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DIFFERENTIAL MICRORNA AND METABOLIC EXPRESSIONS IN NEUROENDOCRINE AND CERVICAL CARCINOMAS

Patrick Scicluna

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Differential microRNA and metabolic expressions in neuroendocrine and cervical carcinomas

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Patrick Scicluna

Principal Supervisor:
Associate Professor Weng-Onn Lui
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor(s):
Professor Catharina Larsson
Karolinska Institutet
Department of Oncology-Pathology

Professor Björn Andersson
Karolinska Institutet
Department of Cell & Molecular Biology

Opponent:
Professor Xiao-Feng Sun
Linköping University
Department of Biomedical and Clinical Sciences

Examination Board:
Associate Professor Mikael Lindström
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Professor Kjell Öberg
Uppsala University
Department of Medical Sciences

Associate Professor Agné Kulyté
Karolinska Institutet
Department of Medicine Huddinge

“The truth is, we are all human, and we all make mistakes”

Mike Coffman

“När man är tjugo år, har man löst världsgåtan, vid trettio börjar man tänka över den, och vid fyrtio finner man den olöslig”

August Strindberg

ABSTRACT

MicroRNAs (miRNAs) are important regulators of many biological processes involved in cancer development and progression. However, the functional roles of miRNAs in some cancer types are still poorly understood. The aim of this thesis was to investigate the expressions, targets and functions of specific miRNAs in cervical, adrenocortical and Merkel cell carcinoma.

In Paper I, we characterized the functional roles of *miR-944*, a miRNA with higher expression in cervical cancer. We showed that *miR-944* promotes cell proliferation, migration and invasion. Using Ago2 PAR-CLIP sequencing, we identified 58 candidate targets of *miR-944* and validated *HECW2* and *S100PBP* as direct targets of *miR-944* by luciferase reporter assays. This study reveals the oncogenic role and novel targets of *miR-944* in human cervical cancer cells, suggesting its important role in cervical cancer development and its potential implications as a biomarker of cervical carcinoma or as a therapeutic target.

In Paper II, we showed that the *IGF2-H19* locus was consistently deregulated in adrenocortical carcinoma (ACC). We used available proteomic data, to identify a subset of proteins inversely correlated to miRNAs of the *IGF2-H19* locus. Interestingly, several of the proteins are involved in mitochondrial respiration, such as NDUFC1, a subunit of mitochondrial respiratory complex I. NDUFC1 was observed to be down-regulated in our ACC cohort and was a predicted *miR-483-5p* target. Inhibition of *miR-483-5p* in ACC cells increased NDUFC1 expression and reduced both glycolysis and mitochondrial respiration, suggesting that *miR-483-5p* controls major energy metabolism and its high expression is required to fuel cellular activies in ACC cells.

In Paper III, we demonstrated that over-expression of *miR-375* suppressed cell growth and migration, induced cell cycle arrest and apoptosis in Merkel cell polyomavirus-negative (MCPyV-) Merkel cell carcinoma (MCC). Inhibition of *miR-375* suppressed cell growth and increased apoptosis in MCPyV-positive (MCPyV+) MCC. Additionally, we showed that LDHB is a target of *miR-375* in MCC as shown by its inverse relationship. Low levels of LDHB are required to maintain cell growth and viability in MCPyV+ MCC, while high levels are required for MCPyV- MCC.

In Paper IV, we revealed that MCPyV oncoproteins suppressed LDHB and promoted glycolysis. Intriguingly, LDHB overexpression could revert the growth-promoting effect of sT or truncated LT, indicating that a low LDHB expression is important for maintenance of cell growth and viability in MCPyV+ cells. Inhibition of glycolysis reduced cell growth and induced apoptosis in MCPyV+ MCC cell lines, whereas the MCPyV- MCC cell lines rely on oxidative phosphorylation for cell growth and viability. These data suggest targeting metabolism as a therapeutic strategy in MCC.

Taken together, this thesis work provides new insights into the crucial roles of miRNAs in the molecular mechanisms of cervical, adrenocortical and Merkel cell carcinomas.

SAMMANFATTNING

I denna avhandling studeras hur förändringar av mikroRNA (miRNA) kan bidra till cancerutveckling genom att styra uttrycket av gener som påverkar biologiska processer av betydelse i cancer. Detta är ett nytt och lovande forskningsfält som redan bidragit till ökad förståelse och klinisk användning inom diagnostik och som har potential för utveckling av nya behandlingsstrategier. Samtidigt är många frågeställningar ännu obesvarade särskilt avseende vilka underliggande molekylära mekanismer som är involverade. Målet för denna avhandling var att karaktärisera förändringar i miRNA uttryck och hur det påverkar målgener och cellulära funktioner i livmoderhalscancer, binjurebarkscancer och Merkel cells cancer (MCC).

Arbete I fokuserades på funktionen av *miR-944* som tidigare visat ett ökat uttryck i livmoderhalscancer. Vi visade nu att uttryck av *miR-944* ger ökad cellproliferation, migration och invasion av cancer celler i experimentella modellsystem. Med hjälp av den banbrytande metoden PAR-CLIP-sekvensering kunde vi upptäckta 58 gener som är möjliga målgener för *miR-944* reglering. Två av dessa (*HECW2* och *S100PBP*) kunde sedan verifieras som direkta målgener för *miR-944*. Arbetet visar att *miR-944* kan ha en tumördrivande roll genom att interagera med specifika målgener. Resultaten talar för att *miR-944* har en viktig roll i utveckling av livmoderhalscancer och skulle kunna användas för utveckling av kliniska markörer samt utgöra en måltavla för ny behandling.

I Arbete II studerade vi uttrycket av miRNA och mRNA från *IGF2-H19*-lokuset som är karaktäristiskt förändrat i ACC. Här använde vi befintliga globala proteinprofileringsdata från mass spektrometrianalys för att identifiera proteiner vars uttryck var omvänt korrelerat med miRNA uttrycket. Flera av dessa proteiner visade sig ha en känd funktion i mitokondriens andningskedja den s.k. elektrontransportkedjan. Bland dessa detaljstuderades NDUFC1, som ingår i andningskedjans komplex I. NDUFC1 befanns vara nedreglerad i ACC tumörer och är en känd målgen för *miR-483-5p* som genereras från IGF2. Inhibering av *miR-483-5p* i odlade binjurbarkscancerceller ledde till ökat uttryck av NDUFC1 samt minskad nedbrytning av glykos och mitokondriell andning. Detta talar för att *miR-483-5p* kontrollerar energimetabolism och att ett högt uttryck är nödvändigt för ökade cellulära aktiviteter i binjurebakscancer-celler.

I Arbete III visade vi att överuttryck av *miR-375* i virusnegativa (MCPyV-) MCC celler kan hämma celltillväxt och migration samt leda till stopp i cellcykeln. I motsats till detta ledde hämning av *miR-375* i virus-positiva (MCPyV+) tumör celler till minskad celltillväxt och ökad apoptos. Vi visade vidare att *LDHB* kan vara en målgen för *miR-375* i MCC, i enlighet med det inversa sambandet mellan uttryck av *miR-375* och *LDHB*. Låga nivåer av LDHB krävdes för att bibehålla celltillväxt i MCPyV+ MCC celler, medan höga nivåer krävdes för MCPyV- celler.

I det sista och fjärde arbetet studerade vi sambandet mellan virala onkoproteiner (LT och sT) och LDHB. Vi fann att LT och sT kan hämma LDHB och öka glykolysen. Överuttryck av LDHB kunde återställa den tillväxtbefrämjande effekten av sT och trunkerat LT, vilket talar för att lågt LDHB-uttryck är viktigt för celltillväxt i MCPyV+ celler. Hämning av glykolys med

specifika hämmare minskade celltillväxten och inducerade apoptos i MCPyV+ cellinjer, medan oxidativ fosforylering var avgörande för celltillväxt i MCPyV- celler. Resultaten indikerar att påverkan på tumörcellernas metabolism kan vara en möjlig ny terapeutisk strategi för vid MCC.

Sammanfattningsvis påvisas i denna avhandling hur förändringar i miRNA uttryck och funktioner har stor betydelse för att kontrollera molekylära mekanismer som bidrar till utveckling av livmoderhalscancer, binjurebarkscancer och Merkelcellscancer. Flera observationer har potential att användas för utveckling av biomarkörer och som måltavlor för nya behandlingsstrategier.

LIST OF SCIENTIFIC PAPERS

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

I. Hong Xie, Linkiat Lee, **Patrick Scicluna**, Ersen Kavak, Catharina Larsson, Rickard Sandberg, Weng-Onn Lui. Novel functions and targets of *miR-944* in human cervical cancer cells.
Int J Cancer, 136(5): E230-41 (2015).

II. **Patrick Scicluna**, Stefano Caramuta, Hanna Kjellin, Cheng Xu, Robin Fröbom, Monira Akhtar, Magnus Kjellman, Malin Almgren, Anders Höög, Jan Zedenius, Tomas J. Ekström, Weng-Onn Lui, Catharina Larsson. Altered expression of the *IGF2-H19* locus and mitochondrial respiratory complexes in adrenocortical carcinoma.
Manuscript

III. Satendra Kumar, Hong Xie, **Patrick Scicluna**, Linkiat Lee, Viveca Björnhagen, Anders Höög, Catharina Larsson and Weng-Onn Lui. *MIR-375* regulation of LDHB plays distinct roles in polyomavirus-positive and -negative Merkel cell carcinoma.
Cancers, 10(11): 443 (2018).

IV. Satendra Kumar, **Patrick Scicluna**, Jiwei Gao, Hao Shi, Viveca Björnhagen, Anders Höög, Catharina Larsson, Weng-Onn Lui. Merkel cell polyomavirus oncoproteins suppress LDHB and promote glycolysis for cell growth.
Manuscript

LIST OF OTHER PAPERS

1. Suhas Vasaikar, Giorgos Tsipras, Natalia Landázuri, Helena Costa, Vanessa Wilhelmi, **Patrick Scicluna**, Huanhuan L. Cui, Abdul-Aleem Mohammad, Belghis Davoudi, Mingmei Shang, Sharan Ananthaseshan, Klas Strååt, Giuseppe Stragliotto, Afsar Rahbar, Kum Thong Wong, Jesper Tegner, Koon-Chu Yaiw and Cecilia Söderberg-Naucler (2018). Overexpression of endothelin B receptor in glioblastoma: a prognostic marker and therapeutic target?
   BMC Cancer 18(1):154 (2018). doi: 10.1186/s12885-018-4012-7

2. Koon-Chu Yaiw, Tim Schulte, Alice Assinger, Lisa Simonsson Nyström, Olov Wahlsten, Helena Costa, Huanhuan L.Cui, Hudson Pace, Vanessa Wilhelmi, Belghis Davoudi, Abdul-Aleem Mohammad, **Patrick Scicluna**, Chato Taher, Lynn Butler, Afsar Rahbar, Fredrik Höök, Per-Åke Nygren, Kum-Thong Wong, Adnane Achour and Cecilia Söderberg-Nauclér. The endothelin B receptor mediates entry of human cytomegalovirus into endothelial cells.
   Submitted manuscript

CONTENTS

LIST OF ABBREVIATIONS

4SU	4-thiouridine
6SG	6-thioguanosine
ACC	Adrenocortical carcinoma
Ago 2	Argonaute 2
ADC	Adenocarcinoma
ASC	Adenosquamous cell carcinoma
BCL2	B-cell lymphoma 2
B-CLL	B cell chronic lymphocytic leukemia
BIC	B-cell integration cluster
CIN	Cervical intaepithelial neoplasia
CLIP	Crosslinking and immunoprecipitation
c-Myc	MYC proto-oncogene, bHLH transcription factor
Ct	Cycle threshold
DGCR8	DiGeorge Syndrome Critical Region 8
DMSO	Dimethyl sulfoxide
E2F1/2/3	E2F transcription factor 1/2/3
ECAR	Extra cellular acidification rate
EdU	5-ethynyl-2'-deoxyuridine
$FADH_2$	Flavin adenine dinucleotide (reduced)
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT	Glucose transporter
H19	H19 imprinted maternally expressed transcript
HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2
HIF-1	Hypoxia-inducible factor 1
HITS-CLIP	High-throughput sequencing-CLIP
HMGA2	High mobility group-hook-2
HOTS	H19 opposite tumor suppressor
HPV	Human Papilloma virus
HR-HPV	High-risk human papilloma virus
HSIL	High-grade squamous intraepithelial lesion
IGF2	Insulin-like growth factor 2
LDHA/ B	Lactate dehyrogenase A/ B
lncRNA	Long non-coding RNA
LR-HPV	Low-risk human papilloma virus
LSIL	Low-grade squamous intraepithelial lesion
LT	Large T antigen
m^7G	7-methyl guanosine
MCC	Merkel Cell Carcinoma

MCPyV	Merkel Cell Polyoma virus
miRLC	miRISC loading complex
mt	Mitochondrial
MRE	microRNA recognition element
MTOR	Mechanistic target of rapamycin
NADH	Nicotinamide adenine dinucleotide (reduced)
ncRNA	Non-coding RNA
NDUFC1	NADH ubiquinone oxidoreductase subunit C1
NF-KB	Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells
nt	Nucleotides
OCR	Oxygen consumption rate
Oxphos	Oxidative phosphorylation
PACT	Protein activator of PKR
PAR-CLIP	Photo activatable ribonucleoside-enhanced-CLIP
PDCD4	Programmed cell death 4 (neoplastic transformation inhibitor)
PEP	Phosphoenolpyruvate
PI3K	Phosphatidylinositol 3-kinase
PDK1	Pyruvate dehydrogenase kinase 1
Pol II	Polymerase II
PTEN	Phosphate and tensin homolog
pri-miRNA	Primary microRNA
RT-qPCR	Quantitative real-time – polymerase chain reaction
RBP	RNA binding protein
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
RNP	RNA-containing ribonucleoprotein complex
RNU6B	RNA U6 small nuclear 2
S100PBP	S100P binding protein
SCC	Squamous cell carcinoma
SDH	Succinate dehydrogenase
sT	Small T antigen
T Ag	T antigen
TRBP2	HIV-1 TAR RNA-binding protein 2
UTR	Untranslated region
XPO5	Exportin 5

1 INTRODUCTION

The story of ribonucleic acid has been evolving since the first discoveries of rRNA and tRNA in the 1950s and later RNA polymerases, ribozymes and RNA splicing. This prompted the hypothesis of an 'RNA World' where RNA was the precursor molecule able to self-replicate, hold information and carry out catalytic function (Joyce and Orgel, 1999). As more discoveries were made, RNAse P (Stark et al., 1978), snRNA (Yang et al., 1981), *7SL* RNA (Walter and Blobel, 1982) and *XIST* RNA (Brown et al., 1991), RNA took a more central role in biology. The Human Genome and ENCODE projects revealed that only ~22,000 genes (<2%) were translated into protein, emphasizing the significance of non-coding genes in organism complexity (The ENCODE Project consortium, 2004; Moraes and Goes, 2016). With the advent of high-throughput sequencing it become apparent that most of the genome is transcribed into non-coding RNA or ncRNAs (Mattick and Makunin, 2006) and this discovery has expanded our understanding in regulation of gene expression.

The first hint of the RNAi mechanism was observed when researchers inserted trans-pigmentation genes in *Petunia hybrida*, only to observe no effect or ectopic pigmentation. This phenomenon was termed 'co-suppression' and could only be explained once the RNA interference (RNAi) mechanism was identified by Fire and colleagues in 1998 (Napoli et al., 1990; van der Krol et al., 1990). Since then, the ncRNA family has grown further with the emergence of new RNA species that are broadly categorized as small ncRNAs (sRNA) or long ncRNAs (lncRNA). Small ncRNAs are <200 nucleotides (nt) long and include tRNAs, microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), vault RNAs (vtRNAs) and small vault RNAs (svRNAs). Less well known species include promoter-associated small RNAs (PASRs), antisense termini-associated short RNAs (aTASRs), transcription start site associated RNAs (TSSa-RNA), splice-site RNA (spliRNA) and snoRNA-derived RNA (Wang et al., 2011a; Kapranov et al., 2010; Taft et al., 2010). lncRNAs are large (>200 nts) poorly conserved mRNA-like transcripts, often exhibiting a 5'cap and a poly-A tail (Feng and Fullwood, 2016). These include rRNAs (*18S*; *28S*) and long intergenic ncRNAs (lincRNA) such as *HOTAIR* (HOX antisense intergenic RNA), *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) and *ANRIL* (Antisense Non-Coding RNA in the INK4 Locus) (Rinn et al., 2007; Tano and Akimitsu, 2012).

The work presented here focuses on miRNA biology but also extends into the tumor metabolic activities that had been observed since the 1920s (Warburg et al., 1927). Metabolic reprogramming has seen a renaissance in the last two decades and is now considered a fundamental characteristic of cancer, contributing to the onset and maintenance of the tumorigenic state. miRNA derangement and tumor metabolism are the two main aspects addressed in this thesis work, in order to deepen our knowledge in the molecular mechanisms of cancer development and progression.

1.1 MIRNA DISCOVERY

In 1993, Victor Ambros and colleagues cloned the *Caenorhabditis elegans lin-4* locus. This locus did not encode a protein product instead, the *lin-4* gave rise to a 61 and a 22 nt RNAs that aligned to the 3'UTR of *lin-14* transcript. The LIN-14 protein is highly expressed in the very early and late larval developmental stages but the *lin-14* transcript is constantly expressed indicating posttranscriptional regulation. Before the discovery of the RNAi mechanism, there was already enough evidence indicating that the *lin-4* product could interact with the 3'UTRs of the *lin-14* and *lin-28* transcripts to inhibit their translation (Arasu et al., 1991; Lee et al., 1993; Wightman et al., 1993; Ambros, 2001).

Later, Fire and colleagues demonstrated that dsRNA was more effective in regulating gene expression and suggested a mechanism for RNA interference (RNAi) in nematodes, invertebrates and vertebrates (Fire et al., 1998). Further reports started to uncover this mechanism involving small RNAs that guide mRNA cleavage in *Drosophila* cells (Hammond et al., 2000; Zamore et al., 2000). In the year 2000, a second gene *let-7* was discovered to transiently express the *let*-7 miRNA to regulate proteins involved in *C. elegans* late larval to adult development (Reinhart et al., 2000). Unlike *lin-4*, *let-7* expression was found to be conserved across the Ecdysozoan, Lophotrochozoan and Deuterostomia superphyla in metazoans (Pasquinelli et al., 2000).

miRNA are boardly expressed in mammals, plants, unicellular organisms and viruses (Baulcombe, 2004; Pfeffer et al., 2004; Lee et al., 2007; Molnar et al., 2007; Friedman et al., 2009). It is estimated >60% of the protein-coding genes in humans are modulated by miRNAs (Friedman et al., 2009) to coordinate physiological (development, hematopoiesis, cell growth and apoptosis, anti-viral defense) and pathological processes [cancer, vascular and immune disorders] (Ambros, 2001; Voinnet, 2001; He and Hannon, 2004; Li et al., 2009). The current number of known miRNAs has reached 38 589 pre-miRNAs and 48 860 mature miRNA sequences from 271 species. Of these, the human genome codes for 1917 pre-RNAs and 2654 mature miRNAs (Kozomara et al., 2019).

