A genome-wide search for tumor suppressor microRNAs in ovarian cancer

A Dissertation Presented to
the Faculty of the Department of Biology and Biochemistry
University of Houston

In Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy

By
Anadulce Hernández Herrera

August 2014

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ABSTRACT

Ovarian cancer is one of the most lethal cancers among women. The Cancer Genome Atlas (TCGA) is a collaborative effort, which seeks to characterize the complete set of molecular changes associated with cancer and provide a public resource that will allow the development of new therapies and better diagnostic tools for cancer. Much of the focus is on protein coding genes and our understanding of the contribution from non-coding RNAs is lagging behind. MicroRNAs are small non-coding RNAs that can bind and repress hundreds of gene targets to regulate gene networks. Therefore, defining and understanding the miRNA-regulated genes offer new insights that can be clinically applied for many of the disease. In order to identify new tumor suppressors for ovarian cancer and downstream targets that drive key aspects of this disease such as drug resistance and metastatic spread, 3 candidates were selected from the microRNA-mRNA bioinformatic analyses from the TCGA. A combination of molecular and functional studies confirmed that miR-29a that can regulate genes from the histone modifier and cell cycle pathways, inhibit proliferation and moderately increase cisplatin response in the p53-WT HEYA8; miR-509-3p which targets genes from the ECM/EMT networks, inhibits cell proliferation in p53-WT HEYA8 and p53-mut OVCAR8 and correlated with improved overall survival when analyzed by *in situ* hybridization in an independent cohort; miR-130b increases apoptosis by 3-fold in p53-mutant OVCAR8 and p53-wild-type HEYA8 and significantly induces TAp63 and BCL2L11 (BIM). Forced expression of TAp63 decreases cell viability by 60-80% and miR-130b-ABT-737 (BCL2L11-mimetics) combination increases apoptosis by 9-fold suggesting TAp63 and BIM are

critical effectors of the tumor-suppressive mechanisms driven by miR-130b, and can be used to develop new therapeutic strategies that will target p53 WT and p53 mutant tumors.

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LIST OF ABBREVIATIONS

3’ UTR	Three prime untranslated region
5’ UTR	Five prime untranslated region
Ago	Argonaute
AKT	Protein kinase B
ALDH	Aldehyde dehydrogenase
ATG	Autophagy related gene
CML	Chronic myeloid leukemia
Crasi-RNA(s)	Centrosome associated RNA(s)
CRC	Colorectal cancer
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EOC	Epithelial ovarian cancer
Exp5	Exportin 5
FDA	Food and drug administration
FDR	False rate discovery
GDP	Guanosine diphosphate
GOF	Gain of function
GTP	Guanosine-5’- triphosphate
HGSOC	High grade serous ovarian carcinoma

iPSCs	Induced pluripotent stem cells
MET	Mesenchymal to epithelial transition
MiR(s)	MicroRNA(s)
MiRNA(s)	MicroRNA(s)
MM	Multiple myeloma
moRNA(s)	MicroRNA-offset RNA(s)
mRNA	Messenger RNA
MSY-RNA(s)	MSY-2 associated RNA(s)
MTOR	Mammalian target of rapamycin
MTS	CellTiter 96® AQueous One Solution Cell Proliferation Assay
NC	Negative control
nt	Nucleotide
NT	Non-treated
OD	Oligomerization domain
OS	Overall survival
OSE	Ovarian surface epithelium
PAR(s)	Promoted associated RNA(s)
PSF	Progression free survival
PI3K	PI3 kinase
piRNA(s)	Piwi-associated RNA(s)
Pol II	Polymerase II
Pol III	Polymerase III

Pre-miR	Precursor microRNA
Pri-miR	Primary microRNA
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA(s)	Ribosomal RNA(s)
SCNA	Somatic copy number alterations
sdRNA(s)	Sno-derived RNA(s)
siRNA(s)	Silencing RNA(s)
snoRNA(s)	Small nucleolar RNA(s)
SNP	Single nucleotide polymorphism
TAD	Transactivator domain
TCGA	The cancer genome atlas
Tel-siRNA(s)	Telomere-small RNA(s)
tRNA(s)	Transfer RNA(s)
VEGF	Vascular endothelial growth factor
Wild type	WT
XPO5	Exportin 5

