DEPARTMENT OF ONCOLOGY-PATHOLOGY

Karolinska Institutet, Stockholm, Sweden

MICRORNA EXPRESSION AND FUNCTION IN VIRUS-ASSOCIATED HUMAN CANCERS

Hong Xie

谢 虹

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Faculty Opponent

Anders H. Lund, Professor
Biotech Research & Innovation Centre, Centre for Epigenetics
University of Copenhagen, Copenhagen, Denmark

Examination Board

Andor Pivarcsi, Docent
Department of Medicine-Solna
Karolinska Institutet, Stockholm, Sweden

Torbjörn Ramqvist, Docent
Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

Afshin Ahmadian, Docent
School of Biotechnology
Royal Institute of Technology, Stockholm, Sweden

Supervisors

Weng-Onn Lui, Assistant professor
Department of Oncology-Pathology, KI

Catharina Larsson, Professor
Department of Oncology-Pathology, KI

External Mentor

Harvest F. Gu, Docent
Department of Molecular Medicine and Surgery, KI

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Front cover: WaGa cells stained with SQSTM1/p62 (green) and cytokeratin (MNF116, red) upon silencing of MCV T-Ag (right) and control (left). /Linkiat Lee, Vladana Vukojevic and Hong Xie

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“The only way to do great work is to love what you do.

If you haven’t found it yet, keep looking. Don't settle.”

Steve Jobs

To myself and my family

Thesis Defense

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ABSTRACT

microRNAs (miRNAs) are small non-coding RNAs that play important roles in gene regulation. It is now clear that miRNAs participate in tumor development and progression. Despite many studies have reported specific miRNA expression signatures in various tumor types, the functional roles of these deregulated miRNAs in specific tumor types remain to be determined. The general aim of this thesis work was to investigate the expression and functional roles of miRNAs in cervical carcinoma and Merkel cell carcinoma (MCC).

In **Paper I**, we describe the functional roles and targets of *miR-205* in human cervical cancer cells. Using *miR-205* over-expression and suppression experiments, we show that *miR-205* regulates cell proliferation and migration in cervical cancer cells. Using a CLIP-Chip approach, we identified a set of candidate *miR-205* targets functionally associated with cell proliferation and migration. Among them, *CTGF* and *CYR61* were further validated by Western blot and Ago2 CLIP-qRT-PCR analyses. Both genes are also downregulated in human cervical cancer tissues. Our findings suggest that *miR-205* and its targets may play important roles in the pathogenesis of cervical carcinoma.

In **Paper II**, we show that *miR-944* functions as an oncogene in human cervical cancer by promoting cell proliferation, migration and invasion. We identified a set of novel *miR-944* targets using the PAR-CLIP sequencing approach. Among them, *HECW2* and *S100PBP* were further validated as direct targets of *miR-944* by luciferase reporter assays. Our findings reveal novel functions and targets of *miR-944* in human cervical cancer cells, which may provide new insights of its role in cervical carcinogenesis.

In **Paper III**, we report miRNA expression patterns associated with Merkel cell polyomavirus (MCV) status and clinical outcomes in MCC. In addition, we show that *miR-203* overexpression inhibits cell growth and induces cell cycle arrest in MCV-negative MCC cells. We also demonstrate that *survivin* expression is regulated by *miR-203* in MCV-negative MCC cells or by MCV T-antigen(s) in MCV-positive MCC cells.

In **Paper IV**, we demonstrate that *miR-375* functions as a tumor suppressor by inhibiting cell growth and cell migration, as well as promoting cell cycle arrest and apoptosis in MCV-negative MCC cells. In addition, we show that *miR-375* is epigenetically regulated in MCC, and different epigenetic mechanisms may contribute to *miR-375* transcription regulation in MCV-positive and -negative MCC cells.

Overall, this thesis work illustrate the high value of Ago2 CLIP approach for miRNA target identification, and provides evidence for the role of miRNAs in human cervical cancer and Merkel cell carcinoma.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which will be referred to throughout the text by their Roman numerals.

I **Xie H***, Zhao Y, Caramuta S, Larsson C, Lui WO*.
miR-205 expression promotes cell proliferation and migration of human cervical cancer cells.
PLoS ONE, 2012; 7(10): e46990.

II **Xie H***, Lee L, Scicluna P, Kawak E, Larsson C, Sandberg R, Lui WO*.
Identification of *miR-944* targets in human cervical cancer cells using PAR-CLIP sequencing.
Manuscript

III **Xie H***, Lee L, Caramuta S, Höög A, Browaldh N, Björnhagen V, Larsson C, Lui WO*.
microRNA expression patterns related to Merkel cell polyomavirus infection in human Merkel cell carcinoma.
J Invest Dermatol, 2013; DOI: 10.1038/jid.2013.355. [Epub ahead of print]

IV **Xie H***, Lee L, Höög A, Larsson C, Lui WO*.
Functional role of *miR-375* in human Merkel cell carcinoma.
Manuscript

* Corresponding author

LIST OF RELATED PUBLICATIONS

1 Caramuta S, Lee L, Özata DM, Akçakaya P, Georgii-Hemming P, **Xie H**, Amini RM, Lawrie CH, Enblad G, Larsson C, Berglund M, Lui WO.
Role of microRNAs and microRNA machinery in the pathogenesis of diffuse large B-cell lymphomas.
Blood Cancer J, 2013; 3:e152. DOI: 10.1038/bcj.2013.49

2 Caramuta S, Lee L, Özata DM, Akçakaya P, **Xie H,** Höög A, Zedenius J, Bäckdahl M, Larsson C, Lui WO.
Clinical and functional impact of TARBP2 over-expression in adrenocortical carcinoma.
Endocr Relat Cancer, 2013; 20(4): 551-564.

3 Huang Z, Rahman M F-U, Jiang, L, **Xie H**, Hu H, Lui WO, Li N.
Thrombin induces de novo protein synthesis of stromal cell derive factor-1a but not angiostatin in human platelets.
J Thromb Haemost, 2012; 10(10): 2202-2205.

4 Özata DM, Caramuta S, Velázquez-Fernández D, Akçakaya P, **Xie H**, Höög A, Zedenius J, Bäckdahl M, Larsson C, Lui WO.
The role of microRNA deregulation in the pathogenesis of adrenocortical carcinoma.
Endocr Relat Cancer, 2011; 18(6): 643-655.

5 Akçakaya P, Ekelund S, Kolosenko I, Caramuta S, Özata DM, **Xie H**, Lindforss U, Olivecrona H, Lui WO.
miR-185 and *miR-133b* deregulation is associated with overall survival and metastasis in colorectal cancer.
Int. J Oncol, 2011; 39(2): 311-318.

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LIST OF ABBREVIATIONS

4-SU	4-thiouridine
6-SG	6-thioguanosine
Ago2	argonaute 2
ATCC	American Type Culture Collection
cDNA	complementary deoxyribonucleic acid
CDS	coding sequence
CLIP	crosslinking immunoprecipitation
CLL	chronic lpmphocytic leukemia
Co-IP	co-immunoprecipitation
CTGF	connective tissue growth factor
CYR61	cysteine rich 61
DNA	deoxyribonucleic acid
EDU	5'-ethynyl-2'-deoxyuridine
FFPE	formalin-fixed paraffin-embedded
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2
HPV	human papillomavirus
IHC	immunohistochemistry
LT-Ag	large T antigen
MCC	Merkel cell carcinoma
MCV	Merkel cell polyomavirus
mir	precursor microRNA
miR	mature microRNA
miRNA	microRNA
mRNA	messenger RNA
ncRNA	non-coding RNA
PAR-CLIP	photoactivable ribonucleoside enhanced-CLIP
PARP	poly ADP ribose polymerase
PCR	polymerase chain reaction
PI	propidium iodide
pre-miR	precursor miRNA
pri-miR	primary miRNA

PS	phosphatidylserine
qRT-PCR	quantitative reverse transcription PCR
RNA	ribonucleic acid
RISC	RNA indiced silencing complex
SAM	significant analysis of microarray
sT-Ag	small T antigen
TSA	trichostatin A
UTR	untranslated region
UV	ultraviolet
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2-H-5-tetrazolio]-1,3-benzenedisulfonate

1 INTRODUCTION

According to the typical central dogma of molecular biology, which was first described in 1958 and re-stated in 1970 (Crick, 1970), ribonucleic acid (RNA) only plays the mediator role between deoxyribonucleic acid (DNA) and protein, while protein plays the major role in almost all biological pathways. Surprisingly, the protein-coding genes represent less than 2% of the human genome sequence, while at least 90% of the genome is actively transcribed (Stein, 2004; Taft *et al.*, 2010). The understanding of the role of RNA within the cells has changed dramatically in the last two decades. Some novel findings in RNA field expanded our understanding of RNA molecules. For example, catalytic RNA (also called ribozyme, ribonucleic acid enzyme) was first reported by Thomas Cech in 1980s (Kruger *et al.*, 1982), RNA interference (RNAi) was reported in 1998 (Fire *et al.*, 1998) and non-coding RNAs were described in 2001 (Eddy, 2001). Non-coding RNAs (ncRNAs) are functional RNA molecules that are not translated into protein. Recent evidences suggest that ncRNAs play major biological roles in cellular development, physiology and pathologies (Brosnan and Voinnet, 2009). NcRNAs can be divided into two major classes based on the transcript size: Small non-coding RNAs which are generally less than 200 nucleotides in length such as microRNA (miRNA), small interfering RNA (siRNA) and PIWI-interacting RNA (piRNA); and Long non-coding RNAs (lncRNAs) which are mRNA-like transcripts ranging in length from 200 nucleotides to around 100 kb, *e.g.* Xist (Wutz, 2007) and HOTAIR (Gupta *et al.*, 2010).

miRNA, ~19-25 nucleotides in length, is the most abundant class of small RNAs in mammals and are currently the best described ncRNAs involved in human cancers. The expression and function of miRNAs have been documented in many tumor types. However, little is known about their roles in human cervical carcinoma and Merkel cell carcinoma. This thesis work focused on the biological and clinical role of miRNAs in these two cancer types.

1.1 DISCOVERY OF MIRNA

The first miRNA was discovered in 1993 in *Caenorhabditis elegans* (*C. elegans*). This miRNA *lin-4* regulates the timing of larval development by translational repression of *lin-14* through imperfect complementarity between *lin-4* and the unique repeats within

the 3' untranslated region (3'-UTR) of the *lin-14* mRNA (Lee *et al.*, 1993; Wightman *et al.*, 1993). This discovery was thought to be unique to *C. elegans*, because no *lin-4* homolog was found in other species. The second miRNA *let-7* was discovered seven years later (Reinhart *et al.*, 2000). Similar to *lin-4*, *let-7* controls the developmental timing in *C. elegans*. However, unlike *lin-4*, *let-7* expression was detected in a wide range of animal species (Pasquinelli *et al.*, 2000). This finding indicated that small RNAs are not restricted to *C. elegans* and that small RNA-mediated gene regulatory functions may exist in other species. Many researchers were inspired to search for similar types of tiny RNAs. In 2001, three laboratories independently identified more than 100 tiny RNAs, which they termed miRNAs, from flies, worms and human cells using a small RNA cloning method (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Following these discoveries, the miRNA field has expanded rapidly. To date, thousands of miRNAs have been identified in many species *e.g.* human, primates, viruses, and worms (Enright *et al.*, 2003; Kiriakidou *et al.*, 2004; Krek *et al.*, 2005; Lewis *et al.*, 2005; Rhoades *et al.*, 2002). The latest update of miRBase (Release 20, June 2013; http://www.mirbase.org) contains a total of 24,521 hairpin precusor miRNAs expressing 30,424 mature miRNAs were annotated from 206 species. Currently, there are 2,578 mature miRNAs known in human.

1.2 BIOGENESIS OF MIRNA

miRNAs are most frequently located within intergenic regions (distance from annotated genes) or introns of protein-coding genes, and less commonly within exons or antisense transcripts. Many miRNA precursors are also found to be clustered in the human genome (Lagos-Quintana *et al.*, 2003; Rodriguez *et al.*, 2004). Examples of miRNA locations in relation genes are shown in Figure 1.1.

miRNAs that are located in introns of genes and which have the same orientations as the host transcripts are generally co-transcribed with their host genes; thus their expression levels are strongly correlated with their host transcripts (Aravin *et al.*, 2003; Lai *et al.*, 2003; Lim *et al.*, 2003). Intergenic miRNAs are transcribed as independent transcription units with their own promoter/transcriptional regulatory region.

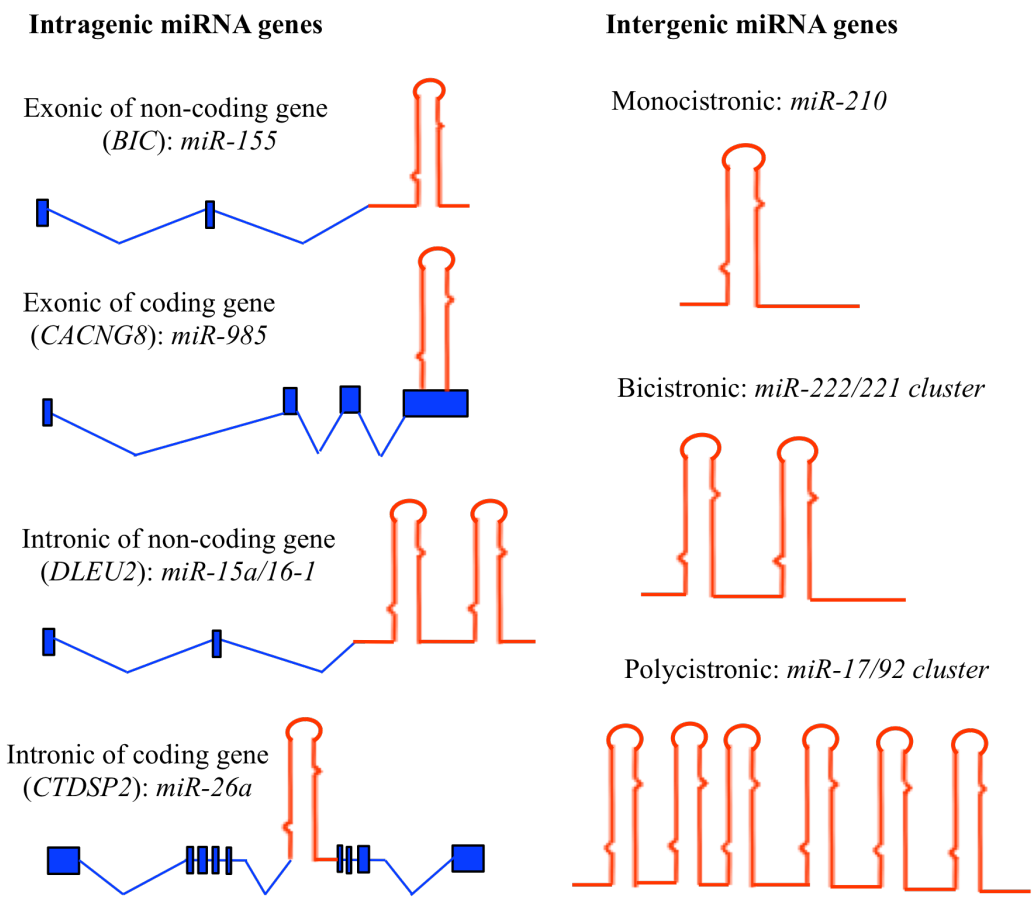

Figure 1.1 Genomic localization of miRNA genes within (left) or between (right) genes [Adapted from (Di Leva *et al.*, 2013)].

The biogenesis of miRNA generally involves both nuclear and subsequent cytoplasmic cleavage events performed by two ribonuclease III endonucleases, Drosha and Dicer (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Hutvagner *et al.*, 2001; Lee *et al.*, 2003; Lee *et al.*, 2002). However, there are some exceptions where one of these RNase-dependent cleavage events may be bypassed. Both canonical and non-canonical biogenesis pathways are involved as illustrated in Figure 1.2, and described below.

1.2.1 Canonical biogenesis pathway

miRNAs are transcribed by RNA Pol II or Pol III to produce a long primary miRNA transcript (pri-miRNA) with a stem-loop structure (Borchert *et al.*, 2006; Han *et al.*, 2006; Lee *et al.*, 2004). The miRNA maturation process begins with cleavage of pri-miRNA to a precursor miRNA (pre-miRNA) by a protein complex composed of the RNase III endonuclease Drosha and the double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8) in the nucleus (Denli *et al.*, 2004;

Gregory *et al.*, 2004; Han *et al.*, 2004; Lee *et al.*, 2003; Lee *et al.*, 2002). Subsequently, pre-miRNAs are exported to the cytoplasm by the nucleocytoplasmic transporter containing Exportin-5 (XPO5) and RanGTP (Bohnsack *et al.*, 2004; Okada *et al.*, 2009; Yi *et al.*, 2003; Zeng and Cullen, 2004). In the cytoplasm, pre-miRNAs are further processed by Dicer and TARBP2 complex to miRNA duplex (Chendrimada *et al.*, 2005). One strand (the leading strand) of the duplex is then loaded into argonaute-containing protein complex and the other strand (the passenger strand) is usually degraded.

