Short Conceptual Overview

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The microRNA-200 family: still much to discover

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Abstract: In the last decade, microRNAs (miRs or miRNAs) became of great interest in cancer research due to their multifunctional and active regulation in a variety of vital cellular processes. In this review, we discuss the miR-200 family, which is composed of five members (miR-141, miR-200a/200b/200c and miR-429). Although being among the best investigated miRNAs in the field, there are still many open issues. Here, we describe the potential role of miR-200 as prognostic and/or predictive biomarker, its influence on motility and cell migration as well as its role in epithelial to mesenchymal transition (EMT) and metastasis formation in different tumour types. Recent studies also demonstrated the influence of miR-200 on drug resistance and described a correlation between miR-200 expression levels and overall survival of patients. Despite intense research in this field, the full role of the miR-200 family in cancer progression and metastasis is not completely understood and seems to differ between different tumour types and different cellular backgrounds. To elucidate these differences further, a finer characterisation of the role of the individual miRNA-200 family members is currently under investigation.

Keywords: cell migration; drug resistance; epithelial to mesenchymal transition; microRNA-200; prognostic biomarker; ZEB1.

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Introduction

MicroRNAs (miRs or miRNAs) are 21–25 nucleotide stretches of non-coding RNA, which regulate gene expression post-transcriptionally by base pairing with the 3′untranslated regions (3′UTRs) or open reading frames within the target gene (1, 2). This base paring results in target degradation or repression of miR translation (1). Through imperfect base pairing of the specific miR, the target degradation is mediated by a stretch of 6–11 nucleotides. As a consequence, one miR can target hundreds of mRNAs (3). Bioinformatics tools like miRBase (http://www.mirbase.org) predict that more than 1800 miRNAs exist in the human genome. In addition, most multi-cellular organisms rely on miRNA-mediated control mechanisms including plants.

As regulatory molecules, miRs are involved in nearly all processes including cell proliferation, apoptosis or epithelial to mesenchymal transition (EMT) (3). With regard to tumourigenesis, alterations in the miR profile are related to tumour development, progression and metastasis (4). Occasionally, genetic alterations of miR encoding genes can create amplifications or deletions of these miRNAs. In addition, defects in the miRNA processing machinery often lead to functional imbalances, especially deletions of Dicer or DGCR8 proteins, which result in changes of proliferation and malignant properties (5). Due to its haploinsufficient oncosuppressor function where deletion of one gene could lead to malignancy, the Dicer complex is very sensitive to deletion. In addition, not only changes in the miR processing machinery may result in functional imbalances, but also changes in the 3′UTR binding site of mRNA, which can delete or even introduce miRNA binding sites (6). Conversely, cancer development fundamentally alters the cell's miRNA profile over time with direct impact on protein synthesis (7).

In this review, we focus on the miR-200 family, which consists of five highly conserved members (miR-141, miR-200a/200b/200c and miR-429) (8) and is one of the best investigated miRNAs in the field.

The miR-200 controversy

Dysregulation of these miR family members was shown to increase cell growth and tumour progression in different

tumour types. In the majority of studies, miR-200 family members were downregulated and decreased cell proliferation (2, 9–11). Further, miR-200 family is known to directly affect tumour development, apoptosis and anoikis (2, 9, 12–17) in many other tumour types like gastric, breast, lung and brain cancer. This miR-200 suppression is discussed in detail in the following sections. In addition to cancer, miR-200 members are also known to be involved in other diseases like diabetes type 2 (18), Hirschsprung disease (10) or endometriosis (19).

In contrast to miR-200 loss, Guo and co-workers observed that overexpression of miR-200 activated protein kinase B (AKT) phosphorylation and inactivated the phosphorylation of ribosomal protein S6 kinase β-1 (p70S6K). More importantly, they found that the insulin receptor signalling protein (IRS-1) was increased by miR-200, which causes the inactivation of p70S6K in lung cancer cells resulting in feedback activation of AKT (12).

Thus, a differential role of the miR-200 family in different cellular contexts may not be excluded and continues to stir controversies.