CHAPTER 1: INTRODUCTION

Every cell of a given organism contains an identical copy of the genome. However, in complex multicellular organisms cells from different tissues express specific sets of genes that allow them to differentiate into tissues and organs with diverse functions. To maintain genome integrity and to express specific sets of genes at the appropriate level in the correct place and time, two critical aspects need to be tightly regulated for organisms to develop and function. In order to achieve these goals, organisms have evolved elegant mechanisms to monitor the genome stability and control gene expression through complex regulatory networks. In recent years, it has been discovered that a larger percentage of these mechanisms are RNA-dependent more so than previously envisioned.

Non-coding RNAs are generally classified by size, in small RNAs (< 40 nt) and long non-coding RNAs (> 40 nt). Long non-coding RNAs are involved in several biological processes including transcription (Clark and Mattick, 2011), splicing (Tripathi et al., 2010), translation (Muddashetty et al., 2002), protein localization, imprinting (Brown et al., 1991), and cell cycle (Mourtada-Maarabouni et al., 2008).

The small non-coding RNAs classification and some of their functions are shown in Table 1 (Taft et al., 2010).

Table 1. Classification and functions of non-coding small RNAs

Type of small RNA:	Function:	Reference:
Small Interfering RNAs (siRNAs)	Small RNAs 21-22 nt long, produced by Dicer cleavage of complementary dsRNAs duplexes. siRNAs form complexes with Argonaute proteins and are involved in gene regulation, transposon control viral defense	(Ghildiyal and Zamore, 2009; Malone and Hannon, 2009)
microRNAs (miRNAs)	Small RNAs approximate 22 nt long produce by Dicer cleavage of imperfect RNA hairpins encoded in long primary transcripts or short introns. They associate with Argonaute proteins and are involved in post-transcriptional gene regulation	(Ghildiyal and Zamore, 2009; Winter et al., 2009)
PIWI-interacting RNAs (piRNAs)	Dice-independent small RNAs approx. 26-30 nt long, principally restricted to the germline and somatic cells bordering the germline. They associate with the PIWI-clade Argonaute proteins and regulate transposon activity and chromatin state	(Ghildiyal and Zamore, 2009; Malone and Hannon, 2009)
Promoter-associated RNAs (PARs)	A general term encompassing a suite of long and short RNAs, including promoter-associated RNAs (PARs) and transcription initiation RNAs (tiRNAs) that overlap promoters and TSSs. These transcripts may regulate gene expression	(Belostotsky, 2009; Taft et al., 2009)
Small nucleolar RNAs (snoRNAs	Traditionally viewed as guides for rRNA methylation and pseudouridylation. However, there is emerging evidence that they also have gene-regulatory roles	(Matera et al., 2007)
X-inactivation RNAs (siRNAs)	Dicer-dependent small RNAs processed from duplexes of two lncRNAs, Xist and Tsix which are responsible for X-chromosome inactivation in placental mammals	(Ogawa et al., 2008)
Sno-derived RNAs (sdRNAs)	Small RNAs, some of which are Dicer-dependent, which are processed from small nucleolar RNAs (snoRNAs). Some sdRNAs have been shown to function as miRNA-like regulators of translation	(Ender et al., 2008)

Continued

Type of small RNA:	**Function:**	**Reference:**
microRNA-offset RNAs (moRNAs)	Small RNAs approximate 20 nt long, derived from the regions adjacent to pre-miRNAs, their function is unknown	(Langenberger et al., 2009)
tRNA-derived RNAs	tRNAs can be processed into small RNA species by a conserved RNase (angiogenin). They are able to induce translational repression.	(Thompson and Parker, 2009)
MSY2-associated RNAs (MSY-RNAs)	MSY-RNAs are associated with the germ cell-specific DNA/RNA binding protein MSY2. Like piRNAs, they are largely restricted to the germline and are 26-30 nt long. Their function is unknown	(Xu et al., 2009)
Telomere-small RNAs (tel-sRNAs)	Dicer-independent approximate 24 nt long RNAs principally derived from the G-rich strand of telomeric repeats. May have a role in telomere maintenance	(Cao et al., 2009)
Centrosome-associated RNAs (crasiRNAs)	A class of 24-42 nt small RNAs, derived from centrosome that show evidence of guiding local chromatin modification	(Carone et al., 2009)

MicroRNAs

The main focus of this thesis research is to carry out a genome-wide search for tumor suppressor miRNAs for ovarian cancer and to understand the role of 3 potential tumor suppressor miRNAs in and ovarian cancer model with different p53 background, thus miRNAs biogenesis and regulation will be discussed in further detail.

