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FUNCTIONAL STUDIES OF microRNAs IN DEVELOPMENT AND CANCER

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TO MY LOVING FAMILY

"Eventually, everything connects."
– Charles Eames

ABSTRACT

MicroRNAs (miRNAs) COMPRISE a large family of small (~23 nucleotide in length), endogenous RNAs that regulate gene expression at the posttranscriptional level. Functional studies have indicated that miRNAs participate in the regulation of nearly all cellular processes investigated so far, including differentiation, apoptosis, and proliferation. Further, the deregulation of miRNA expression greatly contributes to human diseases, and is associated with many human pathologies, such as cancer.

The studies in this thesis have focused on miRNA expression and regulation in various forms of malignancies. Specifically, we wanted to provide mechanistic insights into the role of miRNAs in tumorigenesis. In parallel, we hoped to discover new therapeutic targets that could be exploited clinically to treat childhood and adult cancer. In the work presented, we describe the functional consequences of miRNA perturbations in three distinct neoplasias: (1) chronic lymphocytic leukemia (CLL), the second most common type of blood cancer in adults; (2) neuroblastoma (NB), an embryonal malignancy of the sympathetic nervous system that is derived from primordial neural crest cells and occurs almost exclusively in infants and young children; and, (3) basal cell carcinoma (BCC), a basal cell-derived malignancy of the epidermis, which ranks as the most commonly diagnosed human cancer among fair-skinned individuals.

Our CLL studies revealed that the *DLEU2* transcript functions as a regulatory host gene for the miRNAs *miR-15a* and *miR-16-1*. These miRNAs were shown to target the G_1 cyclins D1 and E1 for translational repression, resulting in a prominent cell cycle arrest. Further, ectopic expression of *DLEU2* inhibited the colony-forming capacity of tumor cell lines, suggesting a tumor-suppressive function for miR-15a and miR-16-1. We also demonstrate that *DLEU2* is transcriptionally regulated by the oncoprotein c-MYC, providing a novel mechanism by which MYC can regulate the G_1 cyclins in a posttranscriptional manner. Functional loss of *DLEU2* may thus constitute an important step in CLL tumorigenesis and various c-MYC-dependent cancers.

In our analysis of *MYCN*-amplified neuroblastoma (NB), we investigated the molecular consequences and functional outcome of abnormal miRNA regulation and discovered that miR-17~92 cluster-derived miRNAs potentiate the tumorigenic behavior of this childhood cancer. Importantly, we could show that miR-18a and miR-19a target and repress the expression of estrogen receptor-α (ESR1), a ligand-inducible transcription factor implicated in neuronal differentiation. We propose that ESR1 represents a previously undescribed MYCN target in NB and demonstrate a unique oncogenic circuitry in which the repression of ESR1 through MYCN-regulated miRNAs may play a fundamental role in NB tumorigenesis.

Finally, based on our genome-wide miRNA expression analysis of a non-melanoma skin cancer, we found that the skin-specific miRNA, miR-203, is preferentially lost in BCC. Functional analyses demonstrated that the inappropriate activation of the Hedgehog and MAPK pathways in BCCs may contribute to cancer progression via severely reduced expression of miR-203, which dramatically facilitates the misexpression of genes involved in the regulation of cell proliferation and cell cycle, including c-JUN and c-MYC. In this respect, miR-203 constitutes a 'gatekeeper' miRNA controlling keratinocyte proliferation. The molecular reconstitution of miR-203 could therefore serve as a novel therapeutic strategy in the treatment of BCC tumors.

LIST OF PUBLICATIONS

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I. Mikael Lerner, Masako Harada, **Jakob Lovén,** Juan Castro, Zadie Davis, David Oscier, Marie Henriksson, Olle Sangfelt, Dan Grandér, and Martin M. Corcoran. (2009)
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**, 2941–2952.

II. **Jakob Lovén**, Nikolay Zinin, Therese Wahlström, Inga Müller, Petter Brodin, Erik Fredlund, Ulf Ribacke, Andor Pivarcsi, Sven Påhlman, and Marie Henriksson. (2010)
MYCN-regulated microRNAs repress estrogen receptor-α (ESR1) expression and neuronal differentiation in human neuroblastoma.
Proceeding of the National Academy of Science (PNAS), **107**, 1553–1558.

III. Eniko Sonkoly*, **Jakob Lovén***, Tianling Wei, Ning Xu, Florian Meisgen, Petter Brodin, Viljar Jaks, Maria Kasper, Takashi Shimokawa, Masako Harada, Johann Heilborn, Mari-Anne Hedblad, Andreas Hippe, Dan Grandér, Bernhard Homey, Peter Zaphiropoulos, Marie Arsenian-Henriksson, Mona Ståhle, Andor Pivarcsi. (2010)
miR-203 functions as a bona fide tumor suppressor microRNA in basal cell carcinoma.
Manuscript.
*Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
5'UTR	5' untranslated region
AGO	argonaute
AKT	v-akt murine thymoma viral oncogene homolog
ATXN1	spinocerebellar ataxia type 1 protein
BARD1	BRCA1 associated RING domain 1
BCC	basal cell carcinoma
BCL2	B-cell CLL/lymphoma 2
BCNS	basal cell nevus syndrome
bHLH	basic-helix-loop-helix
C.elegans	*Caenorhabiditis elegans*
CCND2	cyclin D2
ChIP	chromatin immunopreciptation
CLL	chronic lymphocytic leukemia
CNS	central nervous system
D.melanogaster	*Drosophila melanogaster*
DGRC8	DiGeorge syndrome critical region
DLEU2	deleted in lymphocytic leukemia 2
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EcR	ecdysone receptor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
endo-siRNA	endogenous small interfering RNA
ESR1	estrogen receptor-α
EXP5	exportin-5
FFL	feedforward loop
GLI	glioma-associated oncogene family zinc finger 1
HCC	hepatocellular carcinoma
HCV	hapatitis C virus
HES	hairy e(spl)
HH	hedgehog
HOX	homeobox

IFE	interfollicular epidermis
IHC	immunohistochemistry
JNK	Jun N-terminal kinase
lin	lineage abnormal
LNA	locked nucleic acid
MAPK	mitogen-activated protein kinase
MAX	MYC-associated protein X
MDR	minimal deleted region
mESC	mouse embryonic stem cells
miRISC	miRNA-programmed RISC
miRNA	microRNA
MIZ1	MYC-interacting zinc finger protein 1
MNT	MAX-interacting protein
MXD	MAX dimerization protein
MYC	myelocytomatosis
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived
ncRNA	noncoding RNA
NGF	nerve growth factor
PACT	protein activator of PKR
piRNA	Piwi-interacting RNA
pre-miRNA	miRNA precursor
pri-miRNA	primary miRNA
PTCH1	patched homologue 1
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
RB	retinoblastoma
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNA Pol	RNA polymerase
RNAi	RNA interference
ROBO2	roundabout, axon guidance receptor, homolog 2
ROS	reactive oxygen species
SA	sympatho adrenal
SHH	sonic hedgehog
SMO	smoothened
snoRNA	small nucleolar RNA
SNS	sympathetic nervous system
SOP	sense organ precursor
stRNA	short temporal RNA
TAR	trans-activation responsive
TF	transcription factor
TFBS	transcription factor binding site
TRBP	TAR RNA binding protein
TRKA	tyrosine kinase receptor A
tRNA	transfer RNA
TSG	tumor suppressor gene
TSS	transcriptional start site

1. INTRODUCTION

IN THE LAST decade, the biology of ribobnucleic acid (RNA) has witnessed a transformation unmatched by any other area in medical research. The discovery that RNA molecules act as versatile regulators of eukaryotic gene expression has reshaped our understanding of gene regulation and function. At present, any transcript, regardless of coding potential, may have an intrinsic function as an RNA. Whether long or short, noncoding RNAs (ncRNAs) have been found to regulate some of the most important levels of genome function, including chromatin structure, transcription, RNA processing, RNA stability and translation.

In 1986, Walter Gilbert proposed the RNA world hypothesis, a theory about the origin of life based on the view that the most critical event is the emergence of a self-replicating molecule, a molecule that can both copy itself and mutate and, hence, evolve to more efficient copying (Gilbert, 1986). RNA is such a molecule. During the very early stages of life on Earth, proteins were not yet engaged in biochemical reactions and RNA carried out both the information storage task of genetic information and the full range of catalytic roles necessary in a very primitive self-replicating system.

Despite this functional versatility, RNA was long considered a docile molecular entity, largely operating as an inert intermediary between gene and protein. However, the finding that most of the genomes of complex organisms are transcribed in a regulated fashion along with the discovery of several classes of regulatory, noncoding RNAs (including microRNAs) has challenged this assumption (Mercer et al., 2009). Recent advances have revealed unexpected diversity of function for various ncRNAs, suggesting that RNA has continued to evolve and expand alongside DNA and protein.

The work presented in this thesis focuses on a particular class of small noncoding RNAs termed microRNAs, or miRNAs, and their functional role in developmental pathways and cancer etiology.

IN BIOLOGY, HETEROCHRONY refers to changes, over evolutionary time, in the rate or timing of developmental events (Moss, 2007). Differences in the relative timing of developmental events have long been believed to be a major force in the evolution of morphology. Early attempts to identify heterochronic phenotypes in *Caenorhabditis elegans* (*C. elegans*) revealed a collection of genes that, when mutated, execute stage-specific cell fates at inappropriately early or late time-points (Chalfie et al., 1981). These so-called 'heterochronic' genes were identified among a larger group of lineage-abnormal (*lin*) mutants, and revealed a developmental timing mechanism that functions independently of other types of developmental regulation, including growth, induction and differentiation (Moss, 2007).

Among the first heterochronic mutants to be described was *lin-4*. Mutations in *lin-4* caused the first larval stage (L1) to reiterate at later developmental stages (*figure 1*) (Chalfie et al., 1981). The converse phenotype was later observed for the *lin-14* mutant, which arose spontaneously in a culture of *lin-4* mutant animals. Omission of the L1 cell fates and premature development into the L2 stage are observed in worms that are deficient for *lin-14* (Ambros and Horvitz, 1984). Following extensive mutagenenic analyses, it was realised that *lin-4* encodes a pair of small untranslated RNAs measuring 61 and 22 nucleotides (nt) in length (Lee et al., 1993). Surprisingly, these lin-4 RNAs had antisense complementarity to multiple sites of the 3'-untranslated region (3'UTR) of the lin-14 gene (Lee et al., 1993). *lin-14*, on the other hand, encodes a nuclear protein, downregulation of which at the end of L1 initiates the developmental progression into the second larval stage (L2) (Lee et al., 1993; Ruvkun and Giusto, 1989). In addition to a functional *lin-4* gene, the negative regulation of the LIN-14 protein requires an intact 3'UTR (Lee et al., 1993; Wightman et al., 1991).

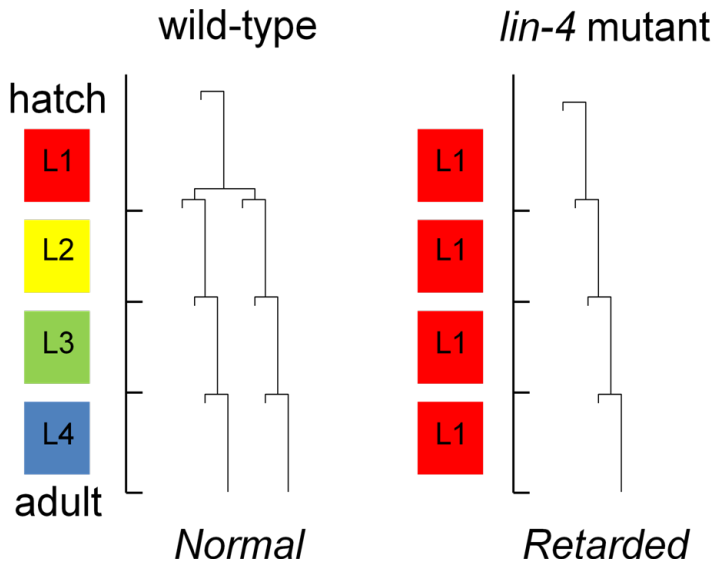

Figure 1. The heterochronic phenotype of the *lin-4* mutant in C.elegans. Worms with mutations in *lin-4* do not develop normally and cell divisions characteristic of larval stage 1 (L1) reiterate throughout development.