Biological and functional differences between the miR-200 family gene members

Five different members of miRNA family 200 are known, i.e. miR-200a, miR-200b, miR-200c, miR-141 and miR-429. The whole family is highly conserved in higher vertebrates and normally expressed in epithelial tissue (8). MiR-200s are divided into two different genomic clusters located on different chromosomes (chr 1 and chr 12), but also into two functional groups, which share the same seeding sequence (2). This sequence, located at positions 2–7 from the miR 5′ end (20), is decisive for the binding properties of the miRNA to the target mRNA. The difference between the two functional groups is the third nucleotide in the seed sequence, either a C or a U (Figure 1) (functional group 1 including miR-141/200b/200c: AAUACUG; functional group 2 including miR-200a/429: AACACUG) (2). Through various modifications of the promoter region of these five miRNAs, their expression can be regulated by histone modification on chr 1 or promoter methylation on chr 12 (21). These differences in seed sequence and genomic clustering allow for a dynamic and differential regulation of the five miR-200 family members upon very different stimuli.

In various cells, Magenta and colleagues showed that oxidative stress enhances the expression of the miR-200 family members (22). In detail, miR-141 and miR-200c from the same functional cluster were significantly upregulated, whereas the other three clustered miR-200 family members, i.e. miR-200a/b/429, were induced only at lower levels. Batista and colleagues demonstrated the link of the regular PTPN6 polyadenylation signalling to allow the downstream transcription of miR-200c/141 and that the promoter of this gene physically interacts via a 3D DNA loop with the miR transcription unit (21). Various studies showed the ZEB1/ZEB2 axis and p53 as important regulators for the whole miR-200 family (23).

On the other hand, miR-200 members target and downregulate ZEB proteins in a feedback loop together with at least 100 other genes [a comprehensive listing to be retrieved in reference (2)].

Taken together, this broad spectrum of regulation together with the localisation of the miRNA-200 family members on different chromosomes provides large opportunities in controlling and fine-tuning different crucial signalling pathways, resulting in tumourigenic behaviour and finally metastatic development.

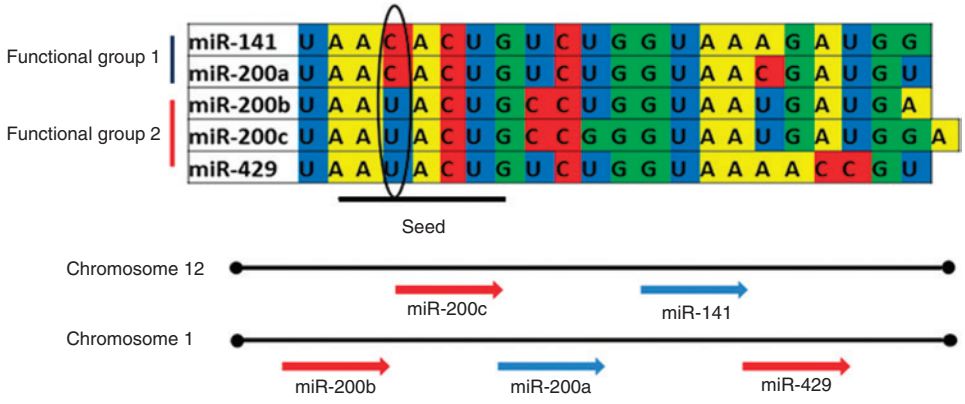

Figure 1: Chromosomal location, classification and sequences of the miR200 family members.

EMT and intravasation

EMT is a reversible process observed in organ development and metastasis (8, 24). In higher vertebrates, EMT is accountable for embryonic development especially for gastrulation and the establishment of the neural crest and the formation of other tissues and organs. Although EMT is not very frequent in adults, the process is involved in wound healing.

EMT is considered as a crucial step in invasion and metastasis assuming that cancer cells from the primary tumour undergo EMT at the invasive front (8). During EMT, the epithelial cell loses its apico-basal polarity and the tight cell-cell adhesion resulting in the gain of mesenchymal and fibroblast phenotype and enhanced migratory abilities (25). The loss of tight cell-cell contacts is elicited by the downregulation of E-cadherin and upregulation of mesenchymal markers like vimentin and N-cadherin (2, 26).