MicroRNAs biogenesis

A total of 1881 microRNAs and/or microRNA hairpins sequences have been deposited in the public microRNA database, miRBase (www.mirbase.org) (Kozomara and Griffiths-Jones, 2013). MiRNAs genes are transcribed by RNA polymerase II (Pol II) or polymerase III (Pol III), to produce a primary miRNA transcript (pri-miRNAs). This transcript may contain 5' and 3' modifications identical to those present in mRNA transcripts. The majority of miRNAs are expressed from non-coding intergenic or intronic regions in the genome. Some of the miRNA-coding sequences are located within or overlap with annotated genes for mRNAs or other RNAs, which are referred as "host genes" for the miRNAs (Cullen, 2004).

When a miRNA gene is situated within or near another known gene, it is assumed that both of the genes will be transcribed together, however it is possible that the miRNA has its own promoter and the transcription of the miRNA and its host gene are independent events.

Human miRNAs can be divided into two classifications based on how the pri-miRNAs are processed to produce the mature miRNAs.

During the canonical pathway the pri-miRNA is cleaved by the Drosha-DGCR8 complex, producing a precursor miRNA (pre-miRNA) or hairpin structure of approximate 60-70 nt. The pre-miRNA is then exported to the cytoplasm by Exportin5 (Exp5) associated with its cofactor Ran-GTP (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Once in the cytoplasm the replacement of GTP by GDP induces the release of the miRNA from Exp5.

The pre-miRNA is further processed by Dicer, to produce a miRNA duplex intermediate of approximate 22 base pairs. Following Dicer processing an Argonaute (Ago) protein binds to the duplex and incorporates the mature single stranded RNA into the Ago:RNA complex also called the RNA-induced silencing complex (RISC) (Hammond et al., 2001; Hutvagner and Zamore, 2002; Mourelatos et al., 2002). In most of the cases, both of the strands from the miRNA duplex have the potential to act as mature miRNAs, the mechanisms by which the cell makes the decision for the preferential use of one strand over the other are not well understood, however it is known that the thermodynamic instability of the RNA duplex plays an important role in the mechanism involved in deciding which strand to load into the RISC (Gu et al., 2011) (Figure 1).

The non-canonical miRNA biogenesis includes Drosha or Dicer-independent pathways, which are described in Figure 2 (Curtis et al., 2012).