1.1.1 The canonical miRNA pathway

MicroRNAs (miRNAs) are a class of small ncRNA that are biochemically analogous to small interfering RNAs (siRNAs). Genomically miRNAs can reside within intergenic regions as a sole or a cluster of genes. miRNAs can be transcribed as a polycistronic miRNA transcript harboring several hairpin structures (Ambros et al., 2003). Alternatively, miRNAs can originate from introns of coding or noncoding genes under the control of their host gene promoter. In exceptional instances, miRNAs can be of exonic origin, typically generated from sites overlapping an exon and an intron (Rodriguez et al., 2004).

Mature miRNAs are single-stranded ~22 nt long molecules, derived from hairpin-shaped dsRNAs (Ambros, 2003; Kim, 2005). They are endogenously transcribed as long primary

miRNAs (pri-miRNA) by RNA polymerase II (pol II) or RNA pol III (Lee et al., 2004; Borchert et al., 2006). Newly transcribed pri-miRNA usually contains a 5', 7-methyl guanosine cap and a poly-A tail (Ambros et al., 2003; Lee et al., 2002; Lee et al., 2004). The maturation process starts by the action of the microprocessor complex, containing the RNase III-type endonuclease Drosha and the DGCR8 co-factor (Figure 1). This complex trims pri-miRNAs at the lower stem start-site in the nucleus to yield a miRNA precursor (pre-miRNA) hairpin molecule of 60 to 70 nt in length, with a 2 nt overhang at the 3' end (Lee et al., 2003; Han et al., 2004; Han et al., 2006). The pre-miRNA is then exported to the cytoplasm by the nucleocytoplasmic protein Exportin 5 (XPO5)/Ran complex, in a GTP-dependent manner (Yi et al., 2003). The pre-miRNA is further processed by Dicer, another RNase III-type enzyme (Bernstein et al., 2001; Hutvagner et al., 2001), to produce a miRNA-duplex (~22 nt) transient molecule by cleaving the hairpin precursor across the terminal loop structure with a 2 nt overhang (Lima et al., 2009). Products of Dicer typically have a 5' phosphate and a 3' -OH group (Ambros, 2003). Dicer requires TARBP2 (HIV-1 TAR RNA-binding protein 2) and PACT (protein activator of PKR) cofactors for the stable formation of miRISC loading complex (miRLC) with the Argonaute 2 (Ago2) protein (Chendrimada et al., 2005; Maniataki & Mourelatos, 2005; Lee et al., 2006; MacRae et al., 2008). The miRLC loads the mature miRNA into the Ago2 component of the RISC (Schwarz et al., 2003; MacRae et al., 2008). The strand entering the RISC (RNA induced silencing complex) is selected on the basis of the binding strength at the 5'end of the miRNA duplex. The mature miRNA is then incorporated into the RISC as a guide strand whereas the passenger strand (miRNA*) is degraded (Schwarz et al., 2003; Bartel, 2009). In some cases the passenger strand can be loaded in the Ago complex and become functionally active (Czech et al. 2009) and can facilitate miRNA loading (Shin, 2008). miRNAs then mediate gene silencing post-transcriptionally by mRNA degradation, translation inhibition or both, in an ATP-independent manner (Meister et al., 2004; Rand et al., 2005; Shin, 2008).

1.1.2 Non-canonical miRNA biogenesis

Mature miRNAs can be generated through alternative mechanisms to produce Drosha- or dicer-independent miRNAs (Figure 1). Mirtrons are an example of Drosha-independent miRNAs, originating as a direct by-product of mRNA splicing that fold-back into a hairpin pre-miRNA structure without the microprocessor catalytic activity, yet exhibiting a 2 nt overhang at the 3'end (Berezikov et al., 2007). Non-canonical pre-miRNAs are exported by the XPO5 complex where they join the canonical miRNA biogenesis pathway. Most mirtrons originate from the 3' arm of their precursors (Okamura et al., 2007). Other Drosha-independent miRNAs include the snoRNA ACA45-derived and small vault RNAs that possess miRNA-like function (Ender et al., 2008; Persson et al., 2009) and viral-encoded miRNAs (Cazalla et al., 2011; Rosewick et al., 2013). An exclusive maturation process is that of *miR-451,* which circumvents Dicer processing. The pre-miR is loaded and cleaved by the Argonaute, yielding a *pre-miR-451* intermediate (Cheloufi et al., 2010) which in turn is

processed by the 3' exonuclease activity of the poly (A)-specific ribonuclease (PARN) to generate the mature *miR-451* (Yoda et al., 2013).

1.1.2.1 *miRNA editing*

Alternative isoforms of miRNAs 'isomiRs' are generated by ribonucleoside post-transcriptional modifications of miRNAs such as adenylation in mammals. Adenylations are catalyzed by PAPD4/5 (Poly (A) polymerase associated domain containing 4/5) on mature canonical miRNAs; MTPAP (mitochondrial PAP) polyadenylates miRNAs and mitochondrial transcripts; TUT1 (terminal uridylyl transferase 1) was found to be responsible for 3' adenyl and 3'uridyl additions to *miR-31* and *miR-200a* respectively (Burroughs et al., 2010; Wyman et al., 2011). Some pri-miRNA transcripts are subjected to modifications by *A*denosine *D*eaminases *A*cting on *R*NA enzymes (ADAR1 and ADAR2). ADAR-editing occurs in dsRNA by an adenosine-specific deamination to inosine (A to I) which is common in primates (Eisenberg et al., 2005). A to I editing is responsible for at least 16% of the modifications in human pri-miRNAs, resulting in alterations in the pri-miRNA stem structure and stability, due to the replacement of A-U pairs to I•U wobble pairs. Indeed, altered seed sequences affect target-gene silencing or prevent microprocessor processing if substitutions are proximal to the cleavage-site. pri-miRNA transcripts with multiple A•I pairs are degraded by Tudor-SN (Tudor Staphyloccal nuclease), a ribonuclease component of the RISC. Thus, miRNA editing disrupts base complementarity with far-reaching consequences in miRNA regulated gene expression (Caudy et al., 2003; Blow et al., 2006; Yang et al., 2006; Kawahara et al., 2008).

1.2 MECHANISMS OF ACTION

1.2.1 Target recognition and regulation

Metazoan miRNA targets are located in the main functional elements of mRNA transcripts, but predominantly in the 3' untranslated region (UTR) and does not require perfect complementarity (Lewis et al., 2003). In contrast, target sequences in plants often match their target with perfect complementarity and can be located in any region along the transcript (Rhoades et al., 2002; Grimson et al., 2007).

miRNAs identify their targets via seed-pairing, located at residues 2-8 of the mature miRNAs. Seed complementarity is essential and independent of 3'end pairing. Seed sites can be classified as 5'dominant or 3'compensatory sites and include 5'seed sites, 3'supplementary seed sites, 3'compensatory sites, cleavage sites and centered sites. Supplementary pairing gives miRNAs only minimal advantage, while 3'complementarity by itself does not yield miRNA-mediated targeting (Doench and Sharp, 2004; Brennecke et al., 2005; Bartel, 2009; Shin et al., 2010). The canonical miRNA seed types are described in Table 1 and are commonly used in the miRNA target prediction programs. Centered sites lack both 5'seed and 3'compensatory site-pairing. Instead, these miRNAs possess 11-12 proximally-positioned

nucleotides spanning positions 4-15 that can direct Ago2-directed cleavage in vivo (Shin et al., 2010).

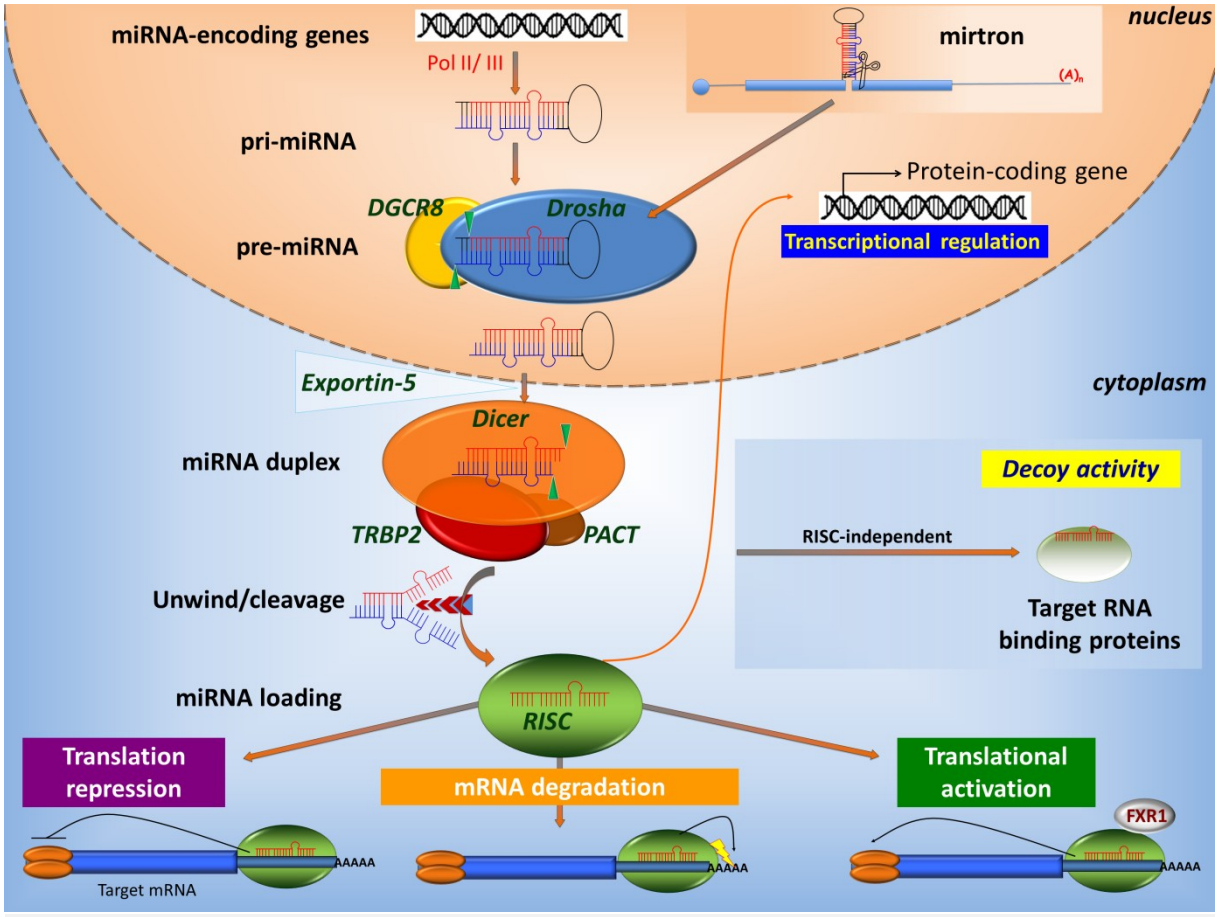

Figure 1 - Canonical and noncanonical miRNA biogenesis pathways: Pri-miRNAs are transcribed by RNA polymerase II/III and trimmed by Drosha/DGCR8 at the cleavage sites (green arrows). Mirtrons arise from spliced introns and form a premirtrons. They are debranched into a precursor hairpins. Both canonical and mirtron precursors are exported by the Exportin5-Ran-GTP complex into the cytoplasm. Precursors are diced to form a miRNA duplex. The red strand represents the mature miRNA with a 5' P and a 3' OH. The duplex is unwound to separate the passenger strand (blue) from the guide strand (red). The miRNA is loaded into the AGO2, the core component of the RISC complex to guide mRNA degradation, translational repression or activation.

Table 1 – Canonical miRNA complementarity sites

Seed type	Description
6mer	Six nucleotide seed match, spanning residues 2 to 7 of the mature miRNA
7mer-m8	Seven nucleotide seed match, spanning residues 2 to 8 of the mature miRNA
7mer-1A	Seven nucleotide seed match, spanning residues 1 to 7 of the mature miRNA, followed by an "A"
8mer	Eight nucleotide seed match, spanning residues 1 to 8 of the mature miRNA

1.2.2 mRNA deadenylation and turnover

This mechanism appears to be dependent on the degree of miRNA-mRNA complementarity. Perfect complementary binding induces mRNA cleavage by the Ago2 endonuclease. This mechanism commonly occurs in plants but rarely in animals (Meister et al., 2004; Yekta et al., 2004; Petersen et al., 2006; Nielsen et al., 2007). Experimental evidence in *Drosophila* suggests that miRNAs activate mRNA degradation via deadenylation and decapping by the CCR4:NOT and DCP1:DCP2 complexes, respectively (Behm-Ansmant et al., 2006).

1.2.3 Translational repression and blocking of elongation

Partial complementarity between miRNA-mRNA interactions generally leads to target silencing via translational repression. Though it is unclear how the mechanism works at the molecular level, one model suggests that translational inhibition takes place at the initiation step, while the other model proposes ribosome drop-off by inhibiting the elongation step in animals (Petersen et al., 2006; Pillai et al., 2005; Zekri et al., 2013).

1.2.4 Atypical targeting mechanisms

Certain miRNAs mediate their action by 5'UTR-targeting to silence or enhance gene expression (Kloosterman et al., 2004; Ørom et al., 2008). Steitz and colleagues reported that miRNA-induced repression in proliferating cells switches to activation in serum-starved non-proliferating cells (Figure 1), implicating a cell cycle-stage determined regulation (Vasudevan et al., 2007). In addition, several miRNAs such as *miR-223* and *miR-320* can mediate transcriptional gene silencing by binding directly on the promoter of their target genes to repress transcription (Kim et al., 2008b; Zardo et al., 2012).

Some miRNAs can act as decoys by direct interaction with the RNA-binding site of RNA binding proteins (RBPs). For example, *miR-328* binds to the translational regulator poly(rC)-binding protein hnRNP E2 that leads to release of *CEBPA* mRNA from hnRNP E2-mediated translation repression during myeloid cell differentiation (Eiring et al., 2010). Likewise, *miR-29* binds to the RNA binding protein HuR that protects the A20 tumor suppressor transcripts from degradation by HuR (Balkhi et al., 2013).

1.3 IDENTIFICATION OF MIRNA TARGETS

To expand and deepen our insights into miRNA functions requires the identification of miRNA targets. The magnitude of this task presents us with the daunting challenge of identifying all these targets in both physiological and pathological conditions in all cell types. Regardless of the complexity, biochemical and bioinformatic methods have been developed to better discern the role of miRNAs in disease.

1.3.1 Bioinformatic approach

Computational biology tools such as miRanda, PicTar, PITA, TargetScan, RNA22 and DIANA-microT provide a useful approach in predicting miRNA:mRNA targets. However,

discrepancies often result due to different algorithms and criteria applied in each tool. For example ‘complementarity to the miRNA seed region’, ‘evolutionary conservation of the MRE’ (miRNA recognition element) and ‘thermodynamic stability’, are considered by different prediction tools (Thomas et al., 2010; Watanabe et al., 2011). The downside of a purely bioinformatic approach is high false discovery rates because the miRNA and its predicted targets may not be simultaneously expressed or the miRNA-mediated target site is not canonical (Watanabe et al., 2011; Pasquinelli et al., 2012). Indeed, distinctive miRNA profiles have been identified based on tissue type or disease (Liang et al., 2007; Lu et al., 2005). For instance, *miR-21* is overexpressed in many cancer types (Chan et al., 2005 ; Iorio et al., 2005; Si et al., 2007; Seike et al., 2009; Özata et al., 2011); multiple miRNAs can target the same transcript (Wu et al., 2010); whereas high expression of certain miRNAs can be organ-specific e.g. *miR-122a* (liver), *miR-124* and *miR-9* (brain), *miR-1b* and *miR-133* (muscle) and *miR-223* (bone marrow) (Baskerville & Bartel 2005). Also, perfect miRNA complementarity is infrequent in metazoa (Lewis et al., 2003), making target prediction more intricate. This level of complexity demands further refinement in prediction tools by incorporating more experimental data. In fact, some databases already contain experimental data, for example miRWalk, miRTarBase, miRecords and miRNA_Targets. Recent refinements of bioinfomatic algorithms take into account RNA secondary structures, non-canonical targeting and Argonaute protein constraints (Khorshid et al., 2013; Agarwal et al., 2015). Yet much remains to be learned about factors that influence target site function *in vivo*.

1.3.2 **Experimental approach**

Early biochemical methods based on RNA immunoprecipitation (RIP) methodology were applied to identify RNA-binding proteins and eventually identify miRNA targets. This method relies on stable physical interactions between miRISC and the target mRNA *in vivo*. Prior to the high-throughput sequencing era, isolated transcripts were identified by a microarray-based approach, known as RIP-chip (Tenenbaum et al., 2000). This method was restricted to targets present on the microarray chip and the RBP-recognition-element could not be specified. In addition, only strong protein RNA interactions could be identified (Tenenbaum et al., 2000; de Silanes et al., 2004). To improve efficiency, crosslinking was applied to covalently bind RNA-protein molecules in the immediate proximity, creating CLIP (Cross-Linking and Immunopreciptiation) (Ule et al., 2003; Hafner et al., 2010; Licatalosi et al., 2010). CLIP methods utilize UV crosslinking since it only induces crosslinks between RNA species and RNA-protein at the molecular contact sites. Another benefit is irreversibility as the errors introduced during cDNA synthesis serve as tags, marking the exact sites of the RNA-protein binding (Darnell, 2010). Further developments coupled CLIP to next-generation sequencing (CLIP-seq) demonstrating its power in identifying miRNA targets and specific binding sites (Ule et al., 2003; Licatalosi et al., 2008; Sanford et al., 2009; König et al., 2010). Among various CLIP-seq methodologies, PAR-CLIP (*P*hoto*A*ctivatable-*R*ibonucleoside-enhanced *C*rosslinking and *I*mmunoprecipitation) and HITS-CLIP (*HI*gh-*T*hroughput

Sequencing of RNA isolated by *C*ross*L*inking and *I*mmuno*P*recipitation) are the most well-known approaches (Chi et al., 2009; Hafner et al., 2010).

In this thesis, the PAR-CLIP method (Figure 2) was used to identify *miR-944* targets in human cervical cancer cells (Paper I). Cells are treated with a photo-activatable ribonucleoside analog, such as 4-thiouridine (4SU) or 6-thioguanosine (6SG), overnight. The ribonucleoside analogs incorporate into mRNA transcripts in living cells and then UV crosslinked at 365 nm prior to cell harvesting. Low energy UV crosslinking increases efficiency and allows identification of the precise target-binding site by T-> C transitions (4-SU) and G->A (6-SG) in sequenced reads (Hafner et al., 2010). HITS-CLIP however, uses higher energy UV crosslinking (254 nm) and no ribonucleoside analogs (Chi et al., 2009) to identify miRNAs targets (Haecker et al., 2012). HITS-CLIP was further improved by applying stringent washes to eliminate background RNA species. Still, these methods are constantly evolving and being refined such as iPAR-CLIP (Grosswendt et al., 2014), CLASH (Crosslinking, Ligation And Sequencing of Hybrids) and CLEAR-CLIP (Covalent ligation of endogenous Argonaute-bound RNAs-CLIP), all employing miRNA-target chimeras in purified AGO complexes, for direct mapping of RNA-RNA interactions (Helwak et al., 2013; Moore et al., 2015).

