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MiRNAs in Cancer

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ABSTRACT

There are many layers of complexity involving the processes through which somatic cells transform into malignant cancers. Historically, cancer was considered to be a disease primarily caused by gene mutations, however it is now well established that the dysregulated expression of the genes leading to the tumorigenic phenotype involves not only mutations but also epigenetic changes. To understand the process of malignant transformation, it is thus important to determine the specific genes targeted by both types of changes.

The studies in this thesis have focused on miRNA expression and its dysregulation in various malignancies and the subsequent role of such dysregulation in tumor pathogenesis. The work includes an analysis of the functional consequences of miRNA alterations in three distinct malignancies, (1) chronic lymphocytic leukemia (CLL), (2) Squamous cell carcinoma (SCC) and (3) Basal cell carcinoma (BCC). Furthermore, acute lymphoblastic leukemia (ALL) was used as a model to describe the role of miRNAs in anticancer treatment. Moreover, we analyzed the effect of the anticancer drug dexamethasone on miRNA expressions and the impact of manipulation of miRNA levels on drug efficacy.

In the CLL study, we demonstrated that the frequently deleted *DLEU2* gene functions as a regulatory host gene for two miRNAs, miR-15a and miR16-1, which negatively regulate the cell cycle by direct targeting G1 cyclins D1 and E1 at the post-transcriptional level, and which, when expressed at high levels in cell line models, lead to the inhibition of colony formation ability. In addition, we demonstrated that the oncoprotein Myc negatively regulates *DLEU2* transcription by targeting the *DLEU2* promoter. These results suggest that the loss of *DLEU2* may be an important pathogenic factor in CLL development.

Our studies on two non-melanoma-skin cancers, SCC and BCC, identified the preferential loss of expression of a skin-specific miRNA, miR-203, in these tumors. Our results further indicate a function of miR-203 in cell cycle regulation, migration and invasion, through the post-transcriptional targeting of the oncogenes *c-JUN* and *c-MYC*, and ultimately leading to an inappropriate inactivation of Hedgehog pathway.

Finally, in the ALL study we demonstrated dexamethasone mediated global down-regulation of miRNAs, in particular the rapid downregulation of *MIR17HG* which occurred following direct binding of the glucocorticoid receptor protein to the *MIR17HG* promoter. The subsequent repression of miR-17 expression aids in dexamethasone cytotoxicity of ALL cells, possibly through de-repression of miR-17 mediated targeting of the anti-apoptotic protein Bim. Analysis of primary B-ALL tumor samples also demonstrated that the cytotoxic efficacy of dexamethasone is associated with its ability to regulate miR-17 levels.

Collectively, these results provide new evidence, not only on the function and importance of microRNAs in tumor pathogenesis, but also suggest the possibility of miRNA targeting to improve the efficacy of existing therapies.

LIST OF PUBLICATIONS

I. Mikael Lerner, **Masako Harada**, Jakob Lovén, Juan Castro, Zadie Davis, David Oscier, Marie Henriksson, Olle Sangfelt, Dan Grandér and Martin M. Corcoran. ***DLEU2*, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1.** *Experimental Cell Research **315**(2009): 2941-2952.*

II. **Masako Harada**, Katja Pokrovskaja Tamm, Stefan Söderhäll, Mats Heyman, Dan Grandér and Martin Corcoran.
Involvement of the miR17 pathway in glucocorticoid-induced cell death in pediatric acute lymphoblastic leukemia.
Leukemia & Lymphoma 2012 Apr 4. [Epub ahead of print]

III. **Masako Harada**, Florian Meisgen, Ning Xu, Lingyun Zhang, Dan Grandér, Veli-Matti Kähäri, Mona Ståhle, Enikö Pivarcsi Sonkoly and Andor Pivarcsi.
Investigation of the role of miR-203 in squamous cell carcinomas of the skin.
Manuscript

IV. Enikö Sonkoly, Jakob Lovén, Ning Xu, Florian Meisgen, Tianling Wei, Petter Brodin, Viljar Jaks, Maria Kasper, Takashi Shimokawa, **Masako Harada**, Johan Heilborn, Mari-Anne Hedblad, Andreas Hippe, Dan Grandér, Bernhard Homey, Peter G. Zaphiropoulos, Marie Arsenian-Henriksson, Mona Ståhle and Andor Pivarcsi. **MicroRNA-203 functions as a tumor suppressor in basal cell carcinoma.**
*Oncogenesis (2012) **1**, e3*

RELATED PUBLICATIONS

I. Mikael Lerner, Moritz Haneklaus, **Masako Harada**, Dan Grandér. **MiR-200c Regulates Noxa Expression and Sensitivity to Proteasomal Inhibitors.**
Accepted by PLoS ONE

II. E Buentke, A Nordström, H Lin, A-C Björklund, E Laane, **M Harada**, L Lu, T Tegnebratt, S Stone-Elander, M Heyman, S Söderhäll, A Porwit, C-G Östenson, M Shoshan, K Pokrovskaja Tamm & D Grandér. **Glucocorticoid-induced cell death is mediated through reduced glucose metabolism in lymphoid leukemia cells**
Blood Cancer Journal (2011) 1, e31

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LIST OF ABBREVIATIONS

16INK4A (CDKN2A)	cyclin-dependent kinase inhibitor 2A
ABL1	V-abl Abelson murine leukemia viral oncogene homolog
AGO	Argonaute
AK	actinic keratosis
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ATM	ataxia telangiectasia mutated
BCC	basal cell carcinoma
bcl-2	B-cell CLL/lymphoma 2
BCR	breakpoint cluster region
bHLHZip	basic-helix-loop-helix-zipper
CAF1	Chromatin assembly factor 1 subunit
CCR4	chemokine (C-C motif) receptor 4
CDK2	Cyclin-dependent kinase2
ChIP	chromatin immunoprecipitation
CIP2A	cancerous inhibitor of PP2A
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
DCP2	decapping enzyme homolog
DLEU2	deleted in lymphocytic leukemia 2
DMNT3a	DNA (cytosine-5-)-methyltransferase 3 alpha
DNA	Deoxyribonucleic acid
E2F1/2/3	E2f transcription factor 1/2/3
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular-regulated kinase
ETV6	ets variant 6
FBW7	F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase
FGF	fibroblast growth factor
FISH	fluorescence in-situ hybridization
GC	glucocorticoid
GLI	glioma-associated oncogene
GSK-3β	glycogen synthase kinase
GTP	Guanosine triphosphate
GW182	glycine-tryptophan protein of 182 kDa
HATs	histone acetyltransferases
HDAC1	histone deacetylase 1
HH	hedgehog
HNRPK	heterogeneous nuclear ribonucleoprotein K
HPV	human papilloma viruses

IGH@	immunoglobulin heavy locus
IgV	immunoglobulin variable region
IL3	interleukin 3
iPS	induced pluripotent stem cells
KDM	lysine demethylase (KDM), an ATPase containing remodeling complex (P-TEFb) and CyclinE/CDK2.
KGF	Keratinocyte growth factor
KMT	lysine Methyltransferase
lincRNA	large intergenic non-coding RNA
LOH	Loss of Heterozigocity
Max	*Myc*-associated factor X
MBL	monoclonal B-cell lymphocytosis
MC1R	melanocortin 1 receptor
MDR	minimally deleted region
MIR17HG	miR-17-92 cluster host gen
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MIZ1	zinc finger, MIZ-type containing 1
MLL	myeloid/lymphoid or mixed-lineage leukemia
mRNA	messenger RNA
MTAP	methylthioadenosine phosphorylase
Myc	myelocytomatosis
NHL	non-Hodgkin lymphomas
NMSC	non-melanoma skin cancers
oncomiR	oncogenic miRNA
p15INK4B (CDKN2B)	cyclin-dependent kinase inhibitor 2B
PBX1	pre-B-cell leukemia homeobox 1
PDGF	platelet-derived growth factor
piRNA	piwi-interacting RNA
PolII/III	RNA polymerase II/III
PP2A	protein phosphatase 2A activator
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTCH1	patched 1
P-TEFb	ATPase containing remodeling complex
PTEN	Phosphatase and tensin homolog
PTGS	posttranscriptional gene silencing
RAS	rat sarcoma viral oncogene
RB	Retinoblastoma
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
RT-PCR	Reverse-transcriptase polymerase chain reaction
RUNX1	runt-related transcription factor 1
S62	N-terminal Serine 62

SCC	squamous cell carcinoma
SHH	sonic hedgehog
SMO	G-protein-coupled receptor, Smoothened
snoRNA	small nucleolar RNA
snRNA	small nuclear RNAs
SP1	specificity protein 1 transcription factor
SWI/SNF	SWItch/Sucrose NonFermentable
T58	phosphorylation of Threonine 58 (T58)
TAp63	tumor protein p63
TCF3	transcription factor 3
TF	Transcription Factor
TGF	transforming growth factor
TP53	tumor protein p53
tRNA	transfer RNA
TSG	tumor-suppressor gene
UPS	ubiquitin-proteasomal system
UTR	untranslated region
VEGF	vascular endothelial growth factor
WHO	World Health Organization
XRN1	5'-3' exoribonuclease 1

INTRODUCTION

This thesis work focuses on the diverse aspects of miRNAs in the context of the cellular and molecular biology of cancer development, diagnosis and treatment. As the thesis covers a variety of molecular events and specific diseases, it will function as a brief introduction to the growing field of research into the biology of microRNAs (miRNAs) and cancer.

