Discussion and future perspectives

The discovery of small RNAs like miRNAs has revolutionized the concept of genes and gene regulation. It is clear that miRNAs play an important role in virtually all biological processes and that their dysregulation is associated with disease. In cancer, widespread changes in miRNAs levels have been observed in virtually all tumor types. Moreover, mouse studies have demonstrated that causal links exist between miRNAs and cancer development [1-4], and miRNAs have been demonstrated to play crucial roles in the response to therapy [5-6].

Given the current interest in miRNAs it is likely that in the following years the role of miRNAs in tumor biology will be better understood. It is likely that in the future the focus will shift from identifying individual miRNAs to describing miRNA networks in healthy and cancer cells [7-10]. High throughput sequencing will enable comprehensive and quantitative analysis of existing and novel miRNAs (as well as different miRNA isoforms) in cancer and normal tissues. However, these approaches generate a lot of data, and the challenge will be to filter out the miRNAs that play a central role in cancer biology. The method we describe in Chapter 2 may help to identify those key miRNAs. We demonstrated that tumor miRNAs that are regulated after DNA damage in primary breast and lung epithelial cells are more likely to modulate the response to genotoxic agents. This method can be extended to other forms of cancer as well. Further studies are necessary to determine whether the regulation of the DDR miRNAs is directly mediated by ATM/ATR signaling and whether these miRNAs affect regulation of the cell cycle and/or repair of DNA lesions.

We have developed an assay to measure the effects of miRNA overexpression on cancer drug sensitivity (Chapter 2, Chapter 5-7). This assay could be used to screen for other miRNAs that play a role in cancer drug sensitivity. In this thesis we have specifically focused on the role of *miR-141* and *miR-634* in the response to platinum-based chemotherapy in ovarian cancer. Avenues for future research and therapeutic options for these miRNAs will be discussed in the remainder of this chapter.

1. The *miR-200* family of miRNAs

The *miR-200* family consists of 5 miRNAs: *miR-200a,b,c*, *miR-141* and *miR-429* (Chapter 4). In Chapter 5 we demonstrated that *miR-141* can induce cisplatin resistance in ovarian cancer cell lines. However, the *miR-200* family has also been associated with the response to paclitaxel, docetaxel, gemcitabine, doxorubicin, imatinib, tamoxifen and erlotinib/gefitinib chemotherapy (Table 1) [11-21]. These drugs have different modes of action. Whereas cisplatin and doxorubicin interact with the DNA [22-23], paclitaxel and docetaxel inhibit microtubules and stimulate the production of ROS [24-26] and gemcitabine is a nucleoside analogue that interferes with DNA synthesis [27]. Imatinib, erlotinib and gefitinib are tyrosine kinase inhibitors [28-29], whereas tamoxifen binds to the estrogen receptor and interferes with estrogen receptor signaling [30]. The question arises how the *miR-200* family can modulate the sensitivity towards these different kinds of drugs.

An explanation for this could be that the *miR-200* family inhibits the Epithelial-to-Mesenchymal transition (through repression of *ZEB1*, *ZEB2* (all members) and *BMI1* (*miR-200b*) [31-34]; see Chapter 4).

