Introduction

1. Protein-coding and non-protein coding genes

Most cellular functions are carried out by proteins, macromolecules composed of chains of amino acids. The information needed to generate these proteins is encoded in the DNA (Deoxyribonucleic Acid) and organized in transcriptional units called ‘genes’. In order to manufacture a protein, the gene is first transcribed into ‘messenger’ Ribonucleic Acid (mRNA), which is translated into protein [1].

For decades, it was thought that non-protein coding DNA had no function, and that the number of protein-coding genes was proportional to the complexity of organisms. However, when the complete human genome sequence was elucidated, it was found that only a fraction (~1-2%) of the human genome consists of protein-coding genes [2]. Furthermore, the number of human protein-coding genes (~22.000 [3]) is comparable to the number of protein-coding genes in the nematode *Caenorhabditis elegans* (*C. elegans*), whereas the human genome is 30x larger [4-5]. Apparently, the non-protein coding DNA is important. The finding that >70% of the DNA is transcribed into RNA [6] has sparkled interest in the function of non-protein coding RNAs.

An increasing number of non-protein coding RNAs has been discovered. Several classes can be distinguished based on function and size (Table 1) [7-15]. One class that has attracted a lot of attention is formed by microRNAs (miRNAs).

Table 1: Some prominent classes of non-protein coding RNAs

Name	Length (Nt)	Main function
nanoRNAs	2-4	Primer initiation of transcription
microRNAs (miRNAs)	19-25	Post-transcriptional gene repression
Small interfering RNAs (siRNAs)	20-25	Post-transcriptional gene repression and transposon silencing
Piwi-associated RNAs (piRNAs)	24-31	Transposon silencing
guideRNAs (gRNAs)	35-78	RNA editing
Transfer RNAs (tRNAs)	73-93	Translation of mRNAs to proteins
Small nucleolar RNAs (snoRNAs)	60-300	Modification of rRNAs
Small nuclear RNAs (snRNAs)	90-220	Splicing
Long non coding RNAs	>200	Regulation of expression of nearby genes
Ribosomal RNAs (rRNAs)	1800-5000	Structural component of ribosome

1.2 miRNAs: Regulators of gene expression

miRNAs are a group of 19-25 nucleotide long RNAs, that are able to regulate the expression of protein-coding genes, via a mechanism that is explained in detail below.

miRNAs can either be located in the DNA as an individual transcriptional unit, form a cluster with other miRNAs (several miRNAs under control of one promoter) or may be part of protein-coding genes (located in introns or exons) [16]. Individual miRNA genes are transcribed as primary (pri-) miRNAs, which are cleaved by the microprocessor (an endonuclease complex composed of Drosha, DGCR8 and accessory proteins) into ~70 nucleotide long precursor (pre-) miRNAs. Following export out of the nucleus by Exportin 5 (XPO5), the pre-miRNA is cleaved by Argonaute 2 (Ago2) and Dicer into a mature ~22 nucleotide miRNA duplex [17-18] (See figure 1, Chapter 4). The biogenesis of miRNAs that are cotranscribed with their host gene or other miRNAs (in case of miRNA cluster) presumably involves similar processing steps, however, some miRNAs (the so-called ‘mirtrons’) may be spliced out of the host gene transcript independent of the microprocessor complex [19].

Another exception are ‘simtrons’, miRNAs which processing requires Drosha, but not Dicer, DGCR8, XPO5 or Ago2 [20].

One (in some cases both) of the strands of the miRNA duplex is incorporated in the RNA induced silencing complex (RISC; composed of amongst other proteins Dicer and Ago2), and the opposing strand is degraded [21-22]. The strand that is incorporated in the RISC complex is used as a template to find complementary binding sites in the 3’UTR of mRNA molecules [23]. The most important target recognition motif is the seed sequence, typically nucleotides 2-8 from the 5’ end of the miRNA [24]. However, the 3’end region may also contribute to effective binding in ~2% of the cases [25] and for some miRNAs target recognition is mediated by a central 11-12 nucleotide region [26].