1.1 WHAT IS CANCER?

Considering that the earliest record of tumors date from approximately 3000 BC in ancient Egypt it is presumed that knowledge and understanding of cancer developed in conjunction with human history and technological development [1]. At present, cancer is recognized as the second leading cause of death worldwide, with 7.6 million people dying worldwide from cancer in 2008, a figure expected to rise to 13.1 million deaths in 2030 [2]. The term "cancer" describes a wide range of distinct diseases originating from all human organs and tissues. Healthy human bodies have a process of homeostatic regulation to maintain tissue mass by means of genetic control of cellular division and death. This system is highly complex but well-organized and controlled, and is responsive to internal and external signals that help the body/tissue to sustain a healthy state. Cancers are the result of the accumulation of cells which have lost this homeostatic mechanism in a way that allows cells to expand in numbers, evading the various evolved anti-tumor mechanisms such as apoptosis and cellular senescence, to eventually form a malignant tumor. These may spread to other organs from their tissue of origin in a process, which is a typical feature for many types of advanced cancers and is the major cause of death in cancer patients.

Causes of cancer are far more complicated than originally imagined. Commonly, a wide range of factors are now known to provoke tumor development and progression, yet the precise causes cannot be determined in many types. Inherited factors are predominant only in 5–10% of cases including many tumor types such as breast, prostate and colorectal cancers. The mutations which cause transformation of normal cells into a malignant tumor result from exposure to various carcinogenic risk factors and result in genetically and/or

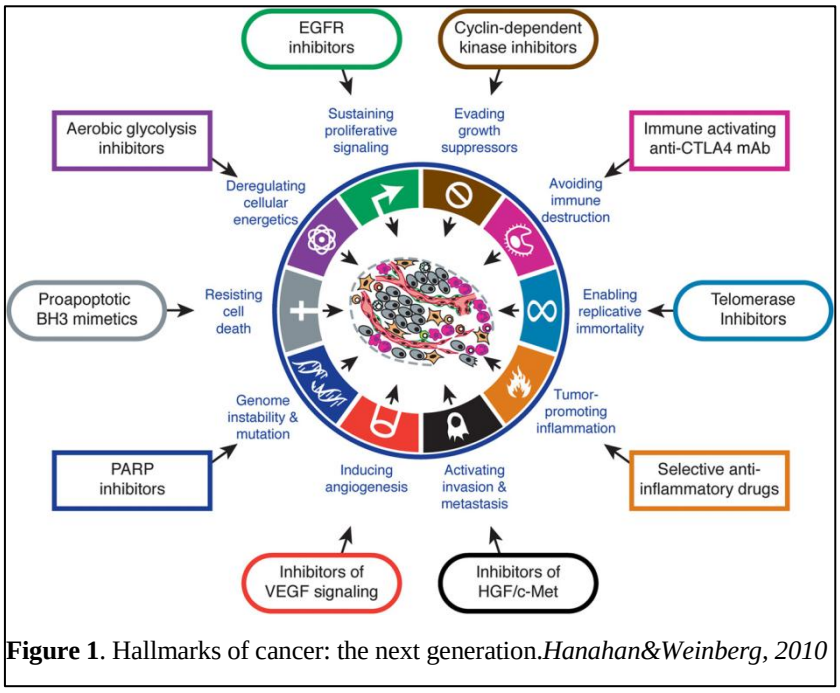

Figure 1. Hallmarks of cancer: the next generation.*Hanahan&Weinberg, 2010*

epigenetically damaged cells that can evade antitumor protective mechanisms and gradually accumulate in number before giving rise to symptomatic disease.
How mutagens promote carcinogenesis is well studied but still incompletely understood. Hanahan and Weinberg proposed a number of "hallmarks of cancer" - novel capabilities acquired by cells resulting in the gradual gain of more aggressive neoplastic features as part of a multistep processes (Fig 1.) [3,4]. Both genetic and epigenetic alterations contribute to these novel tumor promoting traits. Although cancers are multicellular, they are generally thought to develop from a single originating cell. In recent years it has become apparent that some tumors appear to be driven by a type of undifferentiated originating cell, so called cancer stem cells.Tumors also exhibit a type of Darwinian evolution, Lamarckian inheritance, and neutral evolution at the genomic level, driving tumor progression. One of the hallmarks of cancer is genome instability & mutation that drives this successive progression and which gives rise to considerable genetic heterogeneity in the tumor mass [5] and providing the basis for malignant cellular evolution and survival.

1.1.1 THE NATURE OF GENETIC CHANGES

As discussed above, malignant tumors are precipitated by genetic or epigenetic alterations. However random mutations have to hit the correct gene in the genome to give rise to tumor cells. In order for cells to acquire the capability for progressive tumorigenesis, functional mutational events must occur that affect specific classes of genes that play a central role in tumor initiation and progression. The critical genes affected by these alterations are generally classified into two classes; oncogenes and tumor-suppressor genes [6].

1.1.2 ONCOGENES

The term "oncogene" denotes a type of gene which, when overexpressed, can promote tumor development. These genes regulate processes such as cell growth and differentiation in normal cells, and are, in their un-mutated state, called proto-oncogenes. However, once gain-of-function mutations cause them to become hyperactive, they become capable of promoting oncogenesis, and are thus called 'oncogenes'. More than 100 proto-oncogenes have been identified to date with the number increasing as research into the functional analysis of tumor associated genes progresses. The cellular function of proto-oncogenes is diverse and their gene products known to function at many levels of the various signal transduction pathways. Amongst others they include signaling components such as growth factors, growth factor receptors, cytoplasmic signaling molecules, transcription factors and cell-cycle regulators.
So how can proto-oncogenes become activated to exert oncogenic properties?
This can occur by processes such as amplification which produces extra copies of the gene resulting in increased levels of its expression. *ERBB2* and *MYCN* are two well-known examples of oncogenes that are genomically amplified in breast cancers and neuroblastomas respectively [6]. Another mechanism involved in turning proto-oncogenes into oncogenes is the process of point mutation and this is frequently found occurring in the *RAS* family of genes. Ras family proteins mediate signaling by

G-protein coupled receptors following binding to GTP molecules and exerting GTPase activity, and are activated in a wide range of tumors. The point mutation that causes *RAS* activation increases the half-life of the activated Ras-GTP mutant resulting in an excessive response to the signals from the receptor [7]. A third mechanism of activation involves translocation events, which can be sub-divided into two types; translocation creating a novel chimeric gene; and translocations causing co-activation of an oncogene, by placing it under the transcriptional regulation of another, constitutively highly transcribed gene. The former cases are common in hematological malignancies and sarcomas. The first chimeric gene identified in human cancer was *BCR-ABL* – the fusion gene underlying the Philadelphia chromosome, and which is a product of the 3' part of the *ABL* gene on chromosome 9, and the 5' part of the *BCR* gene from chromosome 22. The translocation results in a novel fusion gene, that expresses a tyrosine kinase related to the Abl protein with abnormal transforming properties and occurs in chronic myeloid leukemia (CML) acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) [8].
The final mechanism of oncogenic activation involves the juxtaposition of a highly active promoter upstream of a growth promoting proto-oncogene. Such activation is frequently described in lymphomas, such as Burkitt's lymphoma, where *MYC* oncogene activation occurs by juxtaposing the *MYC* oncogene with various immunoglobulin heavy-chain (H) genes, thereby causing an excess production of the c-Myc transcript [9]

1.1.3 TUMOR-SUPPRESSOR GENES

Another class of genes which plays a critical role in tumorigenesis is the tumor-suppressors, whose normal function is to limit cell growth in particular contexts. Their function is typically inactivated in tumors as exemplified by the Retinoblastoma- (*RB*-), *P53*- and *PTEN* genes. It is noteworthy that mutation/loss of one allele of a tumor-suppressor gene is often not sufficient for malignant transformation of the cells. This was proposed as the "two-hit hypothesis" in 1971 by Knudson, who hypothesized that the recessive property of tumor-suppressor genes at the cellular level requires bi-allelic inactivation for tumor development to occur [10]. Several types of mechanisms behind inactivation of these genes have been found to date, including deletion, mutation and epigenetic silencing through hypermethylation of promoter regions [11]. The specific function of individual tumor-suppressor genes include regulation of apoptosis, cell-cycle checkpoint, DNA repair, senescence and many other cellular processes which are related to cancer development [6].