1.2.2 Alternative biogenesis pathway

As aforementioned, some miRNAs can bypass Drosha or Dicer processing. For examples, mirtrons are a class of intronic miRNA precursors that skip Drosha processing, and pre-miRNAs are directly released by splicing (Babiarz *et al.*, 2008; Berezikov *et al.*, 2007; Okamura *et al.*, 2007; Ruby *et al.*, 2007). Mirtrons are located within short introns and the miRNA precursor is directly adjacent to the splice sites. Examples of mirtrons are *miR-877*, *miR-1224* and *miR-1225*. In human, 243 mirtrons are annotated (http://ericlailab.com/mammalian_mirtrons/hg19/). Besides mirtrons, other miRNAs (or miRNA-like sRNAs) that are independent of Drosha processing include snoRNA-derived miRNAs (Ender *et al.*, 2008), vault RNA-derived sRNAs (Persson *et al.*, 2009), tRNaseZ-mediated processing of murine gamma-herpesvirus 68 miRNAs (Bogerd *et al.*, 2010) and integrator complex-mediated processing of *Herpesvirus saimiri*-encoded miRNA (Cazalla *et al.*, 2011).

To date, *miR-451* is the only miRNA that is processed independent of Dicer. This miRNA is generated from an unsual hairpin structure that is processed by Ago2 cleavage activity (Cheloufi *et al.*, 2010; Yang *et al.*, 2010a). The 3'-end of the Ago2-cleaved *pre-miR-451* intermediate is then trimmed by poly (A)-specific ribonuclease to become the mature *miR-451* (Yoda *et al.*, 2013).

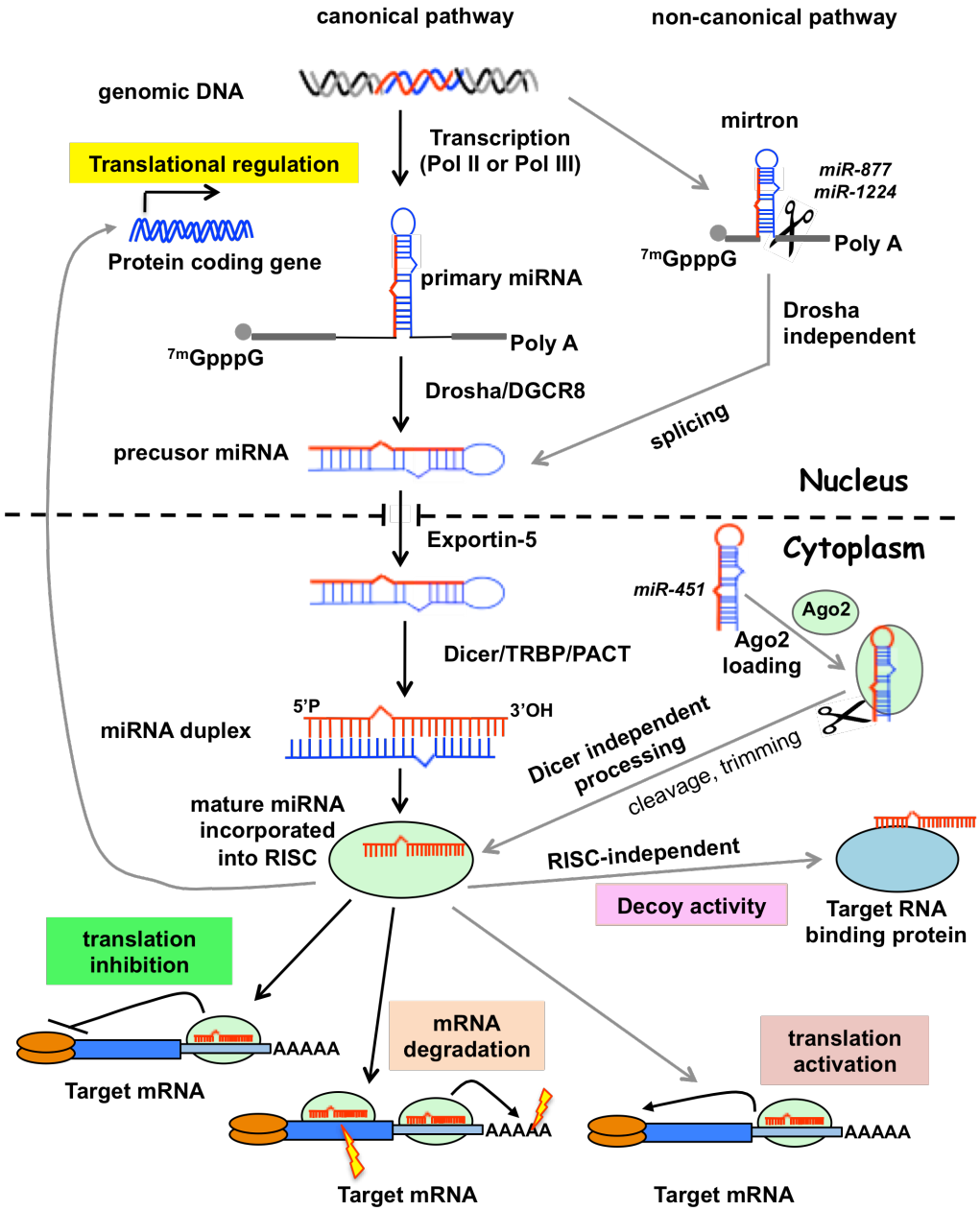

Figure 1.2 Biogenesis and function of miRNAs.

1.3 MIRNA REGULATIONS

miRNAs generally regulate gene expression at the post-transcriptional level by binding to target mRNAs. A single miRNA can regulate several mRNA targets and conversely multiple miRNAs can cooperatively regulate a single mRNA target (Bartel, 2004; Zhou *et al.*, 2013). The target recognition mainly relies on seed pairing (residues 2-8 at the 5'-end of the miRNA). Several different miRNA seed types are known to have important roles for target recognitions, which are shown in Figure 1.3. These seed types are based on evidence of experimentally verified miRNA targets (Bartel, 2009).

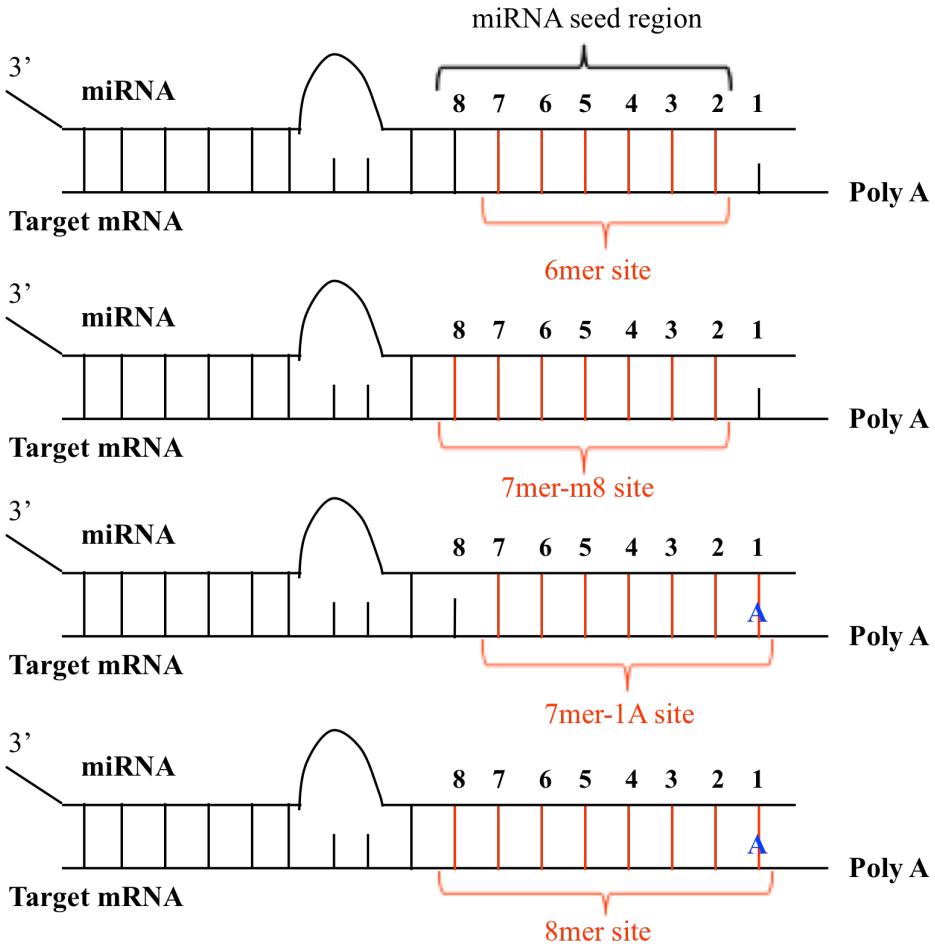

Figure 1.3 Different types of miRNA seed match.

Many experimental evidences support two distinct mechanisms of miRNA-mediated gene silencing, *i.e.* mRNA degradation and translational repression. Some miRNAs can also activate translation, however their mechanisms are still poorly understood (Mortensen *et al.*, 2011; Truesdell *et al.*, 2012; Vasudevan *et al.*, 2007).

1.3.1 Translation repression

When a miRNA binds to its target mRNA with imperfect base-pairing, it generally mediates translation repression. The actual mechanism by which miRNAs inhibit translation is still unclear; however some experimental data support that it occurs at initiation and elongation (Petersen *et al.*, 2006; Pillai, 2005).

One model proposes that miRISC can recruit CCR4-NOT via GW182 proteins that dissociates poly (A)-binding protein (PABP) from the mRNA poly (A) tail, thus disrupting assembly of the translation initiation complex (Zekri *et al.*, 2013). The other

model suggests that miRISC promotes ribosomes drop-off during elongation of translation (Petersen *et al.*, 2006) or stimulating the proteolysis of the nascent peptide (Nottrott *et al.*, 2006).

1.3.2 mRNA degradation

When a miRNA binds to its target mRNA with perfect base-pairing, it generally mediates endonucleolytic cleavage of mRNA by Argonaute (Yekta *et al.*, 2004). This mechanism is common in plants, but not in animals. In animals, the most common mechanism of miRNA-mediated mRNA degradation is removal of the poly (A) tail of the mRNA through recruitment of the CCR4-NOT deadenylation complex (Behm-Ansmant *et al.*, 2006). Loss of the mRNA poly (A) tail makes the mRNA susceptible to exonucleolytic degradation.

1.3.3 Other types of regulation

In an opposite scenario, instead of suppressing gene expression, miRNAs can activate gene expression. The first example is the up-regulation of Hepatitis C virus (HCV) RNA by human *miR-122*. This liver-specific miRNA binds to the 5'-UTR of HCV RNA that protects it from Xrn1 exonuclease degradation (Jopling, 2008; Jopling *et al.*, 2005; Li *et al.*, 2013b), stimulates HCV translation (Henke *et al.*, 2008), and modifies tertiary HCV RNA structures (Mortimer and Doudna, 2013).

Although numerous evidences indicate that miRNAs binding to 3'- or 5'-UTRs of the target mRNA can suppress gene expression (Lytle *et al.*, 2007; Meijer *et al.*, 2013), several studies have also shown that miRNAs can induce translational activation through their interactions with 5'- or 3'-UTRs. Besides the HCV example mentioned above, Orom *et al*. demonstrated that *miR-10a* interacts with the 5'-UTR of ribosomal protein mRNAs resulting in their translational enhancement upon amino acid starvation (Orom *et al.*, 2008). Similarly, Vasudevan *et al.* showed that *miR-369-3p* binds to the 3'-UTR of *TNF-α* mRNA and up-regulates its translation upon cell cycle arrest via recruiment of the *TNF-α* mRNP to heavy polyribosomes by the FXR1/Ago2 ARE complex (Vasudevan and Steitz, 2007; Vasudevan *et al.*, 2007).

In addition to the interactions with protein-coding mRNAs, miRNAs can control their own expression or the expression of other miRNAs. For examples, mouse *miR-709* can

directly bind to a 19-nucleotide *miR-709*-recognition element on the primary *mir-15a~16-1* transcript and prevents its processing into precursor miRNAs (Tang *et al.*, 2012). On the other hand, mature *let-7* binds to the 3'-end of *let-7* primary transcripts and promotes processing of its own primary transcript (Zisoulis *et al.*, 2012).

Besides gene expression regulation, miRNA can also function as a decoy. This was first identified in leukemic blasts. In this case, *miR-328* acts as RNA decoy by binding to hnRNP E2 and releases its translational repression of *CEBPA* mRNA involved in myeloid cell differentiation (Eiring *et al.*, 2010). Another example is *miR-29* that acts as a decoy for HuR to protect the tumor suppressor *A20* transcripts from degradation by HuR (Balkhi *et al.*, 2013).

1.4 ROLE OF MIRNAS IN HUMAN CANCERS

1.4.1 Deregulation of miRNAs in cancers

The first evidence of a role for miRNAs in human cancer came from studies of chronic lymphocytic leukemia (CLL). The expressions of *miR-15a* and *miR-16-1* were found to be down-regulated in 50-60% of CLL cases due to a deletion of chromosomal region 13q14 (Calin *et al.*, 2002). Subsequently, the majority of miRNA genes were found to be located at chromosomal regions that are genetically altered in human cancers (Calin *et al.*, 2004). Following these initial studies many researchers have investigated miRNA expression profiles in various cancer types.

It is now clear that deregulation of miRNA expressions is common in a wide range of human cancers, for example breast cancer (Iorio *et al.*, 2005), lung cancer (Takamizawa *et al.*, 2004), colon cancer (Michael *et al.*, 2003), melanoma (Mueller *et al.*, 2009), gastric cancer (Guo *et al.*, 2009), cervical cancer (Lui *et al.*, 2007; Witten *et al.*, 2010) and Merkel cell carcinoma (Xie *et al.*, 2013).

The observed miRNA deregulation in cancers can be attributed to genomic alterations/mutations, defects in miRNA biogenesis, transcriptional deregulations and epigenetic regulations. Genomic alterations, such as DNA copy number loss or amplification of gene loci-containing miRNA genes, can decrease or increase expression of miRNA genes. In the CLL example, reduction of *miR-15a* and *miR-16-1* in cases with chromosome 13q14 loss, provides a link between genomic alteration and

miRNA expression in cancer. In addition to copy number alterations, germline or somatic mutations have also been reported in the *mir-15a~16-1* gene which leads to low levels of miRNA expression in CLL (Calin *et al.*, 2005).

Evidence for a role of miRNA processing in tumorigenesis was initially demonstrated by Kumar *et al.*, who showed that cancer cells expressing short hairpin RNAs (shRNAs) targeting Dicer, Drosha and DGCR8 (key components of the miRNA processing machinery) reduce mature miRNA levels that enhances cellular transformation and tumorigenesis (Kumar *et al.*, 2007). In addition, mutation and/or deregulation of the key components of miRNA processing factors have been reported in several cancer types (Heravi-Moussavi *et al.*, 2012; Hill *et al.*, 2009; Melo *et al.*, 2009; Merritt *et al.*, 2008).

Several key transcription factors involved in cancers, such as c-Myc and p53, have been shown to regulate miRNA expressions in cancers. For example, the *mir-17~92* cluster is transcriptionally regulated by c-Myc, which is amplified and over-expressed in multiple tumor types. This miRNA cluster has a causative role in lymphomagenesis by coordinating the activation of multiple oncogenic pathways (Jin *et al.*, 2013). Another example is the *miR-34* and *miR-200* families, which are transcriptional targets of p53. Importantly, these p53-regulated miRNAs play important roles in tumor suppression in p53 signaling (Hermeking, 2012).

Epigenetic alterations, such as DNA methylation and histone modifications can also regulate miRNA expression. Numerous studies have demonstrated that miRNAs are deregulated by epigenetic modifications in a variety of cancer types. For example, *miR-203* is hypermethylated in several hematological malignancies (Bueno *et al.*, 2008). In breast cancer, *miR-203* is epigenetically silenced for epithelial-mesenchymal transition and cancer stem cell properties (Taube *et al.*, 2013).

1.4.2 Functions of miRNAs in cancers

miRNAs can suppress the expression of oncogenes (*e.g.* KIT) or tumor suppressors (*e.g.* PTEN), and function as tumor suppressive or oncogenic miRNAs that promote tumor growth by coordinating multiple oncogenic pathways involved in pathways commonly

described as the hallmarks of cancer (Hanahan and Weinberg, 2000, 2011), as illustrated in Figure 1.4.

