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Review

# The role of microRNAs in metastasis and epithelial-mesenchymal transition

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**Abstract.** For a tumour cell to metastasise it must successfully negotiate a number of events, requiring a series of coordinated changes in the expression of many genes. MicroRNAs are small non-coding RNA molecules that post-transcriptionally control gene expression. As microRNAs are now recognised as master regulators of gene networks and play important roles in tumourigenesis, it is no surprise that microRNAs have recently been demonstrated to have central roles during metastasis. Recent work has also demonstrated critical roles for microRNAs in epithelial-mesenchymal transition, a phenotypic change underlain by altered gene expression patterns that is believed to mirror events in metastatic progression. These findings offer new potential for improved prognostics through expression profiling and may represent novel molecular treatment targets for future therapy. In this review, we summarise the multistep processes of metastasis and epithelial-mesenchymal transition and describe the recent discoveries of microRNAs that participate in controlling these processes.

**Keywords.** Micro-RNA, metastasis, cancer, epithelial-mesenchymal transition, ZEB, gene regulation.

## Introduction

The spread and growth of cells from a primary tumour site (known as metastasis) is the most common cause of death for cancer patients and may occur through organ damage caused by growing lesions, paraneoplastic syndromes or treatment complications. Although primary cancers are often treatable by radiation therapy or surgery, metastasis indicates a wider systemic disease and strongly correlates with poor prognosis (reviewed in [1, 2]). At the cellular level, the early stages of metastasis are characterised by the loss of contact with neighbouring cells and an increase in invasive capacity. It is hypothesised these same changes are a recapitulation of the developmental process known as epithelial-mesenchymal transition (EMT), in which epithelial cells acquire a fibroblast-like morphology, increased motility and gene expression patterns characteristic of mesenchymal cells.
MicroRNAs (miRNAs) are endogenous small RNA molecules that act as master regulators of gene expression (reviewed in [3]). Over the past few years, characteristic miRNA expression profiles have been identified that enable tumour classification and prognostication and various individual miRNAs have

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been described as oncogenes or tumour suppressors (reviewed in [4]). Several recent studies have identified specific miRNAs that are central to metastatic progression and EMT. This review will discuss the importance of metastasis in tumour progression, the hypothesised role that EMT plays in cancer and the findings of recent studies that identify roles for miRNAs in these processes.

## Metastasis

For a metastatic lesion to arise, tumour cells must disseminate by intravasating into the blood or lymphatic system. This requires the breaking of local cell-cell contacts and invasion into the surrounding stroma and may be enhanced by neo-angiogenesis into the primary tumour site which is a pre-requisite for continued tumour growth. After intravasation and surviving mechanical stresses associated with circulation, tumour cells then become trapped in capillary beds of distant organs, adhering to endothelial cell surfaces or to an exposed subendothelial basement membrane. Extravasation then occurs into tissue at this secondary site. To survive and grow beyond a size otherwise limited by the diffusion of oxygen, nutrients and growth factors, these so called "micrometastases" must continue evading the immune system and induce neo-angiogenesis to develop a vasculature. This neovasculature may then provide access to the circulation for tumour cells from the secondary site to produce additional metastases [1].

Invasion, which is the initial step in the metastatic process, requires tumour cells to break contact with neighbouring cells and migrate through the extracellular matrix (ECM) and basement membrane. One of the most fundamental features of invasion is therefore the breaking of cell-cell and cell-matrix adhesion. Cell-cell adhesion is mediated by various molecules including cadherins which form interactions between cells via their extracellular domains, whilst their intracellular domains mediate signalling to the actin cytoskeleton [5]. A hallmark of metastatic invasion is a change in cadherin expression from E (epithelial)-cadherin, which is expressed on epithelial cells and inhibits invasion by promoting contact between tumour cells, to N (neural)-cadherin, which is expressed in mesenchymal cells and facilitates tumour cell binding to the stroma [6]. The adherence of cells to the ECM is largely mediated by integrins, a diverse family of heterodimeric receptors which also undergo changes in expression in malignant compared to non-malignant cells [7, 8]. For example, elevated expression of the integrin $\alpha4\beta1$ promotes the growth and spread of melanoma cells and, together with decreased $\alpha6\beta1$ levels, correlates with metastasis [8]. Invasion through the ECM is further enhanced by ECM-degrading proteases, including matrix metalloproteinases and urokinase plasminogen activator, which are frequently upregulated in malignant tumours and are associated with poor prognosis [9]. During the invasive process, cells establish a leading edge from which pseudopod protrusions form, driven by actin polymerisation and assembly into filaments. This pushes the cell membrane forward and establishes new interactions with the ECM. Matrix metalloproteases and other ECM-degrading enzymes are upregulated and recruited to the leading edge, breaking down pericellular ECM and creating a path for the advancing cell. Contraction of membrane-anchored myosin-actin filaments propels the cell body forward, coupled with the simultaneous breaking of existing ECM contacts at the trailing edge [10, 11].

Following detachment from the tumour mass, the tumour cell must avoid anoikis, an apoptotic program activated in anchorage-dependent cells after detachment from the extracellular matrix [12]. Anoikis is believed to contribute to the inefficiency of metastasis, as are other mechanisms including immune surveillance within the stroma and velocity-induced sheer forces after intravasation into the bloodstream [13]. For secondary tumours to arise, the tumour cells that survive must arrest within the bloodstream and extravasate into new sites. This arrest is likely to involve size restriction within narrowing capillaries and both specific and non-specific interactions with various molecules expressed on endothelial surfaces. Certain tumours preferentially metastasize to certain organs [14]. For example, melanoma cells intravenously injected into mice metastasize to the lung or to lung tissue implanted into muscle, but do not form metastases in the kidney or similarly implanted renal tissue [15]. Whilst the characteristics of the tumour cell will contribute in large part to this, with gene expression signatures that mediate metastasis to different sites having been identified [16, 17, 18, 19, 20], the microenvironment at the secondary site also plays a major role. This is probably due both to a distinct repertoire of surface proteins expressed on the endothelial cells of different organs and to the nature of local growth factors. The importance of the tumour microenvironment has been demonstrated recently by comparing mRNA profiles of tumour-associated and normal breast stroma [21]. Here, a tumour-associated stromal signature capable of predicting outcome was identified, which may in future be useful for identifying patients who require aggressive therapy. This provides further demonstration of the "soil and seed" hypothesis initially postulated in 1889 [22], whereby the ability of a tumour cell (the seed) to grow at a

secondary site is dependent upon the microenvironment of that site (the soil). This offers the potential for therapeutic intervention to block metastasis, as demonstrated by depletion of the lung-specific adhesion molecule metadherin which reduced secondary lung metastasis in mice [23].