Figure 1

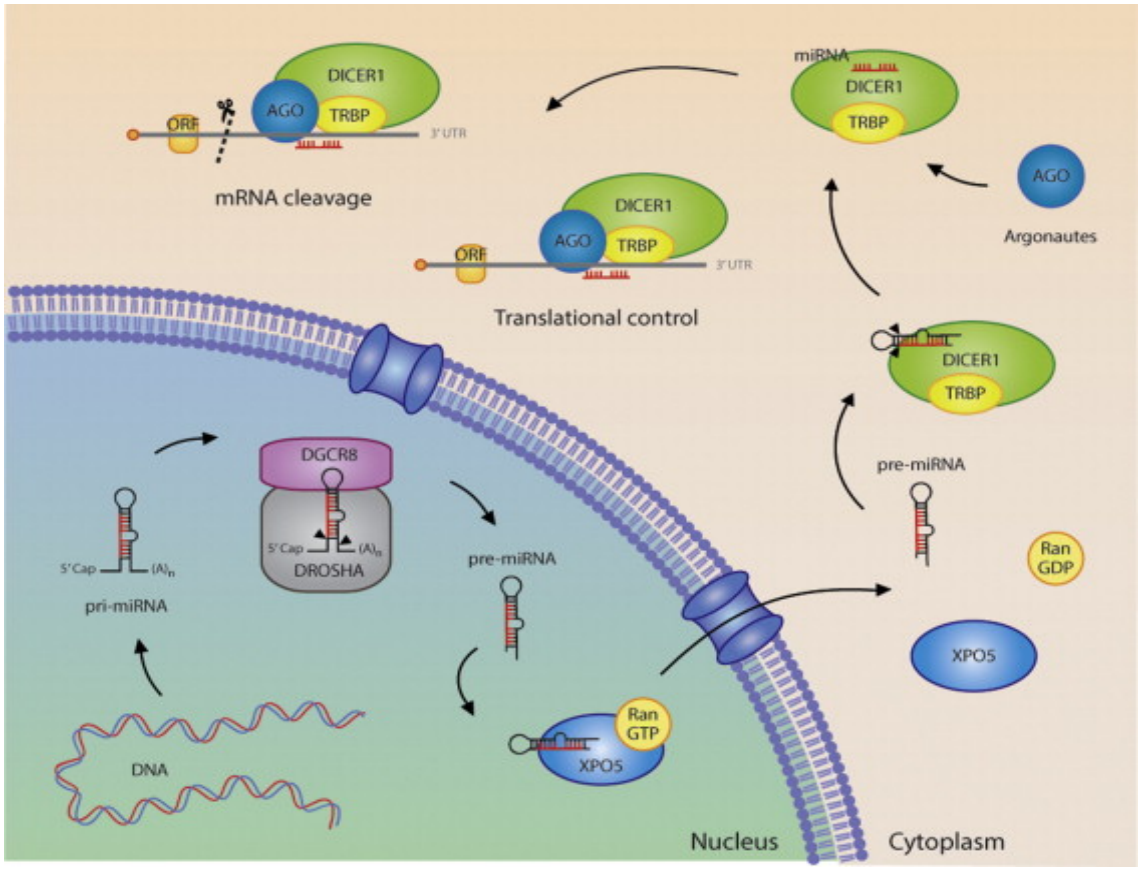


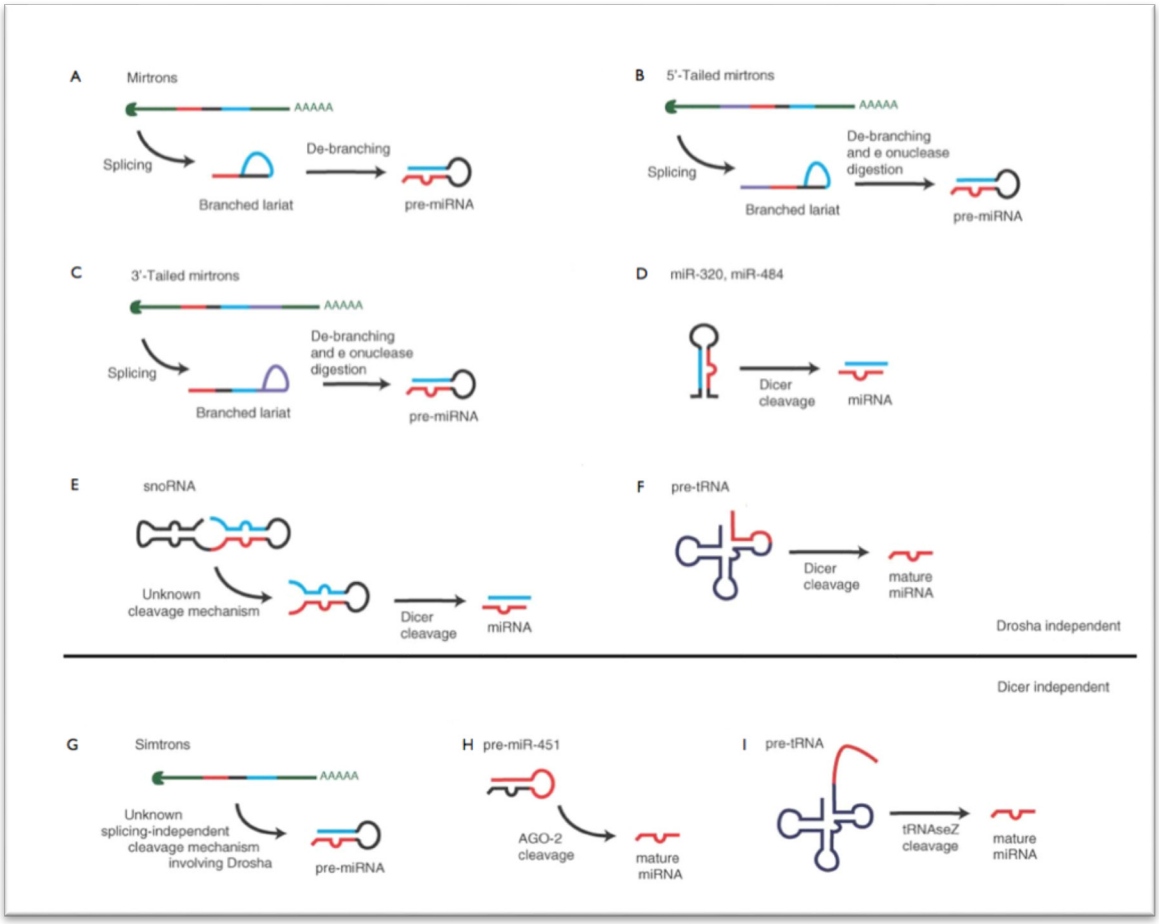
Figure 1. MicroRNA biogenesis.