1.2 MYC

1.2.1 The Myc Oncoprotein – an oncogenic transcription factor

The *MYC* family of transcription factors (TFs) encode a group of well characterized-oncoproteins. The *c-MYC* gene is localized to chromosome 8q24.21 and its dysregulation is observed in more than 50% of cancer cases, which makes Myc an attractive target for cancer therapy. *MYC* was first identified as a cellular homolog to the MC29 avian myelocytomatosis virus gene (*v-myc*) which harbours transforming

capability. *MYCN*, activated in neuroblastoma cells and *MYCL* in small cell lung cancer also belong to the same family and are homologous with v-myc, which share motifs and some function in common [12]. The various Myc proteins are members of the basic-helix-loop-helix-zipper (bHLH-Zip) family that heterodimerizes with the small bHLHZip protein Max and thus have the ability to bind to CACGTG and similar E-box sequences and act as transcription factors [13,14]. Although the mechanism is less defined Myc can influence transcription negatively together with the partner protein Miz [15]. Importantly it has been demonstrated that c-Myc regulates the gene expression of miRNA genes, with both activation and repression of target transcription [16]. This will be mentioned later in the work.

In healthy cells, the proto-oncogene Myc has diverse functions in various cellular pathways, including proliferation, cell growth, energy metabolism and apoptosis. As a transcription factor, Myc has numerous targets in cells that are regulated as a response to both external and internal signaling. It is interesting to note that c-Myc was one of the essential transcription factors required to generate induced pluripotent stem (iPS) cells from human fibroblasts which hints of its potential in neoplastic transformation. [17].

1.2.2 Myc Dysregulation in Cancer

Although *MYC* dysregulation is prevalent in cancer cells, the story is not straight forward. An increasing number of studies using transgenic mouse models demonstrate that c-Myc activation can initiate tumor formation and contribute to tumor progression, however it is clearly evident that additional mutagenic events are required for transformation [18]. To date, *MYC* seems to be the most frequently deregulated oncogene in human cancer and is also found to be a major murine oncogene [19]. Overexpression or aberrant expression of c-Myc is seen in both solid and hematological tumor types including Burkitt's lymphoma, breast cancer, prostate cancer, gastrointestinal cancer, multiple myeloma and myeloid leukemia whereas N-Myc dysregulation is observed only in neuroblastoma and L-Myc only in small cell lung cancer due to their tissue-specific expression [20].

1.2.3 Dysregulation of Myc in Malignancy

Like many other protein encoding genes, the regulation of Myc occurs at multiple levels in normal cells. In this section I will first focus on Myc dysregulation in malignancies.

Translocation

The first and most studied mechanism for Myc dysregulation in tumorigenesis is the *MYC* related translocation found in Burkitt’s lymphoma [21]. Nearly 100% of Burkitt’s lymphoma cases harbor a translocation of *MYC*, located on chromosome 8, juxtaposing it to genomic sequences from chromosome 14, 22 or 3, which harbor the regulatory elements of the immunoglobulin μ heavy chain or λ and κ light chains respectively, resulting in Myc overexpression [20,22].

Gene Amplification

The amplification of the *MYC* family of genes is common in various types of cancers. *MYC* amplification occurs in 9–48% of breast cancer cases, with *MYCL* amplification found in 20%, of lung cancer cases. *MYCN* amplification in found in 4% of Medulloblastoma, 25–30% of Neuroblastoma and 43–67% of Rhabdomyosarcoma cases [14].

Protein Stabilization

Myc is a rapidly degraded short-lived phosphoprotein with a normal half-life of 20-30 min [23]. Increased stability of Myc protein has been reported in several types of cancers such as gastric cancer, head-and-neck squamous cell carcinoma and lymphoblastic leukemia [24]. One well-studied pathway of Myc degradation involves the phosphorylation of the N-terminal Serine 62 (S62) by extracellular-regulated kinase (ERK) followed by phosphorylation of Threonine 58 (T58) by glycogen synthase kinase (GSK-3β), and subsequent targeting by Fbxw7, a ubiquitin E3 ligase, resulting in poly-ubiquitination and subsequent proteasomal degradation [24]. This pathway is regulated at various levels by several other pathways found to be consistently altered in cancer cells. For example, Cip2a, which is the Pp2a inhibitor, works as an endogenous Myc stabilizing protein and the ubiquitin-specific protease Usp28, which is a critical mediator of Myc protein stability, are dysregulated in several cancer types, making them potential c-Myc related therapeutic targets [14,24,25].

1.2.4 Myc in Action

Myc is a transcription factor and is known to work both in the activation and repression of the transcription of target genes. Using an inducible Myc system coupled with ChIP-seq and gene expression change, it was demonstrated that more than 22% of gene promoter regions have c-Myc binding sites [26].

1.2.4.1 Transactivation by Myc-Max heterodimers

Max, a helix-loop-helix zipper protein that forms a complex with Myc, was the first dimerization partner identified for Myc, and functions to promote binding to E-box (CACGTG) sequences in a sequence specific manner [27-30]. Myc activates gene expression by recruiting multiple cofactors such as the ubiquitin-proteasomal system (UPS), SWI/SNF, histone acetyltransferases (HATs), lysine Methyltransferase (KMT), lysine demethylase (KDM), an ATPase containing remodeling complex (P-TEFb) and CyclinE/CDK2. An important feature of Myc-Max activation of gene transcription is that it regulates the activity of all three RNA polymerases, thus controlling both coding and non-coding genes[31].

1.2.4.2 Repression of gene expression by Myc

The Myc oncoprotein can also function in the transcriptional repression of a large set of genes. In this case a different set of cofactors is required from those present during Myc-associated transcriptional activation. Until quite recently it was thought that the repression by Myc is achieved through binding to other transcription factors which by

themselves enhance the transcription of the target genes, such as *MIZ1* and/or *SP1* TFs. Myc binding to these factors instead recruits DNA methyltransferase Dmnt3a and Hdac1, changing Miz1 and Sp1 from activators into transcriptional repressors [32-35]. However, recent reports suggest that Myc-Max heterodimer binding to E-box sequences may also play a role in the repression of transcription, making the role of the Myc-Max dimer less clear [36,37].

1.3 MIRNAS

Historically, few non-coding RNAs were recognized as playing key biological roles in cells. These included transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs), some of which were known to be involved in such processes as protein translation and mRNA biogenesis. Newly discovered classes of RNAs can be classified into two general categories; small non-coding RNAs and large intergenic non-coding RNAs (lincRNAs) [38]. Small non-coding RNA types include piwi-interacting RNAs (piRNAs), antisense RNAs and the focus of this thesis, miRNAs, currently the best described small non-coding RNA known to play a role in posttranscriptional gene silencing (PTGS).

1.3.1 MicroRNA – a class of non-coding RNAs

MicroRNAs (MiRNAs) are small RNA molecules of 20-24 bp in length that are now known to post-transcriptionally regulate gene expression by binding to the 3'UTR of the target gene in a sequence specific manner. MiRNA target sites are often highly evolutionary conserved throughout species, and are partially complementary to the 'seed' sequence of miRNAs - positions 2–7 at the 5' end of the mature miRNA. In general, the result of miRNA binding to the target mRNAs is the decreases in their stability or an inhibition of translation [39]. *Lin-4*, the first miRNA discovered in the nematode *C. elegans*, was found to cause a temporal decrease in *lin-24* protein level and thus regulate the developmental timing of larval stages [40-42]. Today, there are 1,898 mature human miRNA sequences listed in the Sanger updated miRNA registry (miRBase 18), and which are thought to regulate ~50% of protein coding genes. There has been a vast increase in the number of miRNA related publications in last 5-10 years, covering almost all biological processes, indicating the importance of miRNAs within current biological research.

1.3.2 Biogenesis

In the canonical miRNA biogenesis pathway, miRNAs are transcribed by RNA polymerase II (Pol II) from the host genome as long primary transcripts (pri-miRNAs) in the nucleus and which partly fold into stem-loop structures [43,44]. The stem loops within pri-miRNAs are recognized by the microprocessor complex (Drosha-DGCR8), that processes them, using the the nuclear RNase III enzyme Drosha, into precursor miRNAs (pre-miRNAs) that are generally hairpin-loop structures of approximately ~70nt in length [45]. Pre-miRNAs are then exported into the cytoplasm by exportin-5 where another class of RNase III enzyme, Dicer, cleaves the loop part leaving a miRNA/miRNA* duplex. One strand of the miRNA is integrated into the miRNA-induced silencing complex (miRISC) and subsequently directed to a site in 3'UTR of a

target mRNA, causing translational repression or deadenylation and degradation [46-48] (Figure 2). It is worth noting that in miRISC complexes, Argonaute (Ago) protein and glycine-tryptophan protein of 182 kDa (GW182) proteins, which act as downstream effectors in the repression, are key players in the complex and function miRNA-mediated silencing [49]. Alternatively, in the Mirtron pathway, splicing can replace the Drosha cleavage when the excised intron has the appropriate size to form a hairpin-like pre-miRNA [50-52].