After attachment to the vascular endothelium of a target organ, or to the subendothelial basement membrane, tumour cells can extravasate into the tissue, although even at this point metastasis remains an inefficient process. In a melanoma metastasis model, for example, of the injected cells that survived the circulation and extravasated into the liver, only one in 40 formed micrometastases by day three, and of these only one in 100 progressed to macroscopic metastases within 10 days [24]. Again, the tissue microenvironment (the "soil") at the secondary site is critical to metastatic capacity, as is the ability of metastases to stimulate blood vessel growth (angiogenesis).

As metastasis is a multi-step process involving multiple complex pathways, the identification of master regulators of these events (as opposed to isolated genes) is therefore of paramount clinical importance. As will be discussed later, the identification of miRNAs as master regulators of gene expression networks, and recent findings implicating specific miRNAs in metastatic progression, suggest that miRNA manipulation may represent an important treatment strategy.

## Epithelial-mesenchymal transition

Epithelia line body surfaces and cavities, forming protective barriers and regulating secretion and absorption. Epithelial cells establish apical-basal polarity by associating with a laminal layer at their basal surface, known as the basement membrane, and form tightly associated immobile sheets. Mesenchymal cells, together with the ECM, fill the underlying interstitial spaces and form non-polarised loosely associated cells with a high potential for motility. Epithelial-mesenchymal transition (EMT) describes a collective series of transcriptional and protein modification events that result in epithelial cells taking on mesenchymal characteristics, thereby allowing cells to separate, lose apico-basal polarity and gain motility (reviewed in [25, 26, 27]).

EMT is a fundamental process during development that enables cells to move to new localities. The reverse process of mesenchymal-epithelial transition (MET) can subsequently occur to generate polarised epithelia at new sites of tissue formation (reviewed in [28]). Initially, EMT is critical for formation of the three germ layers, a process known as gastrulation, whereby mesenchymal cells migrate along the primitive streak to populate new areas of the embryo that develop into mesoderm and ectoderm. At a later stage of development, epithelial cells from the neural crest undergo EMT to produce mesenchymal cells that migrate to new areas and develop into various mesenchymal cell types including somites and chondrocytes (reviewed in [28, 29]). EMT and MET are also required for palate formation, cardiac valve formation and nephrogenesis and, within the adult, are required for placental formation and fibroblast production during wound healing [30, 31]. One of the fundamental features of EMT is repression of epithelial genes such as E-cadherin, leading to a loss of epithelial cell-cell adhesion. Loss of E-cadherin is also a major factor in the early stages of metastatic invasion [32, 33, 34, 35, 36, 37, 38], prompting speculation that an EMT-like process is associated with metastasis (Fig. 1).

The triggering and progression of EMT is a complex process that may be initiated by multiple pathways including Wnt signalling, Notch signalling and various growth factors (such as TGFβ, FGF, EGF and PDGF) working through their cell surface serine-threonine or tyrosine kinase receptors (reviewed in [27, 39]). There is significant cross-talk between these pathways [40, 41, 42] that ultimately activates a transcriptional program in which epithelial genes are downregulated, and mesenchymal genes upregulated. A key finding in understanding the transcriptional regulation of an EMT program came in 2000 when Snail was identified as a direct transcriptional repressor of E-cadherin [43, 44]. Snail was demonstrated to bind E-boxes within the E-cadherin promoter and repress transcription through recruiting a corepressor complex composed of HDACs 1 and 3 and SIN3A [45]. Since then, the direct repression of E-cadherin has also been demonstrated for other EMT-inducing transcription factors including the zinc-finger protein Snail2 / Slug [46], the two-handed zinc finger / homeodomain proteins ZEB1 and ZEB2 [47, 48] and the basic helix-loop-helix protein E47 [49]. Twist is also known to potently induce EMT and to downregulate E-cadherin expression; however it is unclear whether this occurs by direct binding to E-boxes [50]. These EMT-inducing transcription factors are now known to repress a battery of common epithelial genes associated with cell-cell and cell-ECM interactions and thereby act as master triggers of the EMT transcriptional program (reviewed in [51]). Although typically regarded as transcriptional repressors, at least in some circumstances these same factors may serve as transcriptional activators. For example, although direct repression of epithelial genes by ZEB1 is well characterised, ZEB1