Together, these findings led to the characterisation of a novel regulatory pathway in which *lin-4* RNAs negatively regulate *lin-14* translation by binding to its 3'UTR. The subsequent discovery that another temporally regulated 21 nt small RNA in *C. elegans*, let-7, was readily detected among phylogenetically distinct bilaterians indicated a more general role for small RNAs in developmental regulation (Pasquinelli et al., 2000). It was also established that several additional small RNAs with structural characteristics resembling *lin-4* and *let-7* were detectable throughout metazoan development (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). However, unlike the *lin-4* and *let-7* RNAs, which had been classified as short temporal RNAs (stRNAs) due to their common roles in controlling developmental timing, many of the newly cloned small RNAs did not display heterochronic features. Thus, the annotation stRNA was abandoned and replaced with the term microRNA (miRNA), to denote all small RNAs with similar features but unknown functions (Bartel, 2004).

Since their original discovery, miRNAs have been detected in all metazoans surveyed so far for their presence, including poriferans and cnidarians, and are even present in other distantly related eukaryotuc lineages, such as plants and algae (Grimson et al., 2008). Hence, miRNAs constitute one of the most abundant classes of gene-regulatory molecules in the animal and plant branhces of eukaryota.

microRNA Biogenesis

miRNAs ARE BROADLY defined as small (~23 nt in length), endogenous RNAs that play important gene-regulatory roles by pairing to the mRNAs of protein-coding genes to direct their posttranscriptional repression (Bartel, 2009). MiRNAs belong to a broad class of small RNAs, including endogenous small interfering RNAs (endo-siRNAs) (Reinhart and Bartel, 2002) and the Piwi-interacting RNAs (piRNAs) (Aravin et al., 2007). Together, these RNAs act as sequence-specific guides in RNA silencing pathways. Processed from distinctive hairpin-shaped transcripts, miRNAs act as guide molecules in posttranscriptional processes that involve base-pairing with cognate messenger RNA (mRNA) targets, usually in the 3' untranslated region (3'UTR).

Binding of a miRNA to the target mRNA typically leads to translational repression and/or mRNA destabilasation, although other types of regulation, including translational activation and heterochromatin formation, have been described (Filipowicz et al., 2008; Kim et al., 2008; Orom et al., 2008). At present, the number of confidently identified miRNAs has surpassed 110 in C.elegans, 140 in the fly Drosophila melanogaster and 400 in humans, corresponding to ~ 1–2% of the number of protein-coding genes in these species (Bartel, 2009). Initial efforts based on computational methods estimated that more than one third of human genes appear to be conserved miRNA targets (Lewis et al., 2005), while recent estimates suggest that more than 60% of all human protein-coding genes are under selective pressure to maintain pairing to miRNAs (Friedman et al., 2009).

With regard to their genomic organisation, a sizable portion (~40%) of miRNAs are present within introns of pre-mRNAs, providing a convenient mechanism for the coordinated expression of intron-embedded miRNAs and protein-encoding mRNAs (Bartel, 2004). Furthermore, most mammalian miRNA genes are clustered in the genome, allowing them to be transcribed simultaneously as polycistronic transcription units (Altuvia et al., 2005; Bartel, 2004). Intronic incorporation and genomic miRNA aggregation allows for miRNAs to employ existing functional promoter elements, eliminating the need for *de novo* assembly of transcriptional features, such as promoter-enhancer sequences, transcriptional start sites (TSS), and transcription factor binding sites (TFBS) (Saini et al., 2007; Zhou et al., 2007). Although a majority of miRNA genes are transcribed by RNA polymerase II (Pol II) (Cai et al., 2004; Lee et al., 2004), a subset of miRNAs are processed by RNA polymerase III (Pol III), including the dense cluster of human miRNAs interspersed among repetitive *Alu* elements on chromosome 19 (Borchert et al., 2006). Both RNA polymerases are regulated differently and recognize specific promoter and terminator elements, facilitating a wide variety of regulatory options, thus allowing miRNA genes to be elaborately expressed in particular contexts and cell types.

In the Nucleus

MATURATION OF miRNAs involves several sequential processing steps. In animals, canonical miRNA genes are transcribed by Pol II into primary miRNAs (pri-miRNAs) that are typically several kilobases (kb) long, 5'-capped, polyadenylated, and contain local stem-loop structures (Kim, 2005; Kim et al., 2009). These stem-loop structures serve as substrates for nuclear cleavage of the pri-miRNA by the Microprocessor complex, which contains the RNase III endonuclease Drosha and the cofactor DiGeorge syndrome critical region gene 8 (DGRC8) in humans (or Pasha in *D. melanogaster* and *C. elegans*) (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Lee et al., 2003). The two RNase domains of Drosha cleave the 5' and 3' arms of the pri-miRNA hairpin ~11 bp away from the single-stranded RNA–double-stranded RNA (ssRNA-dsRNA) junction with the aid of DGCR8, which stably interacts with the ssRNA segments and ~33 bp of the stem (Han et al., 2004; Han et al., 2006). This nuclear reaction liberates a ~60-70 nt stem-loop intermediate, known as the miRNA precursor (pre-miRNA) (Lee et al., 2002).

Drosha-mediated cleavage of the pri-miRNA occurs co-transcriptionally and precedes splicing of the protein-encoding or non-coding host RNA containing the miRNAs. Splicing is not influenced by Drosha-mediated cleavage, thereby ensuring both miRNA biogenesis and protein synthesis from a single primary transcript (Kim and Kim, 2007; Morlando et al., 2008). However, the inclusion of miRNA-resembling stem-loop structures in exonic regions of mRNAs may affect transcript stability. Drosha negatively regulates its co-factor DGCR8 via two highly conserved hairpin structures in its 5'UTR and in the coding sequence near the start codon, which results in *DGCR8* mRNA destabilisation following Drosha-mediated cleavage (Han et al., 2009).

Additional observations support a functional role for miRNA–5'UTR/exonic interactions in conveying target-associated repression, implying that putative miRNA-resembling stem-loop structures in non-3'UTR regions of mRNAs may serve as RNA control elements influencing mRNA stability (Duursma et al., 2008; Lee et al., 2009; Lytle et al., 2007). The participation of other, yet-to-be-described nucleases in this mode of mRNA metabolism are also thought to exist (Karginov et al., 2010).

In addition to canonical processing pathways, a subset of miRNAs mature following the completion of splicing. These miRNAs, termed mirtrons, derive from short introns and their biogenesis does not require Drosha processing (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Such non-canonical small RNAs are produced when debranching of lariat-shaped introns results in the appropriate formation of a hairpin structure resembling a pre-miR. Additional 5'- or 3'-end exonucleolytic trimming of mirtron tails is occasionally required before these RNAs are suitable as substrates for nuclear export. Deletion of *DGCR8* in mouse embryonic stems cells (mESC) has also revealed Microprocessor-independent, Dicer-dependent generation of small non-mirtoronic RNAs from other non-coding RNA loci, such as the isoleucine transfer RNA (tRNA) gene, while Dicer-dependent processing of the small nucleolar RNA (snoRNA) ACA45 generates small RNAs with miRNA-like function (Babiarz et al., 2008; Ender et al., 2008). Evidently, multiple non-canonical pathways contribute to a substantial fraction of pre-miR abundance in mammalian cells through Drosha-independent pathways.

In the Cytoplasm

AFTER THE COMPLETION of nuclear processing, pre-miRNAs are exported into to the cytoplasm by Exportin-5, (EXP5), a nuclear transport receptor, in complex with Ran-GTP (Lund et al., 2004). Originally assumed to be a minor export factor of tRNAs, EXP5 predominantly mediates nuclear export of both short-hairpin RNAs (shRNA) and pre-miRs (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2005). EXP5 binds cooperatively to double-stranded RNA (dsRNA) independently of sequence or loop structure (Gwizdek et al., 2003; Zeng and Cullen, 2004), thus ensuring the export of only correctly processed small RNAs with a characteristic stem-motif of 14-16 bp and short 3' overhangs (Lund and Dahlberg, 2006).

Once in the cytoplasm, pre-miRs are cleaved near the terminal loop by the RNase III endonuclease Dicer, releasing ~22 nt miRNA duplexes with ~2 nt 3' overhangs at either end. This cleavage is essential for miRNA processing and has been described in many organisms, including *C. elegans*, *D. melanogaster*, and mammals (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). In humans, Dicer interacts with the dsRNA-binding domain proteins TAR RNA-binding protein (TRBP) and protein activator of PKR (PACT) and the core component Argonaute proteins 1-4 (Ago 1-4) (Gregory et al., 2005; Lee et al., 2006). Together, these proteins contribute to miRNA-programmed RNA-induced silencing complex (miRISC) assembly by forming a

RISC-loading complex (RLC). Although TBRP and PACT are not essential for Dicer-mediated cleavage of pre-miRNAs, RLC formation is facilitated in their presence (Chendrimada et al., 2005; Lee et al., 2006).

After Dicer-mediated cleavage, Dicer and its interactors TRBP and PACT dissociate from the miRNA duplex. The two miRNA strands are then separated and one of the strands associates with an Ago protein within RISC, where it acts as a guide to repress target messages. Strand selection is determined by inherent features of the miRNA duplex, including thermodynamic asymmetry/stability (Khvorova et al., 2003; Schwarz et al., 2003). Typically, the strand whose 5' end is less stably base-paired will be more frequently chosen as the functional guide strand. The other strand (the passenger strand or miRNA*) is presumed to be excluded from miRISC incorporation and subsequently degraded (Carthew and Sontheimer, 2009). However, recent deep-sequencing efforts have revealed that a large number of miRNA* strands can be appreciably detected in Ago complexes, and are in large part functional. Differential sorting of miRNA duplex strands correlates with specific mismatches at positions 9 and 10 of the mature miRNA and the 5' nucleotide identity of the strand (Czech et al., 2009; Okamura et al., 2009; Okamura et al., 2008). In addition, relative levels of the miRNA/miRNA* strands vary widely across tissues (Hu et al., 2009; Landgraf et al., 2007). Together, these findings suggest that miRNA precursors can be bifunctional, with individual strands adopting different fates within small RNA pathways (*figure 2*).

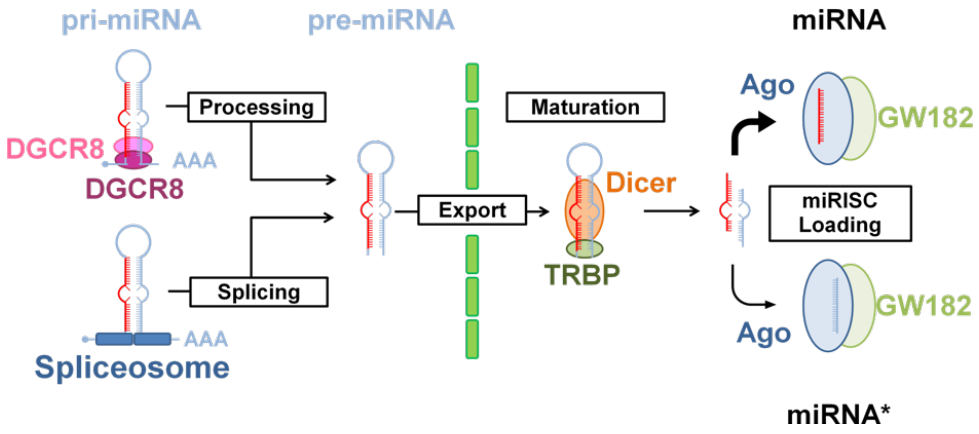

Figure 2. miRNA biogenesis and assembly into miRISC

Once a single strand has been selected, the miRNA acts as an adaptor for miRISC to specifically recognise and regulate particular mRNAs. With the exception of a few aforementioned examples, miRNA-binding sites in metazoan mRNAs lie in the 3'UTR and are present in multiple copies. Most miRNAs base pair imperfectly with their targets, following a set of rules determined by experimental and bioinformatic analyses (Brennecke et al., 2005b; Doench and Sharp, 2004; Grimson et al., 2007; Lewis et al., 2005; Nielsen et al., 2007). The most stringent requirement of miRNAs base-pairing to their targets is a contiguous and perfectly matched Watson-Crick interaction of miRNA nucleotides 2-7, representing the seed region. Other molecular determinants, such as

pairing to the 3' region of the miRNA (3'-supplementary and 3'-compensatory pairing) and UTR accessibility, are known to enhance binding specificity and affinity (Bartel, 2009). Recently, the miRNA targeting code expanded to include functional sites with centered pairing, to describe target sites that lack both perfect seed pairing and 3'-compensatory sites and instead have 11-12 contiguous Watson-Crick pairs to the center of the miRNA (Shin et al., 2010).