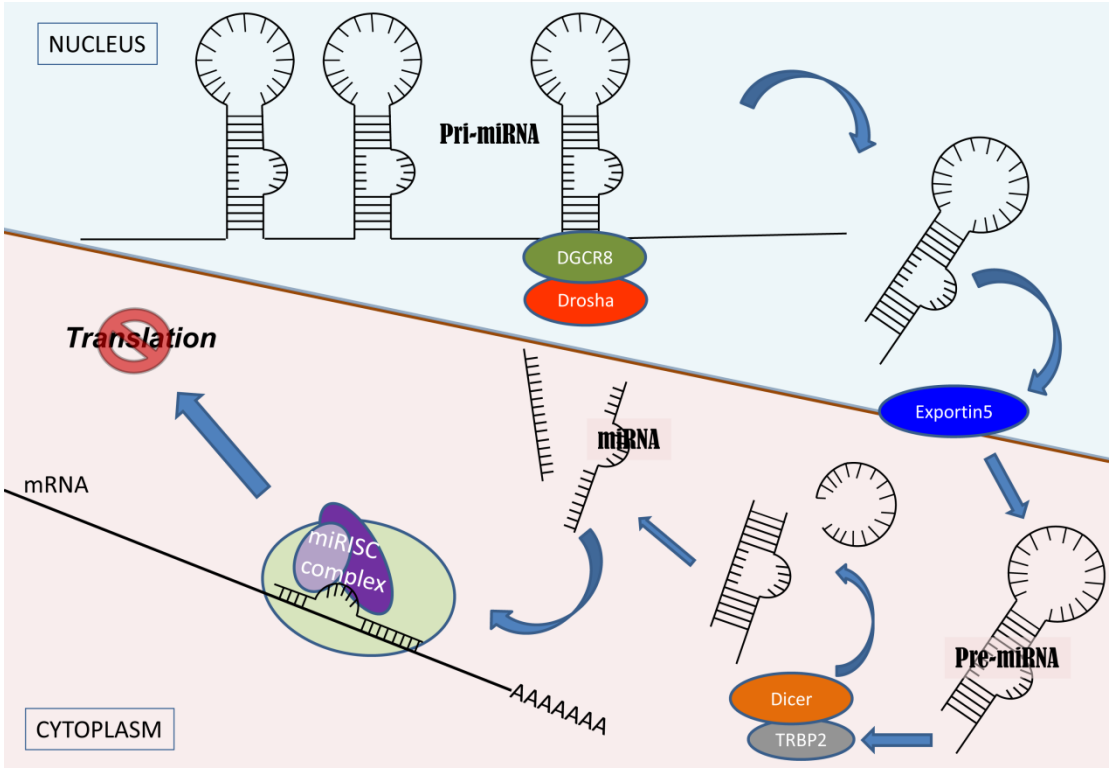

Figure 2. MiRNA processing

1.3.3 The Regulatory Function

1.3.3.1 Target Recognition

Many miRNAs that are expressed in a temporal- and tissue-specific manner are now known to play critical roles in various cellular pathways, including apoptosis, proliferation, migration, cell-cycle and development [53,54]. The regulatory functions of miRNAs in biological pathways are achieved through influencing the stability and the translational capacity of mRNAs. Mature miRNAs contain a characteristic feature, called the "seed" sequences, present between bases 2 to 7, which is the specificity determinant for target recognition [55]. Canonical miRNA targeting sites in target mRNAs often consist of 7 or 8 nucleotides of complementary sequence, however, 6 nucleotide targets can be sufficient to target specific mRNAs for translational inhibition [56]. Although higher levels of complementarity throughout the miRNA will give better efficacy of action, there are several additional determinants that contribute to the efficacy of targeting, including; AU-rich nucleotide composition near the site, proximity to sites for coexpressed miRNAs (which leads to cooperative action),

proximity to residues pairing to miRNA nucleotides 13–16, positioning within the 3'UTR at least 15 nt from the stop codon, and positioning away from the center of long UTRs [57]. Information on putative miRNA targeting can be found in publicly available online bioinformatics databases including TargetScan (http://targetscan.org/) [58], PicTar (http://pictar.mdc-berlin.de/) [59], and MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) [60].

1.3.3.2 Translational Repression

In human cells, mature miRNAs are bound to Ago2 proteins, which binds the mature miRNA, presenting its seed sequence for target recognition [61,62]. Upon recognition of the target RNA, miRISC complexes go through conformational changes, recruiting other components to the target RNA [63]. Complexes of higher complementarity cleave the target mRNA directly, similar to the process of RNAi directed mRNA degradation, however, this type of mRNA cleavage tends to be rare in miRNA-mediated gene silencing. Over the past few years there has been an intensive focus on delineating the molecular mechanism of miRNA-mediated gene silencing [64]. Some experimental evidences suggest that miRNA-mediated translational repression occurs at the post-initiation stage[64]. Petersen and colleagues, discussing a situation where miRNA-mRNA complexes are associated with polysomes which respond to translational inhibitors, alternatively proposed a model in which miRNAs cause ribosomes to dissociate (ribosomal drop off) [65].

1.3.3.3 Target Degradation

The use of RNA transcriptome profiling techniques in recent years, have demonstrated the functional effect of miRNAs in promoting RNA degradation of some mRNA targets [66,67]. , In contrast to perfectly complementary siRNAs, miRNAs tend not to cause direct endonucleolytic cleavage. Instead, the miRNA/RISC complex directs target mRNAs to the cellular 5'-to-3' mRNA decay pathway which involves the CCR4-NOT complexes [68]. Following the deadenylation of the target mRNAs by the CAF1–CCR4–NOT deadenylase complex, they are decapped by the decapping enzyme Dcp2 and additional co-factors and subsequently subjected to degradation by the major cytoplasmic 5'-to-3' exonuclease Xrn1 [54,69].

1.3.3.4 The Changing Transcriptome Landscape

Taking into the account that miRNA directly regulates approximately half or possibly more, of cellular mRNA transcripts it is appropriate to describe them as regulators of most biological processes and the equivalent, in terms of defining cell fate, to many transcription factors (TFs), previously seen as the "key" biological regulators. High-throughput sequencing/screening technologies, such as microarray analysis, deep sequencing, genome-wide ChIP-Seq and RNA-Seq, and the use of various bioinformatics tools, allow an analysis of the interplay between TFs and miRNAs, which in turn adds another layer in the regulatory network of the transcriptome. Several studies to date have indicated combinational and cooperative regulations between TFs and miRNAs, suggesting the importance of miRNAs in gene regulatory networks, however, due to the novelty of the field, these studies should at best be considered

preliminary [70-72]. The next section will touch upon some examples of the role of miRNAs in biological systems, specifically regarding their function in cancer biology.

1.3.4 MiRNAs in Cancer

The first clue that miRNAs play important roles in cancer came from the evidence that many miRNA genes are located at fragile sites and genomic regions that are frequently altered in tumor cells [73]. Indeed, various groups have reported altered expression of miRNAs, or recurrent amplification or deletion of miRNA genes in tumors [73-76]. These early findings have been expanded to validate the individual tumor-altered miRNAs role and function in tumor pathogenesis, with several miRNAs being found to have oncogenic or tumor suppressive functions. The miR-17-92 cluster and miR-155/BIC have been suggested as proto-oncogenes in B-cell lymphomas [77-79] and miR-15a and miR-16-1 are now known to act as tumor suppressor miRNAs (Paper I).

The accumulating experimental evidence indicates that miRNA-mediated gene regulation has a broad impact on gene expression and regulation and that the functional role of this class of post-transcriptional regulators is critical in many human diseases including cancer.
Some of the important aspects of the specific miRNAs described in my studies, in reference to their role in tumor biology, are described in this section.
MiRNAs involved in tumorigenesis can be classified into two broad categories; tumor-suppressor miRNAs and oncogenic miRNAs (OncomiRs).