Canonical miRNAs are transcribed in the nucleus by RNA polymerase II (RNAP II) into pri-miRNAs transcripts. These can be single pri-miRNAs or polycistronic pri-miRNAs. The nuclear microprocessor complex composed by Drosha and DGCR8 further process the pri-miRNA into a 72–80 nt pre-miRNA. Pri-miRNAs are then exported to the cytoplasm by exportin 5 (XPO5)/CRM1 and are processed into mature miRNAs by Dicer1 and TRBP. One of the strands is preferentially incorporated into the RISC complex composed of Dicer1/TRBP/AGO2. The classical function of the mature miRNA incorporated into the RISC complex is to mediate post-transcriptional inhibition by 3′UTR targeting; nonetheless mRNA cleavage is also possible (Melo and Esteller, 2014).

Figure 2. Non-canonical miRNA biogenesis.

A) The mirtron pathway involves splicing of short introns, following debranching of the branched lariat intermediate of the splicing pathway, sequence homology between 5' and 3' ends of the intron allows the formation of pre-miR-like hairpins (Berezikov et al., 2007). B) And C) Tailed mirtrons arise from short introns with hairpin forming potential, however following debranching, the pre-miRNA-like hairpin has a single stranded tail on either the 5' or 3' end which requires exonucleolytic cleavage by a Drosha-independent mechanism (Berezikov et al., 2007; Flynt et al., 2010). D) miRNAs lacking Drosha recognition sequences are process directly by Dicer (Babiarz et al., 2008) E) Some miRNAs map to snoRNAs containing secondary structures that mimic Dicer substrates. Following initial processing by a Drosha-independent mechanism, hairpins structures are process by Dicer into mature miRNAs (Ender et al., 2008) F) tRNA-like secondary structures can be process by Dicer to produce mature miRNAs (Babiarz et al., 2008; Haussecker et al., 2010). G) The simtron route requires Drosha but not splicing, DGCR8 or Dicer (Havens et al., 2012). H) pri-miR-451 is processed by Drosha into a hairpin that is shorter than the canonical Dicer substrates. AGO2 has been found to mediate processing of the pre miRNA into the mature sequence (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). I) tRNAseZ can cleave 3' sequences of precursor tRNAs to release small RNAs that can enter gene-silencing pathways (Haussecker et al., 2010; Lee et al., 2009).