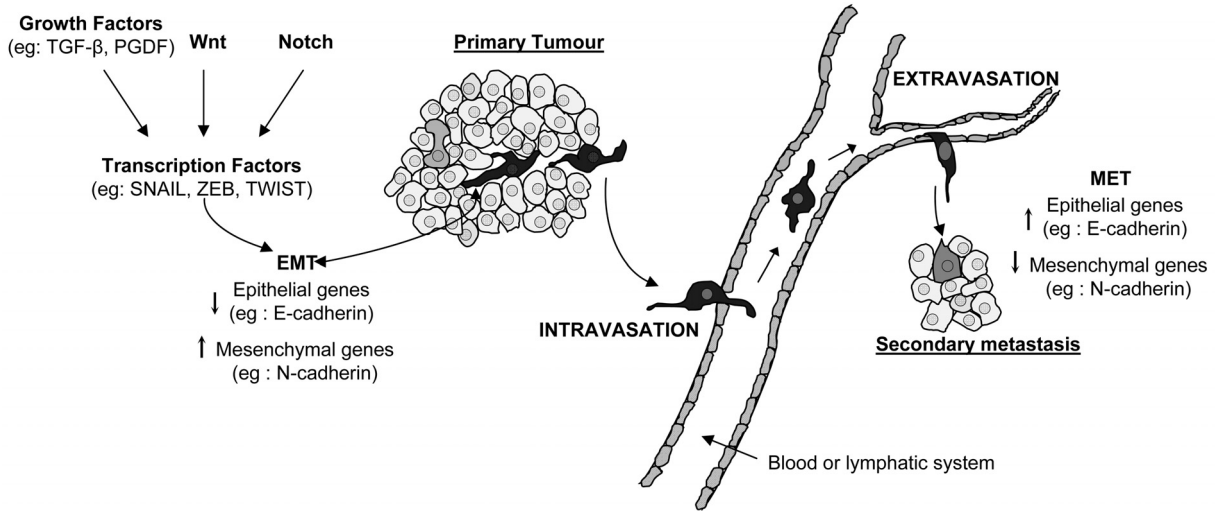


**Figure 1.** Overview of the role of EMT in tumour metastasis. Multiple pathways including growth factor signalling through cell surface kinase receptors, Wnt signalling and Notch signalling can induce various EMT-associated transcription factors including those of the Snail, ZEB and Twist families. This leads to a transcriptional program in which multiple epithelial-specific genes (such as E-cadherin) are repressed and mesenchymal-associated gene expression (such as N-cadherin and fibronectin) are increased. Within a primary tumour, it is hypothesised that the induction of EMT leads to invasion through surrounding stroma and intravasation into the blood or lymphatic system, enabling widespread dissemination. After survival in the bloodstream, cells arrest in capillary beds due to size restriction and / or specific cell surface interactions and extravasate into distant tissue where a subsequent MET enables the formation of secondary metastases with epithelial characteristics.

is also able to form complexes with coactivators (P/CAF and p300) and directly induce gene expression [52, 53, 54]. Twist has also been reported to bind E-box elements within target gene promoters and directly activate gene expression [55]. One noteworthy example is the induction of the mesenchymal marker N-cadherin, either directly or indirectly, which enhances tumour cell motility [56].

**EMT and cancer.** EMT is considered to be a critical process in the progression of epithelial-derived tumours, initially suggested by the observation that the invasiveness of breast cancer cell lines correlates with mesenchymal marker expression, whilst non-invasive cells are characteristically epithelial [32, 57]. It is now well established that the transcription factors that regulate EMT play important roles in tumourigenesis. Snail expression has been specifically detected at the invasive front of squamous cell carcinomas and hepatocarcinomas [58, 59], whilst ZEB1 is expressed at the invasive front of colorectal cancers [60]. Furthermore, Snail [61, 62, 63, 64, 65, 66, 67, 68, 69], Snail2 [64, 70, 71, 72, 73, 74, 75], ZEB1 [76], ZEB2 [77] and Twist [71, 78, 79, 80, 81, 82] expression have been correlated with increased metastases and poor prognosis in a wide range of human tumours. In breast cancer, a basal-like phenotype is associated with highly aggressive metastatic tumours. In a large microarray profiling study of invasive breast carcinomas, it was found that basal-like tumours displayed a characteristic EMT-associated gene expression signature, suggestive of a pro-metastatic phenotype after EMT [83].

The pro-metastatic features of mesenchymal derivates of cancer cells can include increased migratory and invasive potential, increased survival in suspension and resistance to anoikis. However, despite these observations and pro-metastatic features, solid evidence of EMT in clinical carcinoma is still not abundant and remains a topic of controversy [84, 85]. This may be because EMT occurring at the invasive front of a tumour results in the migration of these cells away from this site and, hence, is not readily observable. The importance of EMT to tumours may also be restricted to a small but important subset of cells, such as invasive cancer stem cells, or myoepithelial cells that regulate the microenvironment via cytokine and chemokine production. Because secondary tumours usually display epithelial morphology, it is hypothesised that a subsequent MET must occur during re-establishment of the metastases at secondary sites, which again limits the opportunity for direct observation of an EMT during metastasis. The relationship between EMT / MET and metastasis was supported by experiments using bladder cancer cells from which sub-lines were developed based upon their *in vivo* metastatic ability [86]. The incidence of micrometastases following orthotopic injection was higher in sub-lines that displayed mesenchymal morphology, whilst epithelial sub-lines were the more

effective at tumour formation if injected systemically or directly into a secondary site. This is consistent with the escape from a primary tumour being enhanced by invasive mesenchymal properties, whilst the growth of secondary metastases is enhanced by epithelial characteristics [86]. The requirement for re-epithelialisation in the formation of macrometastases may therefore provide a novel treatment target, preventing the growth of solid tumour metastases by trapping cells in a state of micrometastasis.

It should be noted however that at least in some circumstances, tumour cell invasion may take place in the absence of EMT. Collective cell invasion is one such example, whereby proteolysis and structural rearrangement of the ECM by a leading cell creates channels through which tumour cells may more easily invade. Activated fibroblasts associated with malignant tumours (called cancer-associated fibroblasts) can act as leading cells enabling migration of squamous cell carcinomas through 3D matrices [87, 88]. Collective cell invasion in the absence of EMT may also be enhanced by the small mucin-like protein podoplanin, which induces filopodia formation and is upregulated at the tumour invasive edge [89].

**EMT and cancer-associated stem cells.** It is apparent that subpopulations of cells within heterogenous tumours play significant roles in cancer progression. One important example is tumour-associated stem-like cells (variously referred to as cancer stem cells or tumour-initiating cells, because they are defined by their ability to seed new tumours). These were initially reported in the haematopoietic system [90], and subsequently in human breast carcinoma, from which a stem-like $CD44^+/CD24^-$ subpopulation was isolated capable of self-renewal and the generation of heterogenous progeny [91]. Since then, similar cell populations have been identified in brain and colon tumours [92, 93, 94].

Stem-like $CD44^+/CD24^-$ cells have been derived from a $CD44^-/CD24^+$ population of primary human mammary epithelial cells by successive transfection with the telomerase catalytic subunit, the SV40 small and large T antigens and an oncogenic allele of H-Ras [95]. Interestingly, the stem-like cells had downregulated E-cadherin and upregulated vimentin and fibronectin, suggestive of EMT. Further supporting the link between stem-cell properties and EMT, treatment of $CD24^+$ cells with TGF-β not only promoted EMT, but also led to the appearance of $CD24^-$ cells. In another study, induction of EMT in human mammary epithelial cells by TGF-β, or transfection of the EMT-inducing transcription factors Snail and Twist, led to the upregulation of both mesenchymal and stem-like markers and the acquisition of stem cell-like properties including the enhanced ability to form mammospheres, soft agar colonies and tumours [96]. Similarly, stem-like cells isolated from mammary glands or carcinomas express EMT markers [96].