The degree of miRNA-mRNA complementarity will generally dictate the outcome of the miRNA-mRNA interaction. Perfect complementarity allows Ago-catalysed cleavage of the mRNA strand, although very few examples of miRNA-dependent cleavage have been observed in mammals (Meister et al., 2004; Yekta et al., 2004). More commonly, metazoan miRNAs direct translational repression, mRNA destabilisation, or a combination of both (Filipowicz et al., 2008). The mechanisms by which miRISC regulates translation are not entirely clear; i.e. whether repression occurs at the translational initiation or postinitiation step of mRNA maturation. Several models have been proposed, including repression of initiation at the 7-methylguanosine (m^7G) cap stage, miRISC-dependent obstruction of translationally competent ribosomes at the AUG start codon, or premature ribosome dissociation from mRNAs at the postinititation stage (i.e. the elongation phase) (Carthew and Sontheimer, 2009). The mechanisms associated with mRNA destabilisation, however, are proposed to operate though deadenylation, decapping, and exonucleolytic digestion of the miRNA-bound mRNA, resulting in a significant reduction in mRNA abundance (Bagga et al., 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006). This reaction requires Ago, GW182, and the cellular decapping and deadenylation machinery (Behm-Ansmant et al., 2006). Although the molecular principles underlying the preferred mode of posttranscriptional repression remain elusive, it has been suggested that the number, type and position of mismatches in the miRNA/mRNA duplex play an important role in triggering degradation or translational arrest (Aleman et al., 2007). Suffice to say, miRNAs affect protein production at many different levels with distinct biological outcomes.

The Biological Functions of miRNAs

SINCE THE DISCOVERY that *lin-4* and *let-7* play critical roles in the timing of larval development in *C. elegans*, miRNAs have been implicated in a bewildering array of biological settings, and the importance of miRNAs in select developmental processes in model organisms is indisputable. In particular, miRNAs appear to partake in pathways associated with cell fate determination and differentiation, thereby contributing to the specification of many cell types. Global attenuation of miRNA biogenesis through genetic manipulation of *Dicer* has revealed several clues to miRNA function in developmental contexts. *Dicer* loss of function results in profound phenotypic defects in both zebrafish and mice, emphasising the importance of the miRNA pathway in vertebrate development. However, by in large, early stages of development progress normally in *Dicer* $^{-}/^{-}$embryos. For example, zebrafish embryos lacking *Dicer* develop

without profound defects until maternal stores of *Dicer* are depleted, approximately eight days post-fertilisation (Wienholds et al., 2003). Even *Dicer*-deficient zebrafish embryos lacking maternal *Dicer* (MZ*dicer* mutants) exhibit only mild phenotypic perturbations within the first 24 hours of development (Giraldez et al., 2005), suggesting that miRNAs are not essential for cell fate determination and early patterning, but provide critical functions at subsequent steps in embryonic development. Like zebrafish MZ*dicer* mutants, mouse embryos lacking *Dicer* do not display gross morphological defects before the onset of gastrulation (~embryonic day 7.5), again pointing to an important role for miRNAs in later stages of development (Bernstein et al., 2003).

This notion is further supported by the temporal and spatial expression patterns of conserved vertebrate miRNAs in zebrafish embryos, as well as other model systems, including mouse and chicken (*figure 3*) (Ason et al., 2006; Darnell et al., 2006; Wienholds et al., 2005). For example, *miR-196* and *miR-10* genes of various vertebrates reside in the homeobox (HOX) gene clusters and function at later stages of vertebrate development. Like the *HOX* genes, miR-196 and miR-10 are colinearly expressed in a spatial and temporal manner along the anterior-posterior body axis during development and preferentially target *HOX* mRNAs (Yekta et al., 2008). In mouse embryous, both miRNA families have highest expression in the neural tube and lower expression levels in the trunk mesoderm, with ill-defined anterior limits and broad posterior expression through the tail (Mansfield et al., 2004). The observed expression domains of these miRNA families are in agreement with the patterns that are expected on the basis of their locations within the *HOX* clusters.

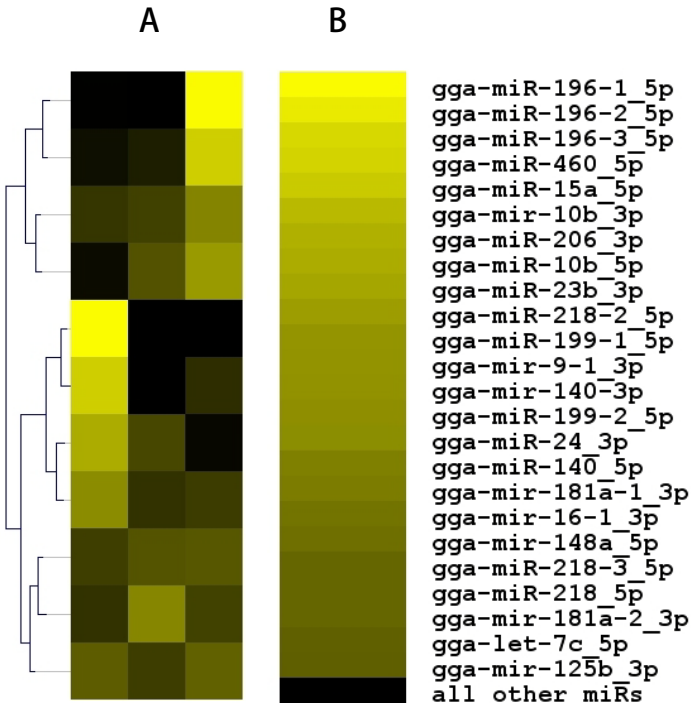

Figure 3. A miRNA catalog of the developing chicken embryo identified by a deep sequencing approach. (*A*) The self-organising tree algorithm (SOTA) diagram on the left illustrates miRNA kinetics in the chicken embryo at embryonic day E4 (left lane), E5 (middle lane), and E6 (right lane). The total number of sequence reads increased over time, demonstrating significant changes in relative miRNA abundance between the three embryonic small RNA libraries. (*B*) A neural-tube enriched miRNA expression profile at embryonic day E5.5. Note the high levels of the miR-196 and miR-10 family members, consistent with their colinear expression and regulation of *Hox* mRNAs.

Although the expression patterns among orthologous miRNAs may vary between species, these studies demonstrate that many miRNAs are expressed with precise tissue specificity late (rather than early) in development, presumably due to their participation in tissue-specific functions and lineage-promoting effects. Indeed, recent studies have shown that the unique modalities by which specific miRNAs exert their widespread function in various developmental settings depend on both the timing and pattern of their expression, as well as the repertoire of co-expressed targets.

miRNAs, Transcription Factors, and Regulatory Networks

GIVEN THAT MORE than half of mammalian mRNAs are under selective pressure to maintain base-pairing interactions with miRNAs (Friedman et al., 2009), most biological processes are likely to be influenced by miRNA function. With such a broad impact on gene regulation, miRNAs have rapidly emerged as one of the most abundant gene regulatory factors in multicellular genomes. In this respect, miRNAs share many regulatory characteristics with transcription factors (TF), which suggests that miRNAs and TFs share a common regulatory logic (Hobert, 2004).

Transcription factors exert their effects by directly (or indirectly) binding DNA at specific genomic loci to control the transcription of nearby genes. In doing so, transcription factors can influence cell fate decisions, functioning as key switches by regulating gene expression programs on a genome-wide level. A single transcription factor can thus, through positively or negatively regulating transcription of numerous genes, execute entire cellular or tissue-level programs. To this end, gene repression has surfaced as an important theme in shaping cell-specific gene regulatory programs, especially in developmental contexts (Bilodeau et al., 2009; Boyer et al., 2006; Hobert, 2004).

However, like miRNAs, TFs rarely operate single-handedly; rather, sets of combinatorially expressed TFs and miRNAs act together to precisely delineate individual cell types and fates (Hobert, 2008; Marson et al., 2008). Cooperativity therefore provides a mechanistic basis for describing the overall output of combinatorial expression patterns of TFs and miRNAs. These combinatorial codes are in turn controlled by more upstream events, such as signaling cascades, which play an integral part in regulatory transactions in cells (Weake and Workman, 2010). Developmental processes can therefore be considered as a succession of hierarchically acting regulatory states (Davidson et al., 2002). Consequently, transcriptional regulatory programs have been placed into well-defined regulatory networks that are characterised by small sets of recurring network motifs that endow the system with specific properties such as signal amplification, dampening, and oscillation (Alon, 2007). As trans-acting factors, TFs and miRNAs frequently associate in small-scale gene regulatory networks with defined topology (Martinez et al., 2008; Tsang et al., 2007). Similar to the network motifs shared by transcription factors, miRNA network motifs are typically composed of feedforward and feedback loops (Herranz and Cohen, 2010). Thus, miRNA regulatory events

interface with known TF and signaling networks that control cell fate and differentiation, modulating their activity through positive and negative feedback loops to reinforce cellular decisions.

Variability, Robustness, and Stochastic Gene Expression

BIOLOGICAL SYSTEMS ARE continuously challenged by an environment that is variable, yet developmental and physiological processes are remarkably stable, resulting in stereotyped outcomes (Hornstein and Shomron, 2006). Such variability, also referred to as 'noise', has multiple sources, including variations in the activity of individual genes, cell-to-cell variations in metabolic activity, or fluctuating levels of an external signal, and must be controlled in living systems in order to maintain phenotypic stability. Gene regulatory networks, involving feedforward and feedback loops, are particularly suited to control the effects of noise, by buffering its impact on gene expression (Raser and O'Shea, 2005).

The ways in which cells and organisms use noise and deal with can greatly influence cellular behavior and phenotypic consequences. By in large, developmental programs are deterministic; however, mechanisms have evolved to buffer stochastic fluctuations, thereby conferring robustness to gene regulatory networks (Hornstein and Shomron, 2006). One form of noise management, termed canalisation, has been selected over the course of evolution to impart developmental pathways with high phenotypic reproducibility. However, some biological processes utilise noise to trigger stochastic developmental decisions (Losick and Desplan, 2008). As miRNAs are frequently embedded in network motifs, their function is often related to the nature and topology of the network to which they belong, suggesting that they confer useful regulatory possibilities to facilitate network decision-making and biological outcomes (e.g. homeostasis, differentiation, and/or lineage specification). In some contexts, miRNAs act as binary switches to help repress target protein output to inconsequential levels while in other settings miRNAs act as rheostats to dampen (tune) protein output to more optimal levels (Bartel, 2009). The ways in which network motifs and miRNAs manage the impact of noise therefore differs from one biological process/circuit to another.

CLASSICAL SWITCH INTERACTIONS shaped the initial paradigm of miRNA targeting, whereby miRNA induction turns off expression of a pre-existing target. In network language, these interactions are examples of feedback (positive or negative) motifs (*figure 4*). Initial examples of switch interactions include *lin-4* targeting *lin-14* and *lin-28*, and *let-7* targeting of *lin-41* (Bartel, 2009; Reinhart et al., 2000). The recent characterisation of *D. melanogaster miR-14* has revealed a different type of regulatory switch that acts by limiting stochastic expression of its target, the ecdysone receptor (EcR). In *Drosophila*, the steroid hormone Ecdysone and its receptor EcR play a key role

in control of developmental timing. Pulses of Ecdysone followed by EcR activation trigger a complex hierarchy of gene expression programs that control the physiological and morphological changes involved in metamorphosis (Thummel, 1996, 2001). An important feature of this process is its all-or-none character, which depends on a positive feedback loop involving transcriptional autoregulation of EcR (Karim and Thummel, 1992; Koelle et al., 1991). Random transcription (noise) is a stochastic event that often occurs in bursts (Raj and van Oudenaarden, 2008). Random transcriptional fluctuations of EcR need to be limited to avoid a self-amplifying response. miR-14 acts directly to reduce EcR levels, and reciprocally, EcR negatively regulates *miR-14*, thereby keeping the circuit in balance. Upon hormonal induction, EcR will negatively regulate *de novo* transcription of *miR-14* and residual miR-14 levels will eventually subside, permitting the cell to distinguish between sustained input of hormone-induced EcR activation and transcriptional fluctuations in EcR levels (Varghese and Cohen, 2007). Thus, while miR-14 prevents inappropriate transitions to occur during development due to noise, it constitutes a developmental switch that allows major transformations in gene expression programs when a certain threshold level is reached.

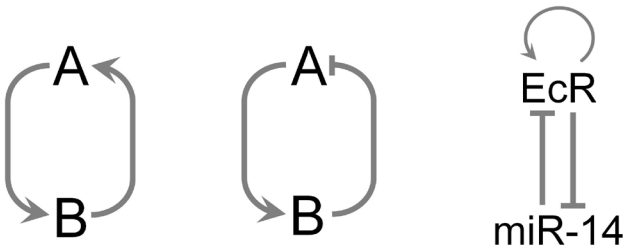

Figure 4. Examples of simple positive and negative feedback loops.
miR-14 acts in a negative feedback loop modulating EcR activity.