Table 1: *miR-200* family and response to anticancer drugs

Cytotoxic agent	Tumor type	*miR-200* family member	Association	Potential target	Reference
Cisplatin	Esophageal cancer	*miR-141*	High levels confer resistance to cisplatin	*YAP1*	[35]
Cisplatin	Esophageal cancer	*miR-200c*	High levels confer resistance to cisplatin	*PPP2R1B*, correlation only	[18]
Cisplatin	Ovarian cancer	*miR-141*	High levels confer resistance towards cisplatin	*KEAP1*	Chapter 5
Cisplatin	Ovarian cancer	*miR-141* and *miR-200c*	High levels correlate with resistance in a subset of patients		[36]
Cisplatin	Breast cancer	*miR-141*, *miR-200b*, *miR-200c*	Expression lower in resistant cells		[37]
Cisplatin	Tongue Squamous Cell Carcinoma	*miR-200b*	Low levels confer resistance towards cisplatin	*BMI1*	[31]
Paclitaxel	Endometrium and ovarian cancer	*miR-200c*	High levels sensitize towards paclitaxel	*TUBB3*, correlation only	[11]
Paclitaxel	Endometrium and ovarian cancer	*miR-200c*	High levels sensitize towards paclitaxel	*TUBB3*	[12]
Paclitaxel	Ovarian cancer	All	High levels sensitize towards paclitaxel		[38]
Paclitaxel	Ovarian cancer	*miR-200a* and *miR-141*	High levels sensitize towards paclitaxel	*p38 MAPK*	[13]
Docetaxel	Lung cancer	*miR-200b*	High levels sensitize towards docetaxel	*E2F3*	[14]
Docetaxel	Lung cancer	*miR-200b*	Expression lower in resistant cells		[39]
Doxorubicin	Breast cancer	*miR-141*	Expression higher in resistant cells		[17]
Doxorubicin	Breast cancer	*miR-200b*	High levels sensitize towards doxorubicin		[40]
Doxorubicin	Breast cancer	*miR-200c*	Expression lower in resistant cells		[41]
Doxorubicin	Lung cancer	*miR-200b*	Expression lower in resistant cells		[42]
Erlotinib/Gefitinib	Bladder cancer	*miR-200c*	High levels sensitize towards Erlotinib/Gefitinib	*ERRFI1*	[19]
Gemcitabine	Cholangiocarcinoma	*miR-141* and *miR-200b*	High levels confer resistance towards gemcitabine	*miR-141 - Clock*, *miR-200b - PTPN12* (correlation only)	[15]
Gemcitabine	Pancreatic cancer	*miR-200b/c*	Expression lower in resistant cells		[43]
Gemcitabine	Pancreatic cancer	*miR-200b/c*	Expression lower in resistant cells		[16]
Imatinib	Chronic Myeloid Leukemia	*miR-141*	Expression lower in resistant cells		[20]
Tamoxifen	Breast cancer	*miR-200*	Expression lower in resistant cells		[21]

In general, mesenchymal cells are more drug resistant than epithelial cells [44], and by converting mesenchymal cells to the epithelial state, *miR-200* family overexpression may enhance drug sensitivity. In addition, *miR-200* members downregulate targets that play a role in cell proliferation and survival, making cells more sensitive for death inducing stimuli. These targets include the cell cycle regulator *E2F3* (*miR-200b*) [14] and the stress response gene *p38 MAPK* (*miR-200a*, *miR-141*) [13]) (Table 1).

However, regulation of these targets is not likely to account for the decreased sensitivity of cancer cells to cisplatin upon overexpression of *miR-141*. Downregulation of YAP1, a protein that plays a role in DNA damage induced apoptosis [45] (Figure 1) may contribute to *miR-141*-mediated cisplatin resistance [35]. Furthermore, we demonstrated in Chapter 5 that the phenotype of *miR-141* is partially mediated by repression of KEAP1, a key regulator of the NRF2-mediated oxidative stress response, the NF-κB pathway and the anti-apoptotic protein Bcl-xL [46-50]). Under normal homeostatic conditions KEAP1 acts as a repressor of NRF2, IKKβ (activator of NF-κB) and Bcl-xL, however, under stress KEAP1 becomes inactivated resulting in derepression. Downregulation of KEAP1 by miR-141 may thus also lead to activation of these pathways, providing a molecular basis for miR-141 induced cisplatin resistance. Accordingly, we showed that *miR-141* overexpression results in activation of NF-κB signaling, presumably through repression of KEAP1 and activation of IKKβ [48-49] (Figure 1). More research is necessary to investigate why KEAP1 downregulation does not result in activation of the NRF2 pathway. It will also be interesting to investigate if activation of NF-κB by *miR-141* affects the response to other chemotherapeutics. Of note, paclitaxel and doxorubicin treatment activate NF-κB signaling [51-54].