In most cases, the binding of a miRNA to a target mRNA results in repression of the target gene. Depending on the degree of complementarity, miRNAs can either promote mRNA degradation and/or inhibit translation. mRNA degradation occurs when there is extensive complementarity via Ago2-mediated RNA degradation [27] or by mRNA deadenylation leading to its degradation [17, 28]. In contrast, translational repression occurs when the complementarity is limited. The mechanisms behind translational repression are not yet fully understood [29-30].

Besides repression of gene expression, some miRNAs can promote translation of their target genes [31-34].

1.2.1 Nomenclature

As of August 2012, 1600 miRNAs have been identified (www.mirbase.org), and individual miRNAs are designated by a number. Identical miRNAs that lie on different chromosomes are given a ‘1’ or a ‘2’ suffix (e.g. *miR-24-1* and *miR-24-2*). Related

miRNAs are indicated by letters (e.g. *miR-200a*, *miR-200b*, *miR-200c*). The strand that is incorporated into the RISC complex is called the mature miRNA (e.g. *miR-141*), and the opposite strand is referred to as the 'star' strand (e.g. *miR-141**). As it is not always clear which strand is functional, the strands can be indicated as '5p' or '3p' (e.g. *miR-369-5p* and *miR-369-3p*), depending on whether the strand is closest to the 5' or 3' end of the miRNA precursor [35-36].

1.2.2 miRNAs and impact on cellular processes

As one miRNA can potentially target hundreds of genes, alterations in the levels of miRNAs may result in widespread changes of gene expression. It has been estimated that miRNAs can regulate >60% of all human genes [37]. Conversely, one mRNA may be regulated by multiple miRNAs, and binding of multiple miRNAs to the 3'UTR of target mRNAs may be necessary for potent gene repression [38], suggesting that miRNA-gene expression networks are complex. It has become clear that miRNAs play a pivotal role in embryonic development and differentiation across invertebrate and vertebrate species [39-42], and dysregulation of miRNAs has been associated with disease (e.g. neurodegenerative disorders [43], cardiovascular disease [44] and cancer [45-49]). miRNAs regulate important cellular processes such as the cell cycle, metabolism and the response to cellular stress [45, 50-52]. In particular, miRNAs have been associated with the DNA damage response [53-54].

2. The DNA damage response and cancer

Every day our body is challenged with various kinds of DNA damage resulting from both endogenous (e.g. production of free radicals as part of metabolism) and exogenous causes (UV-light, Ionizing Radiation (IR)). The DNA lesions (e.g. DNA

base modifications, single- or double strand DNA breaks) form a serious threat to genomic integrity and can disrupt the function of genes. Luckily an elaborate DNA repair system is able to repair most of the damage [55]. Central players in the DNA damage repair pathway are Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3 related (ATR). Detection of DNA damage by ATM and ATR and their binding partners CHK2 and CHK1 activates a signaling cascade that can give rise to chromatin and histone modifications (e.g. phosphorylation of H2AX), activation of transcription factors (e.g. p53 and p21) and upregulation and activation of DNA damage repair genes [56]. One of the immediate consequences of activation of the DNA damage response (DDR) is the induction of a cell cycle arrest. This allows cells to repair DNA damage before the DNA is replicated. However, upon extensive damage, prolonged DNA damage signaling promotes apoptosis (programmed cell death) and/or senescence (a state where cells continue metabolizing but stop proliferating) [57].

Despite the presence of the DNA damage response pathways, some damage is not repaired or erroneously repaired [58]. The accumulating damage in the DNA can lead to cancer formation, for instance if tumor suppressors are mutated (e.g. PTEN or Retinoblastoma protein (Rb)). In addition, mutations in genes that are involved in DNA damage repair occur frequently in cancer (such as *p53* mutations). The development of cancer (carcinogenesis) is a multistep process that is accompanied by the acquisition of traits such as enhanced proliferation signaling, evasion of cell death and replicative immortality. An aberrant DNA maintenance system can promote the successive acquisition of these traits, and is therefore considered a facilitating hallmark for cancer [59].