It is known that members of the miR-200 family inhibit the translation of ZEB1 and ZEB2 via a double negative feedback loop resulting in a stabilised epithelial phenotype and E-cadherin expression (Figure 2). Different studies identified the involvement of the miR-200/ZEB axis in the EMT of several tumour types. Paterson and co-workers showed that primary adenocarcinomas of the colon express higher levels of the miR-200 family than the invasive front of the tumour resulting in loss of E-cadherin (8). Another study on colon cancer revealed the loss of miR-200 family at the invasive front of the tumour, but showed the increased expression of miR-200c/141 in the liver metastasis due to a hypomethylation of the promoter (1). In functional studies, transfection with an miR-200c precursor enhanced cell proliferation, but resulted in reduced migration and invasion (1). A recent investigation linked the miR-200/ZEB1 axis with matrix-dependent tumour invasion and metastasis. The authors found that for ZEB1-dependent EMT, an integrin β1-collagen interaction with the extracellular matrix is needed to mediate focal adhesion kinase (FAK) signalling, which resulted in invasion and metastasis (27) and discovered CRKL as a direct target, which is an adaptor molecule belonging to the CRK family known for interactions with paxillin and p130Cas to relocate sites of integrin-mediated focal adhesion formation (27). Hence, miR-200 modulates the focal adhesion formation via the FAK/Src complex assembly resulting in cytoskeletal reorganisation. CRKL expression was negatively regulated with the miR-200 family and positively associated with ZEB1 and Src signalling leading to poor outcome for patients with high CRKL levels in different tumour types (27).

Chung and colleagues showed recently that grainyhead-like 2 (GRHL2) is a pivotal gatekeeper for EMT via the miR-200/ZEB1 axis in epithelial ovarian cancer. A downregulation of GRHL2 resulted in increased cell migration invasion and motility. Two members of the miR family, miR-200a/b, were identified as direct transcriptional targets of GRHL2, which regulate the epithelial morphology via ZEB-1 and E-cadherin (24). A similar suppression of miR-200 family members in breast cancer was identified recently by Li and co-workers. In this study, the authors discovered that miR-200b can repress breast cancer metastasis via a ZEB1-independent pathway. They used a xenograft orthotropic breast cancer model expressing members of the miR-200b/200c/429 functional group and showed that miR-200b repressed several cytoskeleton associated genes and directly targets moesin. Knock-down of moesin reduced cancer cell invasion and is inversely correlated with miR-200b expression in breast cancer (11).

Additionally to the ZEB/miR-200 negative feedback loop, Gregory and colleagues identified a new autocrine regulatory pathway between the miR-200 family/ZEB and TGF-β (28, 29). An important activator for ZEB1/2 is TGF-β signalling, where a negative correlation was observed between miR-200 family expression and TGF-β.

The process of intravasation is one step in the metastatic cascade and involves detachment of cancer cells of the primary tumour and their active entry into the blood stream or lymphatic system. This could also be achieved during angiogenesis when the newly formed blood vessels are leaky. We have previously shown that the intravasation

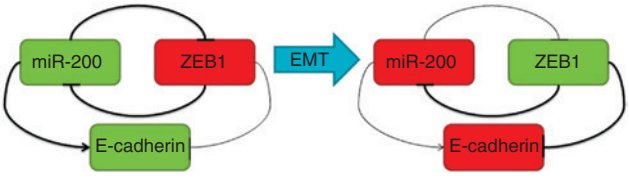

Figure 2: Overview of the regulatory effect of the miR-200 family on ZEB1.
In an epithelial-like cell, miR-200 family members inhibit ZEB1 expression resulting in E-cadherin synthesis. During EMT, cells lose miR-200, which in turn activates ZEB1 with eventual repression of E-cadherin synthesis.

