MicroRNAs in the tumour microenvironment: big role for small players

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Abstract

MicroRNAs (miRNAs) represent a class of small non-coding RNAs with an important regulatory role in various physiological processes as well as in several pathologies including cancers. It is noteworthy that recent evidence suggests that the regulatory role of miRNAs during carcinogenesis is not limited to the cancer cells but they are also implicated in the activation of tumour stroma and its transition into a cancer-associated state. Results from experimental studies involving cells cultured *in vitro* and mice bearing experimental tumours, corroborated by profiling of clinical cancers for miRNA expression, underline this role and identify miRNAs as a potent regulator of the crosstalk between cancer and stroma cells. Considering the fundamental role of the tumour microenvironment in determining both the clinical characteristics of the disease and the efficacy of anticancer therapy, miRNAs emerge as an attractive target bearing important prognostic and therapeutic significance during carcinogenesis. In this article, we will review the available results that underline the role of miRNAs in tumour stroma biology and emphasise their potential value as tools for the management of the disease.

Key Words

- molecular biology
- carcinoma

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Importance of the tumour microenvironment

The tumour microenvironment plays an increasingly appreciated role in tumorigenesis. During neoplastic development, a series of changes occur in the tumour stroma that ultimately promote tumour growth and affect not only the clinicopathological profile of the tumour but also the efficacy of anticancer therapy. Cancer has been postulated to be a physiological response to an abnormal stromal environment (Barcellos-Hoff & Medina 2005). Indeed, certain similarities between carcinogenesis and wound healing have been recognised leading to the suggestion that cancers can be viewed as ‘wounds that do not heal’ (Haddow 1972, Dvorak 1986). Interaction

between the tumour and stromal cells can determine the fate of the tumour cells – whether they remain in a dormant state or progress into invasive and eventually metastatic cancer. Therefore, the establishment of a complex reciprocal network of communication between the cancer cells and cells of the microenvironment, such as fibroblasts, is imperative in order to fuel tumour growth (Kiaris *et al.* 2008).

Various soluble factors that are abundant in the tumour microenvironment have been implicated in the activation of the tumour stroma. The significance of microenvironment has been demonstrated by early

experiments demonstrating that loss of TGF β signalling in fibroblasts leads to development of increased amounts of stromal cells and prostate intraepithelial neoplasia, indicating that loss of TGF β signalling can facilitate neoplastic progression (Bhowmick *et al.* 2004). Stromal interactions with the epithelial cells can be altered by external influences such as radiation therapy. Barcellos-Hoff & Ravani (2000) demonstrated that COMMA-D mammary epithelial cells, which harbour *P53* (*TP53*) mutations in both alleles but which are non-tumorigenic, developed larger tumours more rapidly after implantation into mammary fat pads that had been irradiated compared with sites that had not been irradiated. These data indicate that radiation-induced changes in the stromal microenvironment can influence epithelial cells and contribute to tumour progression *in vivo*, a finding that in turn implies that activation of a paracrine response programme, likely related to DNA damage in stromal cells, is pro-oncogenic. Indeed, recent findings have shown that during chemotherapy treatment, stromal fibroblasts are engaged in a mitogenic programme for which *CDK8* plays a major role and ultimately reduces therapeutic drug efficacy (Porter *et al.* 2012).

Besides changes in the expression profile of the stromal cells, it has been suggested that the genetic changes present in the tumour and stromal compartments are a result of tumour and stromal co-evolution (Wallace *et al.* 2011). To that end, it has been shown that the stromal fibroblasts of human breast and other cancers frequently bear mutations in specific tumour suppressor genes, such as *PTEN* and *TP53*. It is noteworthy that these mutations have occurred in the microenvironment independently of the cancer cells implying different genetic origin from the lesions that may occur in the cancer cells (Kurose *et al.* 2002, Patocs *et al.* 2007, Farmaki *et al.* 2012). These genetic alterations of the stromal cells have been shown to affect tumorigenesis (Kiaris *et al.* 2005, Trimboli *et al.* 2009, Addadi *et al.* 2010, Guo *et al.* 2013) and anticancer drug efficacy considerably (Lafkas *et al.* 2008).