1.3.4.1 Tumor Suppressor miRNAs

- *miR-15a/miR-16-1*

miR-15a and miR-16-1 were the first miRNAs linked to a specific genomic alteration found in tumors, namely the frequent deletion of chromosome 13q14 that is seen in the disease chronic lymphocytic leukemia (CLL) [76]. The 13q14 consensus minimal deleted region (MDR) is lost in more than 50% of B-cell chronic lymphocytic leukemia (B-CLL), monoclonal B-cell lymphocytosis (MBL) patients [80,81] and about 50% of mantle cell lymphoma cases, as well as 16–40% of multiple myeloma patients [76]. Moreover 13q14 MDR was reported in solid tumor such as prostate cancer (60%) [82,83]. Calin and his colleagues identified 2 miRNAs located 30 kb downstream of this deleted region using chromosomal breakpoint mapping of a CLL case with a t(2:13)(q32;q14) translocation. The miRNAs described, miR-15a and miR-16-1 were discovered to be down-regulated in many other cases of CLL that showed allelic loss of 13q14 [76]. The first mRNA target shown to be regulated by these miRNAs was BCL2 which is overexpressed in various cancers, in particular CLL, and which is known to negatively-regulate apoptotic cell death pathway [84]. These findings implicate a role for miR-15a and miR-16-1 in the regulation of apoptosis, with the loss of such regulation contributing to CLL development. The importance of deletion of miR-15a

and miR-16-1 in the pathogenesis strongly suggested that these transcripts function as tumor suppressors.
A more thorough analysis of these miRNAs is presented in paper I and the Results and Discussion section.

- *miR-203*

MiR-203 is another class of miRNA which is now known to function as a tumor-suppressor in tumor cells. It is known as a "skin-specific" miRNA and is preferentially expressed in keratinocytes, which display a gradient of expression within the epidermis, with low expression in the basal layer and high expression in the more differentiated suprabasal layers [85]. The protein p63 is an essential regulator of stem cell maintenance in epithelial stratified tissues and a known target of mir-203, with one report suggesting that miR-203 acts as a switch between proliferation and differentiation, acting predominantly by restricting proliferative potential of progenitors [86]. A primary role for miR-203 is in the repression of the property of "stemness" within skin cells and the loss of miR-203 expression in keratinocyte is hypothesized to lead to various skin disorders including cancer, which is further discussed in paper III and IV.

1.3.4.2 OncomiRs

The term oncomiRs refers to miRNAs with oncogenic activities.
These miRNAs, when constitutively overexpressed, promote tumor cell growth by inhibiting tumor suppressor genes or genes that control cell cycle progression, differentiation or apoptosis [87,88]. For example, miR-21 expression was shown to be strongly elevated in human glioblastomas [89] and later found to regulate cell cycle through the targeting of HNRPK and TAp63 [90]. It is reported that miR-21 level is over-expressed in different types of cancer, such as prostate cancer, colorectal cancer, lung cancer and osteosarcoma and contributes to tumorigenesis by altering the levels of cell cycle and apoptosis genes[91]. The other miRNAs exerting such oncogenic properties include miR-181, miR-222 and miR-155 [92]

- *miR-17 family*

The miR-17∼92 cluster (Oncomir-1) was identified as containing miRNAs that showed potent oncogenic properties, the first such growth promoting miRNAs described [78]. The precursor transcript derived from the mir-17-92 gene, *MIR17HG*, contains six tandem stem-loop hairpin structures that yield six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1 [93]. The 13q31.1 chromosomal region that contains this transcript has been found to be amplified in several hematopoietic malignancies and solid tumors, including diffuse B-cell lymphomas (DLBCLs), bladder cancer, squamous-cell carcinoma of the head and neck, and multiple myeloma [94,95].
The MIR17HG transcript is a primary target for the oncogenic transcription factor, c-Myc, which causes rapid upregulation of the transcription of the gene and the subsequent processing of its contained miRNAs.

Oncogenic activity of miR-17~92 has been reported in lymphomas in which c-Myc associated overexpression of the miR-17~92 cluster accelerates B lymphomagenesis in an *in vivo* mouse B-cell lymphoma model [78]. It is worth noting that *MIR17HG* is an E2F target gene, while at the same time miR-17 and miR-20, two of the miRNAs present within this cluster, target E2F1, while miR-20 additionally targets E2F2 and E2F3, implying a regulatory feedback loop between E2F and these miRNAs [77,96,97]. Diverse biological functions have been reported in relation to individual components of the miR-17~92 cluster and the downstream effect of their expression after drug treatment, in addition to the regulation of the *MIR17HG* host gene, is discussed in paper II.

1.4 CANCER TYPES STUDIED

As the thesis is based on studies relating to various types of tumors, a brief introduction to each tumor entity is described in this section.

1.4.1 Leukemia

1.4.1.1 Chronic Lymphocytic Leukemia

B cell chronic lymphocytic leukemia (B-CLL) represents the most common type of adult hematological malignancy in western society and accounts for ~30% of adult leukemias and 25% of non-Hodgkin lymphomas (NHL) [98]. It affects mostly elderly people with a median age of around 65 years, with less than 10% of the patients being younger than 40 years of age [99]. A characteristic of B-CLL is the clonal expansion of B-lymphocytes that co-express T-cell antigen CD5 and B-cell surface antigens CD19, CD20, and CD23 [100]. There are two major subtypes of CLL; one harbors somatically mutated immunoglobulin variable region (*IgV)* genes, correlating with good prognosis and the other has unmutated *IgV* genes and a less favorable prognosis [100-102]. Moreover, recent reports suggest genomic abnormalities such as 17p and 11q deletions, expression of CD38 and ZAP70, as well as high β-2-microglobulin levels, correlate with chemotherapeutic resistance and a more aggressive clinical outcome [101,103-105]. In CLL patients, genomic aberrations can be identified in about ~80% cases, as determined by fluorescence in-situ hybridization (FISH) using a disease specific probe set. The most common genomic alteration is 13q deletion which is seen in ~55% of the cases followed by ~18% with 11q deletion, ~16% with 12q trisomy and 7% with 17p deletion. However, the pathogenesis of CLL remains to be established because none of the above alterations have been conclusively demonstrated to be critical for developing CLL [80]. Sub-categorizing these genomic abnormalities is important since there is an established correlation between prognosis and the type of abnormality, such as poor survival in patients with 17p deletion and 11q deletion (commonly leading to the deletion of the *TP53* and *ATM* TSGs respectively), or better survival for patients with trisomy 12q, normal karyotype and deletion 13q as the sole abnormality [80]. Due to the high frequency of deletions, both hemizygous and homozygous, sometimes as the sole detectable abnormality, the genomic region of 13q14.2 has been the focus of a search for the presence of a tumor-suppressor gene of importance to the pathogenesis of

CLL. These studies have included a delineation and subsequently a detailed analysis of the minimal deleted region (MDR), that has been shown to include the first exons of the two non-coding genes *DLEU2* and DLEU1 [106-111]. Later Calin and colleagues identified 2 miRNAs, *miR15A* and *miR16-1,* within the intronic region of *DLEU2* [76]. The relationship between *DLEU2* and these two miRNAs are further discussed in paper I.

1.4.1.2 Acute Lymphoblastic Leukemia

Although Acute Lymphoblastic Leukemia (ALL) occurs in both adults and children, the disease is the most common form of cancer in children, with a prevalence peak between 2 and 5 years of age [112]. Infants under 1 year old have a worse outcome compared to older childhood ALL cases [113]. A higher incidence is seen in males than in females in all age groups, and in high income countries the long term cure rate for pediatric ALL is more than 80% with an almost 90% 5-year survival rate [114]. However, ALL remains the leading cause of non-traumatic death in children and young adults in these countries [115]. The World Health Organization (WHO) classification laid out two major sub-groups of ALL, namely precursor B-lymphoblastic leukemia and precursor T-lymphoblastic leukemia [116]. Precursor B-cell ALL accounts for approximately 70% of pediatric ALL cases, with the rest being mostly T-lineage, as diagnosed by immunophenotyping using flow cytometry [117]. Childhood ALL is characterized by a number of recurring cytogenetic alterations which can be divided into 3 sub-groups; chromosomal translocations which result in the creation of novel chimeric fusion genes which in turn express leukemogenic proteins or which lead to the over-expression of oncogenes, established ploidy subgroups characterized by the gain or loss of multiple non-random chromosomes and a miscellaneous subgroup [118,119]. Furthermore, 7 genetic subtypes have been defined by the WHO classification of B lymphoblastic leukemia, which are t(9;22)(q34;q11.2)/*BCRABL1*, *MLL*/11q23 translocations, t(12;21)(p13;q22)/*ETV6-RUNX1*, t(1;19)(q23;p13.3)/*TCF3-PBX1*, t(5;14)(q31;q32)/*IGH@-IL3*, hyperdiploidy and hypodiploidy [119]. A number of risk factors have been identified for pediatric ALL, and risk-adapted therapy is playing an increasingly important role in the optimization of ALL treatment. Despite the relatively high (compared to other malignancies) rate of treatment success, the identification of additional prognostic variables and the discovery of drugs that can overcome resistance to therapy are needed if the remaining 20% of resistant cases are to be tackled [120,121]. Factors such as age, white blood cell count and immunophenotype and the presence of specific genetic aberrations have been associated with patient outcome [118]. Classically, combination based therapy is used to treat pediatric B-ALL, which includes glucocorticoids (GCs), vincristine and third type of drug such as asparaginase and/or anthracycline [112]. GCs have been used as important chemotherapeutic drugs to treat ALL and other lymphoid malignancies for decades [122]. Futhermore, the sensitivity to GCs is a major prognostic factor in childhood ALL [123,124]. However, the mechanism of how GC-induces cell death in ALL cells is still unclear. Paper II will touch upon one possible factor related to GC resistance.