It will also be interesting to look into the mechanisms that regulate *miR-200* expression in response to cisplatin treatment (Chapter 5). It has been reported that p53, a key mediator of the cellular response to DNA damage, can induce expression of *miR-141* and other members of the *miR-200* family [55-56], and overexpression of *miR-141* results in downregulation of p53 [57]. The relation between p53 and *miR-141* signaling in the cellular response to cytotoxics needs to be further elucidated.

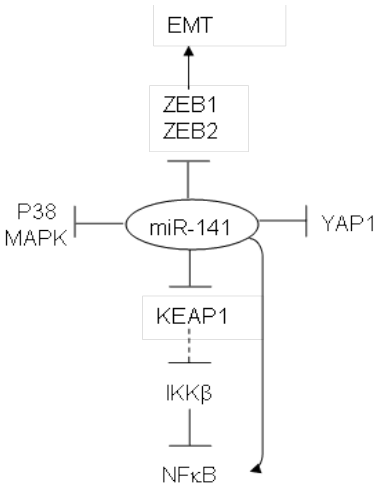

Figure 1: Pathways regulated by miR-141 that may affect the response to anticancer therapy
miR-141 has been reported to inhibit the EMT regulators ZEB1/ZEB2 [32-34] and p38α MAPK [7, 13]. Repression of these targets may result in enhanced chemotherapy sensitivity. Furthermore, repression of YAP1 [7, 35] and KEAP1 (Chapter 5) may enhance resistance. KEAP1 levels may modulate sensitivity to platinum-containing chemotherapeutics (e.g. cisplatin) by regulating the NF-κB pathway. Accordingly, miR-141 overexpression results in NF-κB activation and inhibition of NF-κB partially reverses the miR-141 phenotype. However, further experiments are necessary to demonstrate that these effects are mediated by KEAP1 and IKKβ.

2. *miR-634*

In contrast to the *miR-200* family, *miR-634* has not received a lot of attention. The miRNA was first identified in colon cancer cells [58], and afterwards a study appeared where it was demonstrated that *miR-634* targets the androgen receptor (AR) [59]. Of note, high androgen serum levels are correlated with increased ovarian cancer risk [60] and more than 80% of ovarian cancer tumors express the AR [61].

In chapter 6 we describe that overexpression of *miR-634* results in increased sensitivity towards cisplatin, carboplatin and doxorubicin, but not paclitaxel. Paclitaxel

can promote cell death via at least two mechanisms (i) the inhibition of microtubule dynamics leading to a G2/M arrest [24] and (ii) stimulation of the production of ROS [25-26]. Because the drug exposure time in our assay is only 24 hours (and the population doubling time of the ascites derived cultures is ~48 hours or less) it is conceivable that the effects of paclitaxel on the cell cycle do not contribute to the reduction in viability. Therefore, more studies are necessary to investigate the effect of *miR-634* on paclitaxel cytotoxicity.

We proposed in Chapter 6 that the effects of *miR-634* on drug sensitivity are due to regulation of several components of the Ras-MAPK pathway. In Chapter 7 we further show that repression of one of its predicted targets RSK2, results in enhanced cisplatin sensitivity in ovarian cancer cell lines. In contrast, knockdown of the putative miR-634 target and related kinase RSK1, does not alter cisplatin sensitivity. The fact that RSK2, but not RSK1, is downregulated in response to cisplatin treatment may indicate that this protein plays an important role in the cellular response to cisplatin treatment. The importance of the RSK family in the response to cisplatin treatment is highlighted by the fact that another recent study found that RSK expression levels correlate with cisplatin resistance in a panel of 8 ovarian cancer cell lines [62].