1.4 METABOLISM

Metabolism is a sequence of chemical reactions that transform fuel molecules into smaller molecules that are of a biological useful form while extracting energy. Glucose and glutamine are the two main nutrients providing the carbon intermediates to build various macromolecules. Certain metabolic reactions require an energy input to proceed, called anabolic reactions. The oxidation of glucose and glutamine allows the cells to produce their reducing power in the form of NADH or $FADH_2$ and transfer of electrons to generate adenosine triphosphate (ATP). The triphosphate unit contains two phosphoanhydride bonds that liberate large amounts of energy when it is hydrolysed to adenosine diphosphate (ADP) or adenosine monophosphate (AMP). However, certain reactions can be driven by other derivative nucleotide triphosphates. ATP hydrolysis powers metabolism by shifting the equilibrium of a coupled reaction. In oxidative metabolism, O_2 is the ultimate electron acceptor in the oxidation of carbon-containing molecules into CO_2 and H_2O byproducts. Glutamine also supplies nitrogen for the synthesis of purines and pyrimidines nucleotides, glucosamine-6-phosphate and non-essential amino acids (Pavlova and Thompson, 2016).

Ion gradients across membranes power the majority of ATP synthesis. Food energy is extracted in three stages: A) Breakdown of large molecules into smaller molecules; B) Small energy molecules are broken down into Acetyl CoA; and C) Complete oxidation of the Acetyl CoA by the Kreb's cycle and oxidative phosphorylation. These catabolic pathways are discussed in this section (Figure 3).

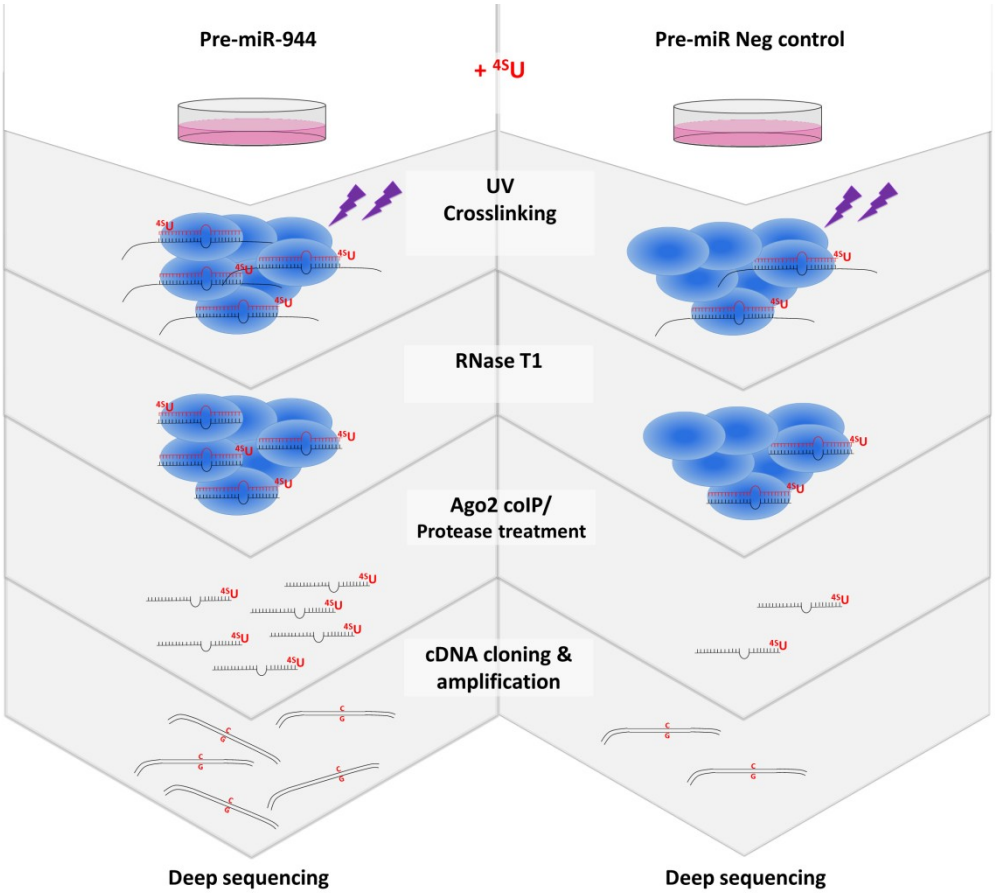

Figure 2 – PAR-CLIP methodology: Cultured cells are transfected with the miRNA mimic of interest. 4SU is added to the culture overnight and cells are UV-crosslinked upon harvesting. Cell lysate is digested with RNase T1 and then immunopurified by Ago2 co-IP. After a second RNase T1 treatment, the Ago2 is digested by proteinase K. Then total RNA extraction is performed, followed by sRNA cloning with Solexa linkers and cDNA synthesis. The library is purified on 3% nusieve gel and the expected band is cut out. The cDNA is extracted and library is then ready for sequencing. Data are analysed bioinformatically, for example by using the Bowtie - PARalyzer pipeline.

1.4.1 Glycolysis and Kreb's cycle

Glycolysis is a cytosolic pathway comprising of ten sequential reactions where one glucose molecule yields two pyruvate molecules (Figure 3). The net energy generated is two ATP molecules per glucose molecule since two ATPs are used in the first reactions yielding fructose 1, 6-bisphosphate. Glycolysis can be split into two stages. In the first stage, glucose enters the cell and is converted into fructose 1, 6-bisphosphate, a compound that can be readily cleaved into two glyceraldehyde 3-phosphate units. In the second stage, glyceraldehyde 3-phosphate undergoes oxidation to pyruvate with generation of ATP. Glycolysis is tightly controlled through three irreversible reactions catalyzed by phosphofructokinase (PFK), hexokinase (HK) and pyruvate kinase (PK) enzymes. Phosphofructokinase function is inhibited by high ATP levels but restored by AMP. Hexokinase is inhibited by the reaction end-product glucose 6-phosphate and by

phosphofructokinase. Pyruvate kinase catalysizes the final reaction of the glycolytic pathway and is allosterically inhibited by ATP, when energy levels are high (Maughan, 2009).

Glucose can also be synthesized from non-carbohydrate sources such as pyruvate, oxaloacetate, phosphoenolpyruvate (PEP), lactic acid, amino acids and glycerol. Lactic acid is formed when the glycolysis rate exceeds the rate of oxidative metabolism and can be interconverted into pyruvate by the lactate dehydrogenase (LDH) enzymes. Glucose can then be synthesized by gluconeogenesis during periods of fasting, starvation or surplus of biosynthetic precursors, ATP and by glucagon (Wasserman et al., 1989; Maughan, 2009).

Glycolysis is an anaerobic process, which harvests only a fraction of the energy available from glucose. The complete oxidation of glucose into carbon dioxide (CO_2) involves a series of oxidative reactions known as the Kreb's cycle or the Citric acid cycle and oxidative phosphorylation. Cellular respiration takes place in the mitochondrion, an organelle responsible for multiple cellular functions (Mayer & Oberbauer, 2003; Nilsson et al., 2009; Wanders et al., 2010; Rudel et al., 2010; Scott et al., 2010; Finkel et al., 2012) and contains its own genome. Mammalian mitochondrial (mt) DNA encodes for mtRNAs (12S rRNA, 16S rRNA and 22 tRNAs) and encodes 13 polypeptides of the oxidative phosphorylation (Solaini et al., 2011).

Fuel molecules enter Kreb's cycle in the mitochondria as acetyl coenzyme A (acetyl CoA) by the oxidative decarboxylation of pyruvate. The cycle consists of eight reactions that harvest high-energy electrons from glucose, amino acids and fatty acids. Every reaction provides the substrate for the subsequent step, starting with the aldol condensation-hyration reaction between oxaloacetate and acetyl-CoA to form citrate. The end-product of every cycle is oxaloacetate and each round yields two CO_2, one ATP, two NADH and one $FADH_2$ molecules. Both NADH and $FADH_2$ drive the electron transport chain in building up a proton gradient against the inner mitochondrial membrane. Isocitrate dehydrogenase (IDH) is a main control point in Kreb's cycle, which is activated by ADP, isocitrate, NAD^+, Mg^{++} and inhibited by ATP and NADH. α-ketoglutarate dehydrogenase is another enzyme that controls the cycle and is inhibited succinyl-CoA, NADH and high energy charge (Taylor et al., 2008; Shi et al., 2011).

1.4.2 The mitochondrion and the respiratory chain

The mitochondrion is a double-membraned organelle. The outer membrane is highly permeable to most small molecules and ions due to the presence of the voltage dependent anion channel or Porin. Porin is key in regulating the metabolic flux by allowing anionic species through (Colombini et al., 1996). The inner mitochondrial membrane has cristae formations that significantly increase the surface area, accommodating more oxidative phosphorylation (oxphos) complexes. The inner membrane is impermeable to almost all charged species but allows transport of specific energy molecules (ATP, pyruvate, citrate). A

membrane potential is build-up across this membrane, with a negative charge inside the matrix and a positive charge in the intermembrane space (Schultz and Chan, 2001).

Kreb's cycle supplies NADH and $FADH_2$ in the mitochondrial matrix, to support electron flow through the respiratory chain and the build-up of a proton gradient fueling ATP synthesis. The oxphos is the oxidation of energy molecules generating ATP, coupled by a trans-membrane proton flux across the inner mitochondrial membrane. Electrons flow from NADH and $FADH_2$ through four protein complexes that ultimately reduce molecular oxygen to H_2O. Protons return to the mitochondrial matrix by flowing through a fifth protein complex, ATP synthase that catalyzes ATP synthesis (Schultz and Chan, 2001).

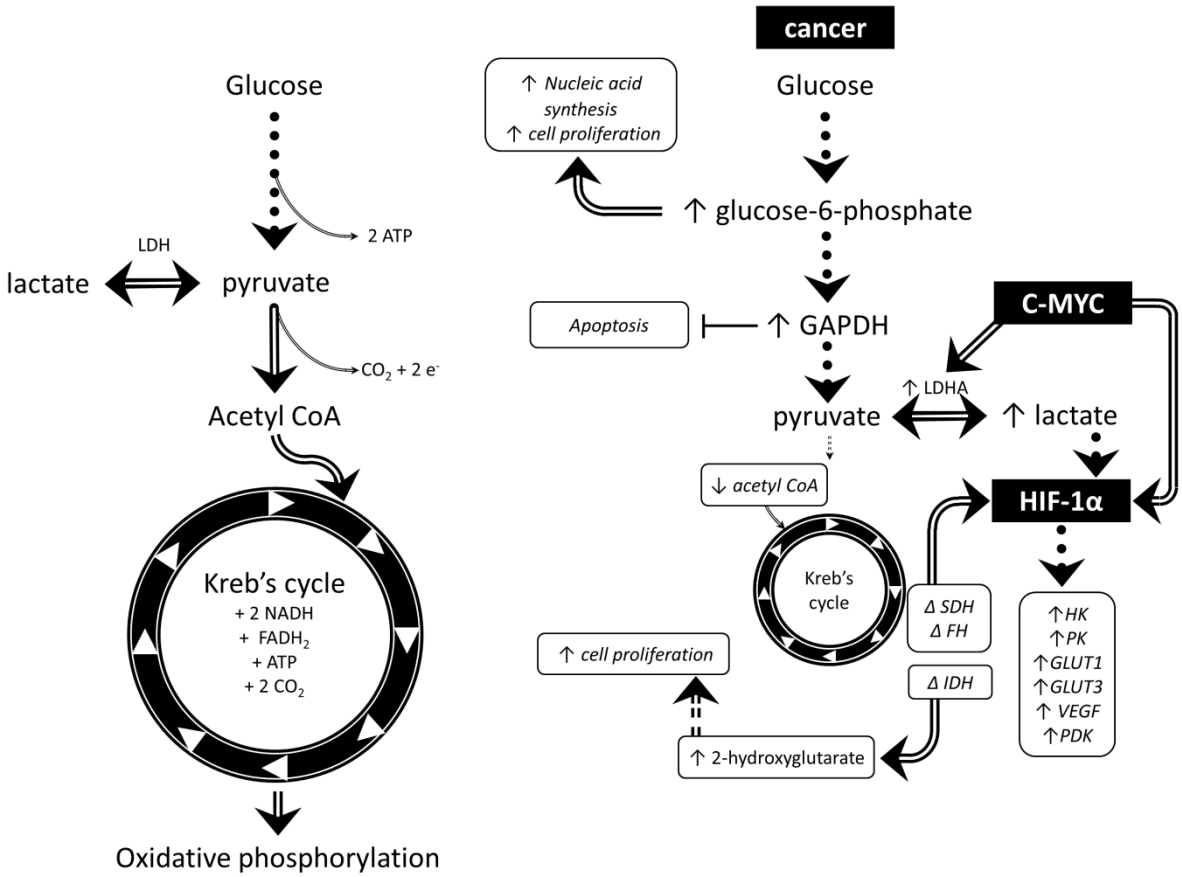

Figure 3 – Glycolysis and Kreb's cycle: Left side shows the catabolic reactions under physiological conditions. Pyruvate is converted to acetyl CoA and fully oxidized to CO_2 and H_2O. **Right side** shows selective metabolic reactions in the cancer cell characterized by high glucose 6-phosphate and GAPDH to support rapid cell growth and evade apoptosis. Rapid cell growth results in hypoxia that induces HIF-1α expression. Mutated *SDH* and *FH* further induce HIF-1α due to accumulation of succinate and fumarate respectively. C-Myc activates HIF-1α directly but also through LDHA overexpression and excess lactate production. In turn, HIF-1α induces overexpression of glycolytic enzymes, glucose transport, simulates vascular growth and inhibits generation of acetyl CoA.

The respiratory chain is made up of four complexes: Three proton pumps; NADH-Q oxidoreductase (complex I), Q-cytochrome C oxidoreductatase (complex III) and Cytochrome C oxidase (complex IV). Another complex, Succinate-Q reductase (complex II) links the oxphos directly to the Kreb's cycle. Electrons flow through these complexes to reduce O_2, and powering the proton flux across the inner membrane. Two electron carriers, coenzyme Q (ubiquinone) and cythochrome C, shuttle electrons from complexes I and II to complexes III and IV respectively. Kreb's cycle is not the only source of NADH, as it can be sourced from fatty acid oxidation or transported from the cytoplasm (Schultz and Chan, 2001).

Complex I is the entry point of high-potential electrons in the respiratory cycle. This complex is a large L-shaped protein complex made of 45 subunits and functions as a proton pump. For every two electrons entering the complex from NADH, four H^+ are pumped out into the mitochondrial inter-membrane space. $FADH_2$ enters the electron transport chain at complex II (Schultz and Chan, 2001; Dieteren et al., 2008). Electrons are then passed to complex III via coenzyme Q and to complex IV via cytochrome C to reduce O_2. For every eight protons, O_2 yields two H_2O molecules and four protons are pumped into the inter-membrane space. Water is a safe by-product of respiration, yet small amounts of dangerous intermediates do occur due to partial reductions. The flow of one electron to O_2 leads to the formation of the superoxide anion (O_2^-) and peroxide ion (O_2^{2-}) if two electrons are accepted. These ions are collectively known as reactive oxygen species (ROS) and has been implicated in cancer and aging. Cells can neutralize superoxide and peroxide damage by superoxidase dismutase and catalase respectively, but also by other antioxidants (Schultz and Chan, 2001; Lenas & Genova et al., 2010).

1.4.2.1 *ATP synthesis*

The respiration is coupled to ATP synthesis through the proton flux, powering the enzyme ATP synthase (complex V). This complex generates most of the ATP in healthy cells and is located in the inner mitochondrial membrane. ATP synthase is made up of two subunits, a cylindrical-shaped F_0 subunit inserted in the inner mitochondrial membrane and a spherical-shaped F_1 subunit that performs the catalytic function (Schultz and Chan, 2001). ADP is the substrate that binds to an ortho-phosphate moiety, to synthesize and release ATP. The complete oxidation of one glucose molecule yields over 30 ATP molecules (Icard et al., 2012).

1.5 CANCER

In 1970, the government of the United States declared a 'war on cancer', which led to the National Cancer Act of 1971 and establishment of the modern National Cancer Institute (National cancer Act, 1971). The last five decades of research have revealed that cancer is a multistep process involving a gradual transformation of non-cancerous cells to malignant cells, by acquiring capabilities that disrupt homeostasis. Cancer can be defined as a disease where cells gain functions prompting uncontrolled proliferation, promoted by mutations that

convert proto-oncogenes into oncogenes and the loss of tumor-suppressor genes function (Hanahan and Weinberg 2000). Initiation and progression of tumor development demands the presence of multiple factors that collectively drive the accumulation of genetic mutations and epigenetic changes that transform normal cells into malignant. Malignant cells harbor multiple genetic lesions that regulate cell growth.

1.5.1 The hallmarks of cancer

In the year 2000, Hanahan and Weinberg proposed six mechanisms that enable cancer cells to bypass the mechanisms controlling cell growth. These mechanisms or hallmarks include: i) evasion of apoptosis, ii) production of growth signals, iii) unresponsiveness to anti-growth signals, iv) local and metastatic spread, v) enhancement of angiogenesis, vi) unlimited proliferation. Later it became apparent that more mechanisms are at play: vii) metabolic reprogramming, viii) genome instability, ix) evasion of immunological surveillance and x) tumor inflammation. Understanding the mechanisms underlying these capabilities is key to uncover the tumorigenic processes (Hanahan and Weinberg, 2011).

1.5.2 Degregulation of miRNAs in cancer

Aberrant miRNA expression was found in many types of human cancers (He et al., 2005; Lu et al., 2005; O'Donnell et al., 2005; Zhang et al., 2006), for example colorectal neoplasia (Michael et al., 2003), lung cancer (Takamizawa et al., 2004), large B-cell lymphoma (Eis et al., 2005), breast cancer (Iorio et al., 2005), cervical (Lui et al., 2007; Witten et al., 2010) and neuroendocrine carcinomas (Soon et al., 2009; Özata et al., 2011; Xie et al., 2014). The first report was published by Croce and colleagues showing down-regulation of *miR-15a* and *miR-16-1* in B-cell chronic lymphocytic leukemia (B-CLL). Both miRNAs are located in the 13q14 chromosomal region, a site frequently deleted in B-CLL. Indeed, most miRNA genes are located at sites already linked to cancer or fragile sites (Calin et al., 2002; Calin et al., 2004). Furthermore, deregulation of the miRNA processing machinery can alter miRNA levels and promote cellular transformation and tumorigenesis (Melo et al., 2010; Hill et al., 2009; Torrezan et al., 2014) of tumors with a highly invasive phenotype (Kumar et al., 2007). Transcription of miRNA genes are also affected by epigenetic changes, such as DNA methylation and histone modifications. For example, *miR-15a*, *miR-16-1* and *miR-29b* are epigenetically silenced by histone deacetylases in CLL (Sampath et al., 2012). *miR-203* is hypermethylated in hematological malignancies (Bueno et al., 2008) and breast cancer (Taube et al., 2013).