Tumour-associated stem-like cells are of particular importance, as the growth of metastases after dissemination from the primary tumour would be expected to require self-renewal capability. It is therefore plausible that EMT is able to enhance metastasis, both through the increased capability for dissemination from the primary tumour and through the promotion of self-renewal capacity, enabling growth of macrometastases.

## MicroRNAs

Microarray studies have identified sets of genes whose expression in primary tumours correlates with metastasis and poor prognosis [18, 97, 98, 99, 100, 101, 102]. These gene signatures are potentially useful diagnostic and prognostic indicators, but in regard to potential therapeutic intervention it is particularly important to identify the regulators of these gene expression networks. The importance of transcription factors has long been suggested in disease contexts and considerable data are now available describing the tumour-regulatory roles of c-Myc, HIF, SMAD and NF-κB, to name a few (reviewed in [103, 104, 105, 106]). MicroRNAs (miRNAs) are particularly attractive candidates as upstream regulators of tumourigenesis and metastasis because they post-transcriptionally regulate large sets of genes [3, 107]. Indeed, each miRNA is predicted to have dozens, if not hundreds, of mRNA targets [108, 109, 110].

The discovery of miRNAs stemmed from the observation in *Caenorhabditus elegans* that the *lin-4* gene encodes a small RNA that binds the 3' untranslated region of the lin-14 mRNA, and inhibits its translation [111]. The widespread importance of miRNAs became appreciated in 2001, with evolutionarily conserved miRNAs being identified and cloned from other organisms including humans [112, 113, 114, 115]. Since then, the list of known miRNAs has expanded, now standing at 733 in human and predicted to increase further [116]. MicroRNA genes can be encoded within their own dedicated transcript, or be spliced from within the introns of protein-coding genes. In both cases the genes are transcribed by RNA polymerase II to generate a primary transcript (termed a "pri-miRNA") that is both capped and polyadenylated [117, 118]. Intra-molecule base-pairing results in pri-miRNAs forming stem-loop structures that are recognised and processed within the nucleus by the DROSHA / DGCR8 complex, which

cleaves pri-miRNAs near the base of their stem loops [119, 120, 121]. This generates the "pre-miRNA" stem-loop of approximately 60 nucleotides that is exported through the nuclear pore by exportin and Ran-GTP [122, 123, 124]. Further processing takes place within the cytoplasm by the RNAseIII enzyme Dicer [125, 126], generating a 21–23nt double stranded RNA duplex, of which one strand is incorporated into the RNA-induced silencing complex (RISC) comprised of dicer, the double stranded RNA binding factor TRBP and argonaute-family proteins [127, 128]. MiRNAs then function within the context of the RISC, base pairing through sequence complementarity to their target mRNAs, typically within the 3' untranslated region (UTR). MiRNAs inhibit translation but can also promote degradation of the target mRNA. The mechanism by which translation is blocked remains controversial, with the question of whether it is translation initiation or elongation that is being blocked still in dispute, raising the possibility that more than one mechanism may be involved (reviewed in [107]). Destruction of the target mRNA is achieved through the recruitment of de-adenylating and de-capping enzymes which thereby expose mRNAs to subsequent exonuclease attack [129, 130, 131, 132, 133]. If there is high sequence complementarity between a miRNA and its target mRNA then direct destruction of the target mRNA by argonaute-2 mediated cleavage occurs [134]. This is a common scenario in plants, but is rare in humans.

**MicroRNAs in cancer.** The initial study establishing a role for miRNAs in cancer came with the observation that two miRNA genes, miR-15a and miR-16a, are deleted in the majority of B cell chronic lymphocytic leukaemia patients [135]. Since then, roles for miRNAs in various cancers have been reported, primarily based upon differential miRNA profiling between cancer and normal tissue or from genetic screens with miRNA expression libraries (reviewed in [4, 136, 137]). The involvement of microRNAs in cancer is perhaps not surprising given the multi-faceted nature of cancer and the wide-range of cancer-associated processes that miRNAs regulate, including cell division [138, 139, 140], apoptosis [141, 142], angiogenesis [143, 144] and EMT [145, 146, 147, 148]. Indeed, many miRNAs have been described as either oncogenes or tumour suppressors based upon the mRNAs that they target. The miR-17-92 cluster, for example, exerts pro-oncogenic effects by inhibiting the key tumour suppressor E2F1 [149], whilst let-7 acts as a tumour suppressor through inhibition of oncogenic RAS [150].

Initial reports correlating miRNA expression with cancer encouraged further profiling of numerous tumours [151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163] with the aim of identifying specific miRNA signatures that can be used in cancer diagnosis, prognosis, or the evaluation of treatment response. Such profiling has shown a remarkable ability to distinguish tumour from normal tissue [155, 157, 158, 164, 165, 166] and miRNA signatures have been associated with survival rates in lung carcinoma [163, 167, 168], chronic lymphocytic leukaemia [153, 169] B cell lymphoma [170], hepatocellular carcinoma [171] and with the aggressive growth and metastasis of pancreatic cancer [161]. This may be of particular benefit for the diagnosis of cancers of histologically uncertain origin, where miRNA profiling was a far more successful diagnostic indicator than mRNA expression [158].

Consistent with their frequent deregulation in cancer, more than 50% of miRNA genes are located in cancer-associated genomic regions or at fragile sites, which are preferential sites for recombination, amplification, deletion, translocation and viral integration [172]. Also consistent with a widespread role for miRNAs in cancer, and potentially explaining the frequent upregulation of key miRNAs associated with tumours, 26 miRNA genes have been found to be increased in gene copy number (and expression level) across a large number of breast, melanoma and ovarian cancer samples [173]. However, these examples of overexpression notwithstanding, the majority of miRNAs are downregulated in cancer [158]. It has been suggested that, due to the more direct influence upon protein expression, miRNA profiling may be of greater significance than mRNA profiling in the ability to diagnose and predict disease progression [174]. This importance is underscored by the fact that any given miRNA is predicted to target many transcripts and thereby act as a master regulator of gene networks.