FAILSAFE INTERACTIONS REFER to switch interactions in which a miRNA is already present when the target is first expressed. For example, sense organ specification in the *D. melanogaster* peripheral nervous system is a stochastic event modulated by *miR-9a*, which inhibits the sporadic production of additional neuronal precursor cells (Li et al., 2006). Sense organ primordia are initially defined as small groups of cells that express a set of proneural basic helix–loop–helix (bHLH) transcription factors of the Hairy-E(spl) (HES) family. Definition of the proneural cluster at this level is deterministic, based on programmed control of gene expression, but fluctuations in the level of the transcription factor Senseless determines which cell adopts the sense organ

precursor (SOP) fate (Herranz and Cohen, 2010). The stochastic selection of a SOP cell leads to an increase in proneural gene expression, which feeds back to increase *Senseless* expression. Senseless also increases expression of the Notch ligand *Delta*, which in turn leads to increased Notch signaling activity in the adjacent cells. Notch activity represses proneural gene expression in these cells, and reinforces the advantage of the *Senseless*- and *Delta*-expressing cell toward becoming the SOP (Jafar-Nejad et al., 2003). As positive feedback loops are inherently labile, additional (failsafe) mechanisms are needed to ensure that random fluctuation in transcription factor levels does trigger positive feedback inappropriately.

In this biological context, *miR-9a* sets a threshold that Senseless transcription must overcome in order to activate the Senseless–proneural feedback loop (Li et al., 2006). Importantly, *miR-9a* expression is under proneural gene control and *miR-9a* levels are at first uniform in the proneural cluster. Following stochastic selection of the SOP, Senseless activity increases proneural gene expression with concurrent reduction of *miR-9a* expression. However, *miR-9a* levels remain high in the surrounding cells, where proneural gene activity is kept low through Notch activity. The opposing regulation of Senseless and *miR-9a* is an integral element of the switch. The transcriptional feedback system is triggered by random fluctuations in gene expression, and *miR-9a* helps to reinforce selection of the SOP cell, thereby bestowing the noise-dependent switch with a failsafe, functionally redundant layer of control (Herranz and Cohen, 2010).

FEEDFORWARD REGUALTION REPRESENTS the second type of network motifs. Feedforward loops (FFLs)have been described in a multitude of gene systems, from bacteria and yeast to mammals (Alon, 2007). Feedforward motifs consist of three components: an upstream regulator (TF) and two targets (e.g. a miRNA and a target gene). Assuming that each of the three regulatory interactions can be either positive or negative, there are eight possible structural types of FFL (Alon, 2007). Further, depending on the nature of the relationships between the components, FFLs can be classified as being either coherent or incoherent (Mangan and Alon, 2003). In a coherent FFL, the miRNA is induced, directly or indirectly, by a TF that repress the target, i.e. the posttranscriptional repression by the miRNA is synergistic with transcriptional inhibition of the same target. In this regard, the coherent FFL can be considered failsafe, as it enhances the fidelity of a genetic program by ensuring that aberrant transcripts do not give rise to consequential amounts of protein. Reciprocally, the TF may activate target transcription and repress miRNA production, thereby removing miRNA-mediated constraints on target gene expression. The impact of coherent FFLs has recently been described in both developmental and oncogenic contexts. For example, miR-7 has been implicated in two coherent feedforward motifs involved in the specification of photoreceptor cell in the *Drosophila* eye (Li et al., 2009). Likewise, *let-7* belongs to a feedforward motif underlying oncogenic transformation in response to inflammatory signals (Iliopoulos et al., 2009).

Incoherent FFLs are less intuitive and have different properties than coherent FFLs, but are just as common as coherent types (Tsang et al., 2007). Incoherent FFLs are particularly suited as triggers for oscillatory systems, enabling more customised expression in different cell types as well as more uniform expression within each cell type. In this scenario, the miRNA is important in fine-tuning the expression level of the target gene. One well-studied example of an incoherent FFL involves c-MYC, *miR-17~92* cluster-derived miRNAs and cell cycle progression (O'Donnell et al., 2005). c-MYC transcriptionally induces the expression of *E2F1*, which in turn regulates an array of cell cycle genes. This is accompanied by the concomitant, c-MYC-dependent induction of *miR-17* and *miR-20a*, which repress *E2F1*. The overall outcome of these interactions results in the precise pulse of c-MYC-induced *E2F1* expression needed for optimal cell cycle progression (*figure 5*).

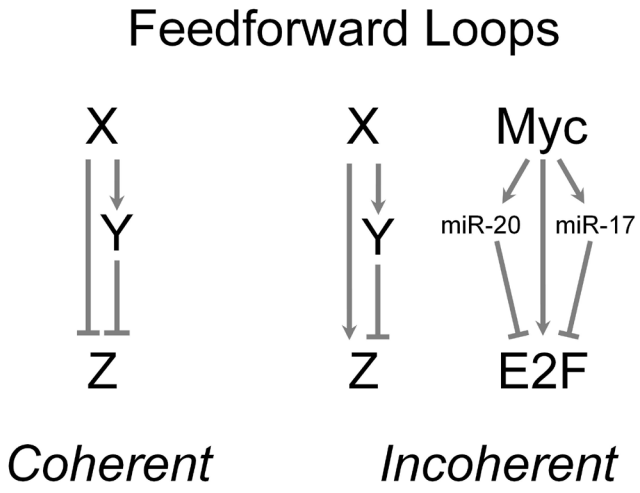

Figure 5. Feedforward motifs where X regulates Z directly and indirectly through regulation of Y. Coherent motifs have the direct and indirect paths from X acting on the target Z in the same direction. Incoherent motifs have the opposite outcomes for the two paths. miR-20 and miR-17 act in an incoherent feedforward motif. c-MYC activates E2F directly while repressing its expression posttranscriptionally via miR-20 and miR-17. This opposing activity sets a precise E2F expression level, and thus promts cell cycle progression.

Network Buffering and Genetic Canalisation

ALTHOUGH miRNAs AND TFs are interconnected in shared regulatory motifs and these regulatory events appear quite similar, their individual importance as regulatory factors is different. Thorough genetic analyses in unicellular and multicellular organisms has firmly demonstrated the importance of TFs in controlling development and homeostasis (Davidson et al., 2002; Kamath et al., 2003). Likewise, the genetic elimination of *Dicer* can produce striking phenotypes, especially defects in later developmental events (Levy et al., 2010; Zehir et al., 2010). However, many miRNAs, even the most highly conserved, can be eliminated individually without causing obvious phenotypes (Miska et al., 2007). In fact, the deletion of 95 *C. elegans* miRNA loci revealed that less than 10% of miRNA knockouts results in clear developmental or

morphological defects (Miska et al., 2007). In contrast, recent RNA interference (RNAi) loss-of-function analysis show that a substantially higher proportion of genes required for viability in *C. elegans* are enriched for TFs, associated with developmental processes, and cause easily discernable phenotypes (Fraser et al., 2000; Kamath et al., 2003). This implies that many miRNAs may be functionally redundant, yet it is difficult to reconcile their extreme conservation with redundancy. Moreover, gene-knockout phenotypes in flies and vertebrates can be very subtle, and are commonly masked by the considerable derepression of only a few conserved targets (Baek et al., 2008; Nakahara et al., 2005; Selbach et al., 2008).

Large-scale proteomic analysis following miR-223 disruption *in vivo* indicated that very few messages in a biologically accurate setting were repressed by more than 50% (Baek et al., 2008). Instead, miR-223 had more modest effects on its endogenous targets (including conserved targets), with individual sites reducing protein output by ~30% (Bartel, 2009). Several reasons may account for these observations. First, the nature of specific miRNA:target interactions may play an important role. Messages with multiple conserved sites and particularly favorable sites are more likely to represent highly responsive miRNA:target interactions, resulting in substantial target repression. This has been illustrated by numerous examples, including the *lin-4:lin-14* interaction, the *let-7:Hmga2* interaction and the *miR-18:ESR1* interaction (Lee et al., 1993; Loven et al.; Mayr et al., 2007; Wightman et al., 1993). However, these types of interactions represent only a minority of preferentially conserved targets, and modest repression appears to be the more common regulatory outcome of a miRNA:target interaction. With more than 90% of conserved miRNA:target interactions involving only a single site to the miRNA, most of these targets would be expected to be downregulated by less than 50% (Bartel, 2009).

In addition, since most messages with a conserved site to one miRNA have at least one other conserved site to an unrelated miRNA, disruption of multiple miRNA:target interaction sites might be necessary before the derepression generated has overt consequences (Bartel, 2009). Mild phenotypes may also be the result of the vast functional diversity of many miRNA targets. Often, ~2-fold protein fluctuations can be tolerated, as evidenced by the rarity of haploinsufficeint phenotypes, even when the targets themselves are regulatory proteins (e.g. TFs). This phenomenon, known as network buffering, may constitute an additional function for miRNAs.

Many regulatory interactions, including many miRNA:target interactions, fall within complex regulatory networks with bifurcating pathways and feedback control that enable accurate response despite a defective node in the network. With this ability to buffer the effects of losing a node, such networks must be perturbed elsewhere before the lost miRNA interaction has discernable phenotypic consequences (Bartel, 2009). In line with their role as genetic buffers, mounting evidence suggests that miRNAs may serve as canalising genes, i.e. that miRNA regulatory networks evolved under natural selection in order to stabilise phenotypes and decrease the variability of specific traits (Hornstein and Shomron, 2006). For example, the muscle-specific miRNA *miR-1* is conserved in sequence and expression pattern throughout the bilaterian lineage. On this

basis, *miR-1* was presumed to play an important role muscle patterning or differentiation. However, the knockout of *miR-1* in *D. melanogaster* resulted in functional muscles that develop normally (Sokol and Ambros, 2005). Only at the onset of rapid larval growth phase does the pheontypic muscle crisis emerge, suggesting that *miR-1* confers robustness to the identity and physiology of muscle cells (Brennecke et al., 2005a). In this setting, *miR-1* is dispensable for muscle differentiation but upon growth stress conditions the lack of *miR-1*-mediated target interactions causes discernable phenotypic consequences and decanalisation of the network (Hornstein and Shomron, 2006).

miRNAs Operate As Pleiotropic RNAs with Diverse Function

WITH SUCH SEEMINGLY diverse modes of regulation and action, it is difficult to assign a generic role to miRNA function. However, a few basic assertions can be made based on the hitherto mentioned examples: (1) miRNAs participate in regulatory networks driven by TF and signaling events that control cell fate and differentiation. miRNAs modulate these events through positive and negative feedback loops to facilitate cellular decisions and reinforce biological outcomes; (2) miRNAs can act as binary switches to help repress target protein output to inconsequential levels or as rheostats to dampen (tune) protein output to more optimal levels. This, in turn, depends on the nature and topology of the network to which the miRNA belongs, and the nature of specific miRNA:target interactions in a particular cell type; and (3) miRNAs are essential for the normal development of animals. However, since miRNAs tend to have highly tissue-specific expression patterns during later stages of development, miRNAs are most likely controlling particular aspects of terminal differentiation programs of individual cell types, i.e. miRNAs do not govern early cell/tissue fate establishment but are critical in later differentiation steps and in the maintenance of tissue identity and integrity.

It is noteworthy to mention that while many miRNAs participate in tissue development and identity, several vertebrate miRNAs partake in cell-autonomous processes associated with cellular physiology. For example, the liver-specific *miR-122* plays an important role in cholesterol biosynthesis and fatty acid metabolism (Esau et al., 2006; Krutzfeldt et al., 2005). Murine *miR-375* is specifically expressed in pancreatic islet β-cells and regulates glucose-stimulated insulin secretion and exocytosis through its interactions with *Myotrophin*. Restricted predominantly to keratinocytes, *miR-203* functions as a switch between proliferative and terminally differentiating compartments in vertebrate skin (Yi et al., 2008), while the miR-23 family members *miR-23a* and *miR-23b* play a more general role in controlling glutamine metabolism reactive oxygen species (ROS) homeostasis (Gao et al., 2009). Given the high number of miRNA genes identified so far, their regulation and diverse expression patterns along with the overwhelming abundance of putative miRNA:target interactions, it is not surprising that miRNAs have been implicated in a broad spectrum of diseases, including cancer.