1.4.2 Skin Cancer

Both basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) belong to the human non-melanoma skin cancers (NMSC) (or keratinocyte carcinomas). This is the most frequently observed type of human neoplasm in the world which is increasing in incidence rate by 3–8% every year in white populations in Europe, the U.S., Canada, and Australia [125,126].

Accumulation of DNA damages by long-term sun exposure is the major cause of NMSC but other risk factors such as male sex, advanced age, immunosuppression (induced or acquired), human papilloma viruses (HPV), chronic inflammation, and genetic variants including somatic mutations in the patched 1 (PTCH1) gene and genetic variants in the melanocortin 1 receptor (MC1R) gene in the skin are also known [127]. Approximately 80% of cases occur in people aged 60 years and older [128]. Although nearly 80% of NMSCs are BCC, and around 20 percent are SCC [129], SCC, which is associated with a substantial risk of metastasis, accounts for the majority of NMSC related deaths. [127].

1.4.2.1 Squamous Cell Carcinoma (SCC)

SCC is the second most common NMSC after BCC, and its incidence has been increasing during recent decades [130,131]. It is a locally invasive, malignant tumor, having metastatic potential, and arising from squamous cells found in the most outer layer of the skin, the epidermis. SCC is more likely to develop in injured or chronically diseased skin such as skin affected by long-standing ulcers, osteomyelitis, radiation dermatitis, vaccination scars and certain chronic inflammatory disorders [127]. The principal precursors of SCC are actinic keratosis (AK) and Bowen's disease (also described as SCC *in situ*), which are both found only in the epidermis and are not metastatic. Solar radiation and immunosuppressive treatment are the most important risk factors [132]. In non-caucasian populations, SCC incidence shows no correlation to solar exposure and chronic scarring processes and chronic inflammation are the most important risk factors [133,134]. Genetic analysis of SCC tumors have demonstrated that the most commonly deleted genomic region in SCC cells is chromosome 9p21 which contains several TSGs such as *16INK4A* (*CDKN2A*), *p15INK4B*, and *MTAP* [135]. Loss of Heterozigosity (LOH) on 3p, 2q, 8p, and 13 and allelic gain of 3q and 8q are also frequently found in SCC, and these can be used as markers in early diagnosis [136].

1.4.2.2 Basal Cell Carcinoma (BCC)

BCC is the most common NMSC which also represents the most common malignancy in the Caucasian population [131]. Historically, it was thought that the cell-of-origin of BCC was from the epidermal basal cell layer, although later studies have favored a follicular derivation of BCC, however the exact cell-of-origin is, to date, uncertain [137]. An autosomal dominant disorder with distinct morphological features including multiple basal-cell carcinomas, results from germline mutations in *PTCH1*, a segment polarity gene (9q22.3) that plays an critical role in development, and frequent mutation of this gene is also detected in sporadic BCC cases [138,139]. *PTCH1* encodes a putative transmembrane protein that acts as a receptor for the diffusible morphogen

protein, sonic hedgehog (Shh), and it has been shown to exert tumor suppressor functions [140]. Mutation of PTCH1 causes reduced suppression of intracellular signaling by the G-protein-coupled receptor, Smoothened (Smo), which targets the Gli family of transcription factors, resulting in sustained activation of target genes [139]. The understanding of pathogenesis of BCC is still partial and more detailed analyses are required.

Recently, it has been proposed that epigenetic alterations contribute to tumorigenesis. Furthermore, the possible involvement of cancer associated dysregulated expression of miRNAs has been described. The skin-specific miRNA miR-203 discussed in paper III and IV, seems to represent one such example of a miRNA that may play an important role in the pathogenesis of skin tumors.

AIMS OF THE THESIS

The overall aim of this thesis was to uncover the function of miRNAs in various types of cancers and the role of microRNAs in drug resistance. The specific aims for each paper are following;

- Paper I: To characterize the regulation and the function of the miR-15a/16-1 locus in relation to the MDR and *DLEU2* host transcript in CLL

- Paper II: To investigate role and mechanism of miRNA regulation in GC-induced apoptotic cell death in ALL and determine the impact of miR-17 regulation in GC-resistance in ALL cell lines and patient samples

- Paper III: To explore the functional role of miR-203 in SCC pathogenesis

- Paper IV: To define the mechanisms of how miR-203 dysregulation may contribute to carcinogenesis of the skin and BCC tumor formation

RESULTS AND DISCUSSION

Paper I

***DLEU2*, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1**

Several groups, including ours, have worked intensively over a number of years to characterize the genes located at the 13q14 chromosomal locus that is deleted in leukemic cells from more than half of CLL patients [106,108-110,141-144]. Our group had described a minimally deleted region or around 10kb encompassing the first exons of the two non-coding genes *DLEU2* and *DLEU1*, indicating that one or more of these genes may be involved in the pathogenesis of CLL. In 2002, Calin and colleagues identified two miRNAs, miR-15a and miR-16-1,located within one of the introns of *DLEU2*, and which were downregulated in CLL, and thus the first miRNAs found to be altered in cancer [76]. Further studies by Calin *et al* demonstrated a germline C→T homozygous substitution in the pri-miR-16-1 transcript in 2 of 75 CLL patients, although this is probably a rare event [49]. Another group described a point mutation in the 3' flanking sequence of the homologous miR-16-1 that correlated with down-regulated expression of miR-16 in New Zealand black (NZB) mice with autoimmune and B lymphoproliferative disease (B-LPD), a condition that is regarded as a model for human CLL [49]. However, the exact role of these two miRNAs in relation to the adjacent genes is still under some debate. In particular the question remained whether miR-15a and miR-16-1 were independently regulated at the transcriptional level or whether they were in fact downstream products of the gene *DLEU2*, thus explaining the relationship between the minimal deleted region and the miRNAs that are located over 30 kb downstream from this MDR.

Our detailed breakpoint analysis revealed that the 13q14 MDR in CLL patients encompasses only 8.5 kb in size, and does not include the miR-15a/16 region but as mentioned above, does include the first exons of *DLEU1* and *DLEU2* [145]. In contrast to the *DLEU1* gene, the *DLEU2* gene is evolutionarily conserved at the nucleotide level although it does not encode a conserved open reading frame, indicating that although there is no protein expressed from the gene, it may function at the RNA level [76]. In Paper I, we investigated whether the *DLEU2* could act as a miRNA host gene and regulate the expression of miR-15a/16-1. In addition, this study included an initial characterization of one biological function of these miRNAs and a description of regulation of the expression of miR-15a/16-1 by the oncogenic transcription factor Myc.

Most miRNAs are transcribed as part of non-coding host genes or from introns of protein-coding genes by RNA Pol II [53]. In almost every case they are transcribed in the same orientation as the predicted mRNAs, suggesting that most of these miRNAs are not transcribed from independent promoters but are instead processed from the introns or exons of their respective host gene. In an effort to establish the link between *DLEU2* expression and miR-15a/16-1, we first assessed if the maturation of miR-15a/16-1 was dependent on the nuclear RNAse III Drosha. RNAi knockdown of

Drosha led to a significant accumulation of a partially spliced isoform of *DLEU2*, but not the fully spliced *DLEU2*, suggesting that *DLEU2* transcripts are processed by Drosha in order to produce functional miRNAs. It had been previously reported that the transcription factor Myc negatively regulates miR-15a/16-1 expression [16]. Together with our finding in relation to Drosha knock down, we hypothesized that miR-15a/16-1 expression was regulated through the *DLEU2* transcript, and that a Myc-mediated repression of *DLEU2* transcription could consequently result in the down-regulation of miR-15a/16-1 expression. Using a doxycycline-regulated model system where Myc expression can be turned off [146], we found that both *DLEU2* and miR-15a/16-1 expression levels were induced following the repression of Myc expression. Furthermore, a chromatin immunoprecipitation (ChIP) assay demonstrated that the reduced expression of *DLEU2* correlates with a direct Myc binding to two *DLEU2* alternative promoter regions. In contrast, Myc binding could not be detected to any loci immediately upstream of the miRNAs, the locus originally proposed by Calin *et al* as the likely promoter of miR-15a/miR-16-1. Taken together, these findings provide evidence that the regulation of miR-15a/miR-16-1 occurs at the two alternative *DLEU2* promoters, located within and upstream of the MDR and therefore that *DLEU2* functions as a host gene for miR-15a/16-1.

Next, we focused on the functional properties of miR-15a/16-1 as tumor-suppressors. Bioinformatic analysis techniques were used to search the putative targets of these miRNAs, resulting in the identification of two cyclins, cyclin D1 and cyclin E1 as conserved high confidence targets.