**MiRNAs and metastasis.** As evidence demonstrating important roles for miRNA in cancer accumulates, characterising the roles that these miRNAs play in specific aspects of tumourigenesis has become a research focus. To this end, several groups have specifically aimed to identify miRNAs with an involvement in metastasis or the regulation of EMT. In many of these cases, the miRNAs identified have also been reported in other cancer-associated contexts, especially from miRNA profiling of tumour versus non-tumour tissue. Studies identifying these miRNAs as differentially expressed between tumour and non-tumour tissue are listed in Table 1.

MiRNA profiling of human breast cancer MDA-MB-231 cells that were selected for being highly metastatic to the bone or lung, in comparison with the parental

**Table 1.** Metastasis / EMT associated miRNAs misregulated in human cancer

| miRNA | Cancer type | Up / Down | Ref |
|---|---|---|---|
| miR-21 | Breast | U | [231] |
| | | U * | [155] |
| | | U | [232] |
| | | U | [162] |
| | | U | [221] |
| | Hepatocellular carcinoma | U | [171] |
| | | U | [205] |
| | | U | [233] |
| | | U | [234] |
| | Colorectal | U * | [235] |
| | | U * | [200] |
| | | U | [162] |
| | Gastric | U | [236] |
| | | U | [162] |
| | Lung | U | [162] |
| | Ovarian | U | [156] |
| | | U | [237] |
| | Uterine leiomyoma | U | [238] |
| | Cervical | U | [239] |
| | Prostate | U | [162] |
| | B cell lymphoma | U | [240] |
| | | U | [170] |
| | Lymphocytic leukaemia | U | [241] |
| | Oesophageal | U | [219] |
| | Pancreatic | U | [157] |
| | | U * | [161] |
| | | U | [162] |
| | Cholangiocyte | U | [159] |
| | Glioblastoma | U | [242] |
| miR-10b | Hepatocellular carcinoma | U | [234] |
| | Ovarian | U | [243] |
| | Acute myeloid leukaemia | U | [244] |
| miR-126 | Cervical | D | [245] |
| miR-205 | Ovarian | U | [156] |
| | Lung | U | [162] |
| | Breast | D | [162] |
| | | D | [221] |
| | Oesophageal | D | [219] |
| | Bladder | U | [220] |
| miR-200 | Ovarian | U | [156] |
| | | U * | [237] |
| | Cholangiocyte | U | [159] |
| | Lung | U | [162] |

* Denotes high miRNA expression correlating with increased distant metastasis and / or poor prognosis

unselected cell population, has identified three miRNAs (miR-335, miR-126 and miR-206) that have lower expression in both metastatic cell lines and in the metastases that developed after the injection of primary human malignant cells into mice [175]. Further strengthening the case that these miRNAs are metastasis suppressors, their overexpression in metastatic MDA-MB-231 cells decreased lung metastases, whilst inhibition of miR-335 in non-metastatic cells was sufficient to increase metastasis to the lung. Overexpression of miR-335 also decreased the migration and invasion of cells *in vitro*. In an effort to identify the downstream targets of these miRNAs that are critical to metastasis suppression, microarray studies were performed looking for mRNAs that are both decreased as a result of miR-335 overexpression and are increased in metastatic cells. Using these criteria, six genes were identified and verified to be direct targets of miR-335, including the extracellular matrix glycoprotein tenascin-C (TNC) and the Sry-like HMG box transcription factor Sox4. Tenascin-C is of particular interest due to its high expression in the microenvironment of many tumours, where it is reported to promote angiogenesis, matrix metalloproteinase expression and tumour invasion and migration [176, 177, 178]. The knockdown of TNC and SOX4 inhibited both cell migration and lung colonisation, consistent with miR-335 exerting anti-metastatic effects through the repression of these genes. Importantly, breast cancer patients with tumours expressing decreased levels of miR-335, miR-206 and miR-126 had a shorter time to metastatic relapse, demonstrating the relevance of this finding to human cancer.

Another study identified miRNAs of interest to cancer on the basis of their differential expression between normal mammary tissue and primary breast carcinoma using microarray profiling data. Among these, Ma et al. identified miR-10b as being highly expressed only in metastatic cells (summarised in Fig. 2b, [179]). Inhibition of miR-10b decreased invasion in matrigel, whilst miR-10b overexpression is sufficient to promote cell motility and invasion in otherwise non-invasive cell lines, and can drive the metastasis of these normally non-metastatic cell lines when they are injected into mouse mammary fat pads. Using target prediction algorithms, Homeobox D10 (HOXD10) was identified as a putative miR-10b target of interest, as it represses the expression of genes associated with cell migration and ECM remodelling and HOXD10 expression decreases with increasing breast cancer malignancy [180, 181]. The authors demonstrated HOXD10 is indeed a target for translational repression by miR-10b, which in turn leads to de-repression of the downstream HOXD10 target RHOC, a pro-metaststic Rho-family GTPase