CANCER

THE TERM CANCER encompasses more than 100 distinct diseases with diverse risk factors and epidemiology which originate from most of the cell types and organs of the human body and which are characterized by relatively unrestrained proliferation of cells that can invade beyond normal tissue boundaries and metastasize to distant organs (Stratton et al., 2009). With such an extensive range of origins and features, it is difficult to pinpoint a single attribute common to all cancers. Nonetheless, cancer can be broadly described as a genetic, disease-causing accumulation of mutations which arises through a multistep, evolutionary process occurring among somatic cell populations within the microenvironments provided by the tissues of a multicellular organism (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 1993). In this respect, malignancy can be considered both a penalty and a potent selection force in the evolution of multicellularity. On one hand, normal physiology requires large, continuously renewing cellular populations, while on the other hand every cell division risks the initiation of evolutionary events leading to a fatal malignancy.

Analogous to Darwinian evolution of species, cancer development resembles evolution on the somatic level, characterised by the continuous acquisition of heritable genetic variation in individual cells by random mutation and natural selection acting on the resultant phenotypic diversity (Stratton et al., 2009). In fact, carcinogenesis can be considered as two evolutionary competing processes: (1) potential cancer-forming cells evolving with adaptations to overcome the tumor suppression mechanisms of the host organism; and (2) the organism evolving strategies to reduce the probability of death from cancer prior to and during reproductive maturity (Gatenby et al., 2010). Constraining the emergence of cancer has thus become an evolutionary imperative in metazoans, especially in large, long-lived organisms with tissues that continuously regenerate (Lowe et al., 2004).

Cell growth, cell division, and the programmed elimination of cells (apoptosis) are tightly controlled processes which act to benefit multicellular organisms in response to environmental cues. The precise regulation of these processes is a requisite to the proper functioning of the organism. Occasionally, a single cell acquires a set of sufficiently advantageous mutations that allows it to proliferate autonomously, invade tissues and metastasize. However, no single mutation is sufficient to cause cancer; rather cancer results from various genetic and epigenetic alterations (i.e. heritable changes in gene expression that are not accompanied by changes in DNA sequence) that eventually confer cells with an ability to escape normal regulation. Most (if not all) malignant tumors acquire the same set of functional capabilities, commonly referred to as the hallmarks of cancer. These include (1) self-sufficiency in growth signals; (2) insensitivity to antigrowth signals; (3) evasion of apoptosis; (4) limitless replicative potential; (5) sustained angiogenesis; and (6) tissue invasion and metastasis (Hanahan and Weinberg,

2000). Once endowed with these properties, a neoplastic cell rapidly gains a proliferative advantage and eventually outcompetes its surroundings in order to thrive and grow (Nowell, 1976).

Adult Cancer

WHATEVER THE ROUTES or mechanistic strategies needed to reach malignancy may be, the progressive conversion of normal cells into cancer cells is fundamentally a function of three aspects: inheritance, the environment, and changes over time (evolution). First, with respect to inheritance, most attributes of tumor development can be explained by genetic alterations of primary DNA sequence. While some mutations are inherited through the germline, others appear *de novo* in somatic cells and are restricted to the neoplastic lineage. Additionally, epigenetic information, encoded as binomial patterns of DNA methylation, also participates in cancer progression (Feinberg and Tycko, 2004; Jones and Baylin, 2007). As a heritable disease, cancer constitutes the propagation of genetic end epigenetic information, either inherited or transmitted, on a cellular and multicellular level. Second, epidemiologic data has demonstrated a clear association between cancer development and environmental factors like smoking, diet, and radiation (Vineis and Berwick, 2006). Numerous studies have substantiated the causal relationship between environmental mutagens and the initiation of cancer (Soto and Sonnenschein, 2010). Finally, cancer is, by in large, an age-related disease that develops over long periods of time. Cancer incidence is known to increase dramatically with age (Vogelstein and Kinzler, 1993) and virtually all individuals over the age of 50 carry some kind of *in situ* carcinoma (Folkman and Kalluri, 2004). Genes and mutations thus evolve in dynamic interaction with the surrounding environment, and cancer development is best described as an evolutionary process within the organism (Gatenby et al., 2010). Accordingly, cancers in adults result from a multistep process and often progress over many years or decades.

Childhood Cancer

THIS IS IN contrast to tumors that develop during childhood (0-15 years of age), which set them apart from adult (solid) tumors. Childhood tumors, including neuroblastomas, Wilm's tumor, retinoblastomas, lymphomas, and CNS malignancies, are fundamentally developmental disorders; the nature of the progenitor cells from which these tumors arise allows these cancers to develop with fewer defects in cell regulatory processes than adult cancers. Much of the cellular behavior (e.g. spontaneous regression) which typically characterises pediatric cancers derives from their developmental nature. Further, processes which typify adult cancers (proliferation, survival, self-renewal, and migration), are all aspects of normal developmental programs. Essentially, cancers that arise during prenatal and postnatal development are driven by normal growth and

differentiation-promoting mechanisms associated with organogenesis, tissue growth, and maturation (Scotting et al., 2005).

The relationship between childhood cancer development and the onset of organogenesis is supported by the fact that the age-specific pattern of these tumors often coincides with the periods of maximum growth of the related normal tissue. Neuroblastoma, Wilms' tumor, and hepatoblastoma occur in children younger than four years of age, whereas bone tumors, germ-cell tumors, and Hodgkin's disease occur predominantly in adolescents (Altekruse et al., 2009). The immature tissue environment represents an additional feature intimately linked to the onset and maintenance of perinatal cancers. In developing tissues, the progeny of most dividing cells are primarily more dividing cells, providing a favorable context for errors in DNA replication and a microenvironment in which proliferation is favored over differentiation (Scotting et al., 2005). Perturbations in the microenvironment can therefore accelerate uncontrolled cell growth and survival of immature cells that retain the ability to undergo high levels of proliferation and migration.

Likewise, the changing environment of the growing host can partially explain the biological and clinical heterogeneity observed in childhood cancers, but not in adult cancers, such as spontaneous regression. It should also be noted that many childhood tumors, with the exception of high-risk neuroblastoma, do not necessarily follow the dogmatic route of transformation during tumor progression, such as chromosomal instability, aneuploidy, or amplification of genetic damage. For example, infant teratomas generally show no signs of gross genetic damage and childhood cancer cells generally have fewer cytogenic defects than adult tumors (Oosterhuis and Looijenga, 2005). With fewer aberrations, childhood malignancies offer more therapeutic avenues and respond more readily to biologically targeted interventions (e.g. retinoic acid treatment for neuroblastoma and acute myeloid leukemia). In summary, childhood tumors represent a unique category of neoplasms that deviate from adult-onset cancers, both in their cell biology and their tissue microenvironment.

Tumor Suppressor Genes and Oncogenes

THE THREE FEATURES of cancer development mentioned in the earlier section (inheritance, environment, and time) have been consolidated in a linear model, which has long served as a standard paradigm of carcinogenesis (Arends, 2000; Fearon and Vogelstein, 1990). Central to this model are the two categories of genes that are affected by mutational events during tumor initiation and progression: oncogenes and tumor suppressor genes (TSG) (Vogelstein and Kinzler, 1993).

Oncogenes are classified as genes that have acquired gain-of-function mutations (or exhibit abnormal expression levels) and are capable of promoting cancer. Under normal circumstances, oncogenes are referred to as proto-oncogenes and function to regulate cell growth and differentiation. Proto-oncogenes are frequently involved in signal transduction pathways, and play important roles in the execution of mitogenic signals.

Upon inappropriate activation however, a proto-oncogene (or its product) may, in the presence of a selective environment, promote cell autonomy and thereby support transformation and tumorigenesis. Examples of proto-oncogenes include c-*MYC*, *GLI*, and *RAS*.

Tumor suppressor genes, on the other hand, represent gene products that can function to counteract the onset of malignancy. Since the cancer-preventive effects of TSGs usually require the presence of only a single functional gene, prototypic tumor suppressor genes are recessive, requiring "two-hit" inactivation of both alleles, a concept formulated by Alfred Knudson in 1971 following studies of inherited and sporadic cases of retinoblastoma (Knudson, 1971; Sherr, 2004). TSGs are commonly inactivated (via deletions, mutations, or epigenetically) in human tumors and participate in a multitude of critical cellular processes, including apoptosis, senescence, and DNA repair. Accordingly, the involvement of TSGs in cell cycle checkpoint control, mitogenic signaling pathways, protein turnover, DNA damage, hypoxia, and other stress responses reflects the broad spectrum of cell-autonomous processes that can be deregulated in cancer cells (Sherr, 2004). Bona fide TSGs include *p53*, *PTEN*, and *RB*, all of which are frequently deregulated in a vast majority of cancers (Harris and Hollstein, 1993; Li et al., 1997).

Since its formulation, the 'standard model' has embodied the efforts of cancer research in its pursuit to describe the multifaceted nature of cancer development. These efforts have primarily focused on identifying the genetic and/or epigentic alterations of protein coding elements in the genome. With the realisation that noncoding RNAs, notably miRNAs, play functional roles in developmental and cancer-associated processes, cancer should now be regarded as a complex genetic disease involving countless abnormalities in both coding and non-coding genes.

The MYC Oncoprotein

COMPARABLE TO THE *p53* gene and its protein product (which are mutated in more than 50% of all human cancers), the prevalence of MYC deregulation in human cancers is staggering (Levine et al., 2004). No category of tumor, whether it be adult or pediatric, solid or hematological, has managed to entirely elude MYC, and the broad spectrum of neoplasms associated with MYC perturbations reflects its central role in the onset and progression of tumorigenesis (Nesbit et al., 1999).

Originally identified as the cellular homologue of the avian acute leukemic (MC29) viral transforming sequence, *c-myc* was the first cellular oncogene shown to be activated through retroviral promoter insertion (Hayward et al., 1981). Unlike the other proto-oncogenes discovered during this pioneering period of cancer research (e.g. RAS), MYC activation was not the result of mutations in its coding sequence. Instead, novel mechanisms, including insertional mutagenesis, chromosomal translocations, and gene amplification, were found to deregulate *MYC* (and other oncogenes), providing new

paradigms for the genetic basis of cancer. The function of MYC, in both normal and pathological settings, has been intensively examined ever since, and the *MYC* gene family ranks among the most exhaustively studied groups of genes in biology (Eilers and Eisenman, 2008).

Briefly, the *MYC* family of proto-oncogenes encodes a number of transcription factors (c-MYC, MYCN, and L-MYC) that heterodimerise with the cofactor MYC-associated protein X (MAX), bind DNA, and activate a substantial portion of coding and non-coding elements in the genome (Cawley et al., 2004; Fernandez et al., 2003). Functionally, Myc-activated genes serve to stimulate cell growth by initiating ribosome biogenesis, protein synthesis, metabolism, and cell cycle progression. Simultaneously, genes involved in cell-cycle arrest, cell adhesion, and cell-cell communication are inhibited. The manner in which gene expression is restricted upon MYC activation is less clear but at least one mechanism has been reported to involve MYC:MAX-dependent displacement of co-factor recruitment to genes bound by the zinc finger transcriptional activator Miz-1 (Herold et al., 2002; Staller et al., 2001). Furthermore, MYC:MAX complexes are counteracted by the MXD family proteins, which anatgonise MYC function by sequestering accessible MAX and, as heterodimers (MNT:MAX or MAD:MAX), promote repression at genomic loci otherwise activated by MYC (Eilers and Eisenman, 2008).

Taken together, MYC posses the capacity to both activate (directly) genes that stimulate growth and deactivate (directly and/or indirectly) genes that abrogate cell cycle inhibition. Thus, by responding to both external and internal cues, MYC proteins modulate and influence a myriad of cellular processes, including proliferation, growth, apoptosis, metabolism, and differentiation, all of which are frequently deregulated in MYC-dependent cancers (Albihn et al., 2010).

miRNAs In Cancer

THE EARLIEST COMPREHENSIVE evidence connecting miRNAs to cancer came from the observation that miRNA genes are greatly enriched in genomic loci known to undergo chromosomal rearrangements, deletion, and amplification (Calin et al., 2004). More direct proof was attained when high-throughput technologies enabled the detection of miRNA expression on a genome-wide level, which revealed that abnormal expression of miRNAs was a common feature of all tumors investigated (Lu et al., 2005; Volinia et al., 2006). Interestingly, these studies reported a global downregulation of miRNA expression in cancer cells, with the exception of a few, highly overexpressed miRNA family members (e.g. members of the miR-17~92 family). These data were consistent with the hypothesis that in mammals, as in *C. elegans*, miRNAs can function to prevent cell division and drive terminal differentiation.