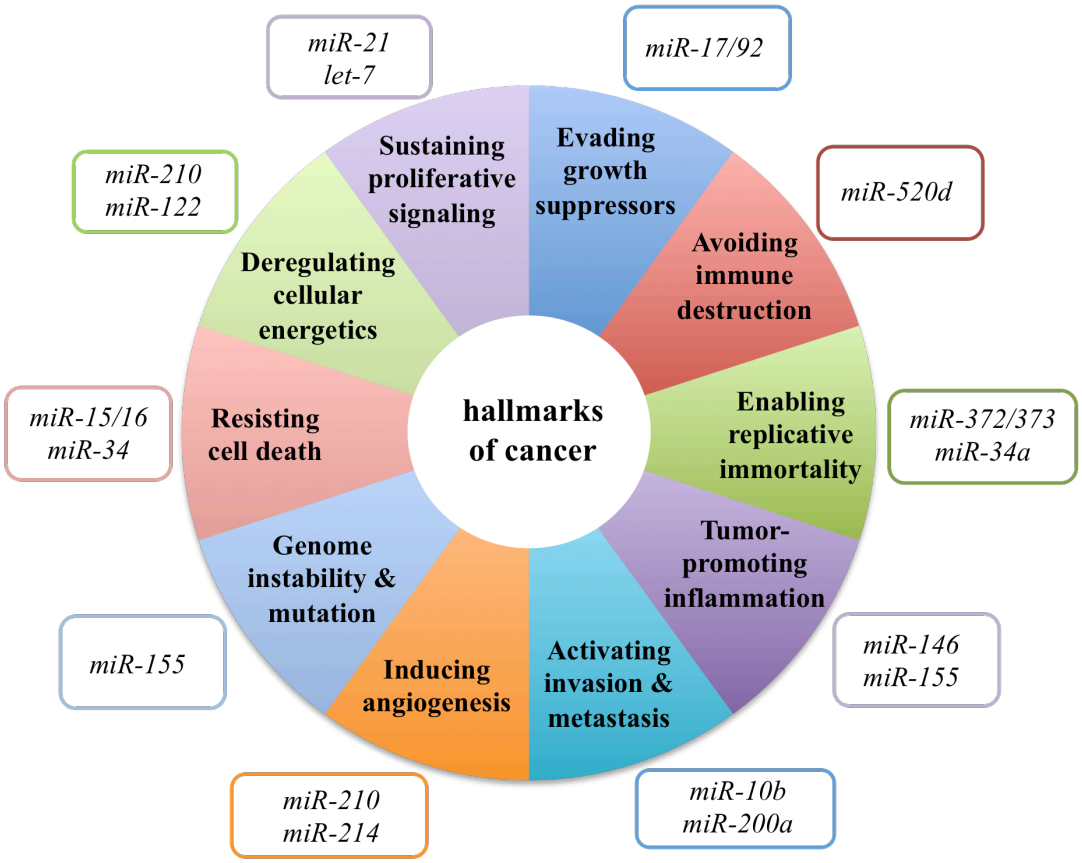

Figure 1.4 Examples of miRNAs involved in cancer.

The list of oncogenic and tumor suppressive miRNAs is growing rapidly, I will give only a few examples here.

1.4.2.1 Oncogenic miRNAs

The first characterized human oncogenic miRNAs come from the *mir-17~92* cluster, which is also known as oncomir-1. This miRNA cluster is located in chromosome 13q31 and transcribed from a single transcript that folds into six stem-loops, including *mir-17*, *mir-18*, *mir-19a*, *mir-20*, *mir-19b* and *mir-92*. These miRNAs can be divided into four different families (*miR-17/20a*, *miR-18a*, *miR-19a/19b* and *miR-92* families) based on their seed sequences. Oncomir-1 has been found highly expressed in many solid tumors and hematological malignancies (Mendell, 2008). The overexpression of *mir-17~92* cluster is either due to gene amplification or activation by c-Myc, as described in previous chapter (O'Donnell *et al.*, 2005). *mir-17~92* cluster was first

demonstrated to accelerate tumor development in a mouse B-cell lymphoma model (He *et al.*, 2005). Depending on cellular conext, this miRNA cluster can promote several aspects of oncogenic transformation, including enhanced cell proliferation (Hayashita *et al.*, 2005), metastasis (Huang *et al.*, 2012), inhibition of apoptosis (Nagel *et al.*, 2009) and senescence (Hong *et al.*, 2010). Interestingly, this miRNA cluster also regulates different signaling pathways in different cancer types. For examples, *mir-17~92* cluster regulates multiple key effectors of the TGF-β pathway in neuroblastoma (Mestdagh *et al.*, 2010), Sonic Hedgehog pathway in medulloblastoma (Uziel *et al.*, 2009), and PI3K/ NF-κB pathways in lymphoma (Jin *et al.*, 2013).

MiR-155 is another oncogenic miRNA, which is commonly up-regulated in several hematological malignancies and solid tumors, including breast, lung, gastric, prostate, colon and pancreatic cancers (Eis *et al.*, 2005; Volinia *et al.*, 2006). *MiR-155* is transcribed from the processing of the B-Cell Integration Cluster (*BIC*), a non-coding transcript expressed in activated B cells, T cells, monocytes and macrophages (Calame, 2007; Tili *et al.*, 2009). Like *miR-17/92* cluster, *miR-155* has multiple roles and targets in different tumor types. For examples, *miR-155* targets histone deacetylase 4 (HDAC4), a co-repressor partner of BCL6, that leads to deregulation of BCL6 transcriptional program for promoting cell survival and proliferation in leukemia/lymphoma (Sandhu *et al.*, 2012). *MiR-155* also reguates HGAL expression and promotes cell motility in lymphoma (Dagan *et al.*, 2012). In breast cancer, *miR-155* directly regulates SOCS1 expression that leads to activation of JAK2/STAT3 pathway and promotes tumor growth (Jiang *et al.*, 2010). In murine mammary gland epithelial cells, *miR-155* is induced by TGF-β/Smad4 pathway and promotes epithelial-mesenchymal transition (EMT), cell migration and invasion by directly targeting of RhoA (Kong *et al.*, 2008). Recently, *miR-155* was shown to promote tumor angiogenesis by targeting tumor suppressor VHL in breast cancer cells (Kong *et al.*, 2013).

1.4.2.2 Tumor suppressor miRNAs

MiR-15a/16-1 are the first identified tumor suppressor miRNAs. The genomic locus of *miR-15a* and *miR-16-1* (13q14) was first reported to be deleted in around 68% of B-cell CLL patients and resulted in decreased expression of *mir-15a~16-1* cluster (Calin *et al.*, 2002). Deletion of this locus and decreased expression of *mir-15a~16-1* cluster have also been reported in several other cancers, including prostate cancer, lung cancer and

pancreatic cancer (Bandi *et al.*, 2009; Bottoni *et al.*, 2005; Klein *et al.*, 2010; Porkka *et al.*, 2011). *miR-15a/16-1* inhibit cell growth, tumor growth and angiogenesis, as well as induce apoptosis and cell cycle arrest (Cai *et al.*, 2012; Dai *et al.*, 2012a; Gao *et al.*, 2011; Luo *et al.*, 2013; Sun *et al.*, 2013). Interestingly, this miRNA cluster has been shown to regulate cell cycle progression in many tumor types by targeting two key cyclin genes, CCND1 and CCNE1 (Bandi *et al.*, 2009; Bonci *et al.*, 2008; Cai *et al.*, 2012; Luo *et al.*, 2013).

MiR-34 family is another example of tumor suppressor miRNA. This family consists of three miRNAs, *miR-34a/b/c*, and was encoded by two transcripts. *miR-34b* and *miR-34c* share one common transcript, while *miR-34a* is encoded by its own transcript. *MiR-34* family members are direct downstream targets of tumor suppressor p53 and their expression frequently downregulated in different cancers due to p53 mutation (Corney *et al.*, 2010). Besides p53 regulation, *miR-34* family can be regulated by promoter DNA methylation (Chim *et al.*, 2010; Lodygin *et al.*, 2008; Tanaka *et al.*, 2012; Wang *et al.*, 2011b). Due to its transactivation by p53, *miR-34* is known to function in p53 signaling (Kim *et al.*, 2011). Interesingly, *miR-34* can also be regulated independently of p53 during oncogene-induced senescence. In this case, *miR-34a* is transcriptionally regulated by ELK1 that targets the MYC proto-oncogene during BRAF-induced senescence (Christoffersen *et al.*, 2010). In non-small cell lung cancer cells, ectopic expression of *miR-34a/c* enhance TRAIL-induced apoptosis and inhibit tumorigenesis (Garofalo *et al.*, 2013). In berast cancer, *miR-34a/c* inhibit breast cancer cells migration and invasion *in vitro* and distal pulmonary metastasis *in vivo* by targeting Fos-related antigen 1 (Fra-1) oncogene (Yang *et al.*, 2013).

It is worth to mention that some miRNAs can function as a tumor suppressor or an oncogene depending on cellular context. An example of such miRNA is *miR-205*, which is described in **Paper I**.

1.4.3 miRNA and viral infection

The first human cancer virus, Epstein-Barr Virus (EBV), was identified in 1964 by Anthony Epstein, Bert Achong and Yvonne Barr from Burkitt lymphoma cells (Epstein *et al.*, 1964). Seven types of viruses are known to be associated with human cancers, including EBV, human papillomavirus (HPV), Hepatitis C virus (HCV), Hepatitis B

virus (HBV), Human T-cell leukemia virus (HTLV-1), Kaposi's sarcoma-associated herpesvirus (KSHV) and Merkel cell polyomavirus (MCV). It is estimated that 15-20% of cancers are associated with virus infection (Parkin, 2006) and appears to be the second most important risk factors for cancer development (zur Hausen, 1991). The majority of tumor virus infected individuals do not develop cancers and the tumor virus may exist in their host cells for several decades. The tumor virus itself is not sufficient for carcinogenesis, additional factors, such as immune suppression, chronic inflammation and host cellular mutations, also contribute to the cellular transformation and tumor development. The complexity of specific interplay between viral factors and host genes is illustrated in Figure 1.5, and the examples are described in the text below.

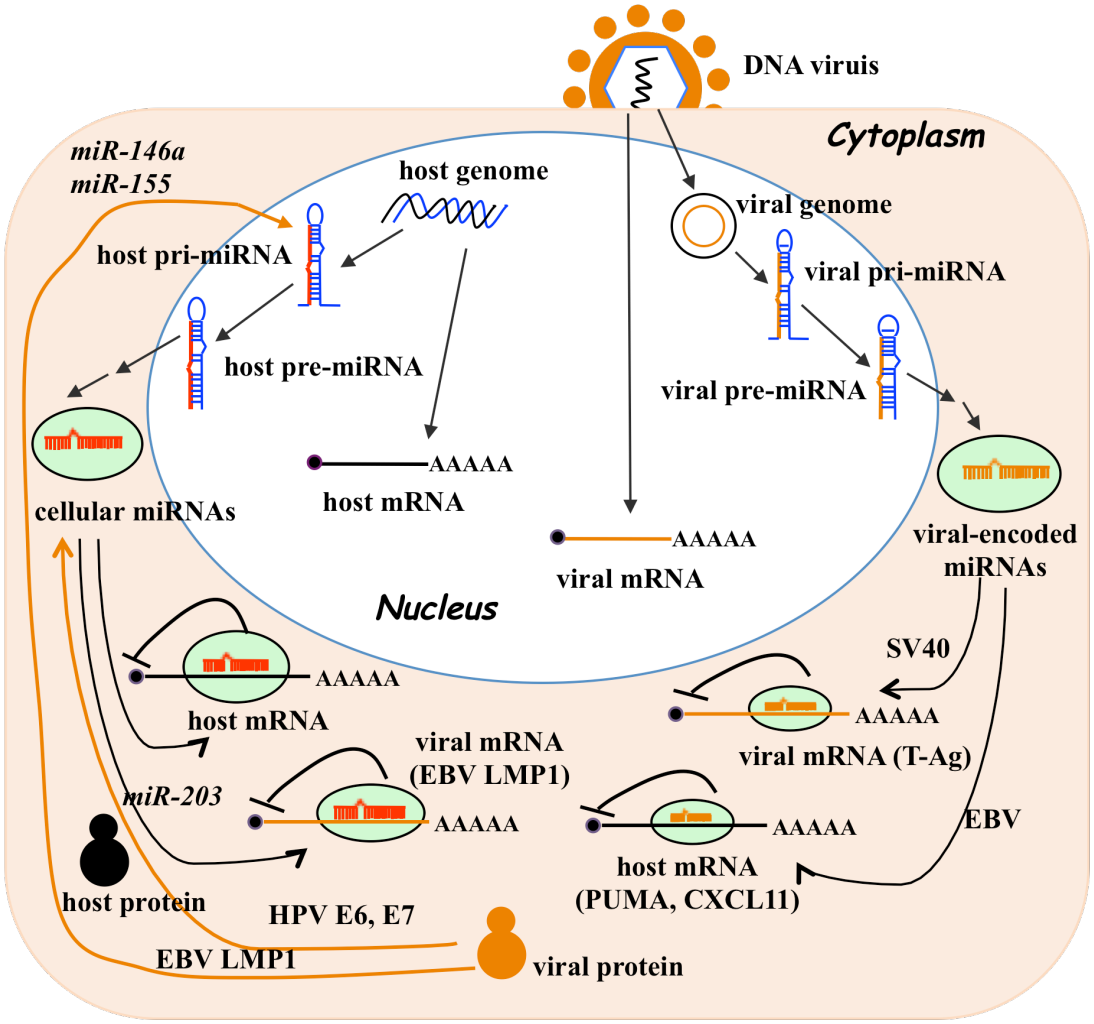

Figure 1.5 The interplays between host and virus.

1.4.3.1 Virus encoded miRNAs

Despite viruses have no miRNA processing machinery, several viruses can encode miRNAs from their viral genome in host cells. The first example of viral-encoded

miRNAs came from cloning experiments in human B-cells latently infected with EBV. These miRNAs were encoded in clusters in two regions of the EBV viral genome (Pfeffer *et al.*, 2004). Since then, several virus types including herpesvirus, polyomavirus, retrovirus and adenovirus, were found to encode miRNAs. Among them, DNA virus accounted for the most viral-encoded miRNAs and members of herpesvirus family contributed the majority of known viral-encoded miRNAs. The first RNA virus encoded miRNAs was identified in bovine leukemis virus (BLV) in 2012. BLV encodes a conserved cluster of miRNAs transcribed by RNA polymerase III (Kincaid *et al.*, 2012). HPV, as a well-studied human tumor virus, is strongly associated with human cervical cancer. Since the discovery of viral-encoded miRNAs, many researchers attempted to hunt for HPV-encoded miRNAs. Despite several studies failed to identify HPV-encoded miRNAs using computational and experimental methods (Cai *et al.*, 2006; Lui *et al.*, 2007), two recent studies have reported HPV encoded miRNAs in cervical tissues and cancer cell lines (Gu *et al.*, 2011; Qian *et al.*, 2013). However, these findings remain controversial and functional studies are required for validation as miRNAs. According to miRBase release 20 (June, 2013) (http://www.mirbase.org/), more than 400 mature viral-encoded miRNAs were identified from 27 different viruses.

Viral-encoded miRNAs are known to modulate both viral and cellular gene expressions. For examples, simian virus 40 (SV40) encoded *miR-S1* located in the antisense strand of viral T antigen mRNAs, which regulates T antigen expression in the late infection (Sullivan *et al.*, 2005). Similar to SV40-encoded *miR-S1*, several other viral miRNAs, *e.g.* CMV-encoded *miR-UL112* and BKV-encoded *miR-B1*, are known to regulate early mRNA expression and viral replications (Broekema and Imperiale, 2013; Grey *et al.*, 2007; Grey and Nelson, 2008; Murphy *et al.*, 2008).

Viral-encoded miRNAs generally target cellular genes involved in cell survival, proliferation as well as antiviral pathways. For examples, EBV *miR-BART5* promotes host cell survival by targeting the PUMA pro-apoptotic gene, a p53-regulated Bcl2 family member (Choy *et al.*, 2008). EBV *miR-BHRF1-3* protects EBV-infected cells from T-cell recognition by directly downregulating the CXC-chemokine ligand 11 (CXCL11) expression (Xia *et al.*, 2008). KSHV miRNAs repress Thrombospondin 1 (THBS1) expression that leads to decreased TGF-β activity (Samols *et al.*, 2007).

1.4.3.2 Interactions of cellular miRNAs and viral proteins

Current evidence indicates that viral proteins can also modulate cellular miRNAs to enhance their replication. For examples, EBV encoded latent membrane protein 1 (LMP1) has been shown to regulate multiple cellular miRNAs, including *miR-29a* (Anastasiadou *et al.*, 2010), *miR-203* (Yu *et al.*, 2012), *miR-10b* (Li *et al.*, 2010), *miR-146a* and *miR-155* (Cameron *et al.*, 2008; Du *et al.*, 2011; Gatto *et al.*, 2008; Motsch *et al.*, 2007) for EBV-induced cellular transformation and oncogenesis. HPV oncoprotein E6 and E7 can inhibit p53 and Rb pathways, respectively. The cellular miRNAs regulated by these two pathways are indirectly influenced by the viral oncoproteins. For example, E6 suppresses *miR-34a* expression through p53 pathway and accelerates cell growth (Wang *et al.*, 2009). Moreover, E6 also reduces *miR-218* expression and subsequently increases LAMB3 expression that enhances cell migration and tumorigenesis (Martinez *et al.*, 2008). Hepatitis B virus X protein (HBx) suppresses *miR-148a* expression to promote cancer growth and cell metastasis in mouse liver cancer model (Xu *et al.*, 2013).

1.5 VIRUS-ASSOCIATED HUMAN CANCERS

This thesis work focused on two types of virus-associated human cancers, which are briefly described here.

1.5.1 Cervical cancer

Cervical cancer is a malignant neoplasm arising from cells originating from the cervix uteri. It is the third most common cancer type among the women worldwide (Jemal *et al.*, 2011). More than 90% of cervical cancers are due to infection and subsequent transformation of cervical cells by specific HPV subtypes (Munoz *et al.*, 2003; Scheurer *et al.*, 2005; zur Hausen, 2002). Persistence infection of high-risk HPV types, such as HPV-16 and -18, appears to be a necessary but not sufficient factor in the development of cervical cancers (Walboomers *et al.*, 1999). With the application of Pap smear test and HPV vaccine, the incidence and mortality of cervical cancer have declined approximately 50% in the United States over the past three decades, but it remains a serious health threat (http://www.cancer.gov/aboutnci/servingpeople/snapshots/cervical.pdf).