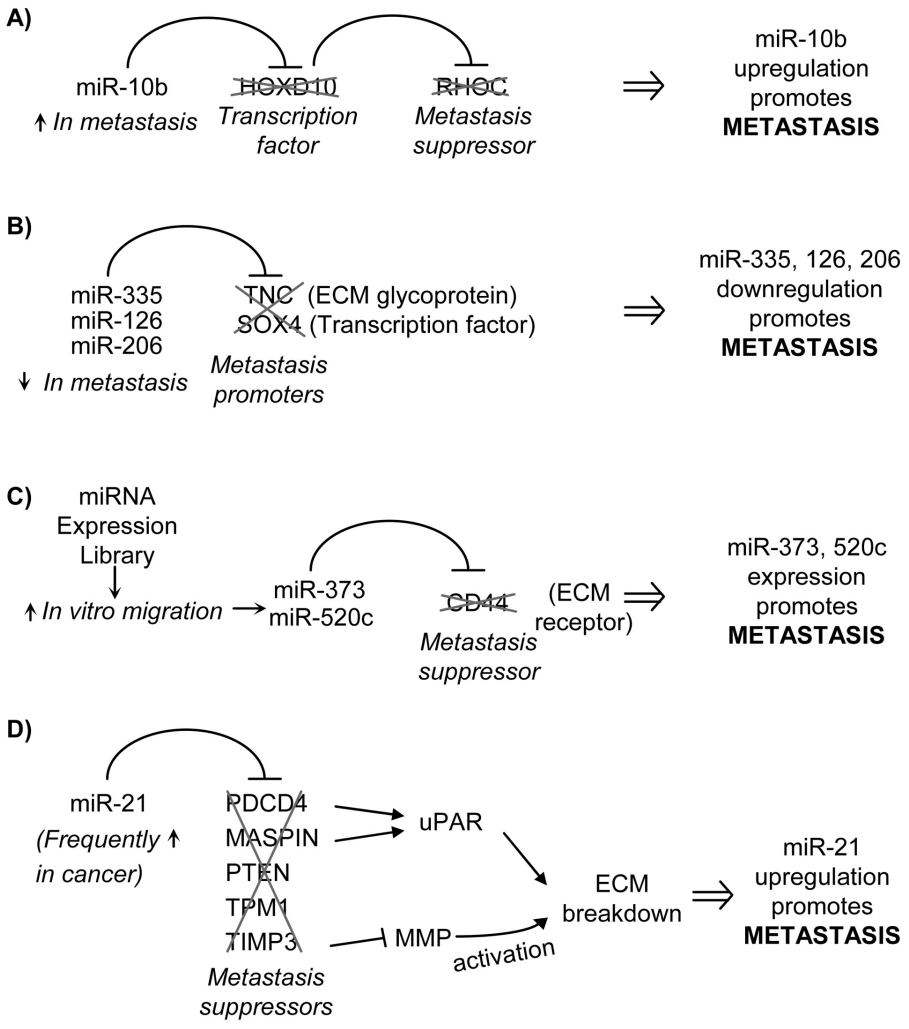


**Figure 2.** Overview of studies identifying roles for miRNAs in metastasis. In two separate studies, cancer-associated miRNAs of interest were identified through microarray profiling comparing normal mammary tissue with primary breast carcinoma (*A*) [179] or mesenchymal breast cancer cell lines selected for their *in vivo* metastatic capacity compared to the unselected parental population (*B*) [175]. In (*A*), the ability of miR-10b to promote metastasis was shown to involve direct repression of the HOXD10 transcription factor and subsequent de-repression of a metastasis suppressor, RHOC. In (*B*), the downregulation of miR-335, miR-126 and miR-206 enabled upregulation of the pro-metastatic genes TNC and SOX4. In (*C*) [193], a miRNA expression library was transduced into an epithelial breast cancer cell line that was then screened for increased *in vitro* cell migration. miR-373 and miR-520c were reported to enhance metastasis through direct repression of a well known metastasis suppressor, CD44. In (*D*), miR-21 is frequently reported to be upregulated in cancer, with multiple studies indicating a direct role in the repression of multiple metastasis suppressors [201, 202, 203, 204, 205].

[182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192]. Thereby, through repression of HOXD10, miR-10b promotes RHOC expression and, hence, promotes metastasis. The clinical significance of this discovery was demonstrated by the reduced miR-10b expression levels noted in metastasis-free patients, whilst 50 % of metastasis positive patients expressed increased miR-10b levels.

To identify miRNAs that participate in cell migration and potentially in metastasis, a different approach was undertaken by Agami and coworkers (summarised in Fig. 2c), where a miRNA expression library was transduced into human breast cancer MCF7 cells, which were then screened for increased cell migration using trans-well migration assays [193]. The overexpression of miR-373 and miR-520c increased *in vitro* invasion through matrigel, and increased *in vivo* metastasis to both the bone and lung when MCF7 cells overexpressing these miRNAs were injected into the tail veins of mice. Conversely, inhibition of miR-373 was sufficient to inhibit *in vitro* migration of otherwise invasive MDA-MB-435 and HCT-15 cells. In order to identify miR-373 and miR-520c target genes, microarrays were performed in MCF7 cells that were transfected with high levels of these miRNAs. Of the genes downregulated by miR-373 and 520c expression, the cell surface receptor CD44 was of particular interest because it is known to suppress metastasis in breast, prostate and colon cancer [194, 195, 196] and its expression correlates with increased survival of breast cancer patients [197, 198]. The pro-invasive activities of miR-373 and miR-520c may therefore be attributable, at least in part, to repression of this metastatis inhibitor. Consistent with this hypothesis, the knockdown of CD44 was sufficient to endow MCF7 cells with a migratory phenotype and conferred increased metastasis to the bone and lung of CD44-depleted cells injected into the tail veins of mice. Expression analysis of primary breast cancer and corresponding lymph node metastases showed significantly higher miR-373 expression in the metastases compared to the primary tumour, whilst CD44

expression was reduced in the metastatic samples. This provides clinical support for the pro-metastatic capacity of miR-373 and is consistent with the hypothesis that a major mechanism through which miR-373 exerts this effect is through inhibition of the metastatis repressor, CD44. In a separate study, miR-373 was also identified in a genetic screen for miRNAs that promote cellular proliferation and tumourigenesis [199].

miR-21 is perhaps the most frequently reported miRNA upregulated in tumours, with several studies (summarised in Fig. 2d) correlating miR-21 overexpression with increased metastasis [161, 200]. Inhibition of miR-21 in MDA-MB-231 cells was found to decrease invasion *in vitro* and decrease lung metastasis of cells injected into the tail vein of mice [201]. Inhibition of miR-21 also decreased intravasation across the chorioallantoic membrane and reduced lung metastases in chicken embryos [202]. Among the reported miR-21 targets are several tumour and metastasis suppressors including programmed cell death-4 (PDCD4), maspin, phosphatase and tensin homolog (PTEN) and tropomyosin I (TPM1) [201, 202, 203, 204, 205]. PDCD4 for example inhibits invasion by breast and colon cancer cells [202, 206], whilst maspin is known to suppress invasion and correlates with improved prognosis [207]. Interestingly, both PDCD4 and maspin regulate expression of the urokinase-type plasminogen activation receptor (uPAR) [208, 209] which breaks down the ECM and hence promotes invasion and metastasis and correlates with poor prognosis [210]. This complements another recent study in which miR-21 was shown to inhibit expression of Tissue Inhibitor of Metalloprotease-3 (TIMP3), thereby de-repressing metalloprotease activity and increasing cancer cell invasion [211]. Taken together, miR-21 overexpression appears to exert pro-tumourigenic effects at multiple levels, including metastasis, through the repression of multiple tumour suppressors.