1.5.2.1 *OncomiRs*

OncomiRs are deregulated oncogenic miRNAs supporting tumor growth by inhibiting tumor-suppressor genes. *miR-21, miR-155* and *miRNAs* originating from the *mir-17~92* cluster (oncomiR-1) are among the most well-known oncomiRs (Figure 4). *miR-21* is highly expressed in tumors of the breast (Yan et al., 2008), lung (Seike et al., 2009), liver

(Gramantieri et al., 2008), pancreas (Dillhoff et al., 2008), colorectal (Asangani et al.,2008) and gastrointestinal (Zhang et al., 2008). It promotes proliferation, invasion, metastasis, inhibits apoptosis (Si et al., 2007; Asangani et al., 2008; Yan et al., 2011; Jin et al., 2013b) and transformation of stromal tissue in squamous cell carcinoma (Nouraee et al., 2013). The *Grhl3* transcription factor is one crucial target with subsequent loss of *PTEN* expression, leading to amplification of the PI3K/AKT/mTOR pathway (Darido et al., 2011) in cervical tumorigenesis (Peralta-Zaragoza et al., 2016), by an intricate positive feedback mechanism (Bhandari et al., 2013). *miR-21* can even induce invasion, metastasis and evasion of apoptosis via *PDCD4* targeting (Asangani et al., 2008; Melnik, 2015). Indeed, *miR-21* is a key promoter of oncogenesis targeting several signaling pathways and cell cycle control (Wang et al., 2009a), particularly in diseases of the skin (Melnik, 2015).

miR-155 was found to be over-expressed in hematological malignancies (Metzler et al., 2004) and solid tumors (Volinia et al., 2006; Gironella et al., 2007; Zhang et al., 2013), and its expression correlates to the *BIC* (B-cell integration cluster) host gene (Zhang et al., 2008). Transgenic mouse models over-expressing this miRNA developed B cell related pathologies (Costinean et al., 2006). The *BIC/miR-155* expression was found to increase in activated T and B cells and other cells of the immune system and seems to be essential for lymphocyte-mediated immune function (Rodriguez et al., 2007). The *miR-155* mediated mechanisms promoting tumor growth are poorly understood however, several targets (Figure 4) were identified in B cell lymphomas/ leukemias, (Costinean et al., 2009; Pedersen et al., 2009), breast cancer (Jiang et al., 2010) and promotion of angiogenesis (Kong et al., 2014).

The *mir-17~92* cluster (*miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1*) is located in chromosome 13q31, a region often amplified in several tumor types (Ota et al., 2004; He et al., 2005; Hayashita et al., 2005; Mendell, 2008) driven by c-Myc and E2F transcription factors (He et al., 2005; O'Donnel et al., 2005; Aguda et al., 2008). E2F and Myc expressions are regulated by positive feedback-loops and simultaneously down-regulated by members of the *miR-17~92* cluster by negative feedback (Aguda et al., 2008; Mendell, 2008). This cluster modulates a vast number of targets (Mogilyansky and Rigoutsos, 2013). Though E2Fs are pro-apoptotic, E2F1 can also be post-transcriptionally regulated by other miRNAs (*miR-106b-25* cluster), leading to increased cell proliferation rather than apoptosis (Hayashita et al., 2005; Mendell, 2008) and to other oncogenic phenotypes (Nagel et al., 2009; Olive et al., 2009; Dews et al., 2010; Huang et al., 2012). In addition, miRNAs from this cluster target: *i)* SMAD2/3/4 (Mestdagh et al., 2010; Dews et al., 2010) of the TGFβ signaling pathway, *ii)* CDKN1A/p21 (Wong et al., 2010), *iii)* inhibitors of PI3K and NFκB pathways (Jin et al., 2013a), *iv)* Bcl-2 interacting mediator of cell death (Tsuchida et al., 2011) and *v)* promote sonic hedgehog-mediated proliferation in medalloblastomas (Northcott et al., 2009).

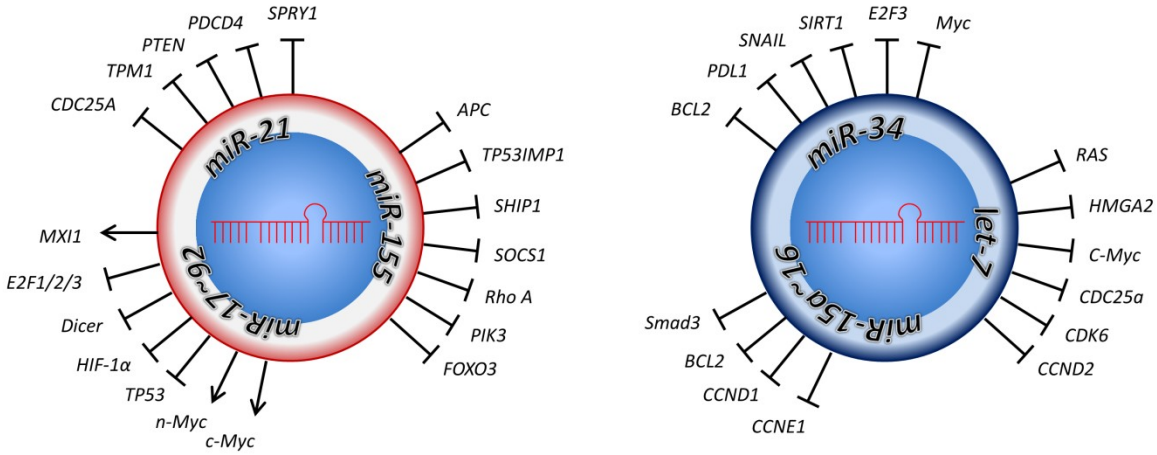

Figure 4 – Oncogenic and tumor suppressor miRNAs: Left - Verified targets of *miR-21*, *miR-155* and the *miR-17~92* cluster oncomiRs. **Right** – Verified targets of *miR-34*, *let-7* and the *miR-15~16* cluster tumor suppressor miRNAs.

1.5.2.2 *Tumor-suppressor miRNAs*

Tumor suppressing miRNAs silence transcripts with oncogenic potential and are often down-regulated in cancer as a consequence of mutations, deletions, epigenetic silencing or disruption in the miRNA maturation process (Calin et al., 2002; He et al., 2005; Park et al., 2009). As mentioned earlier, the *mir-15a~16-1* cluster of tumor suppressor miRNAs are frequently down-regulated in B-CLL, but also in lung (Bandi et al., 2009) and prostate cancers (Porkka et al., 2011). This cluster targets *CCND1* and *CCNE1* (Bonci et al., 2008; Bandi et al., 2009), *Smad3/ACVR2A* (Jin et al., 2018) and *BCL2* (Cimmino et al., 2005) to inhibit cell proliferation, invasion and promote apoptosis (Figure 4).

The *miR-34* family members are tumor suppressor miRNAs whose expression was found to be directly linked to that of p53. Silencing of p53 in human cancer cell lines decreased *miR-34a* levels, while DNA damage restores its expression (Raver-Shapira et al., 2007). It has pro-apoptotic and non-proliferative functions by targeting the apoptosis inhibitors *BCL2* and *SIRT1* (Yamakuchi and Lowenstein, 2009). The 1p36 chromosomal region hosts the *miR-34* family, a site found to be deleted in different cancer types (He et al., 2007; Raver-Shapira et al., 2007; Bagchi and Mills, 2008). Recently, the p53/*miR-34* axis was shown to suppress PDL1 (Cortez et al., 2016) and SNAIL (Siemens et al., 2011). Genetic mutations or epigenetic silencing inactivate p53/*miR-34* in cancer cells, allowing transformation of epithelial to mesenchymal cell transformation, invasion, metastasis and evasion of immunological surveillance (Siemens et al., 2011; Rokavec et al., 2015).

Other tumor suppressor miRNAs include *let-7* and *miR-29*. Various *let-7* isoforms were found poorly expressed in lung and other cancer types (Takamizawa et al., 2004; Barh et al., 2010). *Let-7* is a strict cell cycle modulator showing an anti-proliferative phenotype by targeting

RAS, CDC25a, CDK6, CCND2, HMGA2 (high mobility group at–hook 2), c-Myc and IMP1 (Insulin-like growth factor 2 mRNA-binding protein-1) oncogenes (Johnson et al., 2005 & 2007; Chang et al., 2008; Barh et al., 2010). Members of the *miR-29* family mediate p53 regulation by targeting CDC42 and p85, a subunit of PI3K. Silencing of p85α, upregulates p53 and induces apoptosis. *miR-29* inhibits DNA methyltransferase activity to suppress DNA methylation and also targets MCL1 (myeloid cell leukemia-1) to suppress the Bcl2 protein family (Park et al., 2009). Moreover, *miR-29* has been shown to be under the control of NF-κB - Yin Yang-1 (YY1) interaction and deregulation can lead to rhabdomyosarcoma (Wang et al., 2008). Low *miR-29* levels were reported in CLL (Pekarsky et al., 2006), lung (Yanaihara et al., 2006), prostate (Porkka et al., 2007) and breast cancers (Iorio et al., 2005).

1.5.3 Tumor metabolism and respiratory complexes

In the last two decades, it became more apparent that metabolic reprogramming is an essential malignant transformation and a hallmark of cancer (Hanahan & Weinberg, 2011). Altered metabolism leads oncometabolites that in turn affects gene expression, cellular differentiation and tumor microenviroment. Cancer-associated metabolism can be described by six hallmarks: 1) Deregulated glucose and amino acid uptake, 2) the opportunistic use of modes of nutrient acquisition, 3) the use of glycolysis and Kreb's cycle intermediates for biosynthesis and NADPH production, 4) high nitrogen requirement, 5) altered metabolism-induced gene expression and 6) altered microenvironment (Pavlova and Thompson, 2016). Some of these tumor-related metabolism characteristics are further discussed in this section (Figure 3).

1.5.3.1 *Aerobic glycolysis and altered gene expression*

Aerobic glycolysis is a property of rapidly dividing cells such as tumor cells. These cells exhibit high glucose uptake, high glucose 6-phosphate (G6P) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) combined with high lactate production, even in the presence of oxygen. Aerobic glycolysis was first described by Otto Warburg in the 1920s and is also known as the Warburg effect (Warburg et al., 1927). Tumors with high glucose uptake are often very aggressive and show poor prognosis. The benefits of acidification of the tumor environment are yet elusive but presumably facilitates tumor invasion by evading an immune response (Lardner, 2001). Tumor cells utilize G6P from glycolysis to funnel it into the pentose phosphate pathway to generate reducing power and production of nucleotides (Patra and Hay, 2014).

Aerobic glycolysis diminishes the cell dependence on oxygen for growth, a requirement for the highly proliferative cancer cells. However, hypoxia ensues in unvascularized tumor tissue since blood vessels grow at a slower rate. This induces a metabolic switch by altering which isoform of hexokinase (HK) and pyruvate kinase (PK) are expressed but mainly tumor hypoxia triggers the expression of hypoxia-inducible factor (Wallace, 2005; Luo and Semenza, 2011). Hypoxia inducible factor 1 (HIF-1) induces overexpression of genes

regulating glycolysis, GLUT1 and GLUT3 glucose transporters (Iyer et al., 1998), LDHA, PDK1 (Semenza, 2007) and vescular endothelial growth factor (Jung et al., 2005). Metabolic adaptations allow the cancer cells to survive until blood vessels regenerate.

Mutations in enzymes of the Kreb's cycle (Figure 3), succinate dehydrogenase (SDH), fumarate hydratase (FH), pyruvate dehydrogenase kinase (PDK) and isocitrate dehydrogenase (IDH), promote tumor development by enhancing aerobic glycolysis (Pollard et al., 2005; King et al., 2006; Deberardinis and Chandel, 2016). All these enzymes except for IDH are related to the HIF-1 transcription factor. Under physiological conditions, HIF-1 up-regulates glycolytic enzymes and glucose transporters only under hypoxic conditions (during exercise) and is then hydroxylated to prolyl hydroxylase 2 and destroyed by the proteasome to abolish glycolytic stimulation (King et al., 2006). However, mutations in *SDHx* or *FH* genes induce accumulation of succinate, fumarate and lactate in the mitochondria and cytoplasm which inhibit prolyl hydroxylase 2 and stabilize HIF-1 (Selak et al., 2005; King et al., 2006; Sonveaux et al., 2012). HIF-1 enhances production of PDK to inhibit pyruvate to acetyl CoA conversion and accumulates in the cytoplasm. Mutations in *PDK* also enhance aerobic glycolysis, increase lactate concentration and HIF-1 stabilization (Semenza, 2007). Some pyruvate is still converted into acetyl CoA and used for lipid synthesis in tumor cells, allowing adaptation to metabolic stress and proliferation (Dang, 2013; Munir et al., 2019). Under physiological conditions, IDH catalyzes the converstion of isocitrate into α-ketoglutarate but when mutated, it reduces α-ketoglutarate to 2-hydroxyglutarate, an oncometabolite. 2-hydroxyglutarate alters DNA methylation patterns and promotes uncontrolled cell growth (Dang et al., 2009; Xu et al., 2011).

1.5.3.2 *Dysfunctional oxidative phosphorylation*

Reduced mitochondrial respiration is a feature occurring in many cancer types and linked to aerobic glycolysis (Meierhofer et al., 2004; Hervouet et al., 2008; Calabrese et al., 2013). Decreased activity of complex I and III in cancer was reported to result due to mitochondrial DNA mutations in *ND1* gene of complex I (Bonora et al., 2006; Sharma et al., 2011) or K-ras (Baracca et al., 2010). Heteroplasmic mitochondrial DNA mutations in *ND1* and cytochrome b (complex III subunit) genes have been reported in thyroid carcinoma (Bonora et al., 2006) and lead to high ROS production (Guzy et al., 2005; Ishikawa et al., 2008; Koopman et al., 2010). High ROS levels contribute to tumorigenesis by activating the expression of HIF-1α and other transcription factors while inhibiting expression of PTEN and PTP1B phosphatases (Bertout et al., 2008; Pavlova and Thompson, 2016). Mutations in *SDHx*, encoding a nuclear-encoded mitochondrial protein, has been linked to malignant disease (Linehan and Rouault, 2013; Aspuria et al., 2014; Else et al., 2017). Repression of complex II subunits (*SDHA* and *SDHB*) in cancer contributes to pseudo-hypoxia, HIF-1α overexpression and angiogenesis (Burnichon et al., 2010). *SDHx* and *FH* mutations occur in hereditary tumors.

In addition, the oncoprotein c-Myc promotes a shift from ATP production to aerobic glycolysis, by blocking pyruvate to acetyl-CoA conversion via pyruvate dehydrogenase kinase (PDK) regulation. Myc also increases lactate production through overexpression of LDHA and the lactate transporter MCT1 (Graves et al., 2012; Wahlström & Henriksson, 2015; Pavlova and Thompson, 2016). In addition, the overall oxphos activity correlates to tumor aggressiveness (Simonnet et al., 2002). Indeed, when the oxphos is inhibited by the ATPase inhibitory factor 1 (ATPIF1), an inhibitor of complex V, a shift to aerobic glycolysis is again observed. ATPIF1 also promotes glycolysis and was found highly expressed in breast, lung and colon cancers (Sanchez-Cenizo et al., 2010).

1.5.4 Cervical carcinoma

Cervical cancer is a malignant neoplasm that has the third highest incidence and the third most frequent cancer deaths among women in low/middle income countries. In high-income countries, the incidence has decreased due to the screening programs (Jemal et al., 2011; Torre et al., 2016). The majority of cervical tumors are squamous cell carcinomas (80%), and the remaining (20%) are adenocarcinomas (Durst et al., 1983).

Human Papillomavirus (HPV) infection is prevalent in cervical cancer (Durst et al., 1983; Gissmann et al., 1983). Over 200 types of HPV have been identified (Bzhalava et al., 2014) and categorized as low-(LR) or high-risk (HR) based on their oncogenic potential (Burd, 2003). Indeed, cervical tumors with HR-HPV types 16 and 18 infections are collectively responsible for >70% of invasive cervical carcinomas (Guan et al., 2012). Persistent HR-HPV subtype infections can develop lesions, cytologically described as cervical intraepithelial neoplasia (CIN). Cytological grading is based on the Bethesda system: Low-grade squamous intraepithelial lesion (LSIL) CIN1, and high-grade squamous intraepithelial lesion (HSIL) CIN2 and CIN3. CIN1 and CIN2 represent transformation of a third to 2/3 dysplastic basal epithelium. In CIN3 or cervical carcinoma *in situ*, more than 2/3 of the whole epithelial layer is transformed. Upon invasion of the stromal tissue underneath, the neoplasia is then referred to as cervical cancer (Nayar and Wilbur, 2015; Wang et al., 2014). Though HR-HPV infection is necessary for cancer development, it is not sufficient to develop cervical cancer. Multiple etiologies likely contribute to the disease outcome (Walboomers et al., 1999; Haverkos et al., 2000).

HPV is a small circular DNA (7.9 kb) virus. Its genome is composed of three regions: *i*) The non-coding region that regulates DNA replication, *ii*) the early region that encodes early proteins E1, E2, E4, E5, E6 and E7 necessary for viral replication and oncogenesis, and *iii*) the late region, encoding the L1 and L2 viral capsid proteins. HPVs can be divided into cutaneous or mucosal types depending on the targeted area (Burd, 2003). The HPV early proteins E5, E6 and E7 are the most significant viral oncoproteins with the potential to promote tumor initiation and progression (Moody and Laimins, 2010). However, HPV16 E5 by itself is only partially oncogenic as it is only capable of inducing cervical neoplasia. A

more aggressive tumor results when E5 is expressed in combination with either E6 or E7 by promoting their oncogenicity (Maufort et al., 2010). Recently, the HPV16 E5 gene was reported to downregulate *miR-196a*, resulting in upregulation of its target (Yekta et al., 2004; Liu et al., 2015). Furthermore, an increase in cell proliferation and growth was observed upon *miR-196a* inhibition (Liu et al., 2015). The viral oncogenic protein E6 is well known to target the p53 protein for degradation and immortalization of cells (Scheffner et al., 1990; Niccoli et al., 2012; Togtema et al., 2015). Reduced p53 levels correlate to declined *miR-34a* levels, attributed to E6 that ultimately abolishes the cell cycle checkpoints (Sun et al., 2008; Yamakuchi et al., 2008; Wang et al., 2009b; Wang et al., 2011b; Zuo et al., 2015). In line with this, *miR-34a* expression levels are lower in LSIL, HSIL and cervical cancer than in uninfected cervical epithelium (Li et al., 2010). The E6 forms a complex with E6-associated protein (E6AP), a ubiquitin ligase, that ubiquitinates and degrades p53 in the proteasome (Lechner et al., 1992; Scheffer et al., 1993). E6 also interacts independently with c-Myc transcription factor to promote *TERT* transcription (Veldman et al., 2003). Telomerase activity and expression is further enhanced when E7 is present (Oh et al., 2001). HPV E7-mediated Retinoblastoma (pRb) protein degradation releases E2F1 to drive the cell cycle into S phase (Gonzalez et al., 2001; Roman and Munger, 2013). Unbound E2F1 promotes c-Myb and c-Myc expressions and in turn upregulates the DLEU2 (deleted in lymphocytic leukemia 2) non-coding gene, the region harboring the *miR-15a~16-1* cluster (Zheng and Wang, 2011). Although this region is frequently deleted in malignancy (Calin et al., 2002; Lerner et al., 2009), this cluster is highly expressed in cervical cancer than in matched controls (Wang et al., 2008); still, its significance in cervical cancer is unclear. In addition, the E7 can bind c-Myc directly to promote its DNA-binding and transcription activation capabilities (Wang et al., 2007). HPV E6/E7 hijacking of c-Myc disturbs the expression of many cellular miRNAs, including *miR-17~92* cluster (O'Donnell et al., 2005), *let-7a-1/f-1/d*, *miR-15a~16-1*, *miR-22*, *miR-29a/b* and *miR-34a* (Chang et al., 2008).