2.1 DNA damaging anti-cancer therapy

The defects in the DDR make cancer cells more sensitive to DNA damage than normal cells. This fact is exploited by many DNA damaging cancer therapies. In this thesis we focus on four anti-cancer treatments:

(a) Platinum-based compounds (e.g. cisplatin and carboplatin) are among the most widely used and effective anti-cancer therapies. Although these compounds have affinity for proteins and RNA, the main cytotoxic effect is likely the induction of inter- and intrastrand crosslinks in the DNA [60]. These inter- and intrastrand crosslinks can disrupt gene transcription and cause replication fork stalling, which can lead to DNA Double strand breaks (DSBs) [61]. Cisplatin and carboplatin have a similar mode of action, but cisplatin is more potent whereas carboplatin gives less toxic side effects. Compared to cisplatin, carboplatin induces less nephrotoxicity, neurotoxicity, nausea and vomiting. In contrast, carboplatin is more myelosuppressive than cisplatin [62].

(b) Another commonly used anti-cancer therapy consists of Ionizing Radiation (IR) treatment. IR induces the formation of free radicals, which can induce the formation of single strand breaks (SSBs). If two single strand breaks occur in close proximity on anti-parallel strands, a DSB may be formed (one DSB is formed for every 25 SSBs) [63].

(c) Doxorubicin belongs to the class of anthracyclins and is used for the treatment of a wide range of tumors. It interacts with the DNA by intercalation and this blocks the action of topoisomerase II, an enzyme which unwinds supercoiled DNA to facilitate transcription and replication. In order to unwind the DNA, topoisomerase II creates DSBs, which are afterwards religated. Doxorubicin

stabilizes the topoisomerase II complex after it has cut the DNA, preventing religation of the DSBs [64].

(d) Taxanes like paclitaxel are also highly effective anti-cancer medicines. They stabilize microtubules, which prevents assembly of the mitotic spindle and cell division. Next to effects on microtubules, paclitaxel also stimulates the production of Reactive Oxygen Species (ROS) that can give rise to DNA damage. Studies indicate that the production of ROS contributes to paclitaxel cytotoxicity [65-66].

2.1.1 Therapy resistance

Despite the fact that the above mentioned treatments are among the most successful anti-cancer therapies, the development of resistance, the phenomenon that cells become insensitive to treatment, is a major problem.

The causes of therapy resistance have been extensively studied. Five major mechanisms have been attributed to resistance to anti-cancer therapy [67]:

1. Decreased cellular accumulation of cytotoxics, for instance through altered expression of drug transporters
2. Increased detoxification of cytotoxics
3. Circumvention of the effect of cytotoxics, for instance through mutations in drug targets
4. Alterations in DNA repair
5. Increased proliferation signaling or evasion of apoptosis.

3. miRNAs, the DDR, and cancer

The DDR plays an important role in the formation of cancer and the response to anti-cancer therapy. The role of miRNAs in the regulation of the DDR is just

beginning to be elucidated. A quarter of all miRNAs are significantly induced upon DNA damage in an ATM-dependent manner [68] and it is known that p53 upregulates several miRNAs in response to DNA damage, such as the *miR-34* family, *miR-16* and *miR-215* [69-72]. In addition, several miRNAs have been found to regulate components of the DDR, including *miR-421* and *miR-125b* which regulate *Atm* and *p53*, respectively [73-75].

Increasing evidence suggests that DDR miRNAs play a role in cancer. For instance, *miR-16* and members of the *miR-34* family are dysregulated in many tumors [76-77]. Moreover, miRNA expression levels have been associated with the response to anti-cancer therapy [78-79]. However, most of the studies into the role of miRNAs in the DDR have been performed in cancer cells, which often have an aberrant response to DNA damage.

In this thesis we aim (i) to characterize the miRNA response to DNA damage in 'healthy' epithelial cells, (ii) to profile the miRNA expression in cancer cell lines and to associate expression levels with tumor characteristics, (iii) to examine the expression pattern of DDR miRNAs in tumors and (iv) to identify and functionally characterize miRNAs that play a role in cancer drug resistance. We have focused on three common types of (epithelial) cancer.