Figure 2


A
Mirtrons
AAAAA
Splicing
De-branching
Branched lariat
pre-miRNA
B
5'-Tailed mirtrons
AAAAA
Splicing
De-branching
and e onuclease
digestion
Branched lariat
pre-miRNA
C
3'-Tailed mirtrons
AAAAA
Splicing
De-branching
and e onuclease
digestion
Branched lariat
pre-miRNA
D
miR-320, miR-484
Dicer
cleavage
miRNA
E
snoRNA
Unknown
cleavage mechanism
Dicer
cleavage
miRNA
F
pre-tRNA
Dicer
cleavage
mature
miRNA
Drosha independent
Dicer independent
G
Simtrons
AAAAA
Unknown
splicing-independent
cleavage mechanism
involving Drosha
pre-miRNA
H
pre-miR-451
AGO-2
cleavage
mature
miRNA
I
pre-tRNA
tRNAseZ
cleavage
mature
miRNA

MicroRNA Function

MicroRNAs use a variety of pathways to regulate their target genes including mRNA degradation and destabilization, translational repression, and even gene expression activation (Huntzinger and Izaurralde, 2011). Perfect complementarity between a microRNA and its target site supports endonucleolytic cleavage on the mRNA by Ago (Llave et al., 2002; Yekta et al., 2004). This mechanism is common in plants but is rare in animals. More common mechanisms in animals include the destabilization of the target mRNA by recruitment of deadenylation factors that remove the poly (A) tail and make the RNA susceptible to exonucleolytic degradation (Behm-Ansmant et al., 2006; Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2011; Wu et al., 2006). MiRNAs can also reduce protein levels without impacting mRNA levels, the precise details of this mechanism are not fully understood, however there is evidence for inhibition of translational initiation and elongation as well as for directed proteolysis of the nascent peptide being synthesized from the targeted mRNA (Humphreys et al., 2005; Maroney et al., 2006; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Pillai et al., 2005; Seggerson et al., 2002). On the other hand, there are specific situations in which miRNAs are also able to stimulate the translation of their targets (Orom et al., 2008; Vasudevan et al., 2007), one explanation for this phenomenon is indirect upregulation by silencing of a transcriptional repressor. Overall, miRNAs repress gene expression and it remains to be elucidated whether positive regulation of targets is part of miRNAs general mechanism of action or exceptions to the rule.

Ovarian Cancer

Ovarian cancer by definition is the cancer that forms in tissues of the ovary. In the United States each year 22,000 new cases are diagnosed, and around 16,000 women die yearly from ovarian cancer, making it the most lethal gynecological malignancy (Jemal et al., 2009).

Origin and classification

Despite numerous studies, the mechanisms underneath the lesions that give origin to ovarian cancer development are far from being understood. The prevalent hypothesis suggests that ovarian cancer originates from the surface epithelial layer of the ovaries, which is of mesothelial origin; the main problem with this proposed mechanism is that there are different histological types of ovarian cancers. These subtypes include: endometrial, clear cell carcinomas, mucinous, serous (further divided into low grade and high grade), and Brenner transitional tumors (Lee and Young, 2003). All these cancers have diverse histological characteristics as well as clinical and pathological behaviors, making it unlikely that they all share the same origin (Kelemen and Kobel, 2011).

At the clinical, cellular, and molecular level, ovarian cancers fall into two major groups based on histological grade, molecular genotype, and phenotype (Landen et al., 2008; Shih and Kurman, 2004). Type I includes low grade tumors of serous, mucinous, endometrioid or clear cell histotype, and are often diagnosed in early stage (I or II), grow slowly and may respond to hormonal therapy. The most prevalent type II cancers are high grade tumors of serous, endometrioid, or undifferentiated histotype, these cancers are

usually diagnosed at late stage (III-IV), grow fast and initially respond to chemotherapy but become refractory. The distinction between these cancers provides an insight in the understanding of ovarian cancer heterogeneity (Bast et al., 2009; Vaughan et al., 2011).

One possible explanation for the diversity in ovarian tumors is that progenitor cells for tumor development may come not from the ovary but also from the tissues adjacent to it (Figure 3), such as the fallopian tubes (Kurman and McConnell, 2010); opening the possibility for a two way traffic between the ovaries and the fallopian tubes. Normally oocytes travel from the ovaries to the fallopian tubes, but in some cases cells from the tubes may travel to the ovaries and contribute to malignancies (Mor and Alvero, 2013).