To assess the functional role of miR-15a/16-1, we cloned an alternatively spliced isoform of *DLEU2* that retained part of intron 4 that contained the miRNAs. Using these constructs, rapid over-expression of miRNAs was achieved in transfected cell lines and western blot analysis was used to confirm the down-regulation of both cyclin D1 and cyclin E1 protein levels. In contrast, a *DLEU2* expression construct lacking the miR-15a/16-1 sequence (*DLEU2*Δ-MIR) did not influence the level of these proteins. To test the physiological role of miR-15a/16-1, we performed colony formation assays in tumor cell lines transfected with these constructs, and confirmed that expression of *DLEU2* led to an ~80% decrease in colony numbers compared to mock- or *DLEU2*Δ-MIR- transfected cell lines. The reduction in colony formation was caused by G1 arrest, in line with the effects observed on the G1 cyclins. Additionally, we have performed apoptosis assays on these transfected cells and found no significant induction of apoptosis in contrast to the initial report of miR-15a/16-1 targeting and suppressing Bcl-2 expression [84].

This study delineated a functional role for these miRNAs and provided important new information on the regulation of miR-15a/16-1, demonstrating *DLEU2* as the regulatory host gene of these miRNAs, and also unraveled the mechanism underlying down-regulation of miR-15a/16-1 expression in 13q14 deletion cases with a retention of the miRNA region. Moreover, the notion that the oncoprotein Myc binds and directly regulates the expression of these miRNAs implicates these miRNAs as downstream players of importance in Myc induced transformation. Interestingly, a recent report has shown that restoration of miR-16 expression in a New Zealand black–derived malignant B-1 cell line with impaired miR-16 expression, augments the apoptotic

effects of chemotheutics, nutlin and genistein [147]. These findings implicate miR-15a/16-1 as possible therapeutic targets to improve drug efficacy in human CLL.

Paper II

Involvement of the miR17 pathway in glucocorticoid-induced cell death in pediatric acute lymphoblastic leukemia

GCs have been used as important chemotherapeutic drugs to treat ALL and other lymphoid malignancies for almost 50 years and continue to be significant elements of combination chemotherapy regimens [122]. With intensive multi-agent chemotherapy, the cure rate of childhood ALL is currently more than 80%, however the failure of this treatment in the remaining 20% necessitates the development of improved and novel therapies [114]. Hints to the mechanism of how GCs cause apoptotic cell death in ALL have, in recent years, been revealed using screening methods including gene-expression profiling [148]. Microarray technology led to the discovery of a "signature" list of genes that are dysregulated in GC-sensitive ALL cells [149]. In more mechanistic studies, other groups have demonstrated that GCs induce the expression of the pro-apoptotic protein Bim in various ALL cell lines and primary patient cells, and that Bim induction is sufficient to cause apoptosis [150]. In addition, it was shown that Bim knock-down resulted in the inhibition of GC-induced apoptosis [151]. These reports demonstrate the importance of Bim to the chemotherapeutic function of GCs but the exact mechanism through which GCs induce Bim in lymphoid cells are still to be defined. MiRNAs are capable of altering both the stability of mRNAs as well as their capability to be translated. We hypothesized that GC-mediated cytotoxicity in ALL cells may, in part, be brought about through the regulation of the expression of specific miRNAs. To examine this, we performed an RT-PCR based expression array study, combined with bio-informatic analysis of the role of GC mediated changes in miRNA expression in ALL cells.

In this paper, we observed a trend of general down-regulation of large set of miRNAs after Dexa-treatment prior to the onset of cell death and identified 3 miRNAs which belongs to the same cluster, miR-17-92, that has previously been shown to harbor oncogenic properties. We demonstrated that miR-17 family members target Bim at the post-transcriptional level. We also analyzed the specific mechanism for how GCs downregulate the miR-17-92 cluster. ChIP analysis was used to reveal that the GC-receptor binds directly to the promoter of the miR-17-92 host gene, *MIR17HG*, leading to a suppressed expression of the *MIR17HG* transcript. This in turn caused the downregulation of mature miR-17 expression, and hence the derepression of the miR-17 target gene, Bim. We also showed that the introduction of miR-17 in a B-ALL cell line model system led to the down-regulation of Bim and that the inhibition of miR-17 through the use of anti-miR-17 antagomirs resulted in Bim induction. Furthermore, we demonstrated that over-expression of miR-17 partially inhibits apoptosis whereas inhibition of miR-17 increases the apoptotic rate in GC-treated ALL cell lines, suggesting that modulation of miR-17 expression modulates the sensitivity to GC-induced apoptosis. Confirming these results through an analysis of primary ALL cells, we also found an association between the ex-vivo sensitivity of these cells to GCs and the ability of GCs to downregulate mir-R17 levels. We thus suggest that GC-induced

apoptosis and sensitivity is regulated at least partly through miR-17 mediated regulation of Bim protein levels.

The miR-17-92 cluster plays an important role in promoting cell proliferation and suppressing apoptosis in tumor cells as well as during the normal development of various organs [152]. Like many other miRNAs, the miR-17-92 cluster is located at a fragile site of the genome, and is often amplified in tumor cells including lymphoma [94]. Furthermore, overexpression of miR-17-92 increased tumor progression in the *Eμ-Myc* transgenic mouse model of lymphoma at least in part by inhibiting apoptosis [78]. The downregulation of miR-17-92 is essential for the B cell development at the pro-B to pre-B transition by inducing Bim protein, as a block of this transition is found in miR-17-92−/− hematopoietic precursor cells [153].

Other miRs were downregulated as well during GC treatment of ALL cells. The micoRNA showing the highest levels of downregulation, namely miR-142-3p, has recently been shown to be a direct regulator of the glucocorticoid receptor, GR [154]. This result, in combination with our finding that GC directly regulates the expression of the miR-17-92 cluster, indicates the likelihood that there exists a complex regulatory network involving both positive and negative feedback mechanisms involving the glucocorticoid receptor and multiple miRNAs.

Interestingly, the suppression of miR-15a, which is expressed as a part of the *DLEU2* Pri-miRNA transcript, was not due to the suppression of the primary transcript, although the level of the mature miRNA was suppressed. This suggests the possibility that GC may regulate miRNA expression in various ways and thus further investigation is needed to unravel the different processes by which GCs regulate other miRNAs.

In summary, our findings reveal a novel mechanism of GC mediated regulation of miR-17-92 transcription, and that this regulation plays a critical role in GC sensitivity in ALL. Furthermore, our data supports previous reports demonstrating the importance of Bim in GC-induced apoptotic cell death in human ALL cells. Importantly, these findings also suggest that constituents of the miR-17-92 cluster are potential future therapeutic targets.

Paper III
Investigation of the role of miR-203 in squamous cell carcinomas of the skin

Although having an incident rate much lower than that of BCC, the death rate of SCC is the highest among non-melanoma skin cancer types, accounting for 20% of all skin-cancer related deaths [155,156]. MiR-203 is a skin-specific miRNA which is highly expressed in the squamous cell epithelium [85] and plays an important role during development by repressing ΔNp63 and thus leading to the terminal differentiation of skin stem cells [157]. Recent reports demonstrate a tumor-suppressor function of miR-203 such as G1 phase cell cycle arrest by targeting survivin in laryngeal cancer cells [158] and induction of senescence by targeting E2F3 in melanoma cells [159]. Furthermore, an aberrant regulation of miR-203 expression has been reported in hepatocellular carcinoma [160], bone metastatic prostate cancer [161] and T-cell lymphomas [162]. There are several reports regarding altered miR-203 expression in SCC however knowledge of a detailed mechanism of how miR-203 dysregulation contributes to the SCC pathogenesis is still lacking [163-165]. Saini and his colleagues showed regulation of pro-metastatic genes by miR-203 including ZEB2, Bmi, survivin, as well as bone-specific effectors including Runx2, a master regulator of bone metastasis in prostate cancer [161]. These findings indicate that miR-203 may also play a role in metastasis in SCC cells.

In this paper, we demonstrated that miR-203 expression is progressively dysregulated in relation to the clinical grade in SCC samples as well as in SCC cell lines compared to non-tumor cells, indicating the likelihood that this miRNA participates in the pathogenesis of SCC. Gene expression profiling of SCC cell lines upon miR-203 introduction demonstrated an overrepresentation of expression changes in genes involved in the regulation of cell proliferation as well as differentiation and cell motility. Moreover, gene network analysis showed that many of the genes regulated by miR-203 have been previously shown to be dysregulated in skin neoplasms.

Next, we focused on defining the functional properties of miR-203 as a tumor-suppressor. Bioinformatic analysis was used to search for putative targets of this miRNA and indicated the c-*MYC* proto-oncogene and *COX-2* to be conserved putative targets.