In cervical cancer, integration of HPV genome into the host chromosome(s) leads to overexpression of viral oncoproteins E6 and E7 that can bind to cellular tumor

suppressor p53 and pRB, respectively, which interfere with cell cycle control and DNA repair mechanism (Kessis *et al.*, 1993; Li *et al.*, 2013a; Nor Rashid *et al.*, 2011; Shai *et al.*, 2007). In addition, persistent expression of E6 and E7 can accumulate genetic mutations and promote genetic instability (Duensing and Munger, 2002; Ferenczy and Franco, 2002). Several molecular cytogenetic studies revealed specific recurrent chromosome aberrations, *e.g.* gain of chromosome 3q, in cervical cancer (Heselmeyer *et al.*, 1997; Heselmeyer *et al.*, 1996), implying the importance of genes residing in these chromosome regions are likely to play important roles in cervical tumorigenesis (Huang *et al.*, 2005; Huang *et al.*, 2007; Jalali *et al.*, 2010; Rao *et al.*, 2004; Rodolakis *et al.*, 2012; Scotto *et al.*, 2008a; Scotto *et al.*, 2008b; Wilting *et al.*, 2006). Specific genes frequently altered in cervical cancers include amplification of TERC gene (Andersson *et al.*, 2009; Andersson *et al.*, 2006; Heselmeyer-Haddad *et al.*, 2005), PIK3CA amplification (Bertelsen *et al.*, 2006; Ma *et al.*, 2000; Zhang *et al.*, 2002) and AKT phosphorylation (Bertelsen *et al.*, 2006; Ebert *et al.*, 1999).

Altered miRNA expression profiles have been reported in cervical cancer (Lee *et al.*, 2008; Lui *et al.*, 2007; Wang *et al.*, 2008; Witten *et al.*, 2010). Importantly, several deregulated miRNAs, *e.g.* overexpression of *miR-21* and *miR-205*, are consistently observed in different studies (Lui *et al.*, 2007; Wang *et al.*, 2008), supporting the importance of these miRNAs in cervical tumorigenesis. However, the functional roles of these deregulated miRNAs are not fully understood. Part of this thesis work addressed the functional role and targets of two miRNAs that were found overexpressed in cervical cancer (**Papers I** and **II**).

1.5.2 Merkel cell carcinoma

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine carcinoma of the skin, which was first described as "trabecular tumor of the skin" by Cyril Toker in 1972 (Toker, 1972). Some high risk factors, such as fair skin, a history of extensive sun exposure, immune suppression and over age 50, are strongly associated with the development of MCC. Although it is rare, its incidence has increased 4-fold during 1986 to 2006. Around 1,500 new cases of MCC are diagnosed each year in the United States (Bichakjian *et al.*, 2007; Schrama *et al.*, 2012), and ~50% patients with advanced disease can only survive for nine months or less (Tai, 2008). Histopathological features of MCC are shown in Figure 1.6.

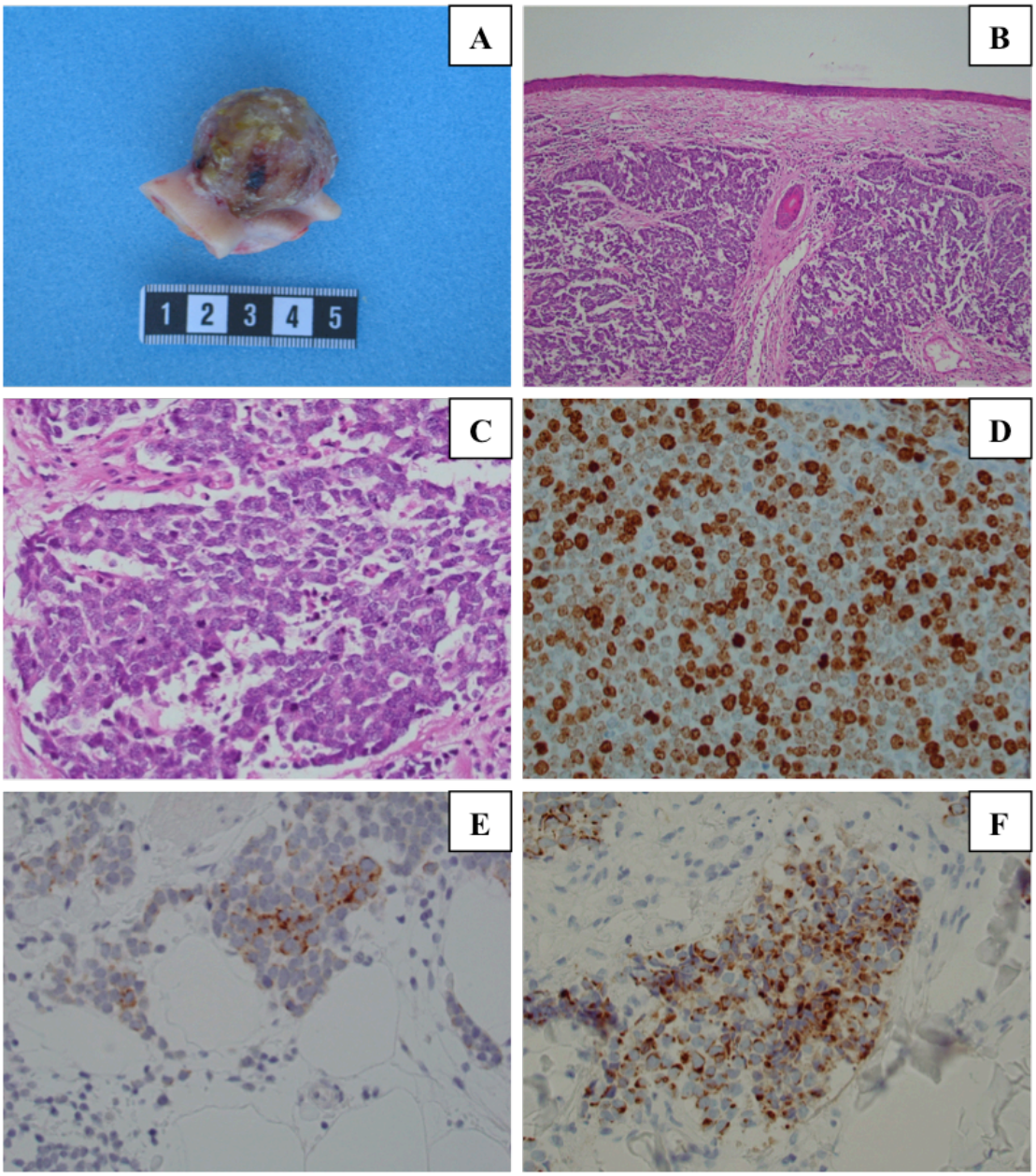

Figure 1.6 Example of Merkel cell carcinoma. **A**: Gross specimen of a Merkel cell carcinoma; **B**: Overview of a MCC tumor area (hematoxylin and eosin staining), 10×; **C**: Larger view of tumor area, 40×; Tumor tissues were stained for **D**: Ki-67, **E**: Chromogranin A, **F**: Cytokeratin 20, 40×.

Around 80% MCC tumors are infected by Merkel cell polyomavirus (MCV) (Feng *et al.*, 2008). This virus is monoclonally integrated into tumor DNA with tumor-specific T-antigen mutations (Feng *et al.*, 2008; Shuda *et al.*, 2008). MCV large T antigen (LT-Ag) was consistently detected in MCC tumors and was required for the maintenance of MCV-positive MCC cell growth (Houben *et al.*, 2010; Shuda *et al.*, 2009; Shuda *et al.*, 2008), suggesting its important roles in MCC tumorigenesis. However, ~20% MCC

tumors have no detectable MCV, indicating different etiologies between MCV-positive and MCV-negative MCCs. Several studies showed the association between MCV status and survival (Bhatia *et al.*, 2010; Laude *et al.*, 2010; Sihto *et al.*, 2009), however no such association could be confirmed by other studies (Handschel *et al.*, 2010; Schrama *et al.*, 2011).

Several reports revealed different genetic and cellular features in MCC tumors. For examples, *L-Myc* amplification is commonly found in MCCs and fewer genomic aberrations are associated with improved survival (Paulson *et al.*, 2009). PI3K/AKT signaling pathway is activated in MCCs and is not associated with MCV status (Hafner *et al.*, 2012; Nardi *et al.*, 2012). Transcriptomic studies identify MCC patients with overexpression of genes associated with cytotoxic CD8+ T lymphocytes have better prognosis (Paulson *et al.*, 2011). Further studies showed that intratumoral immune cells infiltration is associated with favorable survival in MCCs (Sihto *et al.*, 2012), and MCV-specific CD8+ T cells are markedly enriched among the tumor infiltrating lymphocytes as compared with blood (Iyer *et al.*, 2011).

Several studies also showed interactions between viral proteins and cellular molecules. For examples, MCV LT-Ag interacts with the cytoplasmic protein hVam6p resulting its translocation to the nucleus, and sequestering its function in lysosomal trafficking (Liu *et al.*, 2011). The viral small T antigen (sT-Ag) promotes hyperphosphorylation of 4E-BP1 that deregulates cap-dependent translation for cellular transformation and growth (Shuda *et al.*, 2011). sT-Ag also targets the cellular ubiquitin ligase SCF^{Fbw7} to enhance viral replication and to attenuate the degradation of cellular oncoproteins (Kwun *et al.*, 2013). In addition, viral T-antigens also suppress host cell immune response by targeting the NF-κB essential modulator (NEMO) adaptor protein (Griffiths *et al.*, 2013) and Toll-like receptor 9 (TLR-9) (Shahzad *et al.*, 2013). Moreover, Seo *et al.* reported that MCV encoded a miRNA with the ability to autoregulate the virus gene expression using a combination of computational prediction and experimental validation strategies (Seo *et al.*, 2009). The MCV-encoded miRNAs were further confirmed by deep sequencing and qRT-PCR in MCC tumors (Lee *et al.*, 2011). Besides MCV miRNAs, nothing was known about cellular miRNAs in MCCs before our work in **Paper III** (Xie *et al.*, 2013). Recently, Renwick *et al.* showed that specific miRNAs, *miR-375* for MCC and *miR-205* for basal cell carcinoma (BCC), could effectively differentiate between BCC and MCC (Renwick *et al.*, 2013).

However, the functional role of these MCC-specific miRNAs has yet to be explored. The work described in **Paper IV** addressed the functional role of *miR-375* in MCC.

1.6 APPROACHES FOR MIRNA TARGETS IDENTIFICATION

Identification of miRNA targets is important for understanding the functions of miRNAs. However, the methods for identifying miRNA targets are challenging. Here I will discuss several approaches that have been successfully applied for miRNA targets identifications.

1.6.1 Bioinformatic approaches

Several computational programs, such as miRanda, PicTar, PITA, TargetScan, RNA22 and DIANA-microT, have been developed to identify putative miRNA targets. These programs are based on different algorithms such as conservations between species, seed pairing between miRNA and mRNA target sequences, and thermodynamic stability of the miRNA-mRNA interaction (Yue *et al.*, 2009). However, these prediction programs have their inherent limitations. For examples, the complementarity between miRNA and its target mRNA is generally not perfect in animal cells that leads to difficulties for accurate prediction of target sites. Furthermore, most of the prediction programs only consider the binding sites within the 3'-UTR of mRNA, the putative binding sites in the 5'-UTR or CDS will not be detected by these algorithms (Thomson *et al.*, 2011). Emerging evidence indicates that miRNAs can bind to 5'-UTRs and CDS of mRNAs as well as to ncRNAs (Liu *et al.*, 2013; Zhou *et al.*, 2009), several programs have been updated that have the ability to predict miRNA interactions in CDS and 5'-UTR (*e.g.* miR_targets and DIANA-microT-CDS).

In addition, some online databases provide tools to combine putative miRNA-target interactions identified by multiple prediction programs, collection of high-throughput microarray or sequencing data. Some validated targets are also available in some databases, *e.g.* miRWalk, miRTarBase, miRNA_Targets and miRecords.

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1.6.2 Experimental approaches

Apart from the computational approach, several experimental approaches have been developed for miRNA target identification, such as transcriptome analysis, high-

throughput analysis of RNAs isolated from CLIP-based methods (crosslinking immunoprecipitation) and proteomic approaches.

1.6.2.1 Transcriptome analysis

In this approach, the effect of miRNA expression regulation on global gene expression is analyzed directly by microarray or deep sequencing. The first successful example using this approach was reported in 2005 by Lim *et al.* They overexpressed *miR-124* or *miR-1* in HeLa cells and examined the changes of mRNA upon *miR-124* or *miR-1* overexpression. They identified ~100 mRNA transcripts that were significantly downregulated in each case, and motif analysis on the downregulated genes revealed enrichment of the sequences complementary to the 5' seed sequence of each miRNA (Lim *et al.*, 2005). Although this method is straighforward, some of observed changes of gene expression may not be the direct targets of miRNA. Moreover, this method can only detect the targets regulated via mRNA degradation, but not the targets regulated via translational repression.

1.6.2.2 Biochemical approaches

This approach is based on the physical interaction of miRNA-mRNA target in the Ago2-containing complex. A general approach is to purify mRNAs bound to miRNA machinery using Ago2 co-immunoprecipitation (Co-IP), followed by target identification using cDNA microarray or deep sequencing. Several studies, including **Papers I** and **II** in this thesis work, demonstrate the use of this method for identification of miRNA targets (Beitzinger *et al.*, 2007; Hendrickson *et al.*, 2008; Ritchie *et al.*, 2009; Xie *et al.*, 2012).

In 2009, an improved method, called HITS-CLIP (High-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation) was introduced to identifty miRNA targets (Chi *et al.*, 2009). The advantage of this method is capable of identifying specific interaction sites of the direct targets. This method has been applied to identify cellular mRNA targets of viral-encoded miRNA (Haecker *et al.*, 2012). HITS-CLIP is a powerful method, however the use of short wavelength UV (254 nm) for crosslinking has been proven to be inefficient (Hafner *et al.*, 2010). In 2010, a new method called PAR-CLIP (Photoactivable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) was developed in Thomas Tuschl's lab (Hafner *et al.*, 2010). This method is based on the site-specific incorporation of photoreactive ribonucleoside

analogs [*e.g.* 4-thiouridine (^{4S}U) and 6-thioguanosine (^{6S}G)] into RNA transcripts by living cells, followed by UV crosslinking of photoreactive nucleoside-labeled cellular RNAs to interacting RNA binding proteins at 365 nm. This method provides more efficient UV crosslinking and immunoprecipitation, and allows mapping the precise position of crosslinking by mutations (T > C transition, when using ^{4S}U; G >A using ^{6S}G) residing in the sequenced cDNA.

Very recently, another modification of CLIP-sequencing method was applied for identification of miRNA-targets interaction, termed CLASH (crosslinking, ligation and sequencing of hybrids) (Helwak *et al.*, 2013). The advantage of this method is that intramolecular ligation between miRNA and its target is directly performed in the purified miRISC complex, which allows identification of direct targets and their specific interaction sites through chimeric miRNA-mRNA target sequences. Despite this method is specific, only ~2% chimeric reads were obtained based on the published protocol (Helwak *et al.*, 2013). Further improvement of this method is still necessary.

Different from purification of Ago2 complexes, another biochemical approach, which is based on affinity purification of tagged miRNAs (either biotin- or digoxigenin-labeled) associated with their targets (Hsu *et al.*, 2009; Orom and Lund, 2007). This method has been sucessfully applied to identify the targets of *miR-10a* via its interactions in the 5'-UTR of ribosomal protein mRNAs (Orom *et al.*, 2008).

1.6.2.3 Proteomic approaches

Several different proteomic methods have been applied for identification of miRNA targets. In general, these methods can be divided into two groups: (1) direct identification of total protein expressions in cells with and without specific miRNA of interest, and (2) comparison of newly synthesized proteins (by amino acid labeling) that are different between the cells with and without specific miRNA of interest. In the first approach, the pre-existing proteins present in the cells before changes of miRNA expression can complicate the analysis if these proteins have long turnover times. Thus, the advantage of the latter approach is able to measure directly changes in protein synthesis shortly after changes of miRNA expression. An example of such method is called pulsed SILAC (stable isotope labeling with amino acids in cell culture) (Vinther *et al.*, 2006). In this method, different isotopically labeled amino acids are added into growth medium of cells with and without miRNA of interest prior to analysis. Then the

cells are pooled together, digested and analyzed by tandem mass spectrometry. Quantifications of protein expressions are based on the ratio of heavy and light isotopes (Figure 1.7). The application of this method for miRNA target identification has been performed in different cancer types (Ebner and Selbach, 2011; Kaller *et al.*, 2011; Korpal *et al.*, 2011; Lossner *et al.*, 2011; Yan *et al.*, 2011; Yang *et al.*, 2010b).

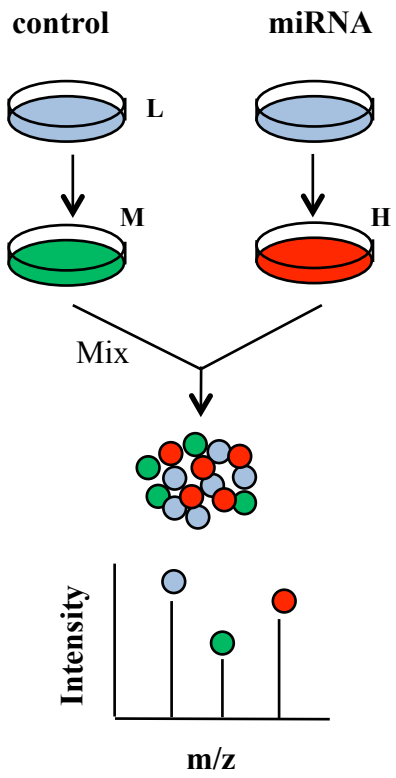

Figure 1.7 pSILAC for miRNA target identification.