Ovarian cancer heterogeneity

In order to gain a better understanding of the tumors behavior, it is important to know their cellular composition; the presence of at least 2 different populations within the same tumor has been reported. Alveros' research group identified two main cell types within epithelial ovarian cancers, including the classical cancer cells characterized by small size and fast cell division defined as Type II ovarian cancer cells. Type I by contrast is comprised of cells that divide slowly and share markers with pluripotent cells, including CD44, MyD88, ALDH1 among others (Mor and Alvero, 2013). Type I cells have the ability to rebuild the original tumor when inoculated on mice, they can differentiate into type II cells, serve as tumor vascular progenitors, and are chemoresistant (Alvero et al., 2009; Alvero et al., 2009; Mor et al., 2011; Steffensen et

al., 2011). Ovarian surface epithelium (OSE) cells with stem properties have been previously described (Szotek et al., 2008), however it is still unclear whether these cells have the potential for long term self renewal. Using the levels of the detoxifying enzyme aldehyde dehydrogenase (ALDH) as discriminatory factor, Flesken-Nikitin et al. were able to isolate an OSE population with high ALDH levels that has self renewal properties. When they looked at mouse ovaries to find the natural occurring place for those cells, they were found at the hilum region the transitional region between OSE, mesothelium, and tuba epithelium. They concluded that the junction/transitional regions contain stem cells that are responsible for OSE regeneration and that are prone to malignant transformation (Flesken-Nikitin et al., 2013).

Figure 3

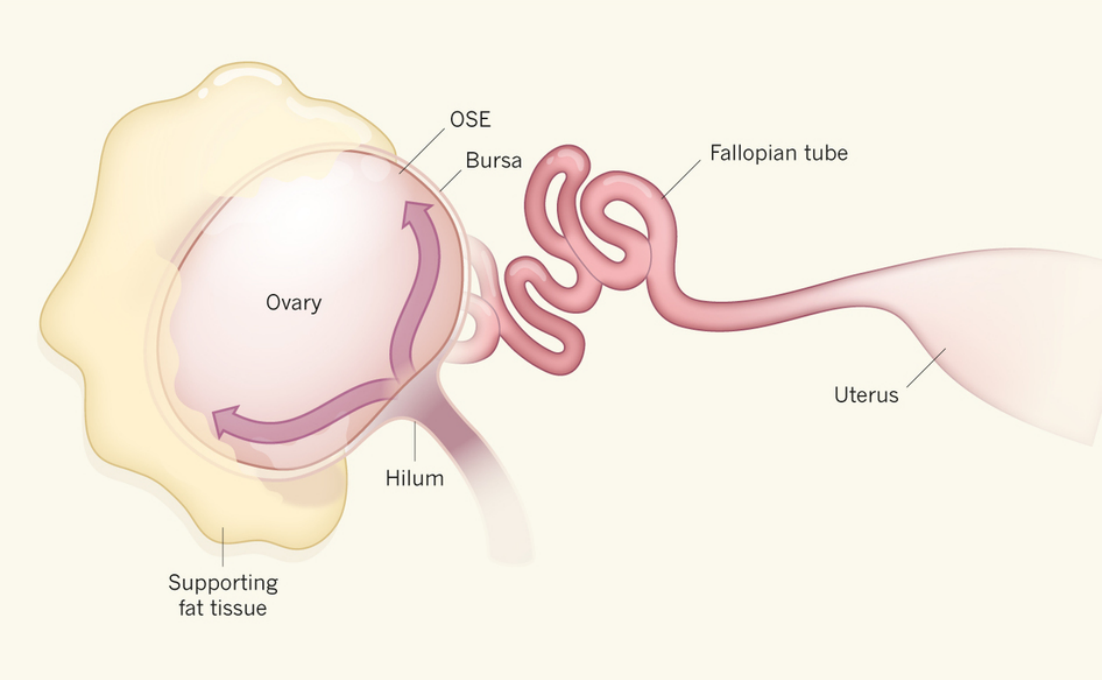


Figure 3. Anatomy of the ovary.