Several studies reported aberrant miRNA expression profiles in cervical cancer (Table 2) (Lui et al., 2007; Wang et al., 2008; Wang et al., 2009b; Li et al., 2011b). Nine miRNAs (*miR-9*, *miR-142*, *miR-642a*, *miR-101*, *miR-3607*, *miR-502*, *miR-378c*, *miR-150* and *miR-200a*), were shown to be of prognostic value in determining patient survival in metastatic cervical cancer. In particular, *miR-200a* expression was associated with good prognosis (Hu et al., 2010; Liu et al., 2016). The *miR-200* family, *miR-141*, *miR-149*, *miR-34a* and *miR-205* were identified as tumor suppressive and anti-metastatic miRNAs (Gregory et al., 2008; Korpal et al., 2008; Pang et al., 2010).

Table 2 – Commonly aberrant miRNA expressions in cervical cancer

Overexpressed in cervical cancer	Underexpressed in cervical cancer
miR-15b	*miR-29a*
miR-16	*miR-34a*
miR-17-5p	*miR-126*
miR-20a/b	*miR-127*
miR-21	*miR-143/145*
miR-93	*miR-218*
miR-106a	*miR-424*
miR-155	*miR-450*
miR-182	*miR-455*
miR-185	
miR-224	
miR-944	
miR-205	

1.5.5 **Neuroendocrine carcinomas**

1.5.5.1 *Adrenocortical carcinoma*

Adrenocortical carcinoma (ACC) is an aggressive neoplasm with an annual incidence of 0.7 – 2.0 cases per million people. Most adrenal masses are benign adenomas (ACA), often detected incidentally, so called incidentalomas (Young, 2007). However, ACCs are often revealed by compression and metastasis or even by excess steroid hormone production (Guillaud-Bataille et al., 2014). Typically, the patient is about 45 years of age, predominantly female and with poor prognosis (Fassnacht et al., 2013). Only a fraction of all adrenocortical tumors (ACT) diagnosed are carcinomas with the majority being adenomas (Giordano et al., 2009). Histopathology is the current method of choice to assess ACT to determine the origin and distinguish benign from malignant disease using the Weiss score criteria (Lau and Weiss, 2009). Unfortunately, histopathological examination proved to be challenging due to inconsistencies among observers (Fassnacht et al., 2013). Indeed, better prognostic and diagnostic markers are required.

Mutations in *TP53*, *CTNNB1* and the *TERT* promoter as well as *IGF2* overexpression and activation of the WNT/β-catenin pathway are common abnormalities in ACC (de Fraipont et al., 2005; Else et al., 2014; Liu et al., 2014). Germline mutations such as R337H in the *TP53* gene are predominant in pediatric ACCs and manifests as Li-Fraumeni syndrome with high predisposition to other cancerous diseases (Faria et al., 2012; Fassnacht et al., 2013). In adult ACC patients, ~6% carry germline *TP53* mutations (Raymond et al., 2013). In addition, somatic *TP53* mutations and loss of heterozygosity of the *TP53* locus are prevalent in adult ACC patients (Else et al., 2014). The WNT/ β-catenin signaling pathway is essential for normal adrenal development and maintenance (Kim et al., 2008a). Accumulation and stabilization of β-catenin in the cell occurs in most ACC cases, by blocking of its degradation complex by members of the Wnt signaling pathway. The protein is then translocated to the

nucleus where it promotes expression of Wnt/ β-catenin target genes that control cell proliferation (He et al., 1998; Tetsu and McCormick, 1999; Bielinska et al., 2009). *CTNNB1* gene mutations are observed in ~25% of both benign and malignant ACTs (Tissier et al., 2005; Rubin et al., 2016).

IGF2 and *H19* are two proximally located imprinted genes clustered on human chromosomal region 11p15.5. *IGF2* is expressed from the paternal allele (DeChiara et al., 1991) and *H19* from the maternal (Bartolomei et al., 1991). The expression of both genes is closely linked and regulated by common regulatory elements (Srivastava et al., 2000). The *IGF2* transcript gives rise to the IGF2 protein and two miRNAs (*miR-483-5p/ -3p*) from intron 7. The *H19* gene is a noncoding gene that harbors *miR-675.* In addition, *H19* locus codes for a protein product located antisense to *H19*, known as *HOTS* or H19 opposite tumor suppressor (Onyango and Feinberg, 2011). *IGF2* overexpression has been extensively studied in pediatric (Wilm's tumor), ACC and other adult tumors (Tricoli et al., 1986; Cariani et al., 1988; Zhan et al., 1994; Vu et al., 2003; de Fraipont et al., 2005). Epigenetic deregulation of the *IGF2*/*H19* domain, known as loss of imprinting (LOI), is known to partly contribute to IGF2 up-regulation. On the other hand, *H19* is lower in ACC compared to normal adrenal glands (Gao et al., 2002), *i.e.* not expressed due to maternal *H19* inactivation (Larsson, 2013). *IGF2*/*H19* domain LOI has also been reported to silence *HOTS* gene in Wilm's tumor (Onyango and Feinberg, 2011). It is clear that deregulation of *IGF2* and *H19* is common in ACCs (Gicquel et al., 1997; 2001), however how these genes contribute to ACC development remains unclear.

miRNA profiling can efficiently classify benign from malignant ACTs (Patterson et al., 2011; Özata et al., 2011). Overexpression of *miR-483* is a common finding in ACC (Soon et al., 2009; Patterson et al., 2011; Özata et al., 2011). *miR-483-3p* inhibits apoptosis by targeting PUMA/BBC3 and enhances cell proliferation in many cancers types (Veronese et al., 2010; Özata et al., 2011). Overexpression of *miR-483-5p* is a distinct feature of ACC (Patterson et al., 2011) and can promote cell proliferation, migration, invasion and metastasis (Özata et al., 2011; Song et al., 2014). Interestingly, *miR-483-5p* can target the 5'UTR of *IGF2* transcript to upregulate *IGF2* expression and promote tumorigenesis in Ewing's sarcoma cells (Liu et al., 2013). Expression of *miR-21* and *miR-210* is higher in ACC than in ACA and *miR-21* promotes cell proliferation (Romero et al., 2008; Özata et al., 2011). Furthermore, HIF-1α was shown to activate *miR-210* expression under hypoxic conditions and in turn targets MNT, a Myc antagonist. As a consequence, c-Myc is activated, to support cell growth (Zhang et al., 2009). Some miRNAs are associated with short-term survival. For example, high expression of *miR-503*, *miR-1202*, *miR-1275* is associated with poor prognosis in ACC patients (Özata et al., 2011); whereas a lower expression of *miR-195* and *miR-497* is consistently found in ACC, but not ACA and normal cortices (Soon et al., 2009; Doghman et al., 2010; Özata et al., 2011). *miR-195* is regarded as a strong differential marker (Soon et al., 2009; Özata et al., 2011; Chabre et al., 2013) that exhibits an inverse correlation with tumor size and cell growth

(Chabre et al., 2013). Moreover, *miR-195* and *miR-497* overexpression induces apoptosis in ACC cells (Li et al., 2011a). The *mir-195/497* cluster is located in chromosomal region 17p13.1, a region often deleted (Gicquel et al., 2001) in many cancers, including ACC (Pinto et al., 2005; Li et al., 2011a). Low expression of *miR-195* and *miR-497* can lead to increased expression of their targets *TARBP2* and *DICER*. Indeed, differential expressions can discriminate malignant from benign adrenocortical tumors based on TARBP2, GRIM-19 (NDUFA13) and several other proteins belonging to mitochondrial complex I (Caramuta et al., 2013; Kjellin et al., 2014). Their aberrant expression can trigger metastatic cell invasion and metabolic reprogamming (Goodarzi et al., 2014; Kjellin et al., 2014).

1.5.5.2 *Merkel cell carcinoma*

MCC is an aggressive neuroendocrine skin tumor, first described in 1972 (Toker, 1972). In most cases, MCC lesions appear on the head and neck area and limbs. The incidence is low but increasing and mortality rate is high (Fitzgerald et al., 2015). Risk factors for MCC include sun exposure, Caucasian ethnicity, UV radiation, specific viral infection and immunosuppression (Schrama et al., 2012). Local and distant metastasis are common, often to the lymph nodes and distant skin (Bichakjian et al., 2007).

MCC is thought to arise from Merkel cells, a type of neuroendocrine cells found in the touch-sensitive epidermal layer, that express epithelial cytokeratins (CKs), specifically CK20, neurosecretory granules (Moll et al., 1992 & 1995; Erovic and Erovic, 2013) and CD56 (Kurokawa et al., 2003). However, the origin of MCC is still unclear as other markers such as CD171 (L1CAM), CD117 (c-KIT receptor) and CD24 (mucin-like adhesion protein) are expressed in the majority of MCC but not in Merkel cells (Su et al., 2002; Deichmann et al., 2003; Feinmesser et al., 2004). Further ambiguous is the fact that MCC cells are highly proliferative and aggressive (Krasagakis et al., 2001), whereas Merkel cells are post-mitotic, terminally differentiated cells indicating a stem cell-like, undifferentiated epidermal origin (Moll et al., 1996). Recent reports showed that most MCC cells express PAX5 (paired box gene 5) and TdT (terminal deoxynucleotidyl transferase) early B-cell lineage markers, adding another layer of perplexity to the precursor of MCC (zur Hausen et al., 2013).

MCC is associated with Merkel cell polyomavirus (Feng et al., 2008). Merkel cell polyomavirus (MCPyV) belongs to the Polyomaviridae virus family carrying a 5.4 kb circular DNA, containing an early and a late region. The early region codes for four T-antigens [Large (LT), small (sT), 57 kDa T, and alternate frame of the LT (ALTO)] and a viral miRNA (*mcv-miR-M1*). The late region encodes viral capsid proteins (VP1, VP2 and VP3). MCPyV DNA is integrated in the MCC tumor DNA (Feng et al., 2008; Feng et al., 2011; Erovic and Erovic, 2013), which encodes a truncated form of the LT (Cheng et al., 2013). The truncated form of the LT is a viral oncogene that lacks the helicase domains required for viral DNA replication (Shuda et al., 2008), but preserves the Rb-binding motif (LxCxE) (Cheng et al., 2013). Rb regulates the E2F transcription factor that controls cell cycle progression from G1 to S

(Cobrink, 2005). The LT oncoprotein interaction with Rb1 disrupts E2F regulation, thereby transforms cells leading to enhanced cell growth and proliferation (Hesbacher et al., 2016). The LT also binds Hsc70, via its functional domains in the cell nucleus, mediated via the HPDK motif located in the DnaJ domain. This interaction was found to be essential to maintain MCC cell growth and proliferation and inactivation of Rb (Kwun et al., 2009; Houben et al., 2015). Additionally, LT binds Vam6p via its MUR (MCPyV T antigen unique region) domain at position 209. This interaction displaces Vam6p to the nucleus and perinuclear area, making it unable to cluster lysosomes (Liu et al., 2011). The purpose of Vam6p re-localization is unknown, however, it was shown to have a role in MCV replication (Feng et al., 2011).

The small T antigen (sT) is another oncoprotein encoded by the MCPyV genome that is able to transform cells and enhance cell proliferation in MCC (Kwun et al., 2013). MCPyV sT enhances LT expression independently of PP2A (sT binding site) and promotes MCPyV DNA replication in synergy with LT (Kwun et al., 2013). sT has been shown to interact with Fbw7 E3 ligase via its LT-stabilization domain (LSD) to diminish LT ubiquitinylation and inhibit degradation of c-Myc and cyclin E, among other cell cycle regulators and tumor suppressors (Kwun et al., 2013). In addition, the LSD domain interacts with downstream factors in the Akt-mTOR pathway by targeting 4E-BP1, a regulator of eIF4E. When active, 4E-BP1 binds to eIF4E to inhibit translation initiation. mTOR1-mediated phosphorylation deactivates 4E-BP1 to detach it from eIF4E and initiate translation. Moreover, 4E-BP1 can be phosphorylated by sT, independently of mTOR1 by an unkown mechanism (Shuda et al., 2011). Further sT interactions have been reported via its cellular phosphatase subunits PP4C and PP2A Aβ to inhibit NF-κB via NEMO (NF-κB essential modulator), rendering it inactive and unable to activate NF-κB-mediated inflammatory pathway (Griffiths et al., 2013). Additionally, the PP4C subunit has been implicated in promoting sT-mediated cell motility, migration and invasion by inhibiting stathmin phosphorylation (Knight et al., 2015).

Only a limited number of reports have characterized the role of miRNAs in MCC. miRNA expression profiling can distinguish MCC tumors based on MCPyV status. Five differentially expressed miRNAs were validated including *miR-30a-5p/-3p*, *miR-375*, *miR-34a* that are up-regulated, whereas *miR-203* is down-regulated, in MCPyV+ tumors (Xie et al., 2014). Functional studies showed that *miR-203* regulates cell growth, cell cycle progression and targets *survivin* (*BIRC5*) in MCPyV-, but not MCPyV+, cell lines. On the other hand, in MCPyV+ MCC cells, the LT regulates survivin expression instead. Comparative analysis of primary versus metastasis tumor profiles, highlighted four miRNAs (*miR-150, miR-630, miR-483-5p, miR-142-3p*) that were differentially expressed (Xie et al., 2014). Very recently, *miR-375* was shown to act as a tumor suppressor and neuroendocrine differentiator by targeting Notch2 and RBPJ in MCPyV- MCC cell lines (Abraham et al., 2016). The role of MCC-specific miRNAs in disease progression and metastasis is yet elusive and further studies are required.

2 AIMS OF THE STUDY

The main objectives of this thesis work were to characterize miRNA regulation, miRNA targets and tumor metabolism in tumorigenesis. Furthermore, we aimed to evaluate the impact of the aberrant miRNAs expression and oncometabolism for prognostic and diagnostic implications in clinical and therapeutic applications. More specifically, we aimed to:

Paper I: Characterize the functions and targets of *miR-944* in cervical cancer

Paper II: Evaluate and characterize the expression of miRNAs located in the *IGF2-H19* locus and identify potential miRNA targets in adrenocortical carcinomas

Paper III: Investigate the functional roles of *miR-375* regulation of LDHB in MCC cells

Paper IV: Determine the involvement of MCPyV T-antigens in regulation of LDHB expression in tumor cell metabolism.

3 MATERIALS AND METHODS

3.1 PATIENT MATERIAL

Studies in this thesis included materials from three human cancers including cervical, Merkel cell and adrenocortical carcinomas. All tumors and their normal counterparts were histopathologically verified before they were added to our cohorts. Ethical approvals were granted prior to commencement of each study by the ethical committee boards.

3.1.1 Cervical carcinomas (Paper I)

Twenty-seven paired frozen cervical tumors and normal cervical specimens were supplied from the Gynecologic Oncology Group Tissue Bank (Colomubus, OH) which included 19 squamous cell carcinoma (SCC); 7 adenocarcinoma (ADC); and 1 adenosquamous cell carcinoma (ASC), as listed in Table 3.

Table 3 – Cervical tumors and normal specimens used in Paper I

Samples	Age at diagnosis (years)	Histological subtype
G013	53	SCC
G603	48	SCC
G702	25	SCC
G026	62	SCC
G243	30	SCC
G507	52	SCC
G601	55	SCC
G531	49	SCC
G612	n.a.	SCC
G699	57	SCC
G623	35	SCC
G645	70	SCC
G529	n.a.	SCC
G648	n.a.	SCC
G576	48	SCC
G850	50	SCC
G871	47	ASC
G613	48	SCC
G652	46	SCC
G575	n.a.	SCC
G220	n.a.	ADC
G428	38	ADC
G659	n.a.	ADC
G696	n.a.	ADC
G761	n.a.	ADC
G691	29	ADC
G547	60	ADC

n.a. - not available; SCC - squamous cell carcinoma; ASC - adenosquamous cell carcinoma; ADC – adenocarcinoma.

3.1.2 **Adrenocortical carcinomas (Paper II)**

A total of 73 fresh-frozen tumor specimens were obtained from the Karolinska University Hospital biobank from patients that were surgically treated for adrenocortical carcinoma (ACC) or adrenocortical adenoma (ACA). This study included 43 ACAs, 30 ACCs and 13 normal adrenal cortices from patients undergoing nephrectomy for reasons other than adrenal diseases. The tumors were classified based on the WHO criteria. Clinical details of the adrenal tumors are specified in Table 4.

Table 4 – Adrenocortical adenoma and carcinoma specimens used in Paper II

Sample	Histological diagnosis	Gender	Age (years)	Tumor size (cm)	Metastasis	Follow-up	
						months[#]	Outcome
Ad 1	Adenoma	F	38	2.5	No	231	Alive
Ad 2	Adenoma	F	63	6.0	No	195	Dead
Ad 4	Adenoma	F	35	4.0	No	93	Alive
Ad 5*	Adenoma	F	42	3.5	No	87	Alive
Ad 6	Adenoma	F	81	2.5	No	23	Alive
Ad 7	Adenoma	M	52	3.2	No	27	Alive
Ad 10	Adenoma	F	80	2.5	No	18	Dead
Ad 11	Adenoma	F	27	4.0	No	39	Alive
Ad 12	Adenoma	F	75	5.0	No	12	Dead
Ad 13	Adenoma	F	40	6.5	No	14	Alive
Ad 14	Adenoma	F	50	2.0	No	258	Alive
Ad 16*	Adenoma	F	29	2.0	No	218	Alive
Ad 17	Adenoma	F	79	2.0	No	112	Dead
Ad 30	Adenoma	F	63	5.0	No	216	Alive
Ad 31	Adenoma	M	63	4.0	No	215	Alive
Ad 32	Adenoma	F	42	2.5	No	89	Alive
Ad 33*	Adenoma	F	64	4.0	No	163	Alive
Ad 34*	Adenoma	F	63	4.0	No	158	Alive
Ad 35*	Adenoma	M	66	4.0	No	117	Dead
Ad 36*	Adenoma	F	54	4.0	No	107	Alive
Ad 37	Adenoma	F	59	2.5	No	104	Alive
Ad 40	Adenoma	M	46	3.5	No	12	Alive
Ad 41	Adenoma	M	66	5.3	No	29	Alive
Ad 42	Adenoma	M	65	3.5	No	43	Alive
Ad 43	Adenoma	F	50	4.7	No	24	Alive
Ca 1	Carcinoma	F	63	20.0	Yes	6	DOD
Ca 2*	Carcinoma	M	78	15.0	No	3	DOD
Ca 3*	Carcinoma	F	72	7.0	No	5	Dead
Ca 4*	Carcinoma	M	30	10.0	Yes	58	DOD
Ca 5	Carcinoma	M	72	11.0	Yes	162	DOD
Ca 6*	Carcinoma	F	40	18.0	No	188	Alive
Ca 7	Carcinoma	F	56	9.0	No	9	Dead
Ca 8	Carcinoma	F	54	15.0	Yes	4	DOD
Ca 9	Carcinoma	M	68	15.0	No	145	Alive

Table 4 cont.

