of delivery of these agents to target cells for achieving therapeutic effects is probably dependent on the specific system under consideration and might be affected by induction of immune responses against released tumor antigens, resulting in death of tumor cells which do not express the therapeutic target.

The miRs discussed in this review were validated in different preclinical metastasis-related models such as experimental metastasis (tail vein injection and lung metastasis), spontaneous metastasis after subcutaneous implantation, metastasis after intracardiac or orthotopic intraprostatic injection, or delivery of tumor cells into the bone. Albeit targets have been identified for the described miRs, in many cases, the precise steps of their interaction in the metastatic cascade remain to be identified. The interpretation of the therapeutic relevance of specific miRs is complicated by the fact that *in vivo* data are restricted to very specific systems and evaluation of *in vivo* data simultaneously covering several metastasis-related models are not available.

What should be the profile of a PC-related anti-metastatic miR-related therapeutic agent? Besides inhibiting the migration and invasion, one of the requirements should be an ablative effect on circulating and disseminated tumor cells such as inhibition of proliferation and induction of apoptosis.

If the agent under consideration does not match these features, increased cytotoxicty of an agent used in combination studies or reversal of therapy resistance would be an additional useful therapeutic feature. Interference with growth of established PC-related metastases is another important feature for the selection of miR-related agents as therapeutic agents. Unfortunately, anti-metastatic miR-related agents have not yet been evaluated in settings with PC-related metastatic burden.

Future experiments will show whether the pitfalls, as outlined, can be tackled successfully.

Conflicts of Interest

The Authors are (AE, FB, UB) or were (UHW) employed by Roche. Roche is interested in targeted therapies.

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Received September 26, 2018
Revised November 8, 2018
Accepted November 23, 2018