The clinical relevance of the findings derived from experimental tumour studies has been shown by analyses of human cancers in which the expression profile of the stromal tissue was sufficient to predict prognosis of the disease and chemotherapeutic drug efficacy. Using laser capture microdissection to isolate breast cancer stroma compared with normal breast stroma at least 2 mm away from the cancer, one study identified a 26-gene stromal gene expression signature as a prognostic indicator independent of other factors such as patient age, tumour grade, oestrogen receptor or human epithelial growth

factor receptor type 2 and lymph node status (Finak *et al.* 2008). Another study found that a stroma-related gene signature was able to predict resistance to neo-adjuvant chemotherapy in breast cancer patients (Farmer *et al.* 2009). These studies indicate the importance of the tumour stroma as it contributes to outcome of the patient as well.

Components of the tumour microenvironment

The tumour microenvironment consists of a multitude of cell types including fibroblasts, endothelial cells and inflammatory cells. Fibroblasts are the most abundant cells in the tumour microenvironment (Micke & Ostman 2005). While normal fibroblasts have been shown to prevent tumour growth and invasiveness in the early stages of carcinogenesis (Paland *et al.* 2009), cancer-associated fibroblasts (CAFs) create an environment that is conducive to tumour initiation and progression (Olumi *et al.* 1999, Hayward *et al.* 2001, Orimo *et al.* 2005, Soon *et al.* 2013). The progressive transition of stromal fibroblasts and their commitment into a cancer-associated state is characterised by specific changes in their secretory profile that includes, among others, the production of an array of chemokines that in turn attract inflammatory cells into the region (Orimo *et al.* 2005) generating a pro-inflammatory microenvironment. The fibroblasts and the inflammatory cells then release growth factors and act either directly on the cancer cells stimulating their proliferation and/or inhibiting apoptosis or indirectly by promoting tumour growth and stimulating angiogenesis (Coussens & Werb 2002). Indeed, the intrinsic relationship between chronic inflammation and cancer has been hypothesised as early as 150 years ago by Virchow (Balkwill & Mantovani 2001). Endothelial cells together with pericytes and smooth muscle cells make up blood vessels that are of prime importance in the tumour microenvironment if the tumour is to grow beyond 2–3 mm or metastasise (Folkman 1971).

While the origin of CAFs is not fully defined, they are thought to arise from several sources. Depending on the exact type of cancer, up to 40% of CAFs can be derived from bone marrow precursor cells (Ishii *et al.* 2003, Direkze *et al.* 2004) that are attracted to the site of the neoplastic growth from the circulation and populate the growing tumour. CAFs may also originate from epithelial cancer cells that have undergone epithelial-to-mesenchymal transition (Petersen *et al.* 2003). If this is the case, as the stromal fibroblasts originate from the neoplastic cells, they are expected to bear genetic lesions in common, a fact that

can profoundly affect their behaviour. In addition, CAFs may arise from resident fibroblasts that have been subjected to a myofibroblast differentiation programme that is marked by the neo-expression of smooth muscle actin (SMA) and represents a hallmark for their commitment into a cancer-associated state (Ronnov-Jessen & Petersen 1993). Recent evidence indicates that specific microRNAs (miRNAs) have emerged as major regulators of this paracrine activity and of the transition of the fibroblasts into the cancer-associated state. As discussed later, miR-21 (Yao *et al.* 2011), miR-31, -214 and -155 (Mitra *et al.* 2012) appear to play a role in differentiation of normal fibroblasts to CAFs.

miRNAs and their role in tumorigenesis

The term miRNA was coined in 2001 to describe a new class of small non-coding regulators (Ruvkun 2001). miRNAs are small, non-coding RNAs that negatively regulate gene expression in a sequence-specific manner. They interact with target mRNAs to induce cleavage and degradation. Alternatively, they may trigger mRNA deadenylation or directly inhibit protein translation. Thus, miRNAs may suppress the expression of their target genes by a variety of mechanisms, by reducing protein expression without altering the mRNA levels (Esquela-Kerscher & Slack 2006) or by reducing mRNA levels. Lee *et al.* (1993), while studying heterochronic genes in *Caenorhabditis elegans*, found that *lin4*, the first miRNA to be identified, was located in an intron of another gene and functioned to negatively regulate *lin14*. A new class of small RNAs was thus discovered. Initially, it was thought that this paradoxical mode of gene regulation was a unique feature of *C. elegans*. However, the subsequent discovery of a second miRNA, *let7*, which negatively regulates *lin41* and *hbl1*, suggested that it reflects a more generalised phenomenon (Pasquinelli *et al.* 2000). *Let7* was found to be phylogenetically conserved (Reinhart *et al.* 2000), which led to the speculation that this new class of RNAs play a major role in gene regulatory networks. Subsequently, many other miRNAs were identified in both plants and animals. Despite the specific discrepancies in their mode of action, miRNAs always operate as regulators of gene expression (Lau *et al.* 2001, Lee & Ambros 2001).