2 AIMS OF THE STUDY

The overall aim of this thesis work was to investigate the expression and function of microRNAs in two types of human virus-related cancers, *i.e.* cervical carcinoma associated with Human Papilloma Virus (HPV) and Merkel cell carcinoma (MCC) associated with Merkel cell polyomavirus (MCV). Specifically, we aimed to:

- Characterize the functional roles of *miR-205* in cervical cancer cell lines and identify its transcriptome-wide targets by CLIP-Chip (**Paper I**).

- Identify functions and targets of *miR-944* in cervical cancer cell lines (**Paper II**).

- Determine specific miRNA expressions associated with MCV status and clinical outcomes of MCC cases, as well as the functional role of specific miRNA in MCC cell lines (**Paper III**).

- Investigate the regulation and functions of *miR-375* in MCC cell lines (**Paper IV)**.

3 MATERIALS AND METHODS

3.1 HUMAN TISSUE SAMPLES

Two types of human cancers, cervical carcinoma and Merkel cell carcinoma, were investigated in this thesis work. All tumor and normal tissue samples used in this thesis work had been re-evaluated and verified by histopathological examination of hematoxylin and eosin-stained paraffin sections.

3.1.1 Cervical carcinoma (Paper I)

Thirty pairs of snap-frozen cervical tumor and matched normal tissues were obtained from the Gynecologic Oncology Group Tissue Bank (Columbus, Ohio). The available clinical information of the cases is given in Table 3.1.

Table 3.1 Clinical information of the 30 paired cervical cancer samples

Sample ID	Age (years)	Diagnosis
G220	NA	ADC
G659	NA	ADC
G696	NA	ADC
G761	NA	ADC
G691	29	ADC
G547	60	ADC
G701	33	ASC
G871	47	ASC
G013	53	SCC
G603	48	SCC
G702	25	SCC
G026	62	SCC
G243	30	SCC
G001	NA	SCC
G428	38	SCC
G507	52	SCC
G601	55	SCC
G727	NA	SCC
G531	49	SCC
G612	NA	SCC
G699	57	SCC
G623	35	SCC
G645	70	SCC
G529	NA	SCC
G648	NA	SCC
G576	48	SCC
G850	50	SCC
G613	48	SCC
G652	46	SCC
G575	NA	SCC

SCC, squamous cell carcinoma; ADC, adenocarcinoma;
ASC, adenosquamous cell carcinoma; NA, not available.

3.1.2 Merkel cell carcinoma (MCC) (Paper III)

Formalin-fixed paraffin-embedded (FFPE) MCC tumor materials (33 samples from 26 patients) were collected for patients operated at Karolinska University Hospital or Stockholm South General Hospital in the period 1986 to 2003. The MCC diagnosis was established by routine histopathological and immunohistochemical analyses at the time of diagnosis. Representative sections from all specimens were histopathologically re-evaluated to confirm high tumor content (>80% tumor cells). The study was approved by the Ethics Committee of Karolinska Institutet. All clinical and histopathological information of the patients are given in Supplementary Table S5 in **Paper III**.

3.2 ESTABLISHED HUMAN CELL LINES

Seven human cervical cancer and six MCC cell lines, as well as HEK293 cell line from human embryonic kidney were used in this thesis. The cell lines are briefly described below, and details about diagnosis and viral status are given in Table 3.2. The culturing conditions are described in the individual papers.

3.2.1 Cervical cancer cell lines

Seven human cervical cancer cell lines were used in **Papers I** and **II**: HeLa, CaSki, SW756, ME-180, SiHa, C4I and C33A. All cell lines were originally purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were kindly provided by Dr. Keng-Ling Wallin (Karolinska Institutet, Stockholm, Sweden). CaSki and ME-180 were derived from metastases, and the other five cell lines were established from primary tumors. The two main cell lines used for functional studies in **Papers I** and **II** (CaSki and HeLa) were recently re-evaluated and verified by short tandem repeats (STR) profiling at Bio-Synthesis, Inc (Lewisville, TX).

3.2.2 Merkel cell carcinoma (MCC) cell lines

Six human MCC cell lines were used in **Papers III** and **IV**. The three MCV-negative MCC cell lines, *i.e.* MCC13, MCC14/2 and MCC26, were purchased from CellBank Australia (Westmead, Australia). The three MCV-positive MCC cell lines, *i.e.* MKL-1, MKL-2 and WaGa, were kindly provided by Drs. Nancy L. Krertt (Northwestern University, Chicago, IL), Roland Houben (University Hospital Würzburg, Würzburg,

Germany) and Jürgen C. Becker (Medical University of Graz, Graz, Austria), respectively.

3.2.3 Control cell line

The HEK293 cell line was used in **Paper IV**, as a MCV-negative non-MCC control, to investigate the effect of MCV LT-Ag over-expression on *miR-375* expression. This cell line was originally purchased from ATCC and kindly provided by Dr. Yingbo Lin (Karolinska Institutet, Stockholm Sweden).

Table 3.2 Cell lines used in this thesis work.

Name	Description	Virus status	Subtype	Paper
CaSki	Cervical carcinoma	HPV16+	SCC	I, II
HeLa	Cervical carcinoma	HPV18+	ADC	I, II
SW756	Cervical carcinoma	HPV18+	SCC	I, II
ME-180	Cervical carcinoma	HPV68+	SCC	I, II
SiHa	Cervical carcinoma	HPV16+	SCC	I, II
C4I	Cervical carcinoma	HPV18+	SCC	I, II
C33A	Cervical carcinoma	HPV-	SCC	I, II
MCC13	Merkel cell carcinoma	MCV-	Variant	III, IV
MCC14/2	Merkel cell carcinoma	MCV-	Variant	III, IV
MCC26	Merkel cell carcinoma	MCV-	Variant	III, IV
WaGa	Merkel cell carcinoma	MCV+	Classic	III, IV
MKL-1	Merkel cell carcinoma	MCV+	Classic	III, IV
MKL-2	Merkel cell carcinoma	MCV+	Classic	IV
HEK293	human embryonic kidney	no HPV, no MCV	NA	IV

HPV, human papillomavirus; MCV, Merkel cell polyomavirus;
SCC, squamous cell carcinoma; ADC, adenocarcinoma; NA, not available.

3.3 MIRNA EXPRESSION STUDIES

3.3.1 miRNA expression profiling (Paper III)

Agilent's human miRNA microarray (version 2; miRBase release 10.1; Agilent, Santa Clara, CA) was used to characterize global miRNA expression profiles of 16 MCC

tumor samples. Total RNA was extracted using a combination of proteinase K treatment and TRIzol reagent giving an appropiate yield suitable for analysis of small RNA.

This microarray platform has a unique design that allows the detection of mature miRNAs with very low amount of RNA input (as low as 100 ng total RNA) and no size fractionation and amplification of RNA is required. The RNA was directly labeled using an enzymatic reaction (T4 RNA ligase) that attaches a single fluorophore-labeled cytosine (pCp-Cy3) to the 3'-end of each miRNA, which is complementary to the additional guanosine (G) at the 5'-end of the hybridization sequence in the probe. This type of probe design together with different lengths of the probes and an incorporation of 5'-end hairpin structure for each probe provide similar melting temperatures for different miRNAs and increase the specificity of the probes to mature miRNAs. The probe design is illustrated in Figure 3.1.

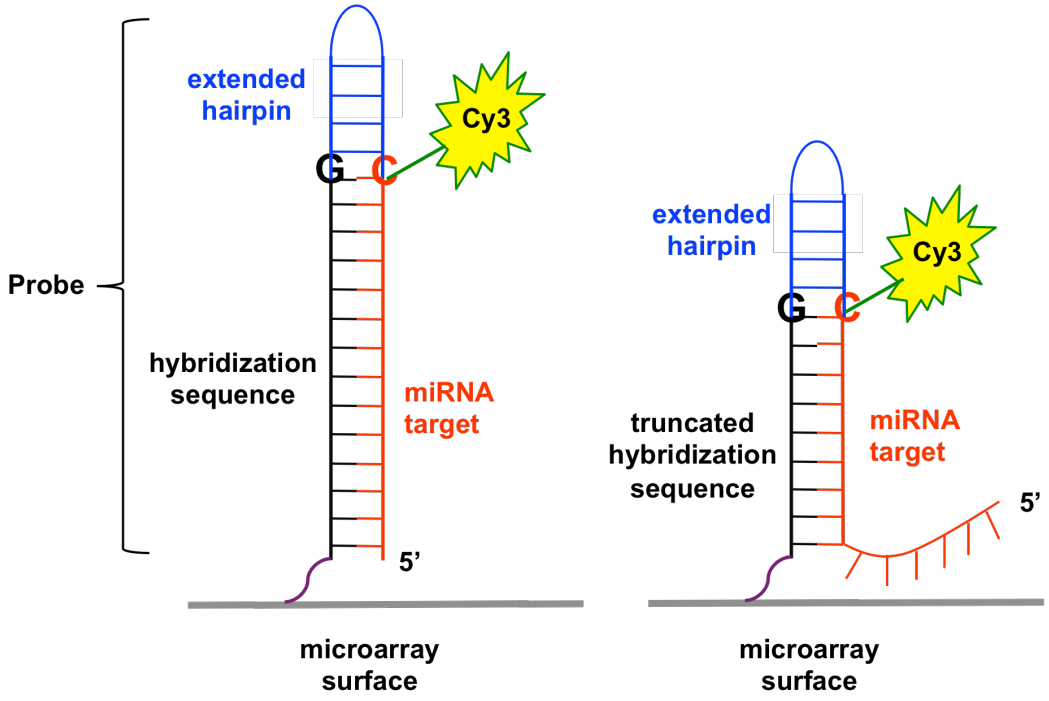

Figure 3.1 Design of Agilent miRNA microarray probe [Modified from (Wang *et al.*, 2007)]. The extended hairpin (blue) at the 5'-end of the probes stabilizes the probe-target interaction (left) and destabilizes the hybridization to non-target RNAs (right).

The experimental procedures are described in detail in **Paper III**. In brief, 200 ng of total RNA was labeled with Cy3 and then hybridized onto the arrays for 20 h at 55°C. After the washing step, the slides were scanned using Agilent microarray scanner. The

images were processed with Feature Extraction Software 10.7.3.1 (Agilent). Intensity values were normalized and median centered using Cluster 3.0. Only normalized miRNAs with less than 20 % missing values across the samples were used for clustering and statistical analyses. Hierarchical clustering was performed based on complete linkage with the Spearman rank correlation using Cluster 3.0 and visualized with Treeview v1.60 (de Hoon et al., 2004).

3.3.2 Quantitative reverse transcription PCR (Papers I-IV)

Quantitative reverse transcription PCR (qRT-PCR) is a method that enables reliable detection and measurement of cDNA in a given sample through a PCR reaction. There are two major types of qRT-PCR methods: TaqMan and SYBR Green assays. The TaqMan method requires a set of specific primers and the TaqMan probe, which is a sequence-specific DNA probe consisting of a fluorphore at the 5'-end and a quencher at the 3'-end. When the fluorophore and the quencher are in proximity, the quencher inhibits any fluorescence signal. During the PCR reaction, the Taq polymerase extends the primer and degrades the TaqMan probe through its 5' to 3' exonuclease activity when it reaches the probe. Degradation of the probe releases the quenching effect of the quencher, thus enables the detection of fluorescence signal. The SYBR Green method is based on the detection of fluorescence signal from the intercalation of the SYBR Green dye into the newly synthesized double-stranded DNA during the PCR reaction. For both methods, the amount of fluorophore released from the TaqMan probe or intercalation is directly proportional to the amount of DNA template present in the PCR, thus provides a quantitative analysis of the gene or transcript analyzed. In this thesis, all qRT-PCR analysis of small miRNA and long mRNAs were performed using the TaqMan-based method. The results were normalized to the expression of control transcripts analysed in parallel, *i.e. RNU6B* for miRNAs and *18S* for mRNAs.

For mature miRNA detection, the TaqMan miRNA assays apply a novel design strategy to include a target-specific stem-loop reverse transcription primer. This primer addresses the short length of mature miRNA, extends the 3'-end of the target to produce a template that can be used in standard TaqMan based real-time PCR. The stem-loop structure in the tail of the primer only allows the specific detection of the mature miRNA, not the primary or precusor miRNAs. The TaqMan miRNA assay design is illustrated in Figure 3.2. For mRNA analysis, the cDNA was synthesized

using random hexamer and qRT-PCR assays with a probe spanning an exon-exon junction were chosen.

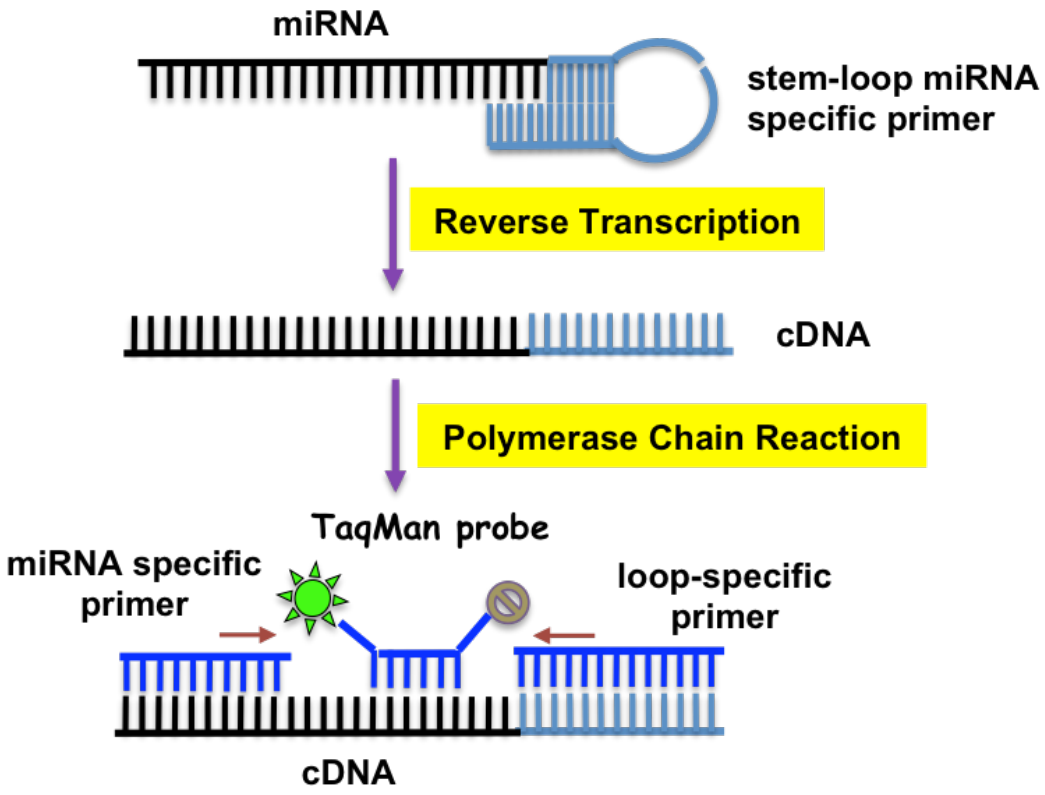

Figure 3.2 Schematic illustration of TaqMan miRNA assays.

In **Paper I**, qRT-PCR was used to quantify expression levels of mature *miR-205* in clinical samples and cell lines and to verify *CYR61* and *CTGF* as candidate targets of *miR-205*. In **Paper II**, mature *miR-944* expresion levels were determined by qRT-PCR in cell lines. In **Paper III**, microarray results were verified by qRT-PCR for selected miRNAs including *miR-203*, *miR-30a-5p*, *miR-30a-3p*, *miR-769-5p*, *miR-34a*, *miR-375*, *miR-148*, *miR-21*, *miR-150*, *miR-146a*, *miR-483-5p* and *miR-630*. *Survivin* expression in MCC cell lines upon *miR-203* over-expression was also evaluated by qRT-PCR. In **Paper IV**, mature *miR-375* expression levels were quantified by qRT-PCR in MCC and HEK293 cell lines with and without treatment of epigenetic inhibitor(s) or MCV T-antigen(s).

3.4 *IN VITRO* TRANSFECTION

Transfection is a process of introducing nucleic acids into eukaryotic cells. This process typically requires transient opening pores in the cell membrane to allow the uptake of

nucleic acids. Transfection can be carried out by calcium phosphate, electroporation or liposomes (achieved by mixing cationic lipids with nucleic acids), which can fuse with cell membrane and deposit the materials into the cells.

There are several transfection methods commonly divided into chemical based and non-chemical methods. ***Chemical-based transfections*** include liposomes, nanoparticles and polymers. Liposome transfection is a very efficient method that packs DNA/RNA and fuses with cell membrane and releases the DNA/RNA into cells due to their similarities to the cell membrane structure. Cationic polymers, such as polyethylenimine and diethylaminoethyl-dextran, can bind DNA/RNA due to their negative charges and are taken up by cells via endocytosis. ***Non-chemical methods*** mainly involve electroporation. Nucleofection is an electroporation method, which uses a combination of cell-type specific reagents with electrical parameters that enables delivery of nucleic acid directly into the nucleus of the cells. This method is suitable for difficult-to-transfect cell lines, primary cells and non-dividing cells. In this thesis work, both chemical-based and nucleofection methods had been applied. A brief description of these transfection methods are mentioned below, and the details are available in **Papers I-IV**.