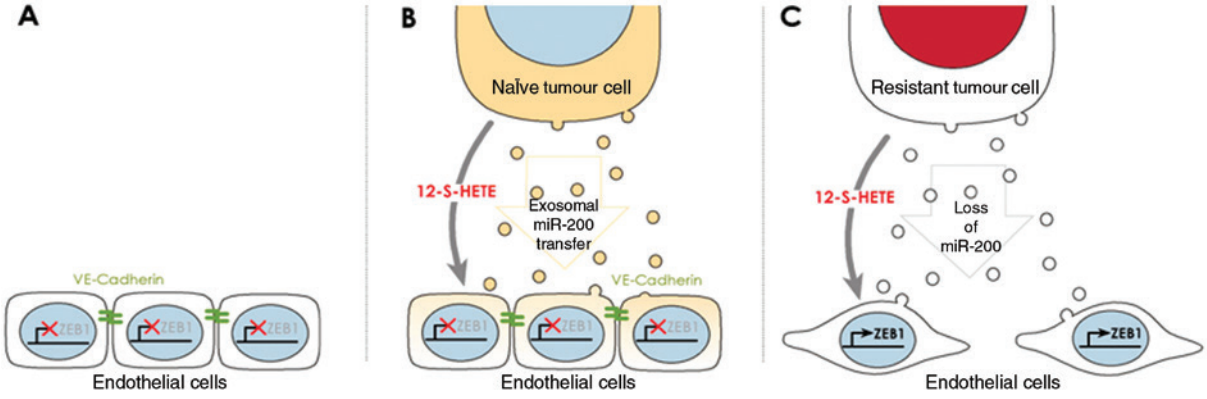

Figure 3: Schematic representation of tumour cell/lymphendothelial cell (LEC) interaction.
(A) In the absence of tumour cells, LEC establishes cell/cell interaction by VE-cadherin (green). (B) In the presence of naïve tumour cells in spatial proximity to LEC, 12-S-HETE (red) is transferred to the endothelium, which would induce the expression of ZEB1. However, simultaneously miR-200 (orange) is transferred via exosomes (small circles), which block the expression of ZEB1. (C) Resistant tumour cells have lost the expression of miR-200; therefore, 12-S-HETE can induce ZEB1 expression, which triggers loss of VE-cadherin in the LEC and results in a motile phenotype via EMT-like mechanisms. As a consequence, tumour cells can pass through LEC layers, which move aside.

of breast cancer emboli through endothelial sheets was accompanied by an induction of ZEB1 in the involved lymphendothelial cells (LECs) (30, 31). Likewise, colorectal cancer spreads across the lymphatic vasculature as we have demonstrated that a loss of the miR-200 family was associated with increased invasiveness through a lymphendothelial layer (32) (Figure 3). Very similar to its relevance in cell motility, intravasation is strongly influenced by different members of the miR-200 family depending on their individual expression levels.

Influence of miRNA-200 family members on cell motility

Cell motility is the collective outcome of a number of orchestrated processes dependent on extensive cytoskeletal rearrangements and assembly of actin bundles (33). Recently, Wong and co-workers demonstrated that miR-200b/c/429, but not miR-200a/141, are involved in cell motility. As an underlying mechanism, overexpression of miR-200b could suppress Rho/Rock signalling in hepatocellular carcinoma metastasis (34).

Further, miR-200b/c is targeting SUZ12/Rock2 and inhibits tumourigenesis, migration and metastasis in cholangiocarcinoma *in vitro* and *in vivo*, and inhibiting the expression of ROCK2 reduces the invasion of cholangiocarcinoma cells *in vitro* (35). As another signalling mechanism, miR200b/c-mediated inhibition of CRKL plays a role in migration as it regulates FAK/Src complex formation, which is required for rapid cell movement (27).

As a first step of metastasis, cells acquire enhanced cell motility. Through dysregulation of different miR-200 family members, the cancer cell can activate pathways resulting in increased cellular motility as one of the initial steps for metastasis formation. This clearly indicates that the miR-200 family is one of the central molecule families regulating cellular migration and motility. Moreover, this seems to be a general mechanism extending to a variety of different cell types including blood and LECs as well as different tumour entities.

MiR-200 family members as biomarker in cancer

Successful cancer management relies on predicting and monitoring response to treatment in cancer patients under therapy, especially to follow-up the tumour progression and changes in tumour biology. Normally, tissue biopsies from solid tumours are sampled only at the time of initial diagnosis to identify the tumour pathogenesis and to deduce the most successful drug treatment. Therefore, the ‘liquid biopsy’ from blood became a focus of interest over the last years as it allows for repeated monitoring of prognostic and predictive biomarkers. Recent approaches in detection of altered expression pattern of miRNAs in different cancer types offer a new and great opportunity of using miRNAs as robust and stable biomarkers for predicting prognosis, diagnosis or drug treatment in cancer (36). Furthermore, miRNAs appear to be remarkably stable outside cells and can be easily detected in extracellular space (37) or in all kind of body fluids such as blood, urine,

breast milk or cerebrospinal fluid (36, 38, 39). Mitchell and colleagues showed that miR-141 is overexpressed in several epithelial cancer types, such as breast, lung, colon and prostate cancer (39). Therefore, the miR-200 family might be a useful biomarker for prognosis and prediction already in the near future (39). Recently, several published studies demonstrated the potential application of miR-200 family members as prognostic (16) or predictive biomarkers (40) in different tumour types, such as ovarian cancer, colorectal cancer, non-small cell lung cancer (NSCLC), gliomas or breast cancer.