To validate these genes as targets of miR-203, we used a pre-miR construct to cause rapid over-expression of this miRNA in SCC cell lines and confirmed the down-regulation of both c-Myc and Cox-2 protein levels using western blot analysis. The physiological role of miR-203 was tested using the same cell lines in colony formation assays. A significant reduction in colony density was observed in the cells transfected with pre-miR-203. A possible mechanism for causing the reduction in colony number was G1 arrest of these cells, which was indeed demonstrated using a cell proliferation assay with the same cells. Next, we assigned the metastatic potential of miR-203 in SCC cells using the wound-healing/scratch assay, demonstrating that miR-203 over-expression slows down the speed of wound-healing, confirmed by both the migration and invasion assays.

The suppression of c-Myc may partially account for the observed reduction of cell proliferation by miR-203. The proto-oncogene *MYC* is well studied due to its high rate of alteration in cancer, leading to dysregulation of cell growth, proliferation, apoptosis and angiogenesis. C-Myc has therefore been considered an attractive future therapeutic target. Interestingly, bioinformatic analysis revealed binding of the c-Myc and E2F1 transcription factors to the miR-203 promoter region, suggesting the existence of an autoregulatory feedback loop between miR-203 and the Myc/E2F1 pathway, similar to the feedback loop suggested for the miR-17 family of miRNAs [77]. Taken together miR-203 may be an upstream regulator of c-Myc, and its dysregulation may thus lead to the disruption of a number of downstream cellular events important to maintaining cellular homeostasis and hence contribute to tumor pathogenesis. Moreover, the direct regulation of Cox-2 by miR-203 also suggests another therapeutic implication, due to the capability of Cox-2 to induce angiogenic factors such as VEGF, bFGF, TGF-1, PDGF and endothelin [166,167]. Therefore we suggest that Cox-2 regulation by miR-203 could be a putative target to inhibit metastasis in SCC for therapeutic application. The mechanism of miR-203 suppression in cancer is still unknown. In addition to a possible regulatory mechanism involving c-Myc and E2F1 there are reports of epigenetic silencing of this transcript in some diseases however a more precise analysis is required to determine the relevance of these finding in SCC.

In summary, our findings show the downregulation of miR-203 in SCC. Furthermore, we found that miR-203 mediated suppression of c-Myc, a factor may play a role in the regulation of proliferation. In addition, our results strongly suggest that, miR-203 plays a role in migration and invasion in SCC through the regulation of Cox-2. The stage-dependent reduction in the miR-203 expression level in the SCC patient material suggests that suppression of this miRNA may have a functional role in sustaining a high proliferation state and metastatic potential of this tumor. Taken together, we suggest that a suppressed expression of miR-203 contributes to oncogenic transformation through activation of proteins with oncogenic properties. Moreover these findings suggest that inducing miR-203 in SCC cells may be of therapeutic potential in this disease.

Paper IV
MicroRNA-203 functions as a tumor suppressor in basal cell carcinoma

Basal cell carcinoma (BCC) represents ~80% of non-melanoma skin cancer, which is the most common human malignancy in the causasian population, with the incidence increasing due to an aging population and current sun exposure habits. Abnormal activation of the Hedgehog (Hh) pathway caused by inactivating mutations in *PTCH1* or activating mutations in *SMO* is indispensable in BCC pathogenesis [168-170]. Animal studies using transgenic mice models demonstrated that forced expression of the *Gli* transcription factors causes the development of BCC-like tumors in these animals [171,172] as well as the forced expression of sonic hedgehog (Shh) [173] and smoothened (Smo) [174], all of which are factors that contribute to the activation of the hedgehog pathway. To date, all investigations on BCC tumor onset and development have focused on mutations and/or expression of protein-coding genes, and although several advances have been made, a comprehensive molecular description detailing BCC pathogenesis is still lacking. At present the role of miRNAs in BCC pathogenesis has not been described.

In this paper, we used a comprehensive, genome-wide analysis of miRNA to compare miRNA expression in human healthy skin and BCCs. Unsupervised hierarchical clustering based on miRNA expression clearly separated BCC tumor samples from healthy skin, and most miRNAs with significant differential expression were found to be expressed at lower levels in BCC, which is in line with previous reports relating to miRNA expression in solid tumors [175]. These findings suggest that the down-regulation of miRNAs may contribute to BCC pathogenesis. Among those suppressed miRNAs, miR-203 was identified as the most significantly and consistently down-regulated miRNA. This particular miRNA is known as a skin-specific transcript that functions to promote epidermal differentiation. *In situ* hybridization using miR-203 specific locked nucleic acid probes showed that miR-203 was preferentially expressed in the suprabasal layer of healthy skin, while in BCCs miR-203 expression was largely absent. A correlation between activation of the Hh pathway and miR-203 suppression was suggested by quantitative real-time PCR, showing that both PTCH1 and GLI1 were significantly upregulated ($p<0.001$) in BCC tumors as compared with healthy skin, in accordance with previously published data. Furthermore, correlation analysis showed a significant negative association between miR-203 expression and GLI1, as well as between miR-203 and PTCH1, suggesting that a loss-of-function of miR-203 may be associated with aberrant Hh signaling in BCC.

Next, we aimed to evaluate the potential mechanisms and/or pathways accounting for miR-203 suppression in BCC. Previous reports suggest that miR-203 is regulated by the protein kinase C/activator protein 1 (AP-1) pathway and suppressed by growth factors such as KGF and EGF in keratinocytes [176]. Recently, it was shown that the EGFR signaling pathway often synergizes with Hh/GLI1 in oncogenic transformation via activation of the MEK/ERK/JUN pathway [177], indicating one possible mechanism for miR-203 suppression in BCC. The miR-203 expression level was examined in primary human keratinocytes treated with inhibitors of EGFR, MEK1/2,

JNK, or Akt in combination with EGF or DMSO alone with the results showing a significant suppression of miR-203 in EGF-treated keratinocytes. Conversely, activation of the EGFR-MAPK signaling pathway represses miR-203 expression, implying a potential role of the EGFR-MAPK signaling pathway in miR-203 down-regulation in BCC. A bioinformatic search for putative miR-203 targets identified several genes in the MEK/ERK/JUN pathway, including the *c-JUN* proto-oncogene which plays a role in cell proliferation. Reporter assays revealed a direct regulation of c-Jun by miR-203 through 3'UTR binding. In line with this, cell-cycle analysis of primary keratinocytes transfected with pre-miR-203 showed a clear inhibition in the G1to S-phase transition of the cell cycle. These results suggest that suppression of c-Jun contributes to the antiproliferative effects of miR-203. In accordance with previous results [178], we also demonstrated that c-Jun in turn suppresses miR-203 expression in primary keratinocytes. Taken together, these results suggest a regulatory circuit in which miR-203 and c-Jun mutually inhibit each other and that the low-levels of miR-203 may be caused by c-Jun activation leading to the basal phenotype in BCC, similar to that seen in basal keratinocytes which also show high expression of c-Jun.

We also showed that activation of the Hh pathway suppress miR-203 expression. These finding suggests the activated Hh pathway in BCCs also contributes to pathogenesis through miR-203 suppression. This also indicates a cross talk with ERK signaling, as the suppression of miR-203 in turn affects the expression of multiple genes involved in the regulation of cell proliferation and cell cycle including c-Jun and other targets.

In conclusion, our study represents the first analysis of miRNA expression and function in a non-melanoma skin cancer. These results indicate a complex molecular network, involving regulatory interactions between potent signaling pathways/oncoproteins and miR-203. The loss of miR-203 expression in BCC suggests that this miRNA could be used as a biomarker of the disease and may also represent a potential therapeutic target for the treatment of BCC. Further investigation will be needed to demonstrate whether the molecular reconstitution of miR-203 may serve as a novel therapeutic strategy in the treatment of BCC tumors.

GENERAL CONCLUSIONS

• *DLEU2* is a non-coding RNA gene that functions as a regulatory host gene for the microRNAs miR-15a and miR-16-1, which negatively regulate Cyclin D1 and Cyclin E1 at the post-transcriptional level.

• *DLEU2* transcripts that contain miR-15a/miR-16-1, exert tumor suppressor activity in proliferation and colony formation assays.

• The oncoprotein c-Myc can directly repress *DLEU2* transcription.

• The miR-17-92 gene (*MIR17HG*) promoter is directly bound by the GC-receptor in ALL cells, through which it causes a transcriptional repression of the pri-miR-17 host transcript.

• GC associated miR-17 repression partially mediates GC-induced cell death in ALL cells, possibly through an abrogated miR-17 mediated suppression of Bim.

• A correlation exists between GC mediated miR-17-5p suppression and GC induced death ex vivo in primary ALL cells.

• miR-203 levels are reduced both in BCC and SCC cell lines and primary tumor cells.

• c-Myc/Cox-2/c-Jun are identified as novel direct targets of miR-203

• miR-203 negatively regulates differentiation, G1/S arrest and migration/invasion, suggesting a putative role for this miR as a tumor-suppressor in non-melanoma skin cancer

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