Currently, miRBase version 20 includes 30 424 mature miRNAs in 206 species with a total of 2578 mature miRNAs described in humans (Kozomara & Griffiths-Jones 2011). In animals, miRNAs are transcribed from intergenic or intronic DNA as large precursors termed pri-miRNAs.

A single pri-miRNA may encode for up to six miRNA precursors. Subsequently, these hairpin loop structures undergo successive enzymatic processing by Drosha, Pasha and Dicer into their mature, usually 22-nucleotide long, double-stranded RNA termed the miRNA:miRNA* duplex. The asterisk (*), or 3p strand, denotes the presence of the 'passenger' strand that is represented at lower levels in the steady state and is usually degraded. The whole process takes place initially in the nucleus and subsequently in the cytoplasm where cleavage by Dicer occurs. This duplex is incorporated into the RNA-induced silencing complex and the mature miRNA strand is preferentially retained (Du & Zamore 2005).

More than 50% of the genomes are likely targets of specific miRNA regulation (Friedman *et al.* 2009). miRNAs have been implicated in a variety of cellular processes such as development, differentiation, growth and apoptosis (Esquela-Kerscher & Slack 2006). There is an increasing recognition that miRNAs are involved in carcinogenesis. Such miRNAs, termed oncomirs, act as tumour suppressor or oncogenes (He *et al.* 2005, Esquela-Kerscher & Slack 2006) depending on whether their specific targets are oncogenic or anti-oncogenic transcripts respectively. A number of malignancies have been associated with characteristic miRNA signatures (Lu *et al.* 2005, Calin & Croce 2006). miRNAs have been found to be dysregulated in cancers compared with normal tissue. For example, miR-143 and -145 have been found to be down-regulated in colorectal (Michael *et al.* 2003) and breast cancers (Iorio *et al.* 2005), while miR-21 has been found to be up-regulated in glioblastomas (Chan *et al.* 2005, Ciafre *et al.* 2005) and breast cancers (Iorio *et al.* 2005). Furthermore, various miRNAs represent targets of specific oncogenes, such as a cluster of Myc-induced miRNAs located on chromosome 13 (miR-17-5p and -20a), which suppresses *E2F1* expression (O'Donnell *et al.* 2005). In addition, miR-145 has been identified as a *TP53* target gene. Considering that there is deregulated expression and genomic alterations of *TP53* in stromal cells, this finding may have important implications on how aberrant expression of this and other miRNAs may play a role in the regulation of the tumour microenvironment (Sachdeva *et al.* 2009).

miRNA-related modulation of gene expression is not limited to the regulation of cell autonomous phenomena that exclusively affect the phenotype of the cells subjected to the differential production of the specific miRNA. Stromal cells also express important regulatory miRNAs that can influence the phenotype of adjacent epithelial cells. Characteristic are the results of a study by Ucar *et al.* (2010) miR-212/-132 null female mice were generated, and

by transplanting WT mammary epithelial cells into fat pads of mutant mice and *vice versa*, they found that miR-212/-132 expression in stromal cells, and not epithelial cells, is essential for normal ductal development of the mammary gland. This study demonstrates that expression of miRNAs by stromal cells influences development of breast tissue. In view of this and various other analogous findings, and considering the important role of tumour stroma in carcinogenesis, it is not surprising that miRNAs produced by cells of the microenvironment, and particularly from stromal fibroblasts, will profoundly affect tumour growth. Indeed, various such stroma-derived miRNAs have been identified and their role in tumorigenesis has begun to be unravelled.