Zuberi and colleagues described a correlation of miR-200a/c expression pattern in serum from epithelial ovarian cancer patients with a significant increase in advanced stages of disease (stages III and IV) when compared with early stages (I and II) (41). In this study, the authors demonstrate a correlation between increased miR-200a/c serum levels which are associated with an aggressive tumour progression combined with poor prognosis in ovarian cancer. Likewise, overexpression of miR-200c and miR-141 in serum of NSCLC and colorectal cancer was associated with poor prognosis and short overall survival (high miR-141: OS 71.7 months; high miR-200c: OS 61.2 months), whereas lower levels of these miRNAs showed a mean overall survival of 136.9 months (42, 43).

In contrast, Men and colleagues observed that in high-grade glioma tissue (grades III and IV), expression of miR-200b was dramatically decreased when compared to non-neoplastic brain tissue. The expression levels of miR-200b were of prognostic significance for progression free and overall survival in high grade glioma patients. This was, however, not the case in grade I and II gliomas (16). Likewise, Chen and colleagues demonstrated that downregulation of miR-200c in breast cancer was correlated with poor response to neoadjuvant chemotherapy (40).

Taken together, circulating miRNAs can serve as prognostic and/or predictive biomarkers in several tumour types in future investigations. To realise the potential of these circulating biomarkers in the clinical setting, several problems need to be overcome, e.g. the lack of conditional miR-200 expression systems in animal models, the lack of well-characterised patient tissue/serum as paired samples and the expensive and/or time-consuming miRNA detection methods.

Therapy and therapy resistance

In a different field, a recent approach in miRNA-200 research is the implementation of a clinical trial entitled 'novel regulators of wound angiogenesis' of the Ohio State University in May 2016. This study will investigate whether elevated levels of wound-edge endothelial miR-200b could be a barrier to wound healing in diabetic patients. The study will follow 60 diabetes food ulcer patients over 14 weeks and the results of the clinical trial will help to provide a better knowledge in developing novel biomarker with the aim of miR-directed therapeutic strategies in general wound healing.

In a 5-fluorouracil resistant (5-FU) colon cancer model, a significant loss of miR-200 family occurred during acquisition of resistance, whereby the miR cluster on chromosome 12 (miR-200c/141) was more affected than the cluster on chromosome 1. The 5-FU resistant tumour cells showed a higher ability to intravasate through a tight endothelial barrier by direct communication via exosomes with the adjacent endothelial cells (32). Similar findings in support of our results using a primary and metastatic CRC cell line from the same patient were published by Tanaka and co-workers who demonstrated that increasing resistance to oxaliplatin resulted in a decreased expression of miR-200. Moreover, the resistant metastasis had decreased levels of miR-200c/141 when compared with the non-resistant one (44).

In line with these results, Brozovic and colleagues mentioned that ovarian cancer cell lines, which are resistant to paclitaxel and carboplatin, were associated with decreased expression of miR-200c/141 combined with a strong EMT phenotype. Transfection with miR-200c/141 inhibitors resulted in increased resistance in the parental cell lines. Nevertheless, reintroducing miR-200c/141 into OVCAR-3 resistant cell line neither restored the epithelial phenotype nor increased the sensitivity to paclitaxel, but it partially did in another resistant MES-OV cell line. Notably, these two cell lines differed in their miR-200 expression (OVCAR-3, specific decrease of miR-200c/141 and MES-OV, general decrease of miR-200 family) (45).