Indeed, global miRNA expression patterns reflect the state of cellular differentiation in tumors, highlighting the potential of miRNA profiling in cancer diagnosis. An

extension of this hypothesis suggested that differential expression levels of certain miRNAs might play a causal role in the generation or maintenance of tumors (Lu et al., 2005). The belief that miRNAs dysfunction can be a causative event in cancer pathogenesis has very recently been addressed in transgenic mouse models (Costinean et al., 2006; Dorsett et al., 2008; Klein et al., 2010; Medina et al., 2010). These studies clearly demonstrate that, similar to prototypic protein-coding oncogenes and TSGs, the misexpression of select miRNAs (even the misexpression of a single miRNA) is sufficient promote and maintain tumor growth and survival *in vivo*. Below, I have highlighted examples of miRNAs with tumor-suppressive activity and miRNAs with oncogenic properties, paying special attention to the cancer forms which I have focused on during my PhD studies.

Tumor Suppressor miRNAs

THE FIRST miRNAs demonstrated to have altered expression in tumor cells and causatively linked to cancer were miR-15a and miR-16-1 (Calin et al., 2002b). These miRNAs are clustered on chromosome 13 within the larger consensus minimal deleted region (MDR) 13q14, a region that is deleted in more than half of B-cell chronic lymphocyte leukemias (CLL) (Dohner et al., 2000). CLL represents the most common B cell-derived malignancy in the adult population, accounting for ~30% of all leukemias (Redaelli et al., 2004). An insidious disease with a chronic course, CLL is charcterised by the clonal expansion of proliferating, neoplastic $CD5^+$ B lymphocytes (Chiorazzi and Ferrarini, 2003).

The course of the disease is variable and while some patients with CLL have a normal life span (indolent CLL), others present with more aggressive disease and die within five years after diagnosis (Rozman and Montserrat, 1995). In cases of disease progression, overexpression of *c-MYC*, deletions of the *Rb* gene, and mutations of the *p53* have been reported (Rozman and Montserrat, 1995). Nevertheless, the genetic cause of CLL remains unknown and its pathogenesis is obscure. On the one hand, CLL is a morphologically homogenous tumor, while on the other, it represents a lymphoma with high clinical heterogeneity. However, there are a few molecular and clinical features which unify CLLs. These include the presence or absence of somatically mutated immunoglobulin variable region (IgV) genes (i.e. pre-germinal versus post-germinal center leukemia), a set of different recurring chromosomal alterations, mainly deletions (including 13q14), and the accumulation of malignant $CD5^+$ B lymphocytes. In view of these characteristics, CLL might result from a multistep process, beginning with an antigen-driven polyclonal expansion of $CD5^+$ B lymphocytes that, in a mutationally conducive setting, would eventually transform into monoclonal proliferation.

The accumulation of malignant cells in CLL has long been considered a consequence of an inherent apoptosis defect, rather than excessive proliferation, since CLL cells frequently overexpress the antiapoptotic protein Bcl-2. In fact, it was suggested that loss of function of miR-15a and miR-16-1 promotes enhanced expression of Bcl-2 and thus

abnormal survival of CLL cells (Cimmino et al., 2005). Lately, these concepts have been challenged. For example, CLLs express significantly shorter telomeres than aged-matched B cells as a result of more frequent cell divisions (Damle et al., 2004). Furthermore, by measuring CLL cell kinetics *in* vivo, the production rate of leukemic cells in patients has been estimated to fall in the range of 10^9 – 10^{12} per day (Messmer et al., 2004), reflecting the high proliferative capacity of CLL cells. Importantly, our careful analyses of miR-15a/16-1 function (see Paper I) strongly suggest a role in cell cycle progression, a finding that was recently corroborated *in vivo* using a *miR-15a/16-1* transgenic mouse model (Klein et al., 2010). Other studies have reached the same conclusion in *miR-15a/16-1*-deficient cancers, including non-small cell lung cancer and prostate cancer (Bandi et al., 2009; Bonci et al., 2008). A more thorough analysis of Paper I and its implications are provided in the Results and Discussion section.

miR-203, A miRNA preferentially expressed in the suprabasal layer of skin, represents another *bona-fide* tumor suppressor miRNA. Early insights into its function revealed that miR-203 acts as a switch between proliferation and differentiation during embryonic skin development (Yi et al., 2008). By altering the embryonic, spatiotemporal expression pattern of miR-203 *in vivo*, it was found that premature expression of miR-203 promoted epidermal differentiation by restricting the proliferative potential of targeted basal stem cells and inducing early cell cycle exit. When miR-203 was inhibited chemically using antagomiR technology (Krutzfeldt et al., 2005) in neonatal mice, antagomiR-203-treated dorsal skin showed clear elevations in epidermal proliferation and atypical expansion of *p63* expression, an essential regulator of stem cell maintenance in epithelial tissue (Yi et al., 2008).

These findings point to an antiproliferative function for miR-203 in the skin and, as evidenced by its tumor-suppressing effects in select hematopoietic malignancies, suggests that loss of miR-203 expression and/or function may promote tumorigenesis (Bueno et al., 2008). As miR-203 is preferentially expressed in the skin, it is highly probable that significant disruption of miR-203 levels in the epidermis may contribute to various types of skin disorders, such as skin cancer. Paper III in this thesis demonstrates that severely reduced expression of miR-203 is a typical feature of basal cell carcinoma (BCC), resulting in a deregulation of critical genes in the Hedgehog (Hh) pathway and subsequent hyperproliferation of transformed keratinocytes.

BCCs, the most common malignancy among persons of European ancestry, are keratinocyte tumors and appear as slow-growing, elevated lesions on sun-exposed skin (Epstein, 2008). Exposure to ultraviolet radiation is generally accepted as the major cause of BCCs, although a rare, familial variant of BCCs frequently occurs in basal cell nevus syndrome (BCNS) patients (commonly referred to as Gorlin syndrome) (Rubin et al., 2005). Despite the high incidence of BCCs among Caucasians, these tumors rarely metastasize; nevertheless, they can cause significant tissue destruction by tissue invasion.

Central to both sporadic and familial BCCs is the inappropriate activation of the Hh signaling pathway, originally identified as a determinant of segment polarity in *D. melanogaster* (Nusslein-Volhard and Wieschaus, 1980). Deregulation of the HH pathway is also common to other tumors, such as medulloblastoma and rahbdomyosarcoma (Taipale and Beachy, 2001). Briefly, secreted Sonic hedgehog (SHH) protein binds the TSG patched homologue 1 (PTCH1), relieving its suppression of smoothened (SMO). SMO signaling culminates in the activation of the Gli family of transcription factors (GLI1, GLI2, and GLI3), which execute SHH-induced signals. As the constituents of the HH pathway are intimately associated with proliferative responses in target cells, proteins belonging to the HH network are frequently misexpressed in BCCs (including loss-of-function mutations of *PTCH1* and gain-of-function mutations of *SMO*). Our work presented in Paper III of this thesis establishes that miR-203 plays an intricate role in fine-tuning key components of the HH network. By assuring the precise protein output of downstream effectors (e.g. *c-MYC* and *c-JUN*) of Hh signaling and other mitogenic pathways, miR-203 constitutes a 'gatekeeper' miRNA controlling keratinocyte proliferation.

Oncogenic miRNAs

IN 2005, TWO seminal papers published in Nature provided the first demonstration that miRNAs are functionally integrated into oncogenic pathways and participate in cancer development (He et al., 2005; O'Donnell et al., 2005). Analyses of a genomic locus (13q31.3) frequently amplified in various lymphomas and solid tumors revealed that *C13orf25*, the only gene found to be upregulated within this amplicon, was a non-coding miRNA polycistron encoding six miRNAs: miR-17, miR-18a, mi-19a, miR-19b-1, miR-20a, and miR-92a. Collectively referred to as the miR-17~92 cluster, enforced expression of these miRNAs hematopoietic stem cells were found to dramatically accelerate the onset of B cell lymphoma in the Eμ-*myc* transgenic mouse model, providing direct evidence that the miR-17~92 cluster has oncogenic activity *in vivo* (He et al., 2005). In the same issue of Nature, O'Donnell et al. reported that the miR-17~92 cluster is transcriptionally activated by the transcription factor c-MYC (O'Donnell et al., 2005). Since then, expression profiling studies have revealed a widespread overexpression of miR-17~92-derived miRNAs in several tumor subtypes, including cancers of the breast, colon, brain, prostate, lung, and nervous system (Volinia et al., 2006). The second paper included in this thesis (Paper II) demonstrates the molecular outcome of abnormal miR-17~92 expression in *MYCN* amplified neuroblastoma (NB), an embryonal tumor derived from the sympathetic nervous system (SNS).

NB is an embryonal tumor derived from the immature cells of the developing SNS and represents the most common cancer of infancy that occurs during the first year of life (Altekruse et al., 2009). NB tumors account for more than 7% of malignancies in patients younger than 15 years and approximately 15% of all pediatric oncology deaths (Maris et al., 2007). The clinical hallmark of neuroblastoma is heterogeneity, with the

likelihood of tumor progression varying widely according to anatomic stage and age at diagnosis. Although great improvements in disease outcome have been observed in certain well-defined subsets of patients, the outcome for children with high-risk NB remains poor, with long-term survival still less than 40% (Maris et al., 2007). In general, children diagnosed before 1 year of age and/or with localized disease are curable with surgery and little or no adjuvant therapy. Intriguingly, some of these tumors undergo spontaneous regression or differentiate into benign ganglionueromas (Matthay, 1999), referred to as stage 4S (S=special) disease. This striking clinical phenotype, which occurs in about 5% of cases, was first described by D'Angio and colleagues, who observed that infants with small, localised primary tumors accompanied by metastases to liver, skin, or bone marrow frequently experience spontaneously regression (D'Angio et al., 1971). In contrast, older children often have extensive hematogenous metastases at diagnosis, and the majority die from disease progression despite intensive multimodal therapy.

The clinical diversity observed in patients correlates closely with several molecular biologic features of neuroblastoma. Activating, germ-line mutations in the anaplastic lymphoma kinase (ALK) oncogene were recently discovered in hereditary NB, and occasionally occur in sporadic NBs (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). The genetic events underlying sporadic neuroblastoma are less clear, although enrichments of single-nucleotide-polymorphisms (SNP) within the non-coding RNA *FLJ22536* and the DNA-repair ligase *BARD1* have been detected in patients with progressing disease (Capasso et al., 2009; Maris et al., 2008). The biological variables that define advanced stage disease include 1p36 deletion (with subsequent loss of the p53-regulated tumor-suppressor *miR-34a*), allelic loss of 11q, 17q22-qter gain. In addition, amplification of the *MYCN* locus, present in ~20 to 30% of all cases, represents the most important genetic aberration, and is strongly related to poor clinical diagnosis (Maris et al., 2007).

Originally cloned in 1983 by identifying an amplified DNA sequence with partial homology to the *c-MYC* gene, *MYCN* amplification is strongly associated with rapid disease progression and low event-free survival (Brodeur, 2003; Schwab et al., 1983). From a pathway perspective, poor outcome neuroblastomas (including all *MYCN*-amplified cases) exhibit elevated signaling through the MYC transcriptional network (c-MYC, MYCN, and L-MYC target genes), along with low expression of lineage marker genes relating to late neuronal differentiation. Additionally, these gene expression traits were not only present in high-risk tumors, but also in patients with tumors initially diagnosed as low or intermediate risk that ultimately had an adverse outcome (Fredlund et al., 2008). In agreement with these findings, NB tumors with high MYC pathway activity also display specific miRNA gene signatures, including elevated expression levels of miRNAs belonging to the miR-17~92 cluster (Mestdagh et al., 2010).

Our findings in Paper II suggest that specific expression changes in miR-17~92 cluster-derived miRNAs may promote tumorigenic behavior of MYCN-amplified NB, the consequences of which are discussed in further detail in the following sections.