This review focuses on the miRNAs involved in the induction of specific changes in the tumour microenvironment, particularly in the CAFs, and their role in inducing tumour progression. A number of studies have revealed the significant role of miRNAs in CAFs and other components of the tumour microenvironment. These studies are detailed below.

Differentiation of fibroblasts to CAFs

Recent evidence indicates that specific miRNAs have emerged as regulators of the transition of the fibroblasts into the cancer-associated state. Yao *et al.* studied primary ovarian CAFs as well as human fetal lung fibroblast cell line MRC-5. Using TGF β or conditioned medium from epithelial cancer cells, which included OVCAR3 (ovarian cancer cell line) and HCT116 (colon cancer cell line), they were able to induce expression of α -SMA, a hallmark of CAFs, in MRC-5 cells. They found that both TGF β and conditioned medium from cancer cells were able to increase expression of miR-21 up to 5.8-fold. They confirmed that over-expression and under-expression of miR-21 in MRC-5 cells resulted in increased and decreased expression of α -SMA in these cells respectively. In addition, they showed by luciferase assay that programmed cell death 4 (*PDCD4*) is a target of miR-21 that induces CAF characteristics in MRC-5 cells (Yao *et al.* 2011).

In another study involving ovarian cancers, Mitra *et al.* discovered that miR-31 and -214 were down-regulated whereas miR-155 was up-regulated in the CAFs compared with normal fibroblasts. Subsequent modulation of the expression of these miRNAs showed that their deregulated expression was sufficient to convert normal fibroblasts to CAFs and *vice versa* and has identified *CCL5* chemokine as a target of miR-214 (Mitra *et al.* 2012).

Cancer-associated fibroblasts

Genetic ablation of *PTEN* in stromal fibroblasts promotes tumorigenesis in the mammary epithelium (Bronisz *et al.* 2012). Analysis of the secretome profile of *PTEN*-null fibroblasts by Bronisz *et al.* identified miR-320 as a major regulator of this paracrine response. miR-320 expression was significantly reduced in *PTEN*-null mouse mammary fibroblasts (MMFs). *PTEN*-null MMFs when injected into immunocompromised mice with a mouse mammary epithelial tumour cell line developed fourfold greater tumour growth when compared with *PTEN*^{+/+} MMFs. This was reversed with restoration of miR-320 expression in *PTEN*-null MMFs. Restoration of miR-320 expression also resulted in less invasive tumours that were less vascular. The authors also examined expression of miR-320 in stromal and epithelial cells of 126 invasive breast cancers and matched normal samples. They found miR-320 expression in both epithelial and stromal compartments, with lower expression in invasive cancer compared with normal breast tissue, consistent with their *in vivo* findings of lower miR-320 expression being associated with a more malignant phenotype. Mass spectrometry analysis of conditioned media from *PTEN*-null MMFs over-expressing miR-320 compared with control *PTEN*-null MMFs revealed 54 proteins that were differentially expressed between the two groups. These were categorised into three groups – proteins that were increased in *PTEN*-null MMFs that returned to near-normal levels with re-introduction of miR-320 – the proteins included matrix metalloproteinase 9 (MMP9), MMP2, bone morphogenetic protein 1 (BMP1), lysyl oxidase-like 2 (LOXL2) and elastin microfibril interface 2 (EMILIN2); proteins that were down-regulated in *PTEN*-null MMPs and that were up-regulated with re-introduction of miR-320 included thrombospondin 1 (THBS1) and secreted frizzled-related protein 1 (SFRP1); and proteins that were unaffected by miR-320 expression which included cathepsin B (CTSB). The human homologues of the 54 murine differentially expressed proteins were able to distinguish tumour from normal stroma and actually correlated with clinical outcome based on stromal gene expression in breast cancer patients. *Ets2* was also identified as a direct target of miR-320. Re-introduction of miR-320 into *PTEN*-null MMFs resulted in a decrease in ETS2 protein levels. In stromal fibroblasts, a *PTEN*-miR-320-*Ets2* tumour suppressor axis therefore exists, which is involved in intercellular communication within the tumour microenvironment and play a role in pathological and molecular events in human breast cancers (Bronisz *et al.* 2012).