Another study stressed the miR-200/ZEB axis as regulator of NSCLC to nindetanib [an angiogenesis and fibroblast growth factor receptor (FGFR) inhibitor] sensitivity. By creating different nindetanib-resistant cell lines, Nishijima and co-workers demonstrated the decrease of the miR-200 family members and an increase of ZEB1 and other EMT markers in the resistant clones. Re-expression of selected miR-200 family members increased the sensitivity to nindetanib in the resistant clones, suggesting a role as predictive biomarker for sensitivity to this novel compound in NSCLC (46). In contrast to the previous study, Tejero and colleagues revealed that high expression of miR-200c and miR-141 is associated with shorter overall

survival in early stage NSCLC adenocarcinomas (43). Via functional analysis, they demonstrated that high levels of miR-200c resulted in a more mesenchymal phenotype and high levels of miR-141 directly reduced expression of the tumour suppressor Kruppel-like factor 6 (KLF6), leading to an increased secretion of vascular endothelial growth factor A (VEGFA) and thereby increasing the blood microvessel density in the tumour. These studies are fine examples illustrating that a positive predictor of response may at the same time be a negative prognostic parameter.

In pancreatic cancer, ZEB1 seems to confer resistance against the antimetabolite gemcitabine. This resistance can be reversed by the histone deacetylase (HDAC) inhibitor mocetinostat, which supresses ZEB1 by inducing miR-203 expression, another player complementing miR-200 family functions at the interplay between tumour biology and pharmacology (47).

A recent investigation showed the importance of miR-200c in claudin-low breast tumours. Claudins are significantly enriched in cancer stem cells (CSC) resulting in a very aggressive phenotype and resistance to chemotherapy. In this study, Knezevic and colleagues modified CSC characteristics, decreased proliferation, increased differentiation and reduced the metastatic ability *in vitro* by transfecting these breast cancer cells with miR-200c. By increasing miR-200c level in these hard to treat tumours, chemosensitivity increased (26).

Taken together, several studies demonstrated the involvement of the miR-200 family and the acquisition to therapy resistance, although in most cases loss of miR-200 expression leads to therapy resistance. In few situations, however, overexpression of the miR-200 family may also trigger chemoresistance stressing the organ environment as a decisive factor for differential miR-200 actions.