Sample	Histological diagnosis	Gender	Age (years)	Tumor size (cm)	Metastasis	Follow-up	
						months[#]	Outcome
Ca 10*	Carcinoma	M	52	11.0	No	132	Alive
Ca 11*	Carcinoma	M	68	12.0	Yes	69	DOD
Ca 12*	Carcinoma	F	84	19.0	Yes	29	Dead
Ca 13	Carcinoma	M	64	21.0	Yes	91	Alive
Ca 14	Carcinoma	M	67	19.0	No	65	Dead
Ca 15	Carcinoma	M	77	11.0	Yes	78	Alive
Ca 18	Carcinoma	F	28	21.0	No	53	Alive
Ca 19	Carcinoma	F	61	14.0	No	51	Alive
Ca 20	Carcinoma	M	60	10.0	No	48	Alive
Ca 22	Carcinoma	F	59	10.0	Yes	22	DOD
Ca 25	Carcinoma	M	49	16.0	Yes	2	DOD
Ca 26	Carcinoma	F	68	12.0	Yes	168	Alive
Ca 27	Carcinoma	F	48	10.0	Yes	18	DOD
Ca 29	Carcinoma	F	35	8.0	No	195	Alive
Ca 30	Carcinoma	F	43	6.6	n.a.	n.a.	n.a.
Ca 31*	Carcinoma	F	35	9.0	No	108	Alive

*Specimens used in the screening series; # Time between surgery and follow-up; F = female; M = male; DOD = Dead of disease.

3.1.3 **Merkel cell carcinomas (Paper III)**

Fifty-four MCC tumor specimens were collected from Karolinska University Hospital or Stockholm South General Hospital between 1986 and 2003. These include 26 formalin-fixed paraffin-embedded (FFPE) tumor specimens and 28 fresh-frozen MCC tumors. All MCC cases were verified by routine histopathology and immunohistopathology (see Table 5).

Table 5 – MCC specimens used in papers III and IV

Sample no.[1]	M/F	Age	Sample analyzed		Viral status[2]	Primary tumor				Follow-up	
			tumor	Type		Size (cm)	Tumor site	L R	Met	Time	Outcome
MCCT_2b	F	91	L. R.	FFPE	-	1.5	face	yes	yes	20	Dead-DOD
MCCT_3a	F	83	P	FFPE	+	1.5	face	no	yes	6	Dead-DOD
MCCT_3b			Met	FFPE	+						
MCCT_4a	M	69	P	FFPE & Frozen	+	3.5	elbow	no	yes	17	Dead-DOD
MCCT_4b			Met	FFPE	+						
MCCT_5a	F	84	P	FFPE	+	1	face	yes	yes	11	Dead-DOD
MCCT_5b			L. R.	FFPE	+						
MCCT_6a	M	74	P	FFPE	+	4.5	elbow	yes	no	113	Dead
MCCT_6b			L. R.	FFPE	+						
MCCT_7a	F	87	P	FFPE & Frozen	+	2.5	face	yes	yes	13	Dead-DOD
MCCT_7b			Met	FFPE	+						

Table 5 cont.

Sample no.[1]	M/F	Age	Sample analyzed		Viral status[2]	Primary tumor		L R	Met	Follow-up	
			tumor	type		Size (cm)	Tumor site			Time	Outcome
MCCT_8	F	85	P	FFPE	+	3.5	forearm	n.a.	n.a.	n.a.	n.a.
MCCT_9	M	67	P	FFPE & Frozen	+	2.3	face	no	no	17	Dead
MCCT_10	F	70	P	FFPE	+	n.a.	thigh	no	no	1	Dead-DOD
MCCT_16a	M	62	P	FFPE	-	3	groin	yes	yes	5	Dead-DOD
MCCT_16b			Met	Frozen	-						
MCCT_18	M	71	P	FFPE	+	3	gluteal region	no	no	60	Dead
MCCT_19	M	76	L. R.	FFPE & Frozen	+	6	gluteal region	yes	no	9	Dead-DOD
MCCT_20	F	46	L. R.	FFPE & Frozen	+	1	arm	yes	yes	230	Alive
MCCT_22	F	63	Met	FFPE	+	5	gluteal region	no	yes	222	Alive
MCCT_23	M	81	Met	FFPE	-	1	scalp	no	yes	46	Dead-DOD
MCCT_24	F	72	Met	FFPE	-	0.7	face	no	yes	82	Dead-DOD
MCCT_26	F	85	Met	FFPE	-	n.a.	face	no	yes	94	Dead
MCCT_27	F	89	P	Frozen	+	4	face (chin)	no	yes	4	Dead-DOD
MCCT_28	M	94	P	Frozen	+	5	scalp	yes	no	17	Dead-DOD
MCCT_29	M	71	P	Frozen	+	2.2	temple	no	yes	36	Alive
MCCT_30	F	75	P	Frozen	+	1	arm	no	no	35	Alive
MCCT_31	M	83	P	Frozen	+	n.a.	arm (wrist)	no	no	31	Alive
MCCT_32	F	87	P	Frozen	+	3.1	temple	yes	yes	5	Dead-DOD
MCCT_33	M	75	L.R.	Frozen	+	0.9	face (cheek)	yes	yes	18	Alive
MCCT_34	F	73	P	Frozen	+	3.5	leg	no	yes	12	Alive
MCCT_35	F	100	P	Frozen	+	n.a.	face (cheek)	yes	yes	4	Alive
MCCT_37	F	89	P	FFPE	+	4.5	chin	no	yes	5	DOD
MCCT_39	F	78	P	FFPE	+	1.5	right lower arm	no	no	40	Dead
MCCT_40	F	90	P	FFPE	-	2.5	right lower arm	no	no	14	Dead
MCCT_41	M	94	P	FFPE	-	2	left chest	no	no	22	Dead
MCCT_42	M	76	P	FFPE	+	3.4	right ear	no	?	13	Dead/DOD?
MCCT_43	M	82	P	FFPE	+	n.a.	left thigh	no	yes	58	Dead/DOD?
MCCT_45	M	91	P	Frozen	+	2,3	scalp	yes	yes	6	DOD
MCCT_46	F	78	P	Frozen	+	1,8	face	no	no	24	Alive
MCCT_47	F	70	P	Frozen		0,2	face	yes	yes	9	DOD
MCCT_49	M	76	P	Frozen	+	1	scalp	no	yes	21	Alive
MCCT_50	M	62	P	Frozen	+	3	back	no	yes	11	Alive
MCCT_51	F	66	P	Frozen	+	2,5	left arm	no	no	20	Alive
MCCT_53a	F	92	P	Frozen	+	4,5	left chest	no	yes	6	Dead
MCCT_53b			Met	Frozen	+						
MCCT_54	F	69	Met	Frozen	+	2,5	right leg	LN	yes	94	Alive

Table 5 cont.

MCCT_55	M	82	P	Frozen	+	n.a.	right elbow	n.a.	n.a.	n.a.	n.a.
MCCT_56	F	81	P	Frozen	+	n.a.	left cheek	n.a.	n.a.	n.a.	n.a.
MCCT_57	M	72	P	Frozen	+	n.a.	right leg	n.a.	n.a.	n.a.	n.a.
MCCT_58	n.a.	na.	n.a.	Frozen	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
MCCT_59	n.a.	na.	n.a.	Frozen	-	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
MCCT_60	M	71	P	Frozen	+	1.5x2	arm	no	no		Alive
MCCT_61	M	67	P	Frozen	+	4.5	thigh	no	no		Alive

1 = a and b indicate primary and recurrent tumor, respectively, of the same patient.
2 = MCPyV status determined by PCR of tumor genomic DNA combined with IHC for MCPyV large T-antigen expression, - = negative; + = positive.
M/F = gender; F = female; M = male; L.R. = local recurrence; Met = metastasis; LN = lymph node; Age and time measured in years; n.a. = not available; P = Primary tumor; DOD = died of disease.

3.2 ESTABLISHED CANCER CELL LINES

All cell lines used in this study were authenticated by short tandem repeats profiling (STR) at Bio-Synthesis, Inc (Lewisville, TX) or by the National Genomics Infrastructure-Uppsala (SciLifeLab, Uppsala University, Sweden) prior to running experiments (Table 6).

Table 6 – STR profiles of cell lines used for functional studies in this thesis work

Locus	CaSki	HeLa	NCI-H295R	MCC 13	MCC 14/2	MCC 26	WaGa	MKL-1	MKL-2
D8S1179	15, 15	12, 13	13	13, 14	10, 14	8, 10	10, 13	10, 10	11, 13
D21S11	30, 30	27, 28	32.2	30, 31	29, 32.2	31, 32.2	28, 30	30, 30	31.2, 31.2
D7S820	8, 11	8, 12	9, 12	10, 10	8, 10	8, 9	10, 10	8, 11	10, 13
CSF1PO	10, 10	9, 10	10, 12	12, 12	10, 11	10, 11	12, 12	11, 12	10, 12
D3S1358	15, 15	15, 18	15, 16	16, 18	16, 16	17, 17	14, 14	16, 16	17, 17
THO1	7, 7	7, 7	9.3	7, 9.3	6, 9.3	9.3, 9.3	9.3, 9.3	9, 9.3	7, 8
D13S317	8, 12	13.3, 13.3	13	12, 12	13, 13	13, 14	8, 13	8, 11	12, 13
D16S539	11, 12	9, 10	11	9, 11	13, 13	11, 13	11, 12	10, 12	10, 12
D2S1338	21, 21	17, 17	25	19, 20	19, 19	23, 26	20, 23	17, 17	17, 23
D19S433	15, 16	13, 14	13	15, 15	12, 14	16, 16	14, 16	14.2, 15.2	13.2, 14
vWA	17, 17	16, 18	17, 18	17, 17	17, 18	16, 18	16, 17	16, 18	16, 17
TPOX	8, 8	8, 12	8	8, 8	8, 8	8, 8	8, 11	8, 8	8, 11
D18S51	17, 17	16, 16	17	16, 17	15, 17	15, 18	10, 13	12, 18	14, 17
AMEL	X, X	X, X	X, X	X, X	X, X	X, X	X, Y	X, Y	X, Y
D5S818	13, 13	11, 12	12	9, 12	13, 13	12, 12	12, 12	11, 12	12, 14
FGA	21, 23.2	18, 21	19.2, 24	19, 20	21, 21	24, 25	19, 19	21, 25	19, 26

3.2.1 Cervical cancer cell lines

Seven cervical carcinoma cell lines including CaSki, HeLa, SW756, ME-180, SiHa, C4I and C33A were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 (Caski and ME-180) and DMEM (SW756, SiHa, C4I, C33A, HeLa)

media. All cell lines were supplemented with 10% FBS and 1% Pen Strep (Gibco, cat# 15140-122) and kept in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

3.2.2 Adrenocortical carcinoma cell line

The NCI-H295R ACC cell line was purchased from the American Type Culture Collection (ATCC; LGC Standards, Middlesex, UK) and used in Paper II. This cell line was established from an invasive metastasizing adrenocortical tumor and was verified to continuously produce over 30 steroidal secretions synonymous with cells from the adrenal cortex (Gazdar et al., 1990). This cell line was cultured in DMEM:F12 (Gibco) supplemented with 2.5 % NuSerum growth medium (Corning 355100) and 1% Insulin-Transferrin-Selenium basal supplement (Thermo Fisher Scientific) in a humified CO_2 incubator maintained at 37°C.

3.2.3 Merkel cell carcinoma cell lines

Six MCC cell lines were used in Paper III and four were used in Paper IV. Of these, three MCPyV+ cell lines (WaGa, MKL-1, MKL-2) were kindly donated by Dr. J.C. Becker (Medical University of Graz, Austria), Dr N.L. Krett (Nortwestern University, IL, USA) and Dr. Roland Houben (University Hospital Würzburg, Germany) respectively. The other three cell lines are MCPvV- (MCC13, MCC14/2, MCC26) and were purchased from CellBank Australia (Westmead, Australia).

3.3 TRANSFECTIONS

A transfection is the process of introducing foreign material, generally nucleic acids of chemically synthesized or recombinant origin, into a eukaryotic cell. In this thesis two transfection methods were used: lipid-based complex transfection and electroporation. Lipid-based complexes mimic the cell membrane phospholipid bilayer to transport their material in a liposome-like manner. The DNA/ RNA of interest are mixed and incubated to from cationic-lipid complexes capable of delivering their cargo with minimal toxicity. Electroporation is an alterative way to deliver genetic material into cell using electrical pulses that allows transfection of primary cultures or cells that are otherwise difficult to transfect. By optimizing the number of electrical pulses, duration and power together with the optimal solutions can yield higher transfection efficiency.

3.3.1 RNA mimics and anti-miRs

miRNA mimics are chemically modified dsRNA molecules that mimic endogenous miRNA duplexes when transfected in the cell. One strand is loaded into the RISC complex while the other strand is a nonfunctional (miRNA*) strand. miRNA inhibitors (antimiRs) are single-stranded RNA molecules exhibiting a 2'-O-methyl (2'-MOE)/ 2'-O-methoxy/ 2'-O-methoxyethyl modification, designed to bind and inhibit endogenously-expressed miRNAs. These modifications stabilize the RNA and enhance their binding affinity. Small interfering RNAs (siRNAs) are chemical synthesized dsRNAs that can cleave a specific RNA transcript

via the RNAi mechanism. All experimental designs included matching negative controls containing scrambled RNA oligos with the same chemical modification but do not target any known human transcripts.

In Paper I, HeLa, CaSki and SW756 cells were transfected with miRNA mimics (pre-miR-944 or pre-miR negative control) or inhibitors (anti-miR-944 or anti-miR negative control) using siPORT NeoFX transfection reagent (Applied Biosystems/ Ambion).

In Paper II, NCI-H295R cells were transfected with mirVana (anti-miR-483-5p, anti-miR-483-3p or anti-miR negative control), using the Amaxa Nucleofector technology (Lonza).

In Paper III, *miR-375* mimic or negative control was transfected into MCPyV- MCC cell lines using Lipofectamine RNAiMAX reagent (Invitrogen) for functional assays. Additionaly two siRNA mimics (siLDHB#1 and siLDHB#2) were used to silence LDHB expression in MCPyV- cell.

3.3.2 Plasmids

Plasmids are small circular dsDNA molecules that occur naturally in prokaryotic cells and replicate separately from chromosomal DNA. Plasmids serve as vectors to clone or transfer specific gene/s of interest (Table 7).

Table 7 – Plasmids used in this thesis work

Plasmid	Description	Source
miR-375sp	miRNA sponge that inhibits miR-375 function	Kumar et al., 2019
miR-375OE	Plasmid expressing miR-375	Kumar et al., 2018
sTco	Plasmid expressing MCPyV sT	Gift from Drs Y Chang and P Moore; Shuda et al., 2011
LTco	Plasmid expressing full-length LT of MCPyV	#40200, Addgene, Cambridge, MA
LT339	Plasmid expressing truncated LT of MCPyV	#28193, Addgene, Cambridge, MA
shTA	Plasmid expressing shRNA targeting exon 1 of MCPyV T-antigens	Xie et al., 2014
shsTA	Plasmid expressing shRNA targeting sT only	Kumar et al., 2019
LDHB-FLAG	Plasmid expressing full-length coding sequence of LDHB with FLAG-tagged on its carboxyl terminus	#OHu08149D, GenScript, Piscataway, NJ

In Papers III and IV two types of plasmids were used: 1) MCPyV T-Ags were cloned in pcDNA6 vector to over-express sTco, LTco and LT339 (pcDNA6.MCV.sTco, pcDNA6.MCV.LTco, pcDNA6.MCV.LT339 respectively) purchased from Addgene except for sTco construct, which was donated by Drs Y. Chang and P. Moore (University of Pittsburgh). These plasmids were transfected using Lipofectamine 2000 (Invitrogen).

In Paper III, short hairpin RNA (shRNA) vectors were electroporated to silence LT and sT (shTA) or sT only (shsTA) in MCPyV+ MCC cell lines, cloned in pcDNA3-U6M2. To silence *miR-375* expression, miRNA sponge sequences containing five tandem miRNA binding sites with a bulged site at miRNA positions 9–12 (to avoid Ago2 cleavage site), were cloned into pcDNA3 vector (miR-375sp) and inserted between *Bam*HI and *Xho*I restriction sites. In addition, *miR-375*-expressing plasmid (miR-375OE) in a pcDNA3 vector was used to stably overexpress *miR-375* in MCPyV- cells and transfected using Lipofectamine 2000.

In Paper IV, a plasmid (pcDNA3.1^{+}/-LDHB-C-(K)-DYK) expressing the full-length LDHB sequence (NM002300.7) with a C-terminal FLAG-tag (DYKDDDDK) was purchased from GeneScript (Piscataway, NJ) and transfected using Lipofectamine 2000 (Invitrogen). In addition, short hairpin RNAs (shRNA) targeting both LT and sT (shTA) or sT only (shsTA) were transfected using the Amaxa Nucleofector.

3.4 FUNCTIONAL ASSAYS

Increased proliferative cell growth, migration, invasion and resistance to cell death are among the principle mechanisms promoting tumorigenesis. In this thesis, several assays were performed to evaluate cell viability, proliferation, apoptosis, migration and invasion.

3.4.1 Cell growth and proliferation

Cell proliferation assays can be divided in four catagories: i) *Metabolic assays:* Cell growth or viability is based on the metabolic activity in viable cells that converts tetrazolium salts such as WST-1 ((4-(3-(4-iodophenyl)-2-(4nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate), MTT, XTT and MTS into a colored formazan dye. The colored dye is measured spectrophotometrically and is proportional to the activity of the mitochondrial succinate-tetrazolium-reductase system in viable cells. Alternatively, one can use Resazurin redox reagent (BioRad). ii) *Cell proliferation-markers:* Certain antigens are specifically expressed in proliferating cells such as Ki-67, proliferating cell nuclear antigen (PCNA), DNA Topoisomerase II α and their detection correlates to cell proliferation (Brustmann and Naudé, 2002). iii) *ATP concentration:* ATP levels are proportional to cell proliferation and its availability is scarce in death or dying cells thus, it can determine cell proliferation status. iv) *Direct measurement of DNA synthesis:* Assays such as 5-Bromo-2'-deoxyuridine (BrdU) or Click-iT EdU assays (Molecular Probes/ Life Technologies) measure the incorporation of Edu in the cellular DNA and detected on addition of a dye-labeled azide group or antibody. Alternatively, Trypan blue exclusion assay can be used. In this thesis work, three different approaches were applied to evaluate cell growth and viability, as described below.

3.4.1.1 *Cell viability*

Cell viability was measured by the WST-1 colormetric assay. This is a robust and widely used method that has the advantage of being soluble, non-toxic and allows continuous monitoring over time. This method was used in Papers I, III and IV.

3.4.1.2 *Trypan blue exclusion assay*

This assay was applied in Papers I and III to discriminate live from dead/ dying cells on the basis of cell membrane integrity by penetrating the degenerating cell membranes of apoptotic or necrotic cells. Cells with intact membranes will thus not take up the stain. Dead (or live) cells are quantified by a hemocytometer or automated cell counter (TC10, Bio-Rad). This method was used as a complement to the WST-1 assay.