miR-148a has been found to be significantly down-regulated in CAFs of endometrial cancer compared with matched normal fibroblasts. Up-regulation of miR-148a expression by lentiviral transfection in CAFs did not alter the growth or morphology of the cells but resulted in a decreased ability of the CAFs to induce migration in endometrial cancer cell lines. Conditioned medium of CAFs expressing miR-148a also decreased the invasive phenotype of endometrial cancer cell lines cultured in three-dimensional culture. *WNT10B* was identified as a direct target of miR-148a, which stimulated migration of endometrial cancer cells (Aprelikova *et al.* 2013). This link between miR-148a and Wnt signalling is also suggested by the identification of this miRNA as a regulator of adipogenesis by suppressing the Wnt pathway (Qin *et al.* 2010).

Aprelikova *et al.* (2010) performed miRNA profiling of five matched pairs of endometrial CAFs and normal fibroblasts and found 11 miRNAs to be significantly differentially expressed between the two groups. Among these, miR-31 was down-regulated, and miR-503, -424 and -542-3p, all located on chromosome Xq26.3, were up-regulated in CAFs compared with normal fibroblasts. Fibroblasts over-expressing miR-31, when co-cultured with endometrial cancer cells, caused decreased migration and invasion but no change in proliferation in the endometrial cancer cells. *SATB2*, a predicted target of miR-31, was found to be over-expressed in CAFs compared with normal fibroblasts. Over-expression of miR-31 in CAFs decreased *SATB2* mRNA and protein levels and luciferase assays confirmed that *SATB2* is a direct target of miR-31. *SATB2* was transduced into normal fibroblasts (which have lower expression of *SATB2*) and co-culture of these fibroblasts with endometrial cancer cells resulted in increased migration and invasion of the cancer cells. Expression of miR-31 in CAFs therefore suppresses cancer cell motility and invasion in part by targeting *SATB2* (Aprelikova *et al.* 2010). Mitra *et al.* (2012) also found that down-regulation of miR-31 and -213 and down-regulation of miR-155 resulted in enhanced CAF migration as well as invasion of ovarian cancer cells co-cultured with these CAFs, an effect mediated by *CCL5*, a target of these miRNAs.

Similarly, Zhao *et al.* performed miRNA profiling of six pairs of matched CAFs and normal fibroblasts from breast cancer patients. They identified three up-regulated (miR-221-5p, -31-3p and -221-3p) and eight down-regulated (miR-205, -200b, -200c, -141, -101, -342-3p, let-7g and -26b) miRNAs in CAFs compared with matched normal fibroblasts (Zhao *et al.* 2012). Interestingly, there

was no overlap with the results found in different CAFs compared with their matched normal fibroblasts, which could in part be explained by tissue specificity of miRNAs (Liu *et al.* 2004).

In situ hybridization analysis of CAFs from prostate cancer suggested that miR-15 and -16 are down-regulated in CAFs compared with normal fibroblasts. CAFs transduced with miR-15 and -16 had decreased rate of proliferation and underwent apoptosis over the course of 1–3 weeks. Fibroblasts transduced with miR-15 and -16, when co-cultured with prostate cancer cells, impaired migration of the cancer cells as well as decreased proliferation by decreasing the proportion of cells in the S-phase and increasing the number of cells in G0–G1 phase. Reconstitution of miR-15 and -16 in CAFs therefore alters the crosstalk between the microenvironment and the cancer resulting in reduced cancer expansion capabilities. CAFs over-expressing miR-15 and -16 were found to express lower levels of *FGF2* and *FGFR1* that act on both the stromal and the cancer cells. Using luciferase assays, these two genes were found to be direct targets of miR-15 and -16. Mice were then inoculated with a mixture of prostate epithelial cells and either empty vector-treated CAFs or miR-15/-16-transduced CAFs. Empty vector-treated CAFs strongly promoted tumour growth compared with miR-15/-16-transduced CAFs. On haematoxylin and eosin staining, tumours generated from cancer cells mixed with empty vector-treated CAFs appeared to recruit stromal cells and developed an extensive invasive front. There was also much less expression of *FGF2* and a reduction in blood vessels in both epithelial and stromal compartments of cancers generated from cancer cells combined with miR-15/-16-transduced CAFs. The data indicate that miR-15/-16 transduction of CAFs affects tumour microenvironment crosstalk (Musumeci *et al.* 2011). Furthermore, miR-16 promoted the differentiation of bone marrow mesenchymal stem cells towards myogenic phenotype, suggesting that interference of this miRNA with the function of stroma is not characteristic only of pathological conditions (Liu *et al.* 2012).