3.4.1 Transfection of miRNA inhibitors and mimics

MiRNA inhibitors are chemically modified [generally 2'-O-methoxy group (2'-OMe) or 2'-O-methoxy-ethyl group (2'-MOE)] single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA molecules. MiRNA mimics are small chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs. These miRNA mimic molecules are modified to ensure that the correct strand, representing the desired mature miRNA, is loaded into the RISC responsible for miRNA activities. The negative controls are similar to the miRNA inhibitor and miRNA mimic molecules with non-targeting random sequences and have been validated not produce identifiable effects on any known miRNA function.

In **Papers I** and **II**, cervical cancer cell lines were transfected with anti-miRs (*miR-205* and *miR-944*), pre-miRs (*miR-205* and *miR-944*) and the respective negative controls for functional studies as well as for target identifications and validations. The

transfection experiments were performed using siPORT NeoFX transfection agent, which is a lipid-based formulation and used to efficiently transfect adherent cells.

In **Papers III** and **IV**, MCC cell lines were transfected with mirVana miRNA mimics (*miR-203* or *miR-375*) and negative controls for functional studies and target identification and validations. The transfection experiments were performed using Lipofectamine RNAiMAX transfection reagent, which is a proprietary RNAi-specific cationic lipid formulation designed specifically for the delivery of siRNA and miRNA into cells.

3.4.2 Plasmid transfection

Plasmids are small circular double-stranded DNA molecules that are commonly used as vectors to multiply or express particular gene(s) of interest. In this thesis work, three types of expression vectors were used, *i.e.* protein expression, short hairpin RNA (shRNA) and luciferase reporter vectors.

In **Papers III** and **IV**, plasmids were used to express the transgenes (*i.e.* wild-type and the truncated MCV LT-Ag) in MCV-negative MCC cells. shRNA vectors were used to express shRNAs targeting the viral T-antigens in MCV-positive MCC cells. All MCV-negative cell lines (MCC13, MCC14/2 and MCC26) were transfected with Lipofectamine 2000 transfection reagent, while the MCV-positive cell lines (WaGa, MKL-1 and MKL-2) were transfected with Nucleofector transfection.

Luciferase reporters are commonly used to monitor transcriptional activity or gene expression in the cells. These reporters contain the gene of interest coupled to a luciferase gene, as a reporter gene. In **Paper II**, the pmirGLO vector, which has an insertion of miRNA target site at the 3'-end of the firefly luciferase gene, was used to evaluate miRNA activity. The interaction between the miRNA and the miRNA target site will lead to reduction of the firefly luciferase expression. This vector also expresses *Renilla* luciferase, which acts as a control reporter for normalization purpose. In this study, the luciferase reporter and *miR-944* mimic were co-transfected using Lipofectamine 2000 transfection reagent to validate the direct interaction between *miR-944* and its candidate targets (*HECW2* and *S100PBP*) in HeLa cells.

3.5 DETECTION OF MERKEL CELL POLYOMAVIRUS (MCV)

3.5.1 PCR and sequencing

Polymerase chain reaction (PCR) is a generally applied biochemical technology to amplify a specific region of a DNA strand. This method consists of repeating cycles of heating and cooling reactions for DNA melting and replication, which allows the detection of a specific DNA sequence also at low abundance in the sample. To determine whether the amplified DNA products are specific to the target sequence of interest, the amplicon can be sequenced to determine its identity. The sequencing method is based on the selective incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerase during *in vitro* DNA replication, and was developed by Frederick Sanger and coworkers in 1977 (Sanger *et al.*, 1977). The sequencing procedure requires a single strand DNA template, a DNA primer, polymerase, normal dNTPs and modified nucleotides (dideoxyNTPs, ddNTPs). ddNTPs lack the 3'-OH group which is required for the formation of phosphodiester bond. The incorporation of ddNTPs into the DNA strand terminates the extension of the DNA strand, which is then recorded by the detector. In **Paper III**, PCR was used to detect the presence of MCV DNA in genomic DNA samples of MCC tumors. A subset of the PCR products was sequenced to verify the MCV genomic sequence.

3.5.2 Immunohistochemistry

Immunohistochemistry (IHC) is used to identify and localize specific proteins in cells on tissue sections. This method is based on specific binding of antibodies to target antigens (*i.e.* proteins) in tissue samples. The antibody-antigen interaction can be visualized by using a secondary antibody conjugated with an enzyme (*e.g.* alkaline phosphatase, AP and horseradish peroxidase, HRP) or a fluorophore.

In **Paper III**, IHC was used to detect expression of MCV LT-Ag in MCCs. Two different antibodies were applied: the commercially available CM2B4 antibody and the Ab3 antibody (kindly provided by Dr. James A. DeCaprio, Dana-Farber Cancer Institute, Boston, MA). The immunoreactivity was scored based on the nuclear staining intensity, and reported as negative (-), weak (+), moderate (++) or strong (+++).

3.6 FUNCTIONAL STUDIES *IN VITRO*

An overview of the functional studies performed in this thesis work is shown in Figure 3.3, and the methods are briefly described in this chapter.

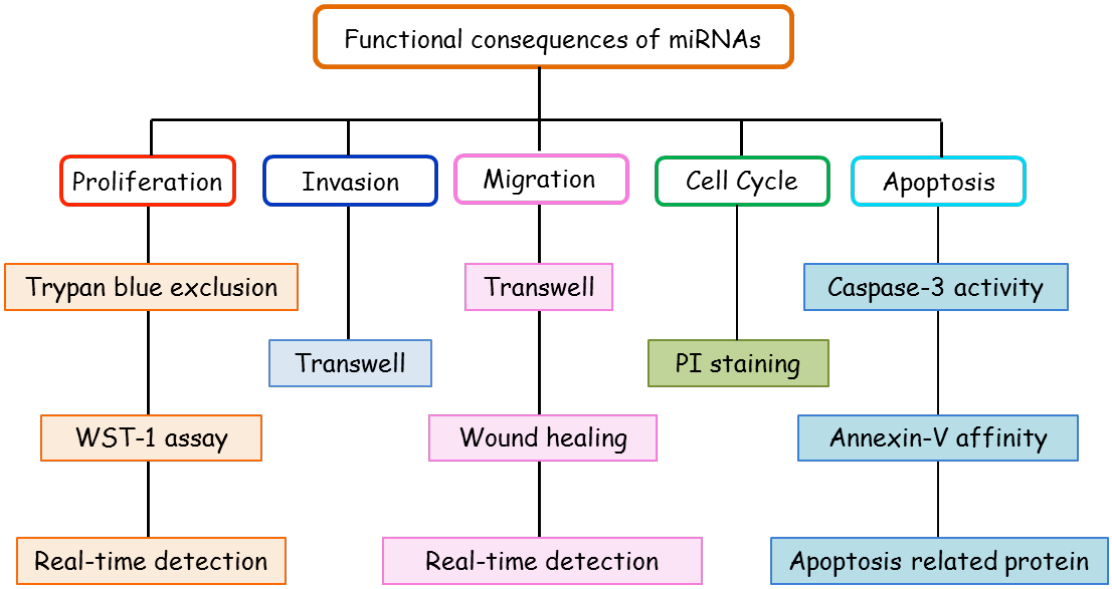

Figure 3.3 An overview of the functional analysis included in this thesis work.

3.6.1 Cell proliferation

There are five major types of cell proliferation detection methods, which are based on different principles: (1) New DNA synthesis through incorporation of a labeled nucleotide, *e.g.* ^{3}H-thymidine, 5-Bromo-2'-deoxyuridine (BrdU) or 5-Ethynyl-2'-deoxyuridine (EdU) incorporation; (2) Metabolic activity of a cellular enzyme from living cells that can reduce tetrazolium salts to colored formazan compounds, *e.g.* MTT, XTT, MTS and WST-1 assays; (3) Antigens associated with cell proliferation, such as Ki-67 and PCNA (proliferating cell nuclear antigen); (4) Measurement of ATP. While ATP is required for biological processes in living cells, dying or dead cells contain little or no ATP; and (5) Trypan blue exclusion. Live cells possessing intact cell membranes have no uptake of trypan blue dye, whereas dead cells absorb the dye. In this thesis, three types of cell proliferation assays were used, as described below.

3.6.1.1 The WST-1 assay

This assay is based on the detection of metabolic activity of viable cells, as mentioned above. WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-

benzenedisulfonate) is a stable tetrazolium salt, which can be cleaved to a soluble formazan by the mitochondrial succinate dehydrogenase of metabolically active viable cells. The amount of formazan dye formed directly correlates to the number of metabolically active cells. Therefore the method is also a cell viability assay. WST-1 is preferable than other tetrazolium salts such as MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). MTT is insoluble in standard culture medium and the formazan crystals produced must be dissolved in dimethyl sulfoxide (DMSO) or isopropanol, whereas WST-1 is a nontoxic reagent that is soluble in culture medium and which can be used for continuous monitoring to follow dynamic changes over time. Due to its robustness, the WST-1 assay was chosen as the primary method for cell growth measurements in this thesis work (**Papers I-IV**).

3.6.1.2 The trypan blue exclusion assay

Trypan blue ($C_{34}H_{28}N_6O_{14}S_4$) is derived from toluidine and commonly used to evaluate cell viability. The dye is only taken up by dead cells where it will appear as a distinct blue color. This method can be used to determine cell viability in a cell population but it can not distinguish between necrotic and apoptotic cells. In **Paper III**, this method was used to complement the WST-1 assay for cell growth analysis.

3.6.1.3 The xCELLigence system for cell proliferation analysis

The xCELLigence system is a cell-based label-free platform. It utilizes the inherent morphological and adhesive characteristics of the cell as a quantitative readout. It is also called real-time cell analysis. The principles of this system are illustrated in Figure 3.4. In this system, cells are placed in wells of a special plate with gold micro-electrodes at the bottom. The interaction of adherent cells with the micro-electrodes leads to the generation of a cell-substrate impedance response that is related to the number of cells in the well, the morphology of the cells and the quality of cell attachment. The instrument measures impedance (electrical resistance) in wells during the entire time course of an experiment. When cells cover the electrodes, impedance increases. The impedance is recorded as the cell index values. This system was used to evaluate the effect of cell proliferation upon *miR-944* inhibition in CaSki cells in **Paper II**.

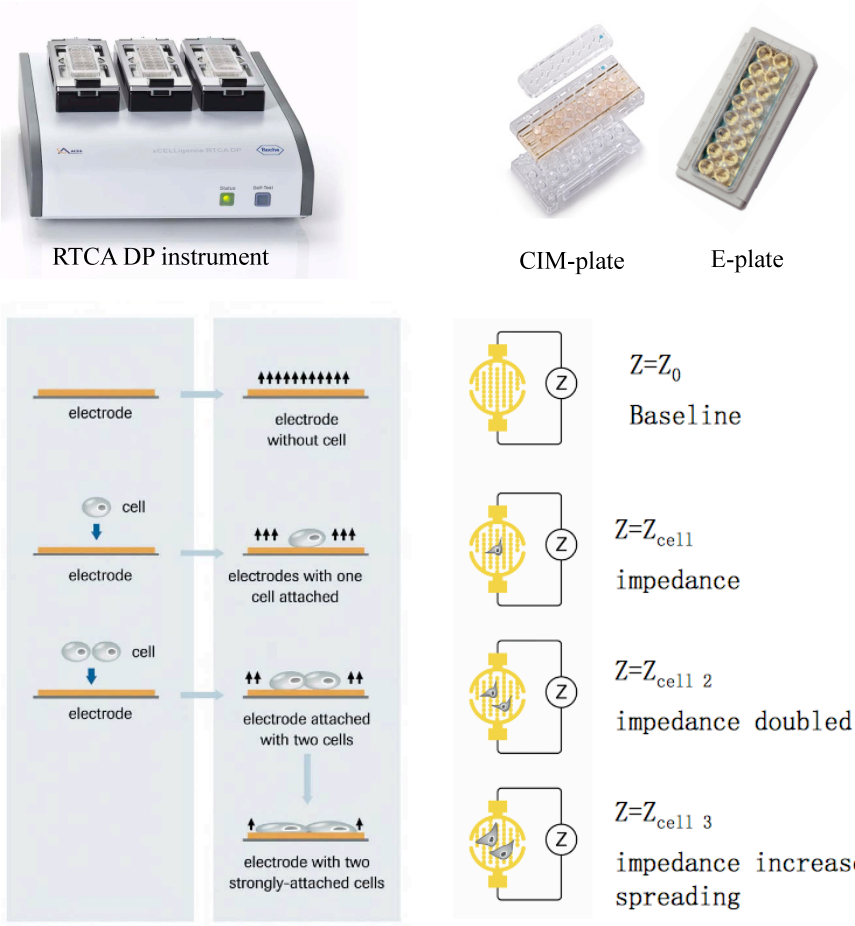

Figure 3.4 xCELLigence Real Time Cell Analysis system (http://www.roche-applied-science.com).

3.6.2 Cell migration and invasion

Cell migration and cell invasion are multi-step processes of importance for the development of metastasis. Two of the most commonly used methods for studying cell migration are the transwell assay and the wound healing assay. The transwell assay can also be used to study cell invasion.

3.6.2.1 The transwell assay (**Papers I** and **II**)

The transwell assay, also called Boyden chamber transwell assay, was originally introduced by Boyden for the analysis of leukocyte chemataxis (Boyden, 1962). It is based on a chamber of two medium-filled compartments separated by a membrane with pores. Cells are seeded in the upper chamber in serum-free medium, while serum or other similar chemoattractants are placed in the lower chamber. After incubation, migratory cells moving through the pores towards the chemoattractants can be fixed, stained, visualised and quantified. Similarly, the cell invasion potential can also be determined by coating the membrane with extracellular matrix protein (such as

matrigel) before seeding cells. Cells with invasive properties can penetrate the matrigel layer and reach the lower surface of the membrane (Figure 3.5).

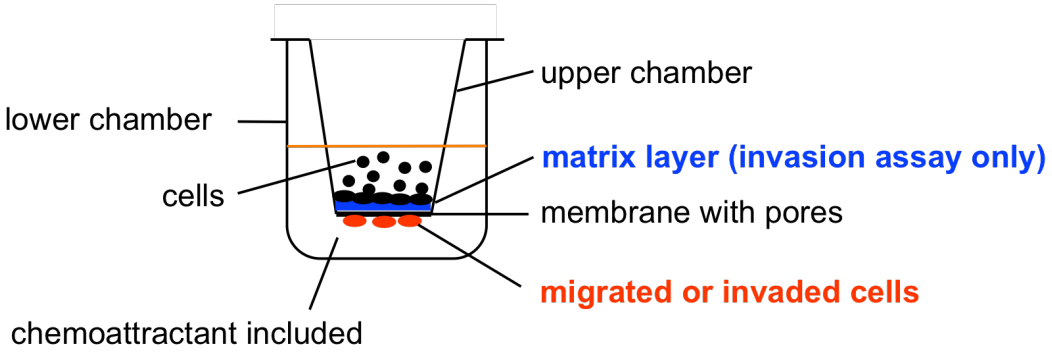

Figure 3.5 The transwell assays. Single cell suspensions are seeded in the upper chamber. At the end of analysis, migrating or invading cells are fixed to bottom side of membrane and stained for visualization and further quantification.

3.6.2.2 The wound healing migration assay (**Papers I, II** and **IV**)

In the wound healing assay, a wound (also called a scratch or a gap) is introduced in a cell monolayer. The open gap is then inspected over time under microscope as the cells will move in and fill the damaged areas. By comparing wound closure at different time points, the relative migration rate of the cells can be calculated.

3.6.2.3 The xCELLigence system for cell migration analysis (**Paper II**)

This system is similar to the cell proliferation measurement (see 3.6.1.3), with the difference that a CIM-plate is used instead of E-plate applied in cell proliferation measurement. The CIM-plate is similar to the transwell assay described above. It has an upper chamber and a lower chamber. The upper chamber is sealed at its bottom by a micropore polyethylene terepthalate (PET) membrane. The bottom side of the membrane contains interdigitated gold microelectrode sensors. The migrating cells that pass through the membrane will generate impedance signals. The readout from this detection shows the kinetic of cell migrations throughout the entire experiment.

3.6.3 Cell cycle analysis

The cell cycle is a series of events involved in cell division and duplication. Cell cycle analysis aims to determine the cell cycle phases. Flow cytometry is generally performed to measure the cell cycle by determining the DNA contents in a cell population, which are stained with a fluorescent dye such as propidium iodide (PI). The

fluorescence intensity of the stained cells correlates with the DNA content of the cells. The DNA content during the G2 and M phases is double to that of cells in the G1/G0 phases because the DNA content of cells duplicates during the S phase of the cell cycle.

In **Papers II-IV**, cell cycle analysis was used to determine the effect of miRNAs over-expression and/or inhibition (*miR-944*, *miR-203* and *miR-375*) in cervical cancer and MCC cell lines. The experimental procedure is described in detail in **Papers II-IV**. In brief, cells were collected and washed with cold phosphate buffered saline (PBS), fixed with 50% ethanol, followed by RNase A treatment and PI staining. The stained cells were quantified by flow cytometry and analyzed by FlowJo software.

3.6.4 Cell apoptosis

Apoptosis, or programmed cell death, is controlled by a diverse range of cell signals providing targets for its evaluation. There are several golden standard methods to study apoptosis including TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay to detect DNA fragmentation, flow cytometry technology to detect mitochondrial membrane potential (*e.g.* JC-1 dye) or membrane associated proteins (*e.g.* Annexin V) and caspase-based detection methods.