Comment [MvJ1]: Ik zou hier nog iets kunnen zeggen over de rol van RSK2 als mogelijke biomarker of toepassing van remmers, maar (a) dit staat al in Hoofdstuk 7 en (b) het effect van RSK2 siRNA op gevoeligheid is klein vgl met miR-634 overexpressie, waardoor een miRNA therapie waarschijnlijk beter zou werken. Ook rem je met miR-634 meerdere targets en vind ik dit dus beter

Intriguingly, the predicted targets of *miR-634* are enriched for genes that play a role in prostate cancer, acute myeloid leukemia, glioma, non-small cell lung cancer, endometrial cancer, clear cell renal cancer, thyroid cancer, colorectal cancer and pancreatic cancer (Supplementary table 4, Chapter 6). Therefore, it will be interesting to study the effect of *miR-634* overexpression on these tumors. Of note, ascites formation is also observed in patients with advanced cancers of other organs in the abdominal cavity, such as lung cancer, colorectal cancer, and pancreatic cancer [63].

Hence, a first step may be to analyze the effects of *miR-634* overexpression on ascites-derived tumor cells in these patients.

3. Towards a miRNA-based therapy

A decade after its discovery miRNAs are rapidly entering the clinic as biomarkers and therapeutic tools [64-65]. The fact that miRNAs can regulate the expression of multiple genes makes them attractive drug targets, as multiple oncogenes can be inhibited at the same time. However, the fact that miRNAs can regulate multiple genes is also a major drawback, because of the risk of unwanted side effects. To reduce side effects, tumor-specific delivery may be necessary.

Several studies have demonstrated that a miRNA-based therapy is feasible. Oncogenic miRNAs can be inhibited by anti-miRNA oligonucleotides (e.g. antagomirs or Locked Nucleic Acids [66-67]) or via constructs containing miRNA target sequences ('sponges' or 'decoys' [68-69]). Tumor-suppressive miRNAs may be repleted by synthetic miRNA molecules or via expression constructs [70-73] Delivery of (anti) miRNAs by 'naked' synthetic oligonucleotides [67, 74-80], oligonucleotides packaged in protein- or lipid based nanocapsules [81-84] or by viral particles [72, 85-87].

Ovarian cancer represents a group of heterologous tumors with different etiology. Since the high grade serous ovarian cancer is the most common histotype and accounts for 70% of the deaths [88], for a miR-based therapy it may be best to focus on this subtype. There is currently only one mouse model that recapitulates the development of human high grade serous ovarian cancer, however, this model is based on knockout of *Pten* and *Dicer* [88]. Since Dicer is required for miRNA biogenesis, studies may be best performed in human ovarian tumor xenografts [89].

To direct expression of miRNAs to ovarian tissue, viral-mediated delivery is probably the best option. Both lentiviral as adenoviral vectors could be used. Lentiviral vectors stably integrate into the genome of the host cell [90], but a disadvantage is that they can disrupt genes. Adenoviral constructs [70, 72] are not incorporated into the DNA of the host cell, but the effect of viral transfection is transient. The tropism of viruses may be altered so that they may bind to cell type specific receptors [91-92]. To direct viruses specifically to ovarian tumor cells the folate receptor (which is expressed in 90% of ovarian tumor cells) may be targeted [93]. Furthermore, fallopian tube specific transcription factors (e.g. Pax8) may be used to confine expression to tumor cells [94].

As a first step towards resensitizing ovarian tumors to chemotherapy, the effects of *miR-634* overexpression on the therapy response of xenograft tumors could be determined. Before *miR-141* can progress towards the clinic, the effect of *miR-141* overexpression on the outcome of combination therapy (ovarian cancer is treated with a platinum-based compound and a taxane) needs to be better understood. *miR-141* can both sensitize ovarian cancer cells for paclitaxel and enhance cisplatin resistance and therefore overexpression may differentially affect the outcome of treatment (Chapter 5, [11-13, 36, 38]. Next to use as a therapeutic target, *miR-141* and *miR-634* may also be exploited as biomarker to predict the response of tumors to chemotherapy.

Altogether, therapies based on *miR-141* and *miR-634* hold promise for the treatment of ovarian cancer.

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