3.4.1.3 *The xCELLigence RTCA system*

The xCELLigence system (ACEA BioScience Inc./ Agilent) provides real-time cell analysis for cell morphology, proliferation and migration monitoring in a 96 well-plate format. Loss and/or gain-of-functions experiments were performed to characterize *miR-944* inhibition in CaSki cells. The instrument utilizes electrical impedance measured by a set of gold microelectrodes embedded at the bottom of every well. When a conductive solution (buffer or culture media) are present, an electric potential (22mV) allows current to flow. However, the presence of adherent cell increases resistance in solution depending on cell shape, size and number. This system can monitor cell behavior over a period of time and does not require any labels or dyes that might otherwise affect the results. This system was used in Paper I to characterize the effect of *miR-944* inhibition on cell proliferation in CaSki cells.

3.4.2 **Cell apoptosis**

Apoptosis is a highly controlled suicidal cell death that occurs as a result of DNA damage and inability to maintain cell homeostasis. However, DNA mutations and genetic instability can lead to an abnormal state where mechanisms intended to keep the cell in-check become ineffective allowing the cell to gain the ability to evade apoptosis and become cancerous. Different assays have been developed including Annexin V assay, caspase-3 assay, TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling), mitochondrial membrane potential (JC-1 dye) and PARP cleavage. Three assays were used in this thesis work.

3.4.2.1 *Annexin V assay*

This is the gold-standard method for detection of the early apoptotic process. In a healthy cell, phosphatidylserine (PS) is located on inside of the cell membrane. In early apoptosis however, PS is translocated “flipped” to the outer side of the cell membrane and thus exposed on the cell surface. Annexin V has a high binding-affinity to PS, making detection possible when labeled with a fluorophores, such as FITC or other conjugates. When combined with Propidium Iodide (PI) or 7-AAD, the assay can distinguish early from late apoptosis or necrotic cells. This assay was used in Papers I and IV.

3.4.2.2 *Caspase-3 colorimetric assay*

A simple and easy method taking advantage of the activation of ICE-family of caspases that activate apoptosis in mammilan cells. Caspase-3 activity cleaves the DEVD motif in the

added p-nitroaniline substrate (DEVD-pNA). If caspases are present, the labeled chromophore p-nitroaniline (pNA) is released, allowing spectrophometric detection at 400-405 nm (Biovision, Mountain View, CA). This method can distinguish necrotic from apoptotic cells and was used in Paper I.

3.4.2.3 *PARP cleavage*

PARP-1 is a nuclear poly (ADP-ribose) polymerase involved in DNA repair following environmental stress. Upon apoptotic signaling, the PARP-1 (116 kDa) is cleaved by caspase-3 into two smaller fragments (~89 and ~24 kDa), abolishing its DNA repair function. Thus, detection of cleaved PARP-1 is commonly used as a marker of the late apoptosis. Cleaved PARP-1 was detected by Western blot in Paper III.

3.4.3 **Migration and invasion assays**

3.4.3.1 *Wound healing assay*

Adherent monolayer cells are seeded in six-well plates and incubated in a cell culture incubator until confluent. A scratch (or wound) is introduced in the middle of the well. The wound closure (or gap) were photographically recorded and compared at different time points, starting at 0 hour. The relative migration rate can be determined at specific time-points in experiment and control cells. Using 0 hour time-point as comparson, the relative migration rate is determined. A cell cycle blocker (e.g. hydroxyurea), is often used to distinguish cell migration from cell proliferation. This method was used in Papers I and III.

3.4.3.2 *Transwell cell migration and invasion assays*

In the migration assay, PET (polyethylene terephthalate) inserts containing a membrane with pores (8.0 μm) were equilibrated in cell culture medium supplemented with 20% FBS. Transfected cells were seeded on the upper side of the insert membrane and incubated for 24h (HeLa and SW756) or 48h (CaSki). For the invasion assay, the PET inserts were filled with a layer of buffered Matrigel Matrix basement membrane (BD Bioscience/ Corning) and pre-incubated at 37°C. A chemo-attractant (20% FBS) was added in the lower chamber, whereas cells are seeded in serum-free medium on top of the matrix basement membrane (Figure 5). After a 48h incubation, cells remaining on top of the membrane or matrix gel were removed whereas the migrated/ invaded cells were fixed and stained in 0.5% crystal violet and the dye-uptake is quantified spectrophotometrically at 570nm (630nm background). Migration or invasion was normalized to the respective negative controls. This method was applied in Paper I.

3.4.3.3 *The xCELLigence system for cell migration analysis*

This system (described in section 3.4.1.3) was also utilized for cell migration using a special CIM-plate. This plate is essentially similar to the transwell assay, consisting of an upper and a lower chamber. The upper chamber has a micropore PET membrane at the bottom of the

chamber, and gold microelectrode sensors are attached at the bottom side of the membrane. The sensors generate signals when cells migrate through the membrane. This assay was used in Paper I.

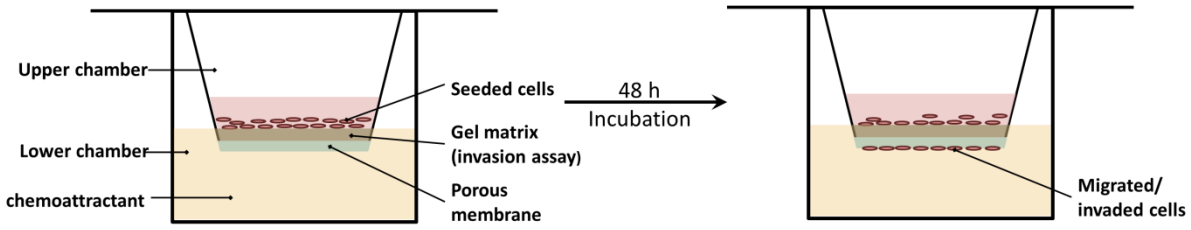

Figure 5 – **Transwell migration and invasion assays:** To test for migratory and invasion assays cervical cancer cells were seeded in PET insert (upper chamber) containing a porous membrane that allows cell to pass through. For invasion assay, a layer of basement gel matrix was included right above the membrane. A chemoattractant was added in the lower chamber and the experiment was incubated for 48h. On harvest time, invaded cells were stained and dye intensity was measured with a plate reader.

3.4.4 Cell cycle analysis

Propidium Iodide (PI) or 7-AAD (7-amino-actinomycin D) are dyes with a high binding affinity to nucleic acids. Combined with RNase A treatment, it allows exclusive DNA staining for DNA studies, cell death or cell cycle analysis. The dye is detected at 650 nm in red side of the visible spectrum upon excitation at 488 nm using a flow cytometer. Fluorescence intensity is proportional to the DNA content in the cell, thus it can segregate cells in G1/G0 phases from cells in the S, G2 or M phases of the cell cycle. This analysis was performed in Papers I and III.

3.5 RNA ISOLATION AND DETECTION

All total RNA extractions were performed using TRIzol reagent (Invitrogen), or mirVana miRNA Isolation kit (Ambion Inc/ Life Technologies Corp.) used as specified by the manufacturers. RNA concentrations were measured by NanoDrop ND-1000 spectrophotometer (Thermo Scientific) or Qubit 2.0 fluorometer (Thermo Fisher Scientific) for small RNA libraries.

3.5.1 Gene expression assay by real-time PCR

The TaqMan assay (Applied Biosystems) is a robust platform for quantifying the expression level of mRNAs with high specificity and sensitivity. The assay utilizes probes labeled with a 5’ fluorescent reporter (FAM-dye, reference ROX-dye) and a 3’ non-fluoresecent quencher. In the presence of the target sequence, the probe and primers anneal to the DNA template. The probe is degraded by the polymerase 3’->5’ exonuclease activity on encountering the DNA polymerase, freeing the fluorophore from quencher proximity. Fluorescent intensity

increases with every successive cycle and is reported in an amplification plot as ΔRn against cycle number. Results can be reported as an absolute amount target or comparative between samples and controls. These assays are normalized to stable endogenous mRNA transcripts that are abundant enough, such as genes supporting the basic cellular functions. Genes frequently used as endogenous control include *GAPDH*, *18S*, *ACTB* and *B2M*. This assay was used quantify *TP63* (Paper I), *IGF2* and *H19* (Paper II) and *LDHB* (Papers III and IV). An alternative method to TaqMan assay is SYBR green, a fluorescent dye that intercalates dsDNA. Fluorescent signal is proportional to the amount of dsDNA present however, not specific to target. This method was not used in this thesis work.

3.5.2 TaqMan miRNA assay

The TaqMan miRNA assay involves two steps: i) cDNA synthesis and ii) real-time target amplification. For reverse transcription a stem-looped primer is used to lengthen the small RNA and allow reverse transcription to start. The cDNA is then amplified and detected using the specific assay probe and primers as explained in section 3.5.1. The assay is normalized to endogenous small ncRNAs such as *RNU6B*, *RNU48*, *RNU44*, *U47* or any specific miRNA that is stable and independently expressed in the experimental conditions. This assay was applied to quantify *miR-944* (Paper I)**,** *miR-483-3p*, *miR-483-5p* and *miR-675* (Paper II) and *miR-375* (Paper III).

3.6 IDENTITIFICATION OF MIRNA TARGETS

As aforementioned, numerous methods have been developed to detect RNA-protein and RNA-RNA binding sites (section 1.3.2). In this thesis, PAR-CLIP (*P*hoto*A*ctivatable *R*ibonucleotide-enhanced *C*ross*L*inking and *I*mmuno*P*recipitation) was employed to identify miRNA targets *in-vitro*.

3.6.1 PAR-CLIP

A photoactivatable nucleoside (4SU or 6SG) is added to cells in culture. These nucleotides incorporate into mRNA transcripts, which are then crosslinked at 365 nm prior to harvesting. This allows identification of targets with high precision due to the presence of T → C or G → A transitions. In addition, the nucleoside analog allows a lower energy level UV cross-linking with high efficiency. Cell lysates are treated with RNase T1 and immunopurified by Ago2 conjugated beads subjected to RNase T1 treatment. After treatment with Proteinase K and total RNA extraction, short RNA sequences are obtained, then cloned and sequenced. This methodology was used in Paper I.

3.6.1.1 *Small RNA cloning*

Short RNAs recovered from the PAR-CLIP experiments were dephosphorylated and ligated to pre-adenylated 3'-adaptor oligonucleotides using T4 RNA ligase 2. The ligated product was purified on 12% denaturing polyacrylamide gel (PAGE) and incubated with T4

polynucleotide kinase and a second 5'-adaptor was ligated using T4 RNA ligase. Reaction was heat inactivated and the ligated products were purified and converted to cDNA and PCR-amplified. The PCR products were gel purified (Nusieve gel) to obtain a 125bp sequencing libraries that were sequenced on the Illumina (HiSeq2000) sequencing platform.

3.6.1.2 *Bioinformatic analysis*

FastaQ Illumina sequencing files were collapsed to FASTA files and aligned to the UCSC human genome (hg19) using Bowtie (v 2.0). Further analysis using the PARalyzer v1.1 tool (http://www.genome.duke.edu/labs/ohler/research/PARalyzer) was performed using the default settings. Reads aligning to tRNA, rRNA and repeat elements (LTR, LINE, SINE) were discarded. Overlapping reads were extracted (1 nt or more) and normalized (read/ total read count) The remaining reads were clustered and annotated based on the UCSC human genome (hg19). Identified targets in our libraries were compared to targets predicted by TargetScan 6.2 (http://www.targetscan.org).

3.7 VERIFICATION OF MIRNA TARGETS

3.7.1 Western blot analysis

This is a fundamental method to confirm protein expression levels in cultured cells and tissue specimens. The tissue or cell specimens are lysed in RIPA (Radio immuno-precipitation assay), Nonidet-P40 (NP40) or other buffers and supplemented with additives on ice to minimize sample degradation. Lysed samples are loaded and separated on denaturing PAGE gel and then transferred to nitrocellulose (or PVDF) membrane. An incubation in blocking buffer prevents nonspecific interactions. The membrane is then incubated with the antibody against the protein of interest and an endogenous control. A short secondary labeled antibody incubation (against the primary antibody's host) allows detection by different methods (radioactive, chemiluminescent, fluorescent or colorimetric). This method requires multiple washing steps after every antibody addition. Signal can be visualized using light or x-ray-sensitive films or CCD-based imaging instruments. Enzyme-labeled antibodies require the addition of a substrate prior to visualization. Fluorescent-labeled antibodies can be visualized directly in instruments equipped with the appropriate excitation and emission filters. This method was used to analyze the targets of *miR-944* (Paper I), *miR-483-5p* (Paper II) and *miR-375* (Paper III). In addition to the assessment of miRNA targets, Western blotting was also applied to detect cleaved PARP in Paper III and to verify the transfection efficiency of MCPyV T-antigens or shRNA in Paper IV.

3.7.2 Luciferase reporter assay

This method was employed to verify direct interaction between miRNA and target mRNA. Generally a fragment of the target gene is cloned downstream in a vector containing the firefly luciferase gene. If the miRNA interacts with the targets site, the transcript is silenced by the RNAi machinery, thus disrupting the firefly luciferase protein expression. However if

the miRNA do not target the gene, the luciferase protein is freely expressed and detected as a luminous signal. As a negative control, a similar reporter with mutations in the target sites interacting with the seed region of miRNA is used.

To verify direct targets of *miR-944* (Paper I), the pmirGLO dual-luciferase miRNA target expression vector (Promega Corp. Madison, WI) was used. The wild-type and mutated *miR-944* binding sites occurring in the 3'UTR of the presumed target transcripts (*HECW2* or *S100PBP*) were cloned in tandem downstream of the *luc2* reporter gene within the *PmeI* and *XbaI* restriction sites and propagated clones were confirmed by sequencing. The reporter constructs were co-transfected with pre-miR-944 mimic or pre-miR-Negative control into HeLa cells in a 96-well plate format. At 24h post-transfection, cells were lysed followed by firefly luciferase and Renilla luciferase activity measurements (Luciferase Activity assay) in a microplate luminometer. The firefly luciferase to Renilla luciferase activity ratios were calculated and normalized to the luciferase activity of the empty pmirGLO vector control.

3.8 METABOLIC PROFILING ASSAYS

To assess metabolic function, the Mito Stress Test and Glycolysis Stress Test kits (Aglilent Technologies Inc.) were used on the Seahorse XF platform (Agilent).

3.8.1 The mitochondrial stress assay

This method allows direct measurement of the cellular oxygen consumption rate (OCR) in real-time. The assay measures several parameters including the basal respiration, ATP production/ proton leak, maximal respiration, spare respiration capacity and non-mitochondrial respiration. Time-delayed sequential injections of the compounds challenge the mitochondrial state and oxygen consumption is measured at each stage. Prior to the experiment it is important that cell culture medium is changed to the XF Base medium (Agilent) and the plate is incubated in a non-CO_2 incubator.

The function of respiratory complexes is regulated by addition of: 1) Oligomycin, an ATP synthase inhibitor, suppressing mitochondrial respiration and OCR; 2) FCCP (carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone), uncouples oxygen consumption and ATP synthesis and disrupts the mitochondrial membrane potential; 3) Antimycin A, a complex III (Q-cytochrome c oxidoreductase) inhibitor; 4) Rotenone, a complex I (NADH-Q oxidoreductase) inhibitor. Both Rotenone and Antimycin A have a negative effect on the OCR. After the basal cell respiration is determined, Oligomycin injection allows ATP production (or proton leak) to be measured. FCCP [Carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone] is then injected to measure the maximal respiration. FCCP disrupts membrane potential allowing electrons to flow uninterrupted, inducing the maximum oxygen consumption by complex IV. This allows the spare respiratory capacity to be calculated. In the final injection, Antimycin A and Rotenone halt mitochondrial respiration completely, permitting measurement of the non-mitochondrial respiration. A mitochondrial stress profile

(Figure 6A) is then generated using the Wave software (Agilent Technologies). This method was used in Paper II.

Alternatively OCR can also be measured using the oxygen-sensitive fluorescence dye, such as the extracellular oxygen consumption reagent (Abcam, Cambridge, UK). The dye is normally quenched by oxygen. During respiration, depletion of oxygen increases fluorescence signal of the dye due to loss of quenching. Unlike the Seahorse platform, this method requires the addition of mineral oil to block atmospheric oxygen from dissolving into the media. This method was used in Paper IV.

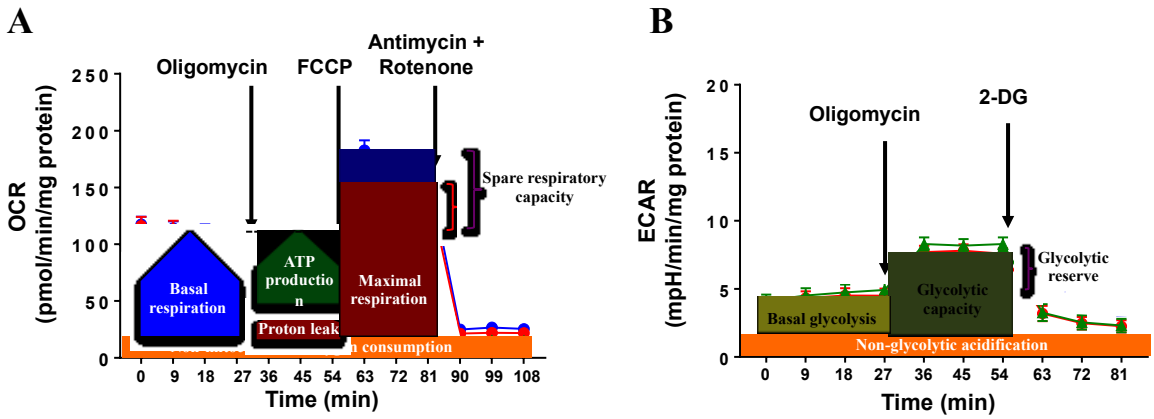

Figure 6 - An example of the Seahorse metabolic assays results: A) Mitochondrial stress assay profile and **B)** Glycolysis Stress test profile. Sequential compound injections and the key parameters of mitochondrial and glycolytic functions are illustrated.

3.8.2 Glycolysis Stress Test

This method employs the direct measurement of the extracellular acidification rate (ECAR) to assess the cellular glycolytic function using the Seahorse XF platform. Glycolytic flux parameters gathered reveal the glycolysis, glycolytic capacity, glycolytic reserve and non-glycolytic acidification. Again, compounds are injected to stimulate the glycolytic capacity of the cells. These include glucose, oligomycin and 2-deoxyglucose (2-DG). In the first stage, the non-glycolytic ECAR is determined in cells incubated in a medium without glucose or pyruvate. In the first injection, cells are stimulated by saturating the cell medium with glucose to determine the ECAR under basal conditions. The second injection, introduces oligomycin to inhibit mitochondrial ATP production, revealing the maximal cellular glycolytic capacity and glycolytic reserve calculation. The final injection releases 2-DG, a glycolysis inhibitor that competes for glucose hexokinase binding. Addition of 2-DG serves as a control to confirm that the ECAR observed is glycolysis-dependent. Data collected is used to generate a glycolytic stress profile (Figure 6B). This method was used in Paper II.

In Paper IV, ECAR was determined by the Glycolysis assay (Abcam), which employs a pH-sensitive reagent that increases its signal when acidification is increased. The ECAR can be calculated by the change in fluorescent signal over time.