Hypoxia

Hypoxia is a key feature of the tumour microenvironment. Tumours develop hypoxia as a result of impaired angiogenesis (Harris 2002). Tumours that exhibit low oxygen tension are associated with poor prognosis and resistance to therapy (Hockel *et al.* 1996, Kulshreshtha *et al.* 2007a). Hypoxia-inducible factors (HIFs) are transcription factors responsible for the expression of a

number of genes responsible for adaptation of cells to low oxygen environment (Harris 2002). By exposing breast and colon cancer cells to hypoxic conditions, Kulshreshtha *et al.* identified a number of miRNAs that were induced by hypoxia. These include miR-21, -23a, -23b, -24, -26a, -26b, -27a, -30b, -93, -103, -103, -106a, -107, -125b, -181a, -181b, -181c, -192, -195, -210 and -213 as well as miRNAs that are down-regulated by hypoxia including miR-122a, -565, -195, -30e-5p, -374, -19a, -101, -424, -29b, -186, -141, -320, -422b, -197, -15b, -16, -20a, -20b, -30b and -224. Many of these hypoxia-regulated miRNAs have also been found to be altered in cancers, suggesting that they play a role in tumorigenesis as well (Kulshreshtha *et al.* 2007b). Of the studies, which have looked at hypoxia-regulated miRNAs, miR-210 has been the single miRNA consistently found to be induced by hypoxia (Kulshreshtha *et al.* 2007b, Camps *et al.* 2008, Fasanaro *et al.* 2008).

Over-expression of miR-210, reflecting hypoxia, in cancers is associated with worse prognosis (Camps *et al.* 2008). Exposing endothelial cells in the microenvironment to hypoxic conditions also results in increased expression of miR-210 via HIF1 α . miR-210 in turn down-regulated Ephrin A3 resulting in decreased capillary-like formation and migration of the endothelial cells (Fasanaro *et al.* 2008).

Extracellular matrix and angiogenesis

The extracellular matrix (ECM), consisting of a complex network of macromolecules, is an important part of the tumour microenvironment (Lu *et al.* 2012). miRNA expression by the cancer cells can alter the tumour microenvironment by inhibiting genes involved in angiogenesis and ECM signalling resulting in inhibition of metastasis. miR-29b has been reported to be down-regulated in hepatocellular carcinomas (HCCs) and associated with worse patient prognosis (Xiong *et al.* 2010). Fang *et al.* found that endothelial cells grown in medium conditioned by HCC cells transfected with miR-29b developed fewer capillary-like structures compared with those transfected with negative control. *In vivo* experiments performed on mice with HCC cell lines transfected with miR-29b or negative control confirmed smaller tumours with lower blood vessel density and fewer and smaller intrahepatic metastases in miR-29b-transfected HCC cells. miR-29b was found to exert its function by suppressing pro-metastatic genes that include MMP2 (Fang *et al.* 2011), ANGPTL4, LOX, MMP9 and VEGFA (Chou *et al.* 2013). miR-29b itself is regulated by

GATA3 (Chou *et al.* 2013), a key regulator of mammary gland formation (Asselin-Labat *et al.* 2007). miR-29b and GATA3 therefore play an important role in inhibition of metastasis.