3.6.4.1 The caspase-3 colormetric apoptosis assay (**Papers I-III**)

Caspases, a family of cysteine proteases with 14 identified members, are the central regulators of apoptosis. Caspase-3 is an important downstream effector of apoptosis. The caspase-3 colormetric apoptosis assay is based on spectrophotometric detection of chromophore p-nitroaniline (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA by active caspase-3. In **Papers I-III**, this assay was performed to determine the effect of cell apoptosis upon *miR-205* and *miR-944* overexpression/inhibition in cervical cancer cell lines (CaSki and HeLa) as well as *miR-203* overexpression in MCC cell lines (MCC13 and MCC14/2).

3.6.4.2 The Annexin V affinity assay (**Paper II**)

Phosphatidylserine (PS) is normally located in the inner membrane leaflet of viable cells. At the early stage of apoptosis, it translocates from the inner membrane to the outer leaflet and leads to membrane-mediated apoptosis. Annexin V has a very high affinity for PS, and is commonly used as a probe to detect early apoptosis. This assay

typically uses a conjugate of Annexin V and a fluorescent (*e.g.* fluorescein isothiocyanate, FITC). The binding between Annexin V and PS is Ca^{2+} dependent. With the combination of cell nucleus PI staining or 7-aminoactinomycin D (7-AAD) staining, we can distinguish the dead cells or the late apoptotic cells from the early apoptotic cells. The detection can be performed by flow cytometry or fluorescence microscopy. In **Paper II**, flow cytometric detection of Annexin V conjugated with FITC and PI staining was used to determine the effects of cell apoptosis upon *miR-944* overexpression and inhibition in cervical cancer cells (Caski and HeLa).

3.6.4.3 Detection of caspase-3 and PARP cleavage products (**Paper IV**)

As mentioned earlier, caspase-3 is a crucial component of apoptotic machinery in many cell types. Activation of caspase-3 is an important apoptosis marker that is widely applied. When the cells undergo apoptosis from mitochondrial or death ligand pathways, pro-caspase-3 is activated and the cleavage of caspase-3 requires proteolytic processing from its inactive full-length form (around 32 kDa) into activated p17 and p12 fragments. Furthermore, active caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates such as caspase-8, caspase-9 and Poly ADP ribose polymerase (PARP). PARP is involved in DNA damage repair. In cells undergoing apoptosis, the full-length PARP (116 kDa) is cleaved by active caspase-3 to 89 kDa and 24 kDa polypeptides during the degradation of cellular DNA, which prevents DNA repair. Based on these features, cells undergoing apoptosis can be identified by detection of active caspase-3 or PARP cleavage products using specific antibodies. In **Paper IV**, Western blot analyses of caspase-3 and PARP cleavage products were performed to evaluate the effect of *miR-375* on cell apoptosis in MCC cell lines.

3.7 IDENTIFICATION OF MIRNA TARGETS

In this thesis work, miRNA targets were identified by CLIP-Chip analysis or PAR-CLIP sequencing, which are briefly described below.

3.7.1 CLIP-Chip analysis

In **Paper I**, CLIP-Chip was used to identify *miR-205* targets in cervical cancer cell lines, as illustrated in Figure 3.6. In this method, mRNAs bound to the miRNA machinery were purified by Ago2 Co-IP. The recovered mRNA targets were then hybridized to cDNA microarrays to assess the miRNP-associated mRNA pool. mRNAs

that show reproducible enrichment in cells over-expressing the miRNA of interest or depletion in cells where the miRNA is inhibited represent candidate targets.

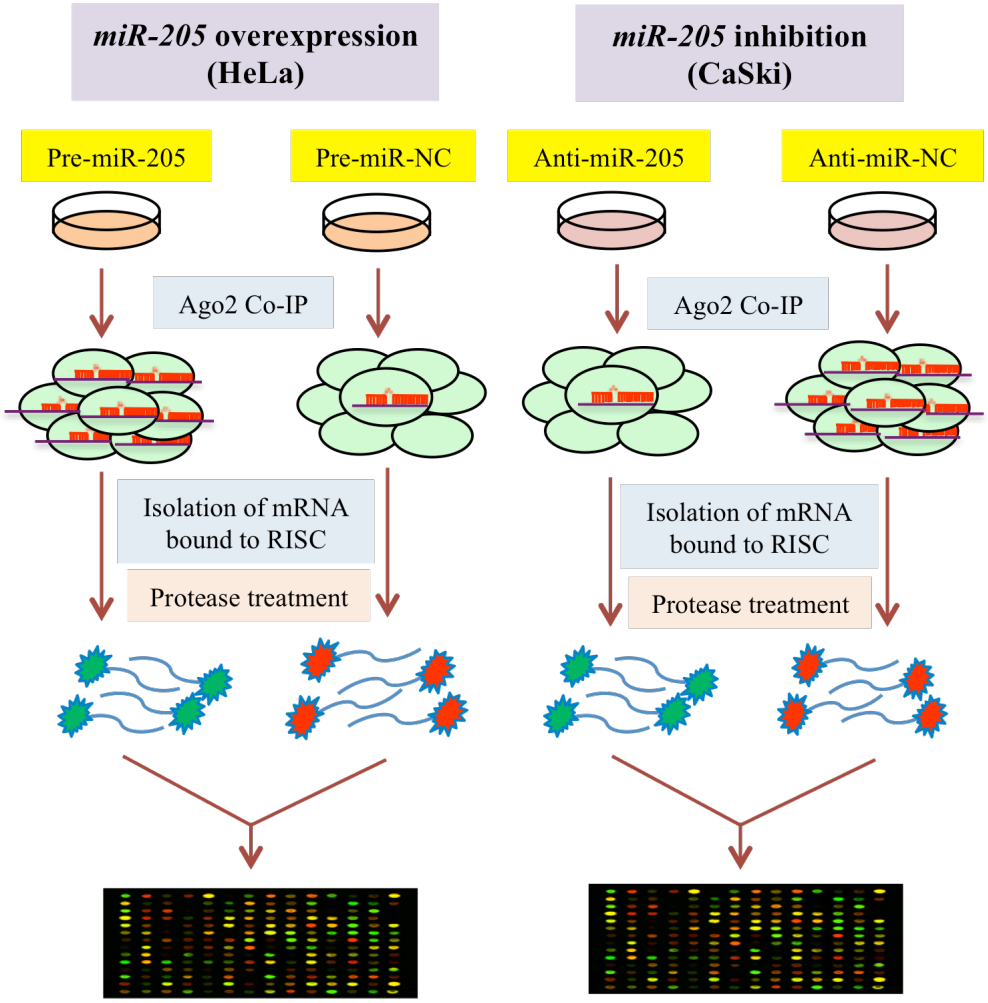

Figure 3.6 Illustration of CLIP-Chip experiment performed in **Paper I** to identify *miR-205* targets.

3.7.2 PAR-CLIP high-throughput sequencing

As mentioned in Chapter 1.6.2.2, PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) is based on the site-specific incorporation of photoreactive ribonucleoside analogs [*e.g.* 4-thiouridine (^{4S}U) and 6-thioguanosine (^{6S}G)] into RNA transcripts by living cells, followed by crosslinking of photoreactive nucleoside-labeled cellular RNAs to interacting RNA binding proteins by UV irradiation. This method provides more efficient UV crosslinking and immunoprecipitation. It also allows identification of the precise position of crosslinking by mutations (T > C transition using ^{4S}U; G > A using ^{6S}G) residing in the sequenced cDNA, which makes it possible to separate them from the background sequences

derived from abundant cellular RNAs. In **Paper II**, the crosslinked RNAs co-immunoprecipited by Ago2 were isolated and converted into a cDNA library, and then sequenced by Illumina HiSeq2000 technology (Figure 3.7).

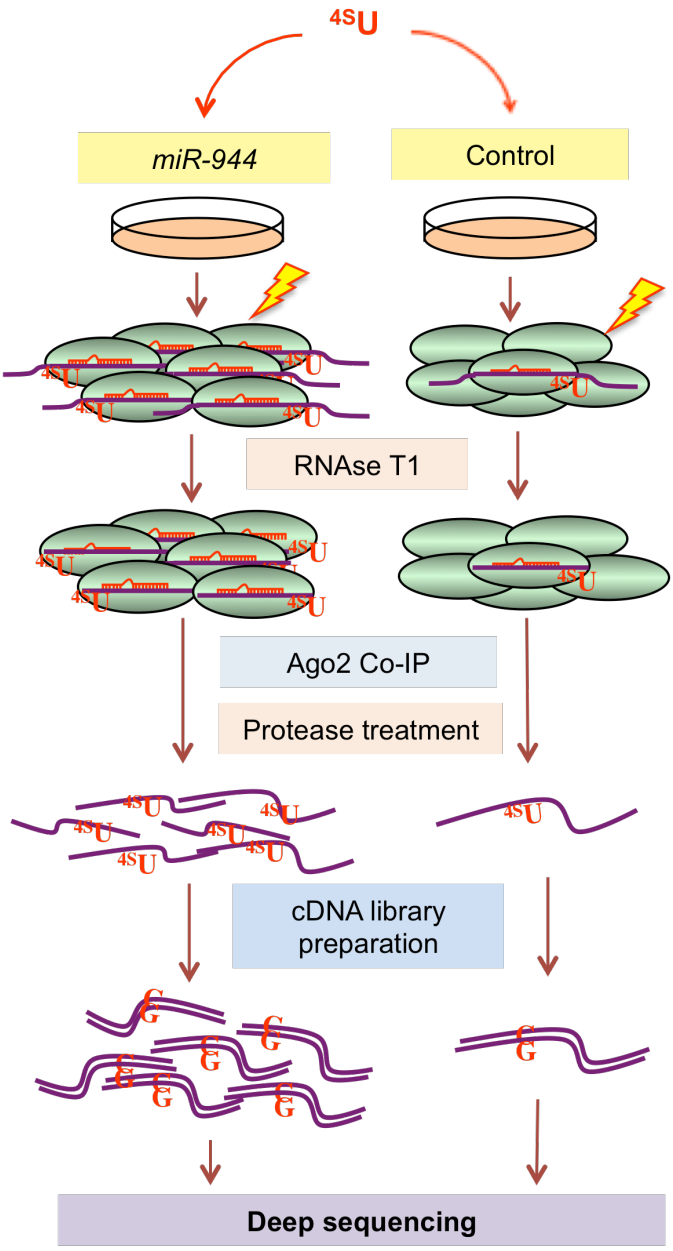

Figure 3.7 Outline of the PAR-CLIP experiment in **Paper II**.

3.8 VALIDATION OF MIRNA TARGETS

3.8.1 Western blot analysis

Western blot, sometimes called protein immunoblot, is a commonly accepted analytical method to detect specific proteins and their sizes in a given sample. This method was originally developed in Harry Towbin's lab (Towbin *et al.*, 1979). It uses gel electrophoresis to separate proteins according to their molecular weight. The proteins are then transfered to a membrane (nitrocellulose or PVDF membrane) and incubated with antibodies specific to the target proteins. After washing away the unbound primary antibody, a species-specific secondary antibody is added and followed by signal detection. There are several detection methods such as colormetric, radioactive, chemiluminescent and fluorescent based on the secondary antibody used. Chemiluminescent and fluorescent detection are most commonly used. At chemiluminescent detection, a HRP-conjugated secondary antibody is used to cleave a chemiluminescent agent and the reaction produces luminescence, which can be detected by photographic films or a CCD camera. For the fluorescent method, a fluorescently-labeled secondary antibody is used, which can be visualized directly using an imaging system with appropriate emission filters.

In **Papers I-III**, Western blot analyses were performed to evaluate the protein expression levels of selected candidate targets of *miR-205*, *miR-944* or *miR-203*. The candidate targets include CYR61 and CTGF for *miR-205* (**Paper I**), HECW2 for *miR-944* (**Paper II**) and Survivin for *miR-203* (**Paper III**).

3.8.2 Quantification of target gene expression by qRT-PCR

As aforementioned, miRNA-mediated gene regulation can be observed at the mRNA and/or protein levels. In this thesis work, two different approaches were applied to assess the effect on the candidate target mRNAs upon modulation of miRNA level.

3.8.2.1 mRNA enrichment analysis of Ago2 Co-IP samples (**Paper I**)

This approach is based on the assumption that miRNAs and their targets mRNA are physically associated within RNA induced silencing comlex (RISC). The isolation of mRNAs bound to miRNP was performed using Ago2 CLIP, as described in above. Enrichment/depletion of selected *miR-205* target genes (*CYR61* and *CTGF*) in Ago2

Co-IP RNAs of cells with *miR-205* over-expression or inhibition was determined by qRT-PCR.

3.8.2.2 mRNA quantifications (**Papers I** and **III**)

In the second approach, the candidate target of a specific miRNA was directly quantified by qRT-PCR. We would expect an inverse expression relationship between the miRNA and its targets, which is different from the approach described above. However, the limitation of this method is that only miRNA-mediated gene regulation involving mRNA degradation, but not mRNA translation repression, can be detected by this method.

3.8.3 Luciferase reporter assay

Luciferase reporter assay is the most commonly used strategy to evaluate the direct interaction between a miRNA and the candidate target. This method is based on an insertion of putative miRNA binding site(s) into the 3'-UTR of the luciferase gene. Both the construct with miRNA binding site(s) and additional construct(s) with mutated miRNA binding site(s) are included to determine the specificity of the miRNA::mRNA interaction. Unlike the two methods described above that do not provide information about its binding site, this method can determine the specificity of miRNA interaction site.

This method was used in **Paper II** to verify *HECW2* and *S100PBP* as direct targets of *miR-944*. The wild-type and mutated *miR-944* binding sites of *HECW2* and *S100PBP* were added into the 3'-UTR of *firefly* luciferase gene in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation, Madison, WI). This vector contains *firefly* luciferase (*luc2*) as primary reporter to monitor miRNA regulation and *Renilla* luciferase (*hRluc-neo*) as a control reporter for transfection normalization and selection. The *firefly* and *Renilla* luciferase luminescence signals were determined 24 hours after co-transfection of reporter construct and pre-miR-944/pre-miR-NC (negative control) in HeLa cells.

3.9 STATISTICAL ANALYSIS

In all four studies, statistical analyses were performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK) or Microsoft Office Excel 2007. Paired student's *t*-test was conducted to

compare *miR-205* expression in paired clinical samples (**Paper I**). miRNA expressions (*miR-203*, *miR-30a-3p*, *miR-30a-5p*, *miR-34a*, *miR-375*, *miR-769-5p*, *miR-148*, *miR-21*, *miR-150*, *miR-146a, miR-483-5p and miR-630*) were compared between groups using unpaired student's *t*-test (**Paper III**). Student's *t*-test was also performed to analyze differences between two transfection groups (**Papers I-IV**). Pearson's correlation analysis was used to determine the association between *miR-205* and *CYR61* or *CTGF* expression levels (**Paper I)**. All analyses were two-tailed and *P*-values < 0.05 were considered as statistically significant (**Papers I-IV**).

In the miRNA microarray data analysis, Significant Analysis of Microarray (SAM, http://www-stat.stanford.edu/~tibs/SAM/) was used to determine the association of miRNAs with MCV status, tumor metastasis and disease-specific survival. Patients who are alive or died of MCC-unrelated reasons were considered as censored (**Paper III**). SAM is a statistical method established in 2001 that identifies statistically significant genes by carrying out gene specific t-test and each gene will be given a score called false discovery rate (FDR) which correlates the strength of the relationship between gene expression and a response variable.

4 RESULTS AND DISCUSSIONS

4.1 *MIR-205* EXPRESSION AND FUNCTIONS IN HUMAN CERVICAL CANCER (PAPER I)

We and others have observed high expression of *miR-205* in cervical cancer samples compared with normal cervices by sequencing-based profiles (Witten *et al.*, 2010) and microarray analysis (Lee *et al.*, 2008; Wang *et al.*, 2008). Here, we validated that *miR-205* expression was higher in cervical cancer samples (n=27) compared to matched normal cervices using qRT-PCR analysis (Figure 1A in **Paper I**). Functional roles of *miR-205* have been reported in breast cancer (Greene *et al.*, 2010; Iorio *et al.*, 2009; Wu *et al.*, 2009), prostate cancer (Gandellini *et al.*, 2009) and melanoma (Dar *et al.*, 2011), but not in cervical cancer. Hence this study was conducted to characterize the functional roles and targets of *miR-205* in cervical cancer cell lines.

4.1.1 *miR-205* affects tumor phenotypes *in vitro*

We evaluated the functional consequences of *miR-205* regulation in human cervical cancer cells using gain-of-function (by ectopically expressing *miR-205*) and loss-of-function (by inhibiting endogenous *miR-205*) experiments. We observed significantly increased cell proliferation and migration in *miR-205*-expressing HeLa and SW756 cells, and decreased cell proliferation and migration in CaSki cells after *miR-205* inhibition (Figure 2 in **Paper I**). However, no significant effect on cell apoptosis was observed in either gain- or loss-of-function experiments. Consistent with our findings, *miR-205* expression has been shown to regulate cell proliferation and migration in other cell types, such as human keratinocytes and multiple cancer cell lines. Taken together, our data and the available literatures suggest a role for *miR-205* in tumor development.

4.1.2 Identification of *miR-205* targets by CLIP-Chip

We performed CLIP-Chip experiments in cells with over-expression or inhibition of *miR-205*, as well as their respective negative controls. We found six clusters (including 270 transcripts/252 annotated genes) in which the expression patterns displayed enrichment after *miR-205* over-expression and depletion after *miR-205* inhibition (Figure S3 and Table S1 in **Paper I**). Several targets are functionally associated with cell proliferation and migration, which is consistent with the results observed in our functional studies. Two of these targets, *CYR61* and *CTGF*, are interesting candidates

because of their aberrant expression in multiple tumor types and their involvement in tumorigenesis. In cervical cancer, both *CYR61* and *CTGF* expression levels were significantly lower in cervical carcinomas compared to normal cervices, and the expressions were inversely correlated with *miR-205* expression (Figure 3 in **Paper I**); further suggesting *CYR61* and *CTGF* as targets of *miR-205*.