3.9 STATISTICAL ANALYSIS

All statistical analyses were performed using Statistica 7.0 or 10.0 (StatSoft, Tulsa, OK), IBM SPSS Statistics 24.0 (IBM Corp., Armonk, NY) or MS Office Excel 2007. Paired Student's *t*-test (Papers I, III and IV) or Wilcoxon matched pairs signed rank test (Paper II) was used to compare between two experimental conditions and one-way ANOVA with post-hoc Tukey test was used to compare three conditions or more. For clinical samples, Mann-Whitney U-test was used to assess the differences between two sample groups. Spearman's rank order correlation was to determine expression correlations between two genes. All analysis were 2-tailed, and *p*-values below 0.05 were considered significant.

4 RESULTS & DISCUSSION

4.1 FUNCTIONS AND TARGETS OF *MIR-944* IN CERVICAL CANCER (PAPER I)

We, and others, have previously identified a subset of differentially expressed miRNAs in cervical cancer as compared to normal cervices (Lui et al., 2007; Wang et al., 2008; Lee et al., 2008; Witten et al., 2010). *miR-944* is one of the differentially expressed miRNAs that is more abundant in cervical cancer (Witten et al., 2010).

miR-944 was first identified in our previous study using small RNA cloning (Lui et al., 2007). This miRNA is located in chromosomal region 3q28, which is frequently amplified in cervical cancer (Heselmeyer et al., 1996 & 1997). In addition, *miR-944* has been demonstrated as a predictive marker for early diagnosis of cervical cancer (Liu et al., 2018b) and as a poor prognostic marker in advanced cervical cancer (Park et al., 2019). Besides cervical cancer, *miR-944* is also associated with tumor development and progression of several tumor types, including melanoma (Stark et al., 2010), colorectal (Christensen et al., 2013) and bladder cancers (Nordentoft et al., 2012). Despite these findings, its function and targets remained unknown. The main focus of this study was to characterize *miR-944* function and identify its targets that could be responsible for cervical carcinogenesis.

4.1.1 Characterize *miR-944* function in cervical cancer cell lines

We assessed the effect of *miR-944* regulation on cell growth, apoptosis, cell cycle, migration and invasion in cervical cancer cell lines. Our results showed that *miR-944* enhances cell proliferation in both HeLa and CaSki cells. *miR-944* also promotes migration and invasion in HeLa, but not CaSki, cells. No effect on apoptosis and cell cycle were observed in both cell lines. One of the explanations for the lack of migration and invasion effect in CaSki cells could due to the higher endogenous *miR-944* in CaSki cells, which could not be completely suppressed by the anti-miR-944 transfection, and the residual *miR-944* expression was sufficient to retain the phenotypes. Another explanation could be due to different histological subtypes between HeLa (adenocarcinoma) and CaSki (squamous cell carcinoma). In summary, our data shows that *miR-944* enhances cell proliferation, migration and invasion, but not apoptosis. Further studies are warranted to address the clinical impact and functional roles of *miR-944* in these two histological subtypes.

4.1.2 Identification and verification of *miR-944* targets

The PAR-CLIP methodology was used for identification of *miR-944* targets. The PAR-CLIP sequencing data revealed a list of 58 candidate targets, of which 19 were also predicted targets by TargetScan 6.2. Two of these targets (*HECW2* and *S100PBP*) have conserved *miR-944* binding sites, therefore these two genes were chosen for further validation. Wild-type and mutated (four mismatches in the seed region) sequences, putative to the *miR-944* binding sites in the 3'UTR of *HECW2* and *S100PBP* were inserted into the pmirGLO dual-luciferase expression vector. Co-transfection of wild-type constructs of *HECW2* and *S100PBP* with pre-

miR-944 resulted in significantly decreased luciferase activties when compared with the pre-miR negative control. The seed mutants rescued the suppression of luciferase activities by *miR-944*. Additionally, the expressions of *HECW2* and *S100PBP* were decreased following *miR-944* overexpression whereas, their expressions were increased upon *miR-944* inhibition. Altogether, our results supported that *HECW2* and *S100PBP* are bona fide direct targets of *miR-944* in human cervical cancer cells.

The main findings in this paper demonstrate that:

- *miR-944* is an oncomir that promotes cell proliferation, migration and invasion in human cervical cancer cells.
- PAR-CLIP sequencing data identified 58 potential *miR-944* targets of which two *HECW2* and *S100PBP* are validated as direct targets.

4.2 ABERRANT *IGF2-H19* LOCUS EXPRESSIONS AND MITOCHONDRIAL DYSFUNCTION IN ADRENOCORTICAL CARCINOMA (PAPER II)

The 11p15.5 chromosomal region harbors *IGF2*, a frequently over-expressed gene in adrenocortical carcinoma (ACC) that may result due to loss of imprinting (Giordano et al., 2009; Laurell et al., 2009; de Reynies et al., 2009). In addition, its overexpression is associated with malignant transformation and tumor growth. *H19* is a gene located downstream of *IGF2* that gives rise to an untranslated transcript often under-expressed in ACC (Liu et al., 1995; Gao et al., 2002). The *IGF2-H19* locus gives rise to three miRNAs: *miR-483-3p* and *miR-483-5p* from intron 7 of *IGF2*, and *miR-675* from exon 1 of *H19*. Both *miR-483-3p* and *miR-483-5p* are overexpressed in several cancer types, including ACC (Veronese et al., 2010; Doghman et al., 2010; Özata et al., 2011; Soon et al., 2009). *miR-483-3p* was shown to promote cell proliferation and inhibit apoptosis by targeting the p53 upregulated modulator of apoptosis (PUMA) (Veronese et al., 2010; Özata et al., 2011), while *miR-483-5p* was demonstrated to promote cell invasion through regulation of the N-Myc downstream-regulated gene 2 (*NDRG2*) (Agosta et al., 2018). On the other hand, *miR-675* is under-expressed in ACC compared to their non-malignant counterparts (Schmitz et al., 2011) and its functions in ACC are as yet unclear. The main focus of this study was to comprehensively characterize the host transcripts and miRNAs originating from the *IGF2-H19* locus in adrenocortical tumors and to identify potential targets of these miRNAs by comparing the proteomic data of these tumors.

4.2.1 Expression of the *IGF2-H19* locus in ACC

Using RT-qPCR, we quantified the expression levels of *IGF2*, *H19*, *miR-483-3p*, *miR-483-5p* and *miR-675* in 30 ACC, 43 ACA and 9 normal adrenals. Our results showed higher expressions of *miR-483-3p*, *miR-483-5p* and their host mRNA *IGF2* in ACC than ACA or normal adrenals. On the other hand, *H19* and *miR-675* were down-regulated in ACC compared to ACA or normal adrenals. Both *miR-483* showed a proportional expression to the *IGF2* transcript whereas *miR-675* expression corresponded to *H19*. Consistently higher *IGF2* and

lower *H19* levels were also observed in 77 ACC compared to 128 adrenals using the Cancer Genome Atlas Program (TCGA) and Genotype-Tissue Expression (GTEx) data sets. These results indicate that the *IGF2-H19* locus is consistently deregulated in ACC, and the host transcripts and miRNAs of this locus may play important functional roles in ACC development.

4.2.2 Potential targets of *miR-483* and *miR-675*

To identify potential targets of these miRNAs, we compared the miRNA expressions to the proteomic profiles previously published by Kjellin et al. (2014). The analysis revealed seven proteins for *miR-483-3p*, 101 for *miR-483-5p* and eleven for *miR-675* that were inversely correlated with their miRNA levels. Among these, several were also predicted as targets by TargetScan 7.1. Notably, we observed that 46 out of 101 proteins inversely correlated with *miR-483-5p* were differentially expressed between ACA and ACC. The gene signature of these 46 proteins could also differentiate between ACC and normal adrenals, using the TCGA and GTEx data sets. Gene ontology analysis of this signature showed a significant enrichment of genes related to mitochondrial function, in particular the mitochondrial respiratory complex I. Interestingly, one of these genes, *NDUFC1*, was also predicted as a target of *miR-483-5p* by TargetScan 7.1. These results prompted us to further characterize mitochondrial metabolism in ACC and the involvement of *miR-483-5p* in regulation of *NDUFC1* and cell metabolism.

4.2.3 Mitochondrial respiratory complexes in ACC

Using Western blot analysis we assessed nine adrenal glands, 10 ACA and 10 ACC tumors for the protein expression of mitochondrial respiratory complexes (ATP5A [complex V]; UQCRC2 [complex III]; MTCO1 [complex IV]; SDHB [complex II]; NDUFB8 [complex I]). Our results show lower expression of complexes I and IV in ACC, compared to ACA ($P = 0.009$ and $P = 0.002$ respectively). On the other hand, expression of complexes II and III was higher in ACC compared to their normal counterparts.

Furthermore, the role of complex I in ACC was assessed by measuring the NDUFC1 protein expression in a larger cohort consisting of 13 adrenal glands; 25 ACA and 29 ACC. Western blot analysis showed a lower NDUFC1 expression in ACC compared to ACA ($P = 0.043$) and normal glands ($P = 0.046$). In addition, we also observed an increase of NDUFC1 protein level upon inhibition of *miR-483-5p*, indicating a potential role of this miRNA in mitochondrial respiration.

4.2.4 Role of *miR-483* in metabolism of ACC cells

Using the Seahorse system, the oxygen consumption (OCR) and extracellular acidification (ECAR) rates were measured upon *miR-483-3p* and *miR-483-5p* inhibition. The OCR profiling showed a decreased basal cell respiration, maximum respiration and ATP production, only in *miR-483-5p* down-regulated cells. However, inhibition of both *miR-483-3p* and *miR-483-5p* showed a reduced glycolysis as indicated in the ECAR profile. Beside glycolysis, the high

endogenous *miR-483-5p* expression in ACC cells regulates mitochondrial respiration, implying a major role in energy metabolism.

The main findings in this paper demonstrate that:

- *IGF2* and its host miRNAs are overexpressed in ACC but inversely correlated to *H19* and *miR-675*.
- Proteomic and Western blot analysis point out protein subunits of the respiratory system as targets for *miR-483*, in particular respiratory complexes I and IV in ACC.
- Metabolic profiling reveals that *miR-483-5p* plays a major role in ACC cell metabolism.

4.3 THE ROLE OF *MIR-375* AND LDHB IN MERKEL CELL CARCINOMA (PAPER III)

miR-375 is highly abundant in MCC (Renwick et al., 2013), and differentially expressed between MCPyV+ and MCPyV- MCCs (Xie et al., 2014). This miRNA has been shown to repress multiple targets, including phosphoinositide-dependent kinase 1 (PDK1), Yes-associated protein (YAP), Janus kinase 2 (JAK2), 14-3-3 protein zeta (14-3-3ζ) and LDHB (Ding et al., 2010; Liu et al., 2010; Tsukamoto et al., 2010; Nakagawa et al., 2011). LDHB is an enzyme catalyzing lactate to pyruvate conversion that can both enhance or suppress tumor growth (Brisson et al., 2016; Liu et al., 2018a; McCleland et al., 2012 & 2013) and was detected in MCC tumors (Shao et al., 2013) however, its function remains unknown. Here, we determined whether *miR-375* regulates LDHB and the functional role of this regulation in MCC.

4.3.1 LDHB expression is regulated by *miR-375*

First, we evaluated the expression levels of *miR-375* and LDHB in MCC cell lines and clinical samples. Our results showed that *miR-375* was highly expressed but LDHB expression was relatively lower in MCPyV+ MCC cell lines, and vice versa in MCPyV- MCC cell lines. Concordantly, an inverse correlation was observed between *miR-375* and LDHB in 54 MCC samples. To determine whether *miR-375* could regulate LDHB, we over-expressed *miR-375* in three MCPyV- MCC cell lines and inhibited *miR-375* expression in two MCPyV+ MCC cell lines. In line with their inverse relationship, overexpression of *miR-375* decreased LDHB whereas its inhibition increased LDHB at both RNA and protein levels, supporting LDHB as a target of *miR-375* in MCC.

4.3.2 Dual roles of *miR-375* regulation in MCC cells

The differential expression of *miR-375* between MCPyV+ and MCPyV- MCC tumors and cell lines led us to speculate that *miR-375* might have distinct roles between the two tumor types. We therefore investigated the functional roles of *miR-375* in both MCPyV+ and MCPyV- MCC cell lines. Our data showed that overexpression of *miR-375* in MCPyV- MCC cells reduced cell viability and wound healing capacity, induced cell cycle arrest and triggered cell apoptosis. In

contrast, inhibition of *miR-375* decreased cell viability and induced apoptosis in MCPyV+ MCC cells. Taken together, these results suggest that *miR-375* has a prominent but distinct role in cell growth and metabolism in both tumor subtypes.

4.3.3 Opposite effect of LDHB in MCPyV+ and MCPyV- MCC cells

To determine if LDHB contributes to the cell growth suppression by *miR-375* inhibition in MCPyV+ MCC cells, we suppressed *miR-375* together with and without silencing of LDHB. We observed a diminished cell viability in *miR-375* only silenced cells and a reversed phenotype in co-silenced MCPyV+ MCC cells. Our results showed that silencing of LDHB could rescue cell growth suppression by *miR-375* inhibition, indicating its tumor suppressor role in MCPyV+ MCC cells. On the other hand, when silencing LDHB in MCVPyV- cells, we observed a reduced cell growth and induced apoptosis *i.e.* a phenocopy effect of *miR-375* overexpression. These findings suggest an oncogenic role of LDHB in MCPyV- MCC cells.

The main findings in this paper demonstrate that:

- LDHB is a target of *miR-375* in MCC.
- Overexpression of *miR-375* in MCPyV- MCC cells reduces cell growth and migration, and induces cell cycle arrest and apoptosis.
- Inhibition of *miR-375* reduces cell growth and induces apoptosis in MCPyV+ MCC cells.
- Silencing LDHB in MCVPyV- cells, reduces cell growth and induces apoptosis. However, *miR-375* inhibition rescues cell growth suppression.

4.4 VIRAL ONCOGENES EXPLOIT LDHB FOR METABOLIC CONTROL (PAPER IV)

A recent report from our group showed that MCPyV T-antigens can regulate *miR-375* (Kumar et al., 2019). In Paper III, we showed the distinct roles of *miR-375* regulation of LDHB in MCPyV+ and MCPyV- MCC cell lines, which could be due to different cellular metabolisms between these two tumor subtypes. This prompted us to investigate the relationship of LDHB and metabolisms between MCPyV+ and MCPyV- cells. The LDH enzymes catalyze the interconversion of pyruvate and lactate and level of LDHB may dictate the metabolic phenotype. In virus-induced tumor cells, glucose to lactate conversion is generally driven by aerobic glycolysis (Warburg et al., 1927; Berrios et al., 2016; Yu et al., 2018). Here we sought out to determine whether MCPyV T-antigens can regulate LDHB and the involvement of MCPyV T-antigens in energy metabolism.

4.4.1 MCPyV T-antigens control glycolysis through LDHB expression

To determine whether MCPyV T-antigens could regulate LDHB, we transfected shTA (silencing both LT and sT) or shsTA (sT only) in MCPyV+ MCC cell lines. Silencing of both LT and sT or sT only, increased LDHB expression at both mRNA and protein levels.

Consistently, ectopic expression of sT and truncated LT (LT339) reduced LDHB mRNA and protein levels, however the full-length LT (LTco) had no effect. Given that LDHB expression is low in MCPyV+ cells, we expected these cells to be glycolytic by shifting the equilibrium to the lactate. Silencing of both viral antigens (shTA) or sTAg only (shsTA) in WaGa and MKL-1 cell lines showed an increase in LDHB expression and a decreased glycolysis rate. We then ectoptically expressed MCPyV antigens (sTco; LTco; LT339) in two MCPyV- cell lines. Cells overexpressing the small antigen (sTco) and truncated large antigen (LT339) showed increased glycolysis and decreased LDHB protein expression but not in the wild-type LT (LTco) expressing cells. Taken together, these results demonstrate that the MCPyV sT and LT oncoproteins down-regulate LDHB expressions and promote glycolysis.

4.4.2 Impact of ectoptic LDHB and MCPyV T-Ags co-expression on cell growth and apoptosis

Next, we tested whether LDHB function is susceptible to MCPyV T-antigens expression. MCPyV- cell lines were transfected with MCPyV T-antigens together with and without LDHB expression. Cells expressing sT and LT339 oncoproteins showed increased cell growth. However, cells co-expressing LDHB neutralized the pro-growth effects of sT and LT339 oncoproteins. Besides, overexpression of LDHB reduced the proportion of apoptotic cells in MCC14/2 cells in a similar way to sT or truncated LT. However, co-transfection of LDHB and sT or truncated LT increased the number of apoptotic cells compared to the cells transfected with sT or truncated LT alone. Together, our findings indicate that suppression of LDHB is important for the maintenance of cell growth and viability in MCPyV+ cells.

4.4.3 Distinctive metabolic mechanisms for cell growth in MCPyV+ and MCPyV- MCC cells

The differential LDHB expression between MCPyV+ and MCPyV- MCC cell lines and the central role of LDHB in metabolism prompted us to investigate whether the growth of these two cell types is dependent on different energy metabolism. We inhibited glycolysis, using oxamic acid, or oxidative phosphorylation, with antimycin A, in MCPyV+ and MCPyV- MCC cell lines. Our results showed that inhibition of glycolysis reduced cell growth only in MCPyV+ cell lines, whereas in MCPyV- cells, cell growth was inhibited by blocking oxidative phosphorylation. These results suggest a different metabolic dependence between MCPyV+ and MCPyV- MCC cells. While the virus-positive cells are dependent on glycolysis for cell growth, the virus-negative MCC cells are reliant on mitochondrial respiration.

The main findings in this paper demonstrate that:

- MCPyV oncoproteins (sT and truncated LT) suppress LDHB expression and increase glycolysis rate.
- LDHB expression counteracts the pro cell-growth effects of MCPyV oncoproteins.

- Distinctive metabolic activities are present in virus-positive and virus-negative MCC cells. MCPyV+ MCC cells are dependent on glycolysis, whereas MCPyV- MCC cells rely on the activity of the oxidative phosphorylation for cell growth and viability.

5 CONCLUSIVE REMARKS

Through this thesis work we have gained deeper understanding of the biological funtions and identification of novel targets of several miRNAs in various tumor types. In particular, the function and targets of *miR-944* were unknown prior to this study.

With these findings presented here, we can conclude that:

- *miR-944* promotes cell proliferation, migration and invasion, suggesting an oncogenic role in cervical cancer. This miRNA may have potential clinical applications as a complementary tool for the diagnosis and prognosis of cervical cancer.

- Applying the PAR-CLIP methodology demonstrates the potential of this method in identifying miRNA targets.

- Altered expression of *IGF2-H19* locus is consistently observed in ACC, indicating the importance of this locus in the development of ACC.

- Deregulation of the mitochondrial respiratory complexes is common in ACC, which may lead to dysfunctional oxidative phosphorylation.

- High expression of *miR-483-5p* is required for maintaining the high metabolic state of ACC cells, and highlights this miRNA as a potential therapeutic target in ACC patients.

- Depending on the viral status, *miR-375* regulation of LDHB plays dual roles in MCC, suggesting distinct cellular metabolisms between MCPyV+ and MCPyV- MCC.

- Dual roles of LDHB is dependent on MCPyV T-antigens. In MCPyV+ MCC cells or cells expressing MCPyV oncoproteins, suppression of LDHB is required to maintain cell growth and viability. This is likely due to the glycolysis-dependent growth in MCPyV+ cells.

- Targeting tumor metabolism can be an effective treatment strategy for MCC patients. However, different strategies should be applied depending on the viral status.

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