miRNAs have also been found to affect endothelial cells, thus likely playing a role in angiogenesis during tumour development. Png *et al.* found that miR-126 suppresses metastatic recruitment of endothelial cells, angiogenesis and colonization via *IGFBP2*, *PITPNC1* and *MERTK*. miR-126 regulates endothelial recruitment to metastatic breast cancer cells regardless of anatomical location. Over-expression of miR-126 strongly inhibited endothelial recruitment while knockdown of miR-126 significantly increased endothelial cell recruitment. miR-126 was noted to suppress eight genes (*ABCB9*, *IGFBP2*, *MERTK*, *PITPNC1*, *PSAT1*, *ITGB4*, *SHMT2* and *VIPR1*), and breast cancers that over-expressed these genes were more likely to develop distal metastasis and have shorter metastasis-free survival. In addition, stage 3 and 4 primary breast cancers were found to significantly over-express a subset of these genes (*IGFBP2*, *MERTK* and *PITPNC1*) compared with stage 1 and 2 cancers. Knockdown of *IGFBP2*, *MERTK* and *PITPNC1* decreased the ability of cells to metastasize as well as reduced metastatic endothelial density and functional vessel content. Over-expression of these three genes in miR-126 over-expressing cells rescued the ability of cancer cells to recruit endothelial cells, indicating that *IGFBP2*, *MERTK* and *PITPNC1* are direct targets of miR-126 and required for metastatic colonisation and endothelial recruitment (Png *et al.* 2012). Consistent with the role of miR-126 in endothelial cells, miR-126 has been found to be down-regulated in colon (Guo *et al.* 2008) and gastric (Feng *et al.* 2010) cancers.

Besides its role in regulating the recruitment of the endothelial cells, miR-126 is also capable of suppressing the recruitment of monocytes and thus inhibiting the development of lung metastases of breast cancers (Zhang *et al.* 2013). Interestingly, this activity is produced by both miR-126 and independently by its passenger miR-126(*). In this model, the anti-metastatic effect is mediated directly by suppressing the expression of *SDF1a* and indirectly by suppressing *CCL2* chemokine by a manner that depends on *SDF1a*.

Immune cells

A number of papers have demonstrated that CAFs play a role in attracting the immune cells to the site of the cancer (Augsten *et al.* 2009, Erez *et al.* 2010, Hembruff *et al.* 2010). Hembruff *et al.* (2010) found that over-expression of the

chemokine CCL2 by fibroblasts and co-culture with 4T1 mammary carcinoma cells *in vivo* resulted in enhanced recruitment of tumour-associated macrophages as well as increased proliferation of the primary tumour and liver metastases. In addition to recruiting immune cells to the site of the cancer, CAFs can also alter the functional activation of these cells resulting in an immunosuppressive tumour-promoting microenvironment (Liao *et al.* 2009, Kim *et al.* 2012). Various chemokines have been identified as the factor(s) secreted by CAFs, which influence immune cells (Raz & Erez 2013). Finally, secretion of miRNAs by CAFs or immune cells may also play a role in the interaction between different cell types of the tumour microenvironment (Chen *et al.* 2012).

Future perspectives

The elucidation of the role of miRNAs in tumour biology is a rapidly growing field in cancer research because it holds promise for application as markers for the prognosis of the disease as well as targets for the development of

novel cancer therapeutics. The advantages of utilising miRNAs as prognostic markers or therapeutic targets have been described in detail elsewhere (Nelson & Weiss 2008, Garzon *et al.* 2010). This notion is particularly true for their role in tumour stroma biology because of the prognostic significance as well as their ability to regulate the crosstalk between CAFs and cancer cells (Table 1 and Fig. 1). While a number of miRNAs and some of their gene targets have been identified to play a role in tumour progression via CAFs, much still remains to be elucidated. In particular, while in many cases the intracellular molecular networks that are affected by the deregulation of miRNAs in CAFs have been established, with a few exceptions, the paracrine mediators of the miRNA-mediated response remain obscure. It is conceivable that understanding the identity of these mediators will provide specific markers for understanding the role of miRNAs as regulators of the paracrine activity and will also suggest novel targets for interfering with the activation of the tumour microenvironment. Currently, how miRNAs are deregulated during the activation of the

Table 1 Overview of specific microRNAs and their reported role in the tumour microenvironment.