2. AIMS

THE OVERALL PURPOSE of this thesis was to explore the role of miRNAs in cancer in order to gain a more complete molecular understanding of the mechanisms underlying select childhood and adult malignancies. More specifically, this thesis aimed to:

- Characterise the regulation and function of the *miR-15a/miR-16-1* host transcript *DLEU2* and examine its role in tumor development, with particular emphasis on CLL (Paper I)

- Investigate the molecular consequences and functional outcome of abnormal miRNA transcription in *MYCN*-amplified NB (Paper II)

- Identify and elucidate the mechanism(s) by which abnormal miRNA expression in the epidermis may contribute to carcinogenesis of the skin and BCC tumor formation (Paper III)

3. 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

IN THEIR EFFORTS to clone a tumor suppressor gene at the 13q14 locus, Calin and coworkers were the first to show a causal link between miRNAs and cancer when they discovered that *miR-15a* and *miR-16-1* reside in region commonly deleted in CLL. Specifically, they claimed that the 13q14 MDR in CLL includes *miR-15a* and *miR-16-1* and occurs in 70% of all CLLs (Calin et al., 2002a). This is in contrast to a recent expression profiling study which demonstrated that only 11% (6 of 56 samples) of all CLL cases analysed had exceedingly low expression of miR-15a/miR-16-1 (Fulci et al., 2007). Furthermore, only three of these six patients showed biallelic loss of 13q14 (Fulci et al., 2007), suggesting that alternative mechanisms contribute to miR-15a/miR-16-1 loss-of-function in CLL. Germline mutations affecting pri-miR-15a/16-1 processing have been detected, but represented only a trivial fraction of patients analysed (2 of 75 CLL cases) and must therefore be considered a rare event (Calin et al., 2005). A similar finding has been reported in New Zeeland Black (NZB) mice that spontaneously develop a lymphoprolifertaive disorder highly reminiscent of CLL. These mice carry a syntenic point mutation in the 3' flanking region of miR-16-1, resulting in decreased expression of miR-16 (Raveche et al., 2007). While biallelic loss and germline mutations negatively impact miR-15a/16-1 expression levels, these genetic perturbations do not account for all cases of *miR-15a* and *miR-16-1* dysregulation in CLL. Moreover, breakpoint analyses of the 13q14 MDR in CLL consistently demonstrate that the *miR-15a* and *miR-16-1* genes are frequently retained following deletion (Lerner et al., 2009; Liu et al., 1997). Instead, the refined 13q14 MDR involves the deletion of portions of *DLEU1* and *DLEU2*, both of which are non-coding transcripts oriented in a sense/antisense fashion. Given that *DLEU2*, in contrast to *DLEU1*, is evolutionarily conserved (as are *miR-15a* and *miR-16-1* positioned

intronically in *DLEU2*), it seems more plausible that *DLEU2* represents the candidate tumor suppressor targeted by deletions and/or deregulation in CLL. In Paper I, we set out to investigate the regulation and function of the *miR-15a/miR-16-1* host transcript *DLEU2* and examine its role in CLL tumorigenesis.

As mentioned previously, most mammalian miRNA genes are clustered in the genome, allowing them to be transcribed simultaneously as polycistronic transcription units (Altuvia et al., 2005; Bartel, 2004). In addition, intronic incorporation and genomic miRNA aggregation allows for miRNAs to employ existing functional promoter elements. To determine if *DLEU2* functions as a bicistronic host gene for miR-15a and miR-16-1, we assessed if the maturation of *DLEU2* RNA was Drosha-dependent using RNAi. Knockdown of Drosha caused a significant accumulation of partially spliced *DLEU2*, yet did not affect the maturation of fully spliced *DLEU2*, suggesting that *DLEU2* transcripts are normally processed by Drosha in order to liberate functional miRNAs.

To further assess the regulatory features of *DLUE2*, we decided to investigate the role of both *DLEU2* promoters and additionally assess the relevance of putative regulatory units just upstream of miR-15a/miR-16-1. As a recent report had demonstrated that MYC transcriptionally represses a substantial portion of miRNAs, including miR-15a and miR-16-1 (Chang et al., 2008), we decided to test if reduction of miR-15a/miR-16-1 expression was associated with concomitant *DLEU2* downregulation. Using a doxycycline-regulated model system in which MYC expression can be turned off (Schuhmacher et al., 1999), we found that both miR-15a/miR-16-1 and *DLEU2* expression levels increased in the absence of Myc. Further, our chromatin immuniprecipitation (ChIP) assays implied that reduced expression of *DLEU2* is the result of direct Myc-mediated transcriptional binding of the two alternative *DLEU2* promoters. In contrast, no binding was detected to any of the loci downstream of the first two DLEU2 exons, including the most conserved region immediately upstream of *miR-15-a/miR-16-1*. These findings conclude that *DLEU2* functions as a host gene for *miR-15-a/miR-16*.

Next, we wanted to analyse the function of miR-15-a/miR-16-1. In order to assess the tumor-suppressive effects of *DLEU2*, we performed colony formation assays in osteosarcoma (U2OS) and breast adenocarcinoma (MCF-7) cells. To this end, we cloned the *miR-15-a/miR-16*-containing *DLEU2* transcript into a cytomegalovirus promoter (CMV)-driven expression vector. As expected, transfection of the DLEU2 plasmid resulted in the rapid upregulation of miR-15a and miR-16-1 in several cell lines, demonstrating that proper miRNA processing occurs after vector expression. We also generated a deletion mutant, DLEU2-ΔMIR that lacks miR-15a and miR-16-1. Ectopic expression of DLEU2 led to an ~80% decrease in colony number as compared to both mock- and DLEU2-ΔMIR-transfected cells. In parallel, DNA histogram analysis performed on HEK293 and U2OS cells transfected with DLEU2 showed a clear G_1 arrest, suggesting an antiproliferative as opposed to an antiapoptotic effect. G_1 arrest in *DLEU2* expressing cells was accompanied by hypophosphorylation of the Rb protein, further indicating that cell cycle entry was abrogated.

Although it was initially reported that miR-15-a and miR-16-1 target the anti-apoptotic gene *bcl-2* (Cimmino et al., 2005), others have reported that miR-15-a/miR-16-1 function to inhibit cell cycle progression (Huang et al., 2007; Linsley et al., 2007), a notion that was recently confirmed *in vivo* using a *miR-15a/16-1* transgenic mouse model (Klein et al., 2010). Using the PicTar and miRBase algorithms, we searched for putative miR-15-a/miR-16-1 targets and identified Cyclin D1 and Cyclin E1 as high confidence predictions. Transfection of *DLEU2* into several different cell lines resulted in prominent downregulation of Cyclin D1 and Cyclin E1 on protein level. RT-PCR and cyclohexamide chase analysis established that the *DLEU2*-mediated repression of the G_1-related cyclin proteins was not the result of a decreased mRNA levels or increased cyclin protein turnover. DLEU2-ΔMIR-transfected cells did not show protein abundance alterations of Cyclin D1 and Cyclin E1. Importantly, luciferase reporter assays established that the *DLEU2*-mediated repression of Cyclin D1 and Cyclin E1 was dependent on the 3'UTRs of these targets.

Taken together, this study delineated a functional role of the *miR-15a/miR-16-1* host transcript *DLEU2*. The identification of *DLEU2* as a regulatory host gene of these miRNAs elucidates how CLL deletions with *miR-15a/16-1* retention may still result in their functional loss. The refined 13q14 MDR suggest that that loss of the promoters (and perhaps the first exons of DLEU2) result in the functional loss of these miRNAs. This notion is supported by the absence of miR-16-1 expression in CLL case 4 that retains *miR-15a/miR-16-1* at the genomic level while deleting both alternative *DLEU2* promoters (Figure 1). We also show that DLEU2 is negatively regulated by MYC, providing yet another mechanism by which *DLEU2* may be deregulated in CLL and other cancers, some of which are Myc-dependent (Bonci et al., 2008). These data also demonstrate how activation of MYC can lead to the induction of multiple G_1 cyclins in a posttranscriptional manner. The exact molecular features relating to miR-15a and miR-16-1 interactions during cell cycle dynamics remain to be addressed. Their constitutive expression across the cell cycle phases (unpublished data) suggests that miR-15a and miR-16-1 may act as an additional regulatory layer to ensure both the precise expression of G_1 cyclins levels during G_0/G_1-S transition and to reduce potentially harmful leaky expression of these cyclins in later cell cycle phases.

PAPER II.

MYCN-regulated microRNAs repress estrogen receptor-alpha (ESR1) expression and neuronal differentiation in human neuroblastoma

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NEUROBLASTOMA HAS LONG been considered a clinical enigma (Brodeur, 2003), owing to the incredibly diverse and often dramatic clinical behavior of NB tumors. NB accounts for disproportionate morbidity and mortality among childhood cancers while conversely representing the cancer with the highest incidence of spontaneous regression and complete tumor involution (Maris, 2010). While low risk and intermediate risk patients have a very high chance of survival, the outcome for children with high-risk NB remains poor, with long-term survival still less than 40% (Maris et al., 2007). A common feature of high-risk NB is *MYCN* amplification, which occurs in ~20% of primary NB tumors and is strongly correlated with advanced stage disease and treatment failure (Maris et al., 2007). While 1p36 deletions have been shown to precede *MYCN* amplification, the mechanisms underlying *MYCN*-mediated NB progression are poorly understood. In Paper II, we wanted to gain further insights into the molecular processes associated *MYCN*-amplified NB, with specific emphasis on deregulated miRNA expression patterns and downstream targets. This, we hoped, would advance our understanding of *MYCN*-amplified NB and uncover novel pathways that could serve as clinical alternatives in NB disease management.

To begin, we employed a genome-wide miRNA expression array in order to identify MYCN-regulated miRNAs, using Tet21N NB cells with doxycycline-inducible *MYCN* expression. Our analyses revealed consistent ~2-fold overexpression of several MYC-associated miRNAs previously identified, including miRNAs from the oncogenic miR-17~92 cluster (e.g. miR-17, miR-18a, and miR-19a) and its paralogs on chromosome 7 and chromosome X (He et al., 2005; O'Donnell et al., 2005). We also noticed a robust decrease of miRNA expression in MYCN expressing cells, many of which exert tumor-suppressive functions in various cancers (Croce, 2009). Further, our chromatin immunoprecipitation (ChIP) assays confirmed that MYCN:MAX associated with canonical E-box sequences upstream of all polycistronic miRNA units assayed, resulting in the transcriptional activation of miR-17~92 cluster-derived miRNAs and its paralogs.

Next, we wanted to analyse the function of a subset of the miRNAs identified in our screen. It had previously been reported that overexpression of the miR-17~92 cluster strongly augments NB tumorigenesis both *in vitro* and *in vivo* (Fontana et al., 2008).
While the oncogenic contribution of miR-17 and miR-20a in relation to NB was described in this paper, the biological significance of other miRNAs residing in the miR-17~92 polycistron (including miR-18a and miR-19a) was not addressed. We therefore decided to interrogate miR-18a and miR-19a function in *MYCN*-amplified NB cells using miRNA-specific inhibitors followed by fluorescence-activated cell sorting (FACS)

analysis. These initial experiments conclusively demonstrated that miR-18a and miR-19a affect cell cycle progression. To further extend this finding, we employed lentiviral constructs encoding specific antisense miRNA sequences designed to target miR-18a and miR-19a function. Long-term inhibition of miR-18a resulted in a dramatic phenotype, characterised by both morphological and biochemical differentiation, while the miR-19a knockdown was more difficult to assess. Nevertheless, our results suggested that miR-18a (and to a lesser degree miR-19a) provide *MYCN*-amplified cells with a proliferative advantage by deregulating messages linked to cell cycle progression and differentiation.

Using the Using the PicTar and Targetscan algorithms, we identified several genes downstream miR-18a and miR-19a that are involved in neural and cancer-associated processes (such as *ROBO2*, *ATXN1*, *CCND2*, and *ESR1*). A literature survey provided the first evidence that estrogen receptor-α (ESR1) could potentially account for the phenotype we observed in our miRNA knockdown experiments. In 1993, Ma and colleagues described the direct involvement of activated ESR1 in a series of morphological and biochemical changes that lead the transfected cells toward a differentiated state (Ma et al., 1993). Unintentionally, the authors used the *MYCN*-amplified cell line SK-N-BE, which, by virtue of its *MYCN* amplification, expresses high levels of miR-18a and miR-19a, potentially leading to the aberrant repression of endogenous *ESR1*. Importantly, upon ligand-dependent activation, ectopic ESR1 in the genetically engineered NB cell line SK-N-E3 resulted in growth arrest (Ma et al., 1993). This led us to hypothesise that MYCN-regulated miRNAs might disrupt estrogen signaling sensitivity in cells derived from SNS precursors through deregulation of ESR1, thereby preventing the normal induction of neuroblast differentiation.

To experimentally assess this idea, we first used luciferase reporter assays to demonstrate that miR-18a and miR-19a repress ESR1 expression through miRNA-binding elements in its 3'UTR. Western blot and quantitative PCR (qPCR) analyses of MCF-7 cells (which express high levels of endogenous ESR1) transfected with miR-18a and miR-19a mimic oligonucleotides confirmed that these miRNAs also target endogenous ESR1. Next, to reaffirm Ma's original work, we used lentiviral constructs expressing *ESR1* cDNA and transduced SK-N-BE(2) cells. Upon lentiviral reconstitution of ESR1, we observed cell cycle arrest and morphological changes indicative of differentiation.
In addition, we were able to demonstrate ESR1 expression in human fetal sympathetic ganglia, suggesting that ESR1 plays an important role during the development of the sympathetic nervous system. Finally, our Kaplan-Meier survival analyses of 251 NB patients indicate that *ESR1*-positive NBs are associated with a favorable disease outcome.