4.1.3 Validation of *CYR61* and *CTGF* as novel targets of *miR-205*

CYR61 and CTGF were further validated as *miR-205* targets using Western blot analysis and quantification analysis of Ago2 Co-IP RNA. In consistence with our CLIP-Chip data, both *CYR61* and *CTGF* were validated as *miR-205* targets in cervical cancer cell lines using both methods (Figure 4 in **Paper I**). However, the direct interaction site(s) of these targets remain to be further investigated by luciferase reporter assays, and their functional roles in cervical cancer have yet to be determined.

In summary, we demonstrate that *miR-205* plays an oncogenic role in human cervical cancer by promoting cell proliferation and migration. Using CLIP-Chip approach, we identified a set of novel *miR-205* targets. Two of them, *CYR61* and *CTGF*, were further validated by Western blot analysis and qRT-PCR analysis of mRNAs enriched in the Ago2 Co-IPs. Interestingly, both *CYR61* and *CTGF* were down-regulated in cervical cancer tissues. Our findings suggest that *miR-205* and its targets may play important roles in cervical carcinogenesis and may provide potential diagnostic tools in clinical pathology.

4.2 *MIR-944* FUNCTIONS AND TARGET IDENTIFICATION IN CERVICAL CANCER CELL LINES (PAPER II)

MiR-944 was first identified by our group in a sequencing-based miRNA profiling study of cervical cancer cell lines and normal cervices (Lui *et al.*, 2007). Its expression is significantly higher in cervical cancer tissues compared with their matched normal cervical tissues (Witten *et al.*, 2010). Several other groups reported its deregulation in melanoma (Jukic *et al.*, 2010; Stark *et al.*, 2010), acute lymphoblastic leukemia (Schotte *et al.*, 2011) and hepatocellular carcinoma (Zhang *et al.*, 2013). High expression of *miR-944* is also associated with tumor recurrence in colorectal cancer (Christensen *et al.*, 2013), poor chemotherapy response and survival in bladder cancer (Nordentoft *et al.*, 2012). Despite its expression patterns, nothing is known about its function and targets. Here, we investigated the functional roles and targets of *miR-944* in cervical cancer cell lines.

4.2.1 *miR-944* exhibits oncogenic features *in vitro*

Using the WST-1 and xCELLigence real-time cell proliferation assays, we showed that *miR-944* over-expression increased and *miR-944* inhibition decreased cell proliferation (Figure 2 in **Paper II**). In the wound healing migration assay, we observed that *miR-944* over-expression enhanced the ability to close the wound in both HeLa and CaSki cells. In transwell assays, significantly enhanced cell migration and invasion were observed in HeLa and/or SW756 cells upon over-expression of *miR-944*. However we did not observe any significant effects on cell migration or invasion in CaSki cells with *miR-944* inhibition or over-expression (Figure 3 in **Paper II**). Furthermore, no significant changes of cell apoptosis or cell cycle were observed in both *miR-944* gain- and loss-of-function experiments (Figure S2 in **Paper II**).

4.2.2 Identification and verification of *miR-944* targets

PAR-CLIP sequencing was performed to identify *miR-944* targets in HeLa cells. We found 58 transcripts present in the *miR-944*-expressing cells, and only two of them (*i.e.*, *CRYAB* and *RRBP1*) were also present in control groups. Among the transcripts identified in PAR-CLIP data of *miR-944*-expressing cells, 25 corresponded to 3'-UTRs, 23 to coding sequences (CDS) and four to 5'-UTRs of protein-coding genes. In addition to mRNAs, we also found six non-coding RNAs (ncRNAs) in our PAR-CLIP data.

Nineteen of the candidate targets identified by PAR-CLIP sequencing were also predicted as *miR-944* targets by TargetScan 6.2 (http://www.targetscan.org/). Two of these targets, *HECW2* and *S100PBP*, have conserved *miR-944* binding sites; which were chosen for validation by luciferase reporter assays and Western blot analysis (Figure 4 in **Paper II**). Using the luciferase reporter assays, we observed a significant reduction of luciferase activities in cells expressing the luciferase construct containing the wild-type *miR-944* binding site of *HECW2* or *S100PBP*, together with *miR-944*. The *miR-944*-mediated suppression of luciferase activity was abolished by the seed mutant constructs. Western blot analysis also showed a significant reduction of HECW2 expression in *miR-944*-expressing cells. Together, these results provide evidence for *HECW2* and *S100PBP* as direct targets of *miR-944* in human cervical cancer cells.

In summary, we show that *miR-944* promotes cell proliferation, migration and invasion in human cervical cancer cells. We identified a set of novel *miR-944* targets using PAR-CLIP sequencing. Among the candidate targets, *HECW2* and *S100PBP* were further validated as direct targets of *miR-944*. Our findings provide evidence that *miR-944* acts as an oncogene in cervical cancer, which may contribute to improved understanding of human cervical cancer development and pathogenesis.

4.3 MIRNA EXPRESSION IN MERKEL CELL CARCINOMA (PAPER III)

The aim of this study was to determine the role of miRNAs in MCC. Here, we characterized miRNA expression profiles of MCC tumors and evaluated the findings against MCV status, clinical features and patient outcomes.

4.3.1 MCV detection in MCC tumors

We performed PCR and immunohistochemistry (IHC) to determine MCV status in a series of 33 MCC samples from 26 patients. For PCR amplification, we used five different primer sets to amplify different regions of the MCV genome. For IHC, we used two different antibodies to detect MCV LT-Ag and the immunoactivity was scored based on nuclear staining intensity. Based on the two methods, we found 12/33 MCV-negative (36.4%) and 21/33 MCV-positive (63.6%) cases in our cohorts.

4.3.2 miRNAs associated with MCV status and survival

To characterize the role of miRNAs in MCC, we performed a genome-wide miRNA expression profiling in 16 MCC tumors (6 MCV-positive *vs.* 10 MCV-negative) using a microarray approach. Using hierarchical clustering analysis, we observed that all except one MCV-positive MCCs were grouped in the same cluster, which were separated from the MCV-negative MCC tumors (Figure 1a in **Paper III**). Using SAM analysis, we identified 56 significant differentially expressed miRNAs between MCV-positive and MCV-negative MCCs (36 over-expressed and 20 under-expressed in MCV-positive MCCs at FDR<30%). Six of the eight selected miRNAs were further validated by qRT-PCR analysis in an extended series of 32 (12 MCV-negative *vs.* 20 MCV-positive) MCC samples (Figure S3 in **Paper III**). By SAM survival analysis, we identified 26 over-expressed and 118 underexpressed miRNAs correlated with shorter survival in MCCs (FDR<12%; Table S4 in **Paper III**).

SAM analysis was also performed to identify the most significant differentially expressed miRNAs between primary tumors (n=9) and metastases (n=5). The analysis revealed that 92 miRNAs had higher expression in metastases compared to primary tumors at FDR<30%. Over-expression of *miR-150* in metastases was validated by qRT-PCR (Figure S4 and Table S3 in **Paper III**).

4.3.3 Functional role of *miR-203* in MCC cell lines

In our microarray analysis, *miR-203* expression was found significantly lower in MCV-positive than MCV-negative MCCs. We further investigated the functional roles of *miR-203* in MCC cell lines.

4.3.3.1 *miR-203* expression inhibits cell growth and induces cell cycle arrest in MCV-negative MCC cell lines

We ectopically expressed *miR-203* using a *miR-203* mimic in four MCC cell lines and investigated its potential effects on cell growth, apoptosis and cell cycle. Interestingly, we found that *miR-203* expression significantly inhibited cell growth and induced cell cycle arrest at G1 phase in all three MCV-negative cell lines (MCC13, MCC14/2 and MCC26), while no significant effects on cell growth or cell cycle progression were observed in the MCV-positive cell line WaGa (Figures 2 and S5 in **Paper III**).

4.3.3.2 Survivin as a putative target of *miR-203* in MCV-negative MCC cell lines

Survivin (also known as *BIRC5*) is a direct target of *miR-203* in several cancer types, including prostate (Saini *et al.*, 2011), laryngeal (Bian *et al.*, 2012) and hepatocellular (Wei *et al.*, 2013) cancers. Interestingly, *survivin* was recently found highly expressed in MCV-positive compared with MCV-negative MCCs (Arora *et al.*, 2012). The inverse expression correlation between *miR-203* and *survivin* expression led us to further investigate whether survivin could also be regulated by *miR-203* in MCC cells. Upon *miR-203* overexpression, we observed a significant decrease of survivin expression at the gene and protein levels in all MCV-negative but not in the MCV-positive cell lines (Figure 3a in **Paper III**).

4.3.4 Survivin is differentially regulated in MCV-positive and MCV-negative MCC cell lines

We observed *miR-203* mediated survivin regulation in MCV-negative MCC cells, and MCV LT-Ag is known to regulate survivin expression in MCV-positive cells (Arora *et al.*, 2012). We therefore asked whether MCV LT-Ag has a different regulation of survivin in MCV-positive and MCV-negative cells. We silenced T-Ag expression in the MCV-positive WaGa cell line and expressed MCV LT-Ag in the MCV-negative MCC14/2 cell line, and determined survivin expression at both protein and mRNA level. In WaGa cells survivin expression was significantly decreased upon T-Ag

silencing using shRNAs, while in MCC14/2 cells we did not detect any significant changes of survivin expression upon LT-Ag (Figure 3b in **Paper III**).

In summary, we identified a set of differentially expressed miRNAs between MCV-positive and MCV-negative MCCs, as well as miRNAs associated with MCC tumor metastasis and disease-specific survival. Functionally, we show that *miR-203* expression inhibits cell growth and induces cell cycle arrest in MCV-negative MCC cells. We also show that survivin expression is regulated by *miR-203* in MCV-negative MCC cells, while in MCV-positive cells it is regulated by MCV T-Ag. Our findings provide new insights into the role of miRNAs in MCC pathogenesis.

4.4 FUNCTIONAL ROLE OF *MIR-375* IN MCC CELL LINES (PAPER IV)

Deregulation of *miR-375* expression has been reported in multiple types of cancer including gastric carcinoma (Ding *et al.*, 2010; Tsukamoto *et al.*, 2010), glioma (Chang *et al.*, 2012) and colon cancer (Dai *et al.*, 2012b; Faltejskova *et al.*, 2012). *miR-375* commonly acts as a potential tumor suppressor that inhibits cell proliferation, migration, invasion and tumor metastasis (Ding *et al.*, 2010; He *et al.*, 2012; Kong *et al.*, 2012; Wang *et al.*, 2011a) by targeting several important oncogenes (He *et al.*, 2012; Hui *et al.*, 2011; Kinoshita *et al.*, 2012; Li *et al.*, 2011; Liu *et al.*, 2010; Nishimura *et al.*, 2013; Nohata *et al.*, 2011; Tsukamoto *et al.*, 2010). However *miR-375* has also been shown to target Sec23A that promotes tumor metastasis in prostate cancer. *MiR-375* is highly expressed in MCC compared to normal skin and other non-MCC skin cancers (Renwick *et al.*, 2013). In **Paper III**, we observed a significantly higher expression of *miR-375* in MCV-positive than MCV-negative MCCs (Xie *et al.*, 2013). Here we investigated the functional roles of *miR-375* and its potential regulatory mechanisms in MCC cells.

4.4.1 Regulation of *miR-375* expression

4.4.1.1 Regulation of *miR-375* expression by MCV T-antigens

Here we investigated whether the MCV oncoproteins could modulate *miR-375* expression. We silenced T-Ag in MCV-positive MCC cells and over-expressed MCV LT-Ag (both wild-type and tumor-specific mutated type) in HEK293 cells and assessed their effects on *miR-375* expression. We found that *miR-375* expression was significantly decreased upon silencing of T-antigens in all three MCV-positive MCC cells. In HEK293 cells, we observed an increase of *miR-375* expression only in the cells expressing the tumor-specific mutated MCV LT-Ag, but not in cells with the wild-type LT-Ag. The findings suggest that MCV T antigen(s) can regulate *miR-375* expression.

4.4.1.2 Epigenetic regulation of *miR-375* expression

The genomic locus of *miR-375* contains two CpG islands spanning the gene and its expression has been shown epigenetically silenced in several tumor types. Hence, we investigated whether *miR-375* expression could be regulated by epigenetic mechanism(s) in MCC cells. We treated MCC cell lines (both MCV-positive and MCV-negative) with a histone deacetylase inhibitor (Trichostatin A, TSA), a DNA

demethylating agent (5'-Azacytidine, 5'-Aza) or a combination of both inhibitors and analyzed their effects on *miR-375* expression. Unexpectedly, we observed different effects in MCV-positive and -negative cells. In MCV-negative cells, we found that *miR-375* expression was significantly increased in cells treated with TSA alone or a combination of 5'-Aza and TSA, but not with 5'-Aza treatment alone. This implies that histone modification rather than DNA methylation contributes to silencing of *miR-375* expression in MCV-negative MCC cells. In MCV-positive cells, we observed that *miR-375* expression was significantly decreased upon treatment with TSA or 5'-Aza alone, but not the combination of TSA and 5'-Aza, suggesting that histone modification and DNA methylation may contribute to the regulation of *miR-375* expression.

4.4.2 *miR-375* acts as a tumor suppressor in MCV-negative cell ines

To investigate the functional roles of *miR-375* in MCC, we ectopically expressed *miR-375* using a miRNA mimic in three MCC cell lines and determined its effect on cell growth, cell cycle, cell migration and apoptosis (Figure 3 in **Paper IV**).

Using WST-1 cell proliferation assay, we observed significantly decreased cell growth in all three *miR-375* over-expressing cells. Cell cycle distribution analysis revealed significant cell cycle arrest at G1 or G2 phases. *MiR-375* over-expression also retarded wound closure, as evaluated by wound healing migration analysis. Using Western blot analysis, we observed an increase of PARP and caspase-3 cleavage products in MCC cells indicating apoptosis. Taken together, our data supports a tumor suppressor role of *miR-375* also in MCV-negative MCC cells.

In summary, we demonstrate that *miR-375* functions as a tumor suppressor in MCV-negative MCC cells, and that different regulatory mechanisms are involved in controlling *miR-375* expression in MCV-positive and MCV-negative MCC cells.

5 CONCLUDING REMARKS

It is clear that miRNAs are deregulated in human cancer and play important roles in tumor development and progression. For the past decade, many studies have reported specific miRNA expression signatures in various tumor types. However, the functional roles of these deregulated miRNAs in specific tumor types have yet to be determined. The aims of this thesis work were to characterize the functional roles of specific miRNAs in cervical carcinoma and Merkel cell carcinoma. The general findings are summarized below:

- Deregulated miRNAs have important biological functions in cancer cells and may function as oncogenes (*e.g. miR-205* and *miR-944*) or tumor suppressors (*miR-203* and *miR-375*) in different cancer types (**Papers I-IV**).

- The Ago2 CLIP biochemical approaches, such as CLIP-Chip and PAR-CLIP sequencing, illustrate the values of a high-throughput method for transcriptome-wide identification of miRNA targets (**Papers I and II**).

- miRNA expression patterns are associated with MCV status of MCC tumors and survival of MCC patients (**Paper III**).

- miRNAs differentially expressed between MCV-positive and -negative MCCs (*e.g. miR-203* and *miR-375*) are regulated by different mechanisms and may have different functions in MCC cells with and without the virus (**Papers III and IV**).

- Survivin (BIRC5) oncogene is commonly deregulated in MCCs, and is regulated by MCV T-Ag in MCV-positive MCC cells and by *miR-203* in MCV-negative MCC cells (**Paper III**).

6 FUTURE PERSPECTIVES

During the past decade, numerous data have advanced our knowledge and understanding of the role of miRNAs in human cancers, including virus-associated human cancers. Still, many questions remain to be addressed. Identification of deregulated miRNAs in cancer tissues may have implications for diagnosis, prognosis, treatment as well as management of the disease. A detailed understanding of their functional roles and their regulatory networks will provide clues to understand the pathogenesis of disease. This thesis work contributes partly to these efforts.

Future investigations are still warranted to evaluate the clinical impacts of the deregulated miRNAs and to elucidate the complete gene regulatory network in these tumor types. In the cervical carcinoma project, it would be valuable to further examine the clinical significance of *miR-205* and *miR-944* (as well as their targets) in tumor progression using liquid-based cytological specimens. Upon confirmation of their clinical values, these miRNAs could be developed into complemetary tools for cervical cancer pathology and cytology. For the Merkel cell carcinoma project, this thesis work has opened up many questions. For example, which are the targets of differentially expressed miRNAs between MCV-positive and -negative MCCs? What interactions occur between viral oncoproteins and cellular miRNAs/genes? And what are the mechanisms of miRNA deregulations? Furthermore, it is known that MCV encodes two mature miRNAs, but their targets and functions remain undetermined. The biochemical approaches used in this thesis work can be applied to identify the targets of MCV-encoded miRNAs. In some other viruses, viral-encoded miRNAs can regulate both viral and cellular genes for the transition from latent to lytic replication and for evading host anti-viral response.

It is clear from this thesis work that the miRNA regulatory system merits close examination as a potentially important contributor to viral oncogenesis. Elucidation of the complex interplay between virus and host, including cellular/viral microRNAs and their targets, will contribute to advanced knowledge of virus-associated cancers.

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