**MicroRNAs and EMT.** EMT is regulated by a number of transcription factors including the zinc-finger proteins ZEB1 (Zinc finger E-box Binding, also called δEF-1 / TCF8 / ZFHX1A) and ZEB2 (also called SIP1 / ZFHX1B) which are of particular interest as they are expressed at the invasive front of tumours and correlate with distant metastases and poor prognosis [60, 76, 77]. Initially, ZEB1 was shown to be suppressed by exogenous miR-200c which in turn promoted E-cadherin expression [212]. miR-200c is one of the five members of the miR-200 family, encoded by two separate dedicated polycistronic transcripts (miR-200b~200a~429 and miR-200c~141) [145, 213]. These are highly conserved miRNAs that fall into two specificity groups (miR-200b, 200c, 429 and miR-200a, 141) based upon their predicted mRNA targets (the two groups have unique, predicted target gene sets due to a difference of one nucleotide within the seed sequence). ZEB1 and ZEB2 have been confirmed as direct targets for repression by miR-200, with each containing multiple sites within their 3' untranslated region for both classes of the miR-200 family [145, 146, 147, 148, 213, 214]. This repression is mediated largely at the level of translational inhibition, as mammalian microRNAs predominantly work in this manner and the overexpression of the miR-200 family had more dramatic effects on the expression of ZEB protein than mRNA [146]. A striking negative correlation also exists between ZEB and miR-200 expression across a panel of 59 human cancer cell lines [148] and a further 10 human breast cancer lines [146], being consistent with the miR-200 family also increasing ZEB mRNA turnover.

The induction of EMT by TGF-β in canine MDCK and mouse NMuMg cells is accompanied by reduced miR-200 and increased ZEB1 expression. Human breast cancer cells of an epithelial phenotype also express high levels of miR-200 but little detectable ZEB1 or ZEB2, whilst cells of a mesenchymal nature express high ZEB1 and ZEB2 but little to no detectable miR-200 [146, 148]. Critically, enforced miR-200 overexpression blocks the ability of TGF-β to induce EMT [146, 147] and inhibition of endogenous miR-200 in epithelial cells is sufficient to reduce the epithelial marker E-cadherin whilst increasing levels of the mesenchymal markers vimentin, ZEB1 and ZEB2 [146, 147, 148]. In human breast and ovarian tumours, a strong correlation exists between miR-200 and E-cadherin expression [146, 148]. Consistent with the pro-invasive properties associated with EMT and the inhibition of EMT by miR-200, inhibition of miR-200 promotes cell migration [146] whilst miR-200 expression reduces migration [148]. These data place miR-200 mediated inhibition of ZEB1 and ZEB2 at the crossroads of the epithelial / mesenchymal phenotype which is likely to be of considerable importance to metastatic progression. As with the aforementioned studies linking ZEB with tumourigenesis, miR-200 overexpression has also been reported in ovarian, cholangiocyte (bile duct) and lung tumours [156, 159, 162, 215], with expression in ovarian cancer correlating with poor prognosis [215]. In light of the fact that miR-200 expression is highly epithelial-specific however, it is possible that alterations in miR-200 expression between tumour and matched normal tissue may be reflecting differences in the epithelial content of the tissues being analysed as opposed to specific upregulation of miR-200 in cancer.

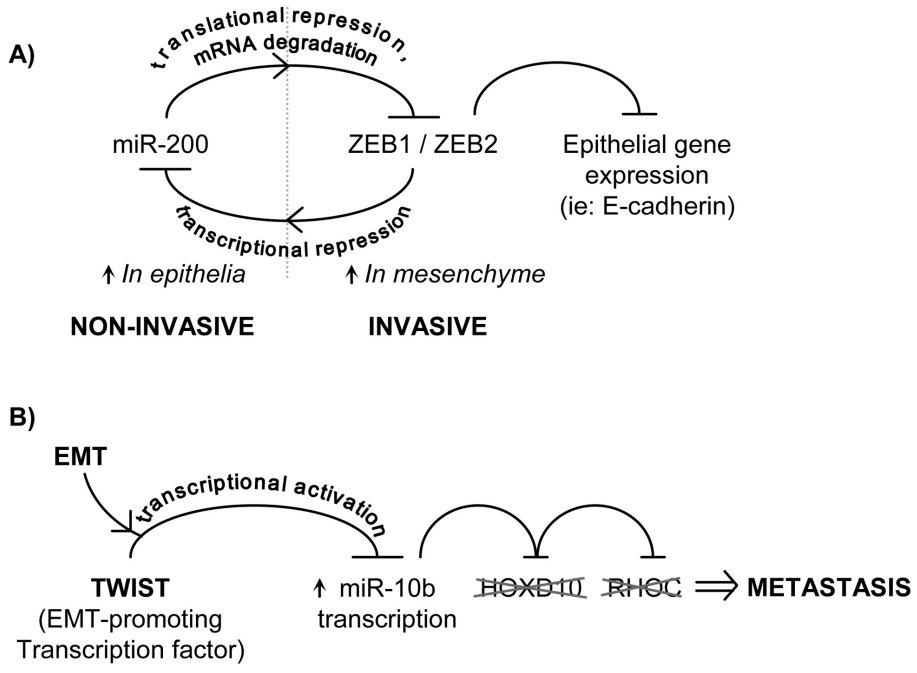


**Figure 3.** Overview of studies identifying roles for miRNAs in EMT. (*A*), The EMT-promoting ZEB transcription factors are expressed in invasive mesenchymal cells but not in non-invasive epithelia where they are directly repressed by the miR-200 family [145, 146, 147, 148]. In turn, the ZEBs directly repress transcription of the miR-200 genes, thereby forming a double-negative feedback loop and ensuring that only in mesenchymal cells are the ZEBs able to repress epithelial gene expression [145, 213]. Such a feedback loop enables maintenance of a stable bi-phasic state whilst retaining the ability to switch between states after an appropriate stimulus. (*B*), miR-10b promotes metastasis through direct repression of the metastasis repressor HOX10 (Fig. 2a). Via direct binding to the miR-10b promoter [179], the EMT-promoting transcription factor Twist upregulates miR-10b expression, thereby promoting metastasis and providing an additional mechanism by which induction of an EMT program may enhance invasion.