In summary, we have uncovered a novel mechanism by which miRNA-mediated deregulation of ESR1 expression potentiates tumorigenesis in human neuroblastoma. On a far more fundamental level of neurobiology, the demonstration of ESR1 expression in human fetal sympathetic ganglia suggests that ESR1 may act as a key constituent in the specification/diversification of the neural crest-derived sympatho-adrenal (SA) lineage.

The importance of estrogen and its receptors during neural development is recapitulated in embryonic neuronal stem cells, which undergo differentiation in response to estradiol exposure (Brannvall et al., 2002) Analyses of ESR1 expression during peripheral nervous system development in chicken embryos show ESR1-immunoreactivity in the dorsal root ganglia (DRG) at cervical, thoracic and lumbo-sacral levels, as well as in the sympathetic ganglia and primary spinal motoneurons (Cui and Goldstein, 2000). Interestingly, it has been shown that ESR1 potentiates mRNA expression of the tyrosine kinase receptor *TrkA* using a cancer cell line derived from a pheochromocytoma (PC12 cells) of the rat adrenal medulla (Sohrabji et al., 1994a; Sohrabji et al., 1994b). TrkA is the high affinity catalytic receptor for the neurotrophin (NGF) and mediates the multiple effects of NGF, which includes neuronal differentiation. Both undifferentiated (naïve to NGF) and NGF-exposed, differentiated PC12 cells (which resemble the sympathetic neuron phenotype) express *ESR1* mRNA. While naive cells express very low levels of *ESR1* mRNA, NGF elicits a significant increase in *ESR1* mRNA. As in sensory neurons, NGF-mediated increases in *ESR1* expression resulted a ~3-fold upregulation of *trkA* mRNA (Toran-Allerand, 1996).

The cooperative interaction between *ESR1*, the neurotrophins and their receptors could be of great significance to the onset and progression of high-risk, *MYCN*-amplified NB. High levels of *TrkA* expression are correlated with younger age, lower stage, and absence of *MYCN* amplification. Furthermore, *TrkA* expression is highly correlated with favorable disease outcome (Brodeur, 2003). Thus, ESR1-positive NBs may represent a potential subgroup of tumors amenable to estrogen treatment, providing a means to force these tumors into spontaneous regression. Likewise, inhibition of miR-18a may represent a promising therapeutic opportunity for *MYCN*-amplified NB patients.

PAPER III.

miR-203 functions as a bona fide tumor suppressor microRNA in basal cell carcinoma

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BASAL CELL CARCINOMA (BCC) is the most common type of malignant cancer in fair-skinned individuals. Common to both sporadic and familial BCCs is the inappropriate activation of the Hedgehog (Hh) signaling pathway, which plays a central role in the pathogenesis of BCC tumors, as well as medulloblastoma (MB) (Wicking et al., 1999). In sporadic BCCs, 10% show *SMO* gain-of-function mutations while ~70% of BCC tumors exhibit inactivating mutations in at least one allele of *PTCH1* (Epstein, 2008). Several murine models support the concept that aberrant Hh pathway activation is sufficient to drive development of BCCs or BCC-like tumors, including mice with constitutive or conditional overexpression of GLI1 or of GLI2, activating SMO mutations, or *PTCH1* haploinsufficiency (Aszterbaum et al., 1999; Grachtchouk et al.,

2000; Nilsson et al., 2000; Xie et al., 1998). To date, all investigations on BCC tumor onset and development have focused on the 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. To this end, we set out to investigate the role of miRNA deregulation in BCC tumors.

To explore the potential involvement of miRNAs in basal cell carcinoma, we performed the first comprehensive, genome-wide analysis of 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 suppressed in BCC. These findings suggest that the altered expression of miRNA has a role in the pathogenesis of BCC and enforces the observation that global downregulation of miRNA expression is a common feature of solid tumors (Lu et al., 2005). Of all miRNAs assayed, miR-203 showed the greatest decrease (~5-fold) in expression in BCC when compared to healthy skin, a finding which was further validated in a lager set of healthy and BCC samples. Expression analyses using *in situ* hybridization with specific locked nucleic acid (LNA) probes demonstrated that miR-203 was preferentially expressed in the suprabasal layers of healthy skin, almost in a gradient-like fashion, while BCC tumors consistently lacked miR-203 expression.

Next, we wanted to explore the potential association between miR-203 downregulation and the activation of the Hedgehog pathway. Quantitative real-time PCR results showed that both *PTCH1* and *GLI1* were significantly overexpressed ($p<0.001$) in BCC tumors as compared with healthy skin, in accordance with previously published data. Further, correlation analysis showed a significant negative correlation 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 Hedgehog signaling in BCC.

Our next aim was to elucidate the potential mechanisms and/or pathways accounting for miR-203 suppression in BCC. Previous signal transduction analyses indicated that miR-203 is regulated by the PKC/AP-1 pathway and suppressed by growth factors, such as KGF and EGF in keratinocytes (Sonkoly et al., 2010). In BCC, EGFR has been shown to synergize with Hedgehog/GLI in oncogenic transformation via activation of the MEK/ERK/JUN pathway (Schnidar et al., 2009). Therefore, we set out to further explore the role of the EGFR signaling pathway in the regulation of miR-203. By measuring miR-203 expression in primary human keratinocytes treated with inhibitors of EGFR, MEK1/2, JNK, or Akt in combination with EGF or DMSO alone, we were able to determine that the EGFR-MAPK signaling pathway represses miR-203 expression, indicating that this pathway may negatively regulate miR-203 levels in BCC.

A bioinformatic search for putative miR-203 targets identified several genes in the MEK/ERK/JUN pathway, including c-JUN, as well as the Hh family member PTCH1. Interestingly, the c-MYC 3'UTR also harbors a miR-203 binding site, albeit poorly conserved. 3'UTR-based luciferase reporter assays subsequently demonstrated that all

genes identified were indeed regulated by miR-203. Moreover, cell-cycle analysis of primary keratinocytes transfected with miR-203 mimic oligonucleotides revealed a clear obstruction in the G_1 to S-phase transition of the cell cycle. Immunohistochemical (IHC) analyses of these targets in BCC tumors confirmed substantial increases of protein expression when compared to healthy skin. Furthermore, IHC analyses in skin collected from the transgenic K5-TreGli1 mouse (which develop skin tumors closely resembling human BCCs) corroborated our findings in human tumors: BCC tumors consistently exhibit severely reduced expression of miR-203, which dramatically influences the expression of genes involved in the regulation of cell proliferation and cell cycle, including key constituents of the Hh pathway (*figure 6*).

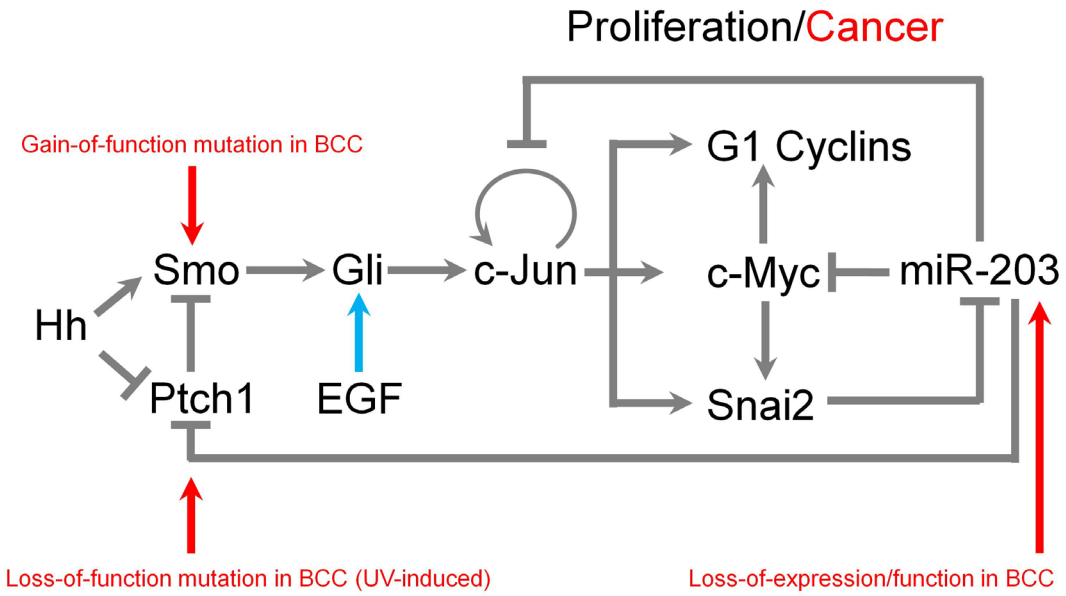

Figure 6. A proposed autoregulatory loop involving the Hedgehog (Hh) and MAPK pathways, the proto-oncogens *c-JUN* and *c-MYC*, and the *bona fide* tumor supressor miR-203. See text for details.

In physiological settings, Hh signaling is required for the proliferation of hair follicle epithelium during development (Chiang et al., 1999; St-Jacques et al., 1998) and postnatal hair cycles, which include a growth phase (anagen), regression phase (catagen), and resting phase (telogen) (Wang et al., 2000). While Hh signaling activity in follicle epithelium is restricted to periods of active growth and is limited by transient levels of Sonic hedgehog (SHH), this pathway is constitutively active in BCCs. Recently, it was demonstrated that BCCs arise from long-lived progenitor cells residing in the interfollicular epidermis (IFE), a stem-cell containing compartment that can give rise to all epidermal lineages in response to appropriate stimuli (Owens and Watt, 2003;

Youssef et al., 2010). Importantly, like other cell lineages of the mammalian epidermis, IFE stem cells respond to Hh signaling, which results in *de novo* hair follicle formation (Silva-Vargas et al., 2005). As BCC tumors have several characteristics in common with immature hair follicles (i.e. stem-cell properties), including similar histology, ultrastructure, and gene expression patterns, it has been proposed that BCC tumors (and other tumors) reflect aberrant organogenesis caused by defects in developmental signaling pathways, such as Hh (Hutchin et al., 2005; Millar, 2002). In this respect, the gradient-like expression pattern of miR-203 in the suprabasal layers of the epidermis may serve as a molecular barrier to limit proliferative cues in replenishing skin. The loss of miR-203 in BCC may thus facilitate the inappropriate activation of Hh signaling, causing the reiteration of molecular programs normally regulating skin and hair development and ultimately leading to tumors which phenotypically resemble immature hair follicles.

In conclusion, our study represents the first analysis of miRNA expression and function in a non-melanoma skin cancer. The loss of miR-203 in BCC suggests that this miRNA could be used as a biomarker of the disease and may represent a potential therapeutic target for the treatment of BCC, especially for the rare cases of metastatic, locally advanced BCCs where surgical treatment options are limited. 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.

4. CONCLUDING REMARKS

CONSIDERING THE FUNDAMENTAL role of miRNAs in organismal development, cellular differentiation, metabolism, viral infection, and oncogenesis, the future of miRNA-based therapeutics holds great potential. In 2008, Elmén et al. demonstrated potent antagonism of miR-122 by the simple delivery of a unconjugated high-affinity LNA-antimiR oligonucleotide in mice and non-human primates (Elmen et al., 2008), providing a new molecular intervention strategy to target hepatitis C virus (HCV) in humans (Jopling et al., 2005). Moreover, the systemic administration of miR-26a in a mouse model of hepatocellular carcinoma (HCC) using adeno-associated virus (AAV) led to induction of tumor-specific apoptosis and dramatic protection from disease progression without measurable toxicity (Kota et al., 2009). Very recently, the first mechanistic evidence of RNAi in humans from an administered siRNA was demonstrated, verifying that RNAi can be used as a gene-specific therapeutic in human solid tumors (Davis et al., 2010). Together, these studies offer reassuring evidence that RNAi-based technologies are feasible in clinical settings and represent toxilogically sound approaches, with sparingly few side-effects reported so far (Bonetta, 2009). To conclude, the work provided herein has identified new miRNA targets that may be exploited therapeutically to treat childhood and adult malignancies. While delivery and specificity remain a challenge for many diseases, especially for cancer, these issues will hopefully be resolved at the same rapid pace as the field of RNAi research has developed in the last few years. The future looks bright.

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PUBLICATIONS & MANUSCRIPTS