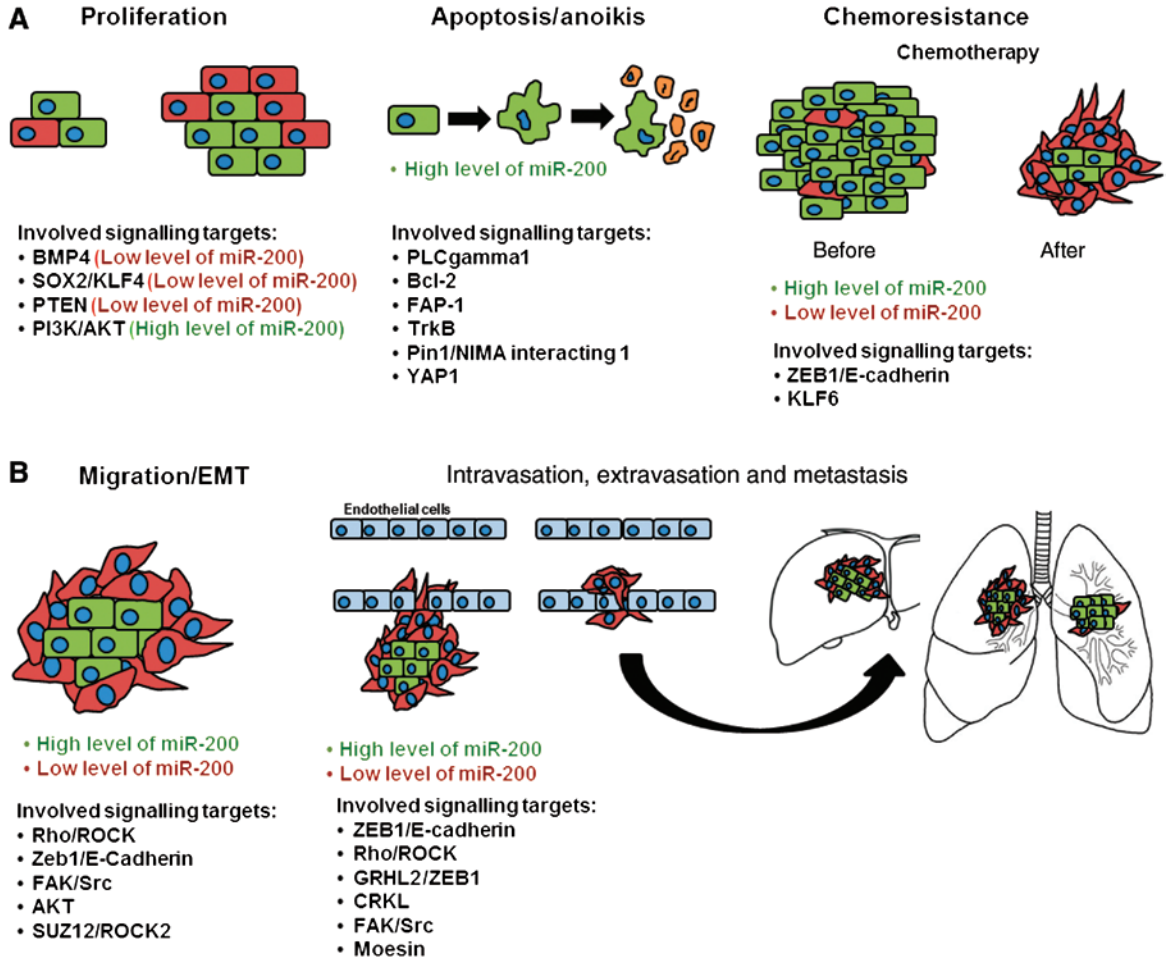

Figure 4: Overview of the different cellular processes in which the miR-200 family is involved.
(A) MiR-200 family is involved in proliferation, apoptosis/anoikis and chemoresistance. (B) MiR-200 is involved in migration, EMT, intravasation and extravasation.

Open issues in current research

Despite intense research in this field, the full role of the miR-200 family in cancer progression and metastasis is not completely understood. In line with their function as a phenotypic switch, levels of the miR-200 family seem to fluctuate during the different steps required for metastasis. When undergoing EMT, the cells lose miR-200 family members resulting in increased motility and metastatic features. After metastatic outgrowth in a different organ, some cells re-express the miR-200 family again. Thus, the role and importance of the miR-200 family differs among tumour types. Although several investigations suggest that a loss of miR-200 is favourable for intravasation in different tumour types, Le and colleagues showed exactly the opposite: exosomes enriched with miR-200 from highly metastatic breast cancer cell lines can transfer their aggressive phenotype to weakly metastatic breast cancer cell lines (48). They also showed that cells which were incubated with exosomes from cells transfected with a miR-200 inhibitor produced a lower number of metastases than cells, which were incubated with exosomes from miR-200 expressing cells. These findings point at another feature of the miR-200 family, when the miR-200 family is required for the colonisation of the lung with poorly metastatic cell lines (48).

Thus, the versatile role of the miR-200 family against different cellular backgrounds depends on a spatio-temporal expression pattern to favour or attenuate malignant progression. Therefore, the packing and transfer of miR-200 in exosomes and their uptake into recipient cells is another upcoming issue. Preliminary data suggest that exosomal miR-200 traffic and reprogramming of target cells might be a key characteristic of the malignant process.

Conclusion

Although all five members belong to the same miR-200 family, they can be divided into two functional groups with different target genes including ZEB1, PTEN, SOX2 and Rho/Rock. As a consequence, miR-200 family is a versatile set of miRNA, which plays relevant roles in several malignant processes (11, 24, 33, 48). Most importantly, these processes are related to cancer progression including apoptotic resistance, contributing to drug-resistance and metastases formation linked to the EMT regulation (Figure 4A and B). To elucidate these differences further, a finer classification of the individual miRNA-200 family members is currently under investigation. This will serve to link the differential expression of the five family members to different functions in their specific cellular context.

Recently, the first clinical trial (phase I) for miRNA-based therapy other than miR-200 in advanced liver cancer was implemented in 2013 (28). To date, this trial is still ongoing. Although the use of miRNA-based therapeutics is still in early stages of research and the potential long-term and side effects needs to be understood and verified, the miR-200 family could be a promising therapeutic target in addition to its proven value as cancer biomarker and regulator of malignant processes.

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