3.1 Breast cancer

Breast cancer is one of the most common forms of cancer, and a leading cause of cancer related death among women [80]. Breast tumors are heterogeneous. Different histological subtypes can be distinguished on the basis of expression of hormone receptors (ER, PR, ERBB2) or gene expression data [81-82]. Breast cancer is usually treated with surgery, which may be followed by IR and/or chemotherapy.

Different chemotherapy regimens exist based on amongst others of doxorubicin and taxanes [83]. Depending on the receptor status of the tumor, patients may also be treated with targeted therapy (e.g. Tamoxifen or Trastuzumab for ER or ERBB2 positive tumors, respectively).

We have profiled the miRNA response to DNA damage in **Chapter 2**. Primary epithelial breast cells were treated with a low dose or a high dose of cisplatin and IR, and miRNA expression was analyzed at different time points (6H, 12H and 24H), thus identifying DNA damage responsive miRNAs. We examined the expression pattern of these miRNAs in breast tumors, and investigated whether these miRNAs can play a role in the response to cisplatin, doxorubicin and paclitaxel chemotherapy.

In **Chapter 3**, we determined the miRNA expression profile of a set of 51 breast cancer cell lines. These breast cancer cell lines have retained most of the molecular characteristics of the breast tumor subtypes [84], and we examined whether miRNAs could discriminate between different subtypes. In addition, miRNA expression profiles were correlated with the mutation status of breast cancer genes (*ERBB2*, *p16*^{INK4A}, *E-cadherin*, *BRCA1*, *PTEN*, *PIK3CA*).

3.2 Lung cancer

Lung tumors are the most common cause of cancer related mortality [80]. Lung tumors are classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC tumors are treated with platinum-based chemotherapy and IR [85-86], whereas NSCLC tumors are more frequently treated with surgery and adjuvant chemotherapy (Platinum-based chemotherapy in combination with amongst others paclitaxel) [87-88].

In **Chapter 2**, we aimed to identify cancer relevant DNA damage responsive miRNAs in primary lung epithelial cells using a similar approach as described for breast epithelial cell lines.

3.3 Ovarian cancer

Ovarian tumors are a group of heterogeneous carcinomas that, until recently, were thought to arise from the ovarian surface epithelium. However, it has become clear that the four major subtypes originate from different non-ovarian cell types. The most common histotype, serous, is thought to originate from epithelial cells in the fallopian tube [89-90]. In contrast, invasive mucinous tumors seem to have a gastrointestinal origin. Two other subtypes, Clear-cell and Endometrioid tumors probably arise from the endometrium and may develop as a consequence of endometriosis (retrograde menstruation) [91].

Although ovarian cancer is 10x less common than breast cancer, it is the fifth most common cause of cancer related death in females [80]. There are two main causes for the high mortality. First of all, ovarian cancer is often detected in a late stage. If ovarian cancer is detected when it is confined to the ovaries (stage 1) the 5-year survival rate is 90%, whereas the 5-year survival rate drops to 30% if distant metastases are present [80].

The second major reason why ovarian cancer has a high mortality is because of the development of therapy resistance. Treatment consists of surgery and combination chemotherapy. As first line treatment, paclitaxel is administered in combination with carboplatin or cisplatin [92-93]. As second line treatment, pegylated liposomal doxorubicin may be used [94].

Recently, miRNAs have also been implicated in ovarian cancer biology and therapy resistance (see **Chapter 4**). In **Chapter 5**, we investigated the role of one of these miRNAs, *miR-141* and its targets in cisplatin sensitivity of ovarian cancer cell lines. In **Chapter 6** we examined the effect of overexpression of another miRNA, *miR-634*, on the response of ovarian cancer cell lines and primary tumor cells to chemotherapy. In **Chapter 7**, we explored the role of the *miR-634* putative targets RSK1 and RSK2 in the response to cisplatin chemotherapy in an ovarian cancer cell line.

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