miRNA	References	Cancer type	Gene target	Effect when miRNA level altered
miR-21	Yao <i>et al.</i> (2011)	Up-regulated in MRC-5 fetal lung fibroblasts by TGF β or conditioned medium of cancer cell lines	<i>PDCD4</i>	Up-regulation of miR-21 in MRC-5 cells resulted in transdifferentiation to a CAF phenotype
miR-31	Aprelikova <i>et al.</i> (2010)	Down-regulated in endometrial CAFs compared with normal endometrial fibroblasts	<i>SATB2</i>	Up-regulation of miR-31 decreases migration and invasion of endometrial cancer cells
miR-15/-16	Musumeci <i>et al.</i> (2011)	Down-regulated in prostate CAFs compared with normal prostate fibroblasts	<i>FGF2</i> <i>FGFR1</i>	Up-regulation of miR-15/-16 impairs migration, decreases proliferation
miR-148a	Aprelikova <i>et al.</i> (2013)	Down-regulated in endometrial CAFs compared with normal endometrial fibroblasts	<i>WNT10B</i>	Up-regulation of miR-148a decreases migration
miR-214	Mitra <i>et al.</i> (2012)	Down-regulated in ovarian CAFs compared with adjacent normal omental fibroblasts	<i>CCL5</i>	Decreases CAF migration and invasion of co-cultured ovarian cancer cells. Down-regulation of miR-214 in normal omental fibroblasts resulted in differentiation of these fibroblasts into CAFs
miR-320	Bronisz <i>et al.</i> (2012)	Down-regulated in <i>Pten</i> -null mammary mouse fibroblasts compared with WT PTEN mammary mouse fibroblasts; also found to be decreased in expression in stromal and epithelial compartments of invasive breast cancer samples compared with normal breast	<i>ETS2</i>	When <i>Pten</i> -null mammary mouse fibroblast co-injected with epithelial cancer cell line <i>in vivo</i> , there was less tumour growth with less invasive and less vascular tumour
miR-126	Png <i>et al.</i> (2012)	Down-regulated in colon and gastric cancer	<i>IGFBP2</i> <i>PITPNC</i> <i>MERTK</i>	Up-regulation of miR-126 suppresses metastatic endothelial recruitment, angiogenesis and colonization

Figure 1

Diagrammatic overview for the possible involvement of microRNAs in the tumour microenvironment. Certain stimuli elicited by the cancer cells cause the modulation of the expression of specific microRNAs in the stromal fibroblasts. These changes in expression of microRNAs are associated with the activation of stromal fibroblasts and the transition into the

cancer-associated fibroblast state, which in turn stimulates the invasion, migration and proliferation of the cancer cells. Cancer cell-independent differential expression of microRNAs in the fibroblasts may also be sufficient for the neoplastic conversion of otherwise normal epithelium.

stroma in carcinogenesis and, in particular, how specific signals from the cancer cells are integrated into the stromal fibroblasts and induce their transition into CAFs remain poorly understood. Interestingly, the majority of the miRNAs known to date that are differentially expressed in CAFs are down-regulated when compared with normal fibroblasts, implying that they are acting as suppressors of stroma activation. Thus, the identification of an array of miRNAs that may act as activators is anticipated and remains to be confirmed.

Further work is urgently required to deepen our understanding of miRNAs not only within the tumour environment but also within the epithelial cancer cells themselves before miRNAs can be thought of as potential therapeutic targets.

Conclusion

Although several lines of evidence are available regarding the actual secreted mediators of the paracrine communication between cancer and stromal cells, the intracellular regulators of this process remain poorly understood. The rapid induction of this non-cell autonomous activity following neoplastic development in combination with its pleiotropic activity that apparently affects a

variety of cellular processes, such as stimulation of cell proliferation, acquisition of resistance to apoptosis, induction of angiogenesis as well as the generation of a pro-inflammatory microenvironment by mobilising cells of the immune system, imposes interesting challenges: It must be a rapid response that should be triggered rapidly following the onset of malignancy, it must be capable of affecting the production of various cytokines and chemokines and at the same time, it should also be very specific as the profile of fibroblasts' activation appears quite homogeneous regardless of the source of the cancer with its variable origin and characteristics. To that end, understanding the regulation of the tumour microenvironment is essential in order to understand basic aspects of tumour biology and may also provide novel targets for therapeutic intervention and tools of prognostic value.

Fibroblasts have been shown conclusively to play a role in tumorigenesis with miRNAs implicated in regulating their transition into the CAF state. Because the tumour stroma, of which CAFs comprise the major component, is able to influence patient outcome, research in the regulatory role of miRNAs in CAFs' biology is needed to unravel the complex interactions between stroma and tumour cells and ultimately to improve overall patient outcome.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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