In counterpoint to the targeting of ZEB1 and ZEB2 by miR-200, ZEB1 and ZEB2 can transcriptionally repress both miR-200 gene clusters by binding paired E-box elements located within their promoters [145, 216]. Thus, miR-200 and ZEB expression are mutually exclusive, with epithelial-specific miR-200 expression and mesenchymal-specific ZEB expression confirmed across a panel of epithelial or mesenchymal human breast cancer cell lines [146]. This mutual repression forms a double negative feedback loop that specifies the epithelial or mesenchymal status of cells and hence their migratory capacity (summarised in Fig. 3a). Such a feedback mechanism supports the existence of mutually exclusive stable states (epithelial or mesenchymal) in the absence of continued stimuli, whilst still retaining the capacity for stimulus-driven interchangeability. Feedback loops are a common feature of genetic pathways involving miRNAs, where they appear to enhance functionality and robustness of gene networks [217].

MiR-205 was also found to be strongly downregulated during EMT [146]. Like the miR-200 family, miR-205 inhibits ZEB1 and ZEB2 expression and was found to be specifically expressed in a panel of epithelial but not mesenchymal breast cancer lines. However, although miR-205 was capable of promoting an epithelial phenotype, unlike miR-200 it is not downregulated in metaplastic tumours and questions regarding the nature of the miR-205 target genes or its role in EMT remain largely unexplored. Intriguingly however, miR-205 has also been implicated in the maintenance of mouse mammary epithelial progenitor cells [218] and therefore may have a role in cancer-associated stem cells with which EMT has been recently linked [96]. Altered miR-205 expression has also been reported in various cancers [156, 162, 219, 220, 221].

Twist is a basic helix-loop-helix transcription factor that has also been shown to promote an EMT program within cells. Twist activates miR-10b transcription by binding to an E-box within the miR-10b promoter; the miR-10b then promotes metastasis via direct inhibition of the metastasis suppressor HOXD10 [179] (summarised in Fig. 3b). The enhancement of cell motility and invasion that results from Twist overexpression is significantly decreased by miR-10b inhibition. On its own, Twist is capable of inducing EMT whereas miR-10b expression alone cannot, so miR-10b is likely to represent an important component of a larger Twist-regulated EMT gene expression program that culminates in increased invasiveness.

A further link between miRNAs and EMT was recently found by miRNA expression profiling of keratinocytes treated with TGF-β, a major EMT inducer. In this study, a novel EMT-specific miRNA signature was reported including the upregulation of miR-21 [222]. Interestingly, induction of miR-21 by TGF-β can occur through a post-transcriptional mechanism involving interaction of SMADs with the Drosha-containing microRNA processing complex [223]. This provides not only a means to upregulate a known pro-tumourigenic, pro-metastatic miRNA, but also suggests a novel mechanism whereby wider effects on miRNA expression are mediated by TGF-β.

## Conclusions

MiRNA expression signatures have already been identified as diagnostic or prognostic markers for cancer whilst the identification of miRNAs acting as oncogenes or tumour suppressors suggests the further possibility that these small molecules may represent future treatment targets. Several obstacles hinder this approach, however. The first is the ability to manipulate miRNA activity *in vivo;* however, this seems achievable given antisense oligonucleotides are now available to inhibit miRNAs within cells and cholesterol-conjugated "antagomirs", or LNA-modified "antimirs" have been developed which enable specific silencing of endogenous miRNAs in mice and primates [224, 225]. An additional hinderance is the identification of which miRNAs to target, complicated by the fact that any one miRNA is predicted to have dozens, if not hundreds of mRNA targets [108, 109, 110]. Techniques to thoroughly query the targeting ability of a miRNA are only now being developed [226, 227, 228, 229]. Whilst the specific inhibition of a given miRNA is clearly achievable, there remain significant obstacles in both correctly targeting tumour sites and achieving specificity of action given miRNAs target multiple genes.

An example of such variability is demonstrated in a recent large scale miRNA profiling of patients comparing metastatic and non-metastatic hepatocellular carcinomas [230]. Sets of miRNAs, both up and downregulated, were identified that correlated with patient survival; however, the fact that none of these miRNAs are those identified in the studies described above (summarised in Figs. 2 and 3) underscores the variability between tumour types and makes the identification of generic miRNAs to target unlikely. A further complicating factor is the role that the tumour microenvironment plays in the establishment of distant metastases. Recent work suggests mRNA profiles of tumour versus non-tumour microenvironment may be an excellent diagnostic predictor [21]. At present, no information is available on the role that miRNAs may play in regulating the tumour microenvironment or the potential prognostic benefits in miRNA profiling of such localities.

Metastasis, be it EMT-related or otherwise, involves multiple steps that may be amenable to therapeutic treatment. Many reports, including some of those discussed above, identify miRNAs of interest either initially using *in vitro* systems or by transfecting miRNA expression libraries into cells that are subsequently injected into mice. Given that these studies bypass a number of steps in the metastatic process, this leads us to question what aspects of metastasis we may be missing. Similarly, comparing expression profiles of malignant to benign samples does not necessarily identify miRNAs key to metastatic initiation. The heterogeneity of tumours and increasing recognition of key tumour cell subpopulations also leads us to question if we are studying the right cells, and what influence miRNAs have specifically with regard to dormant, stem or chemoresistant cancer cells. Questions such as these, the role of the tumour microenvironment and the identification of key miRNA targets from the plethora of those predicted will remain active areas of research in future.

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