Modified from (Brenton and Stingl, 2013)

Current treatments

Surgery

Ovarian cancer is one of the few malignances in which surgeons will undertake cytoreductive operations (surgical excision of a part of a tumor which can not be completely removed). Cytoreduction is considered to be optimal when no macroscopic tumor is left before chemotherapy (Stuart et al., 2011). The success of cytoreduction (Colombo and Pecorelli, 2003) as well as the expertise of the surgeon are consider to be prognostic factors (Giede et al., 2005).

Primary Chemotherapy

The standard chemotherapy treatment for newly diagnoses patients after cytoreductive surgery includes six cycles of carboplatin and paclitaxel. Carboplatin is an alkylating agent that binds covalently to DNA creating cross-links. Paclitaxel binds non-covalently to microtubules stabilizing them and interfering with the formation of the mitotic spindle (Alberts et al., 1996; Armstrong et al., 2006; Markman et al., 2001). Bevacizumab, an antibody against the vascular endothelial growth factor (VEGF), has been used on clinical trials. The combination therapy can improve progression free survival (PFS) but not overall survival (OS) (Burger et al., 2011; Perren et al., 2011).

Second Line Chemotherapy

More than 70% of patients with ovarian cancer will experience disease recurrence within 12-18 months. Retreatment with carboplatin and paclitaxel results in 20-50 % response when this initially platinum sensitive disease recurs more than 6 months after

primary chemotherapy. Recurrent disease is considered to be incurable, however the use of combination therapy, carboplatin with paclitaxel, gemcitabine or liposomal doxorubicin, can prolong survival (Bast and Markman, 2010). Disease that recurs in less than 6 months is considered to be platinum resistant; for those cases, progression-free survival can be achieved in 10-30% of patients with the use of liposomal doxorubicin, paclitaxel, and topotecan.

Molecular pathways

Ovarian cancer is a very complex malignancy that involves alteration in numerous genes and biological pathways (Figure 4). Mutations in the tumor suppressor p53 are present in 96% of high-grade serous ovarian cancers (HGSOC). Consequently several important pathways such as cell cycle and apoptosis are affected. Other important pathways are described as follows.

Notch

The Notch signaling pathway is involved in cellular differentiation, proliferation.and apoptosis. Cells use this pathway to communicate with neighboring cells and take decisions about their cellular fate. As in many other pathways and genes, the role Notch plays can be oncogenic or tumor suppressive depending on the cancer type. In ovarian cancer, upregulation of this pathway seems to be playing an important role in tumor development and survival (Park et al., 2006). Inhibition of the pathway with specific siRNAs can lead to reduction of proliferation and apoptosis induction. Overexpression of Notch has been correlated with chemoresistance and poor prognosis

(Park et al., 2010).

Wnt/β-Catenin

The Wnt/β-Catenin signaling pathway plays a central role in cell to cell communication and other important processes such as adhesion, proliferation differentiation, cell cycle, and polarity (Logan and Nusse, 2004). Ovarian cancer mutations in β-Catenin are associated with the endometroid subtype (Palacios and Gamallo, 1998). Overexpression of proteins belonging to these pathways has been found to be involved in the development of serous ovarian carcinoma (Schmid et al., 2011).

Hedgehog

The hedgehog signaling is an important pathway involved in organs formation, regeneration, stem cell fate and differentiation of somatic tissues. Aberrant activation of this pathway seems to be implicated in the pathogenesis of epithelial ovarian cancer (EOC). High expression of sonic hedgehog is present in 57.7% of ovarian malignancies, especially in endometrial and clear carcinoma, with a lower percentage (25%) in serous and mucinous carcinomas (Liao et al., 2009). Drugs that can inhibit this pathway, such as cyclopamine, suppress proliferation of ovarian cancer *in vitro* and arrest tumor growth *in vivo* (McCann et al., 2011).

PI3K/AKT/mTOR Pathway

This pathway is comprised of three main driving molecules: PI3 kinase (PI3K), AKT, and mammalian target of rapamycin (mTOR). The PI3K/Akt/mTOR pathway is a central regulator of cancer proliferation, metastasis and chemotherapy resistance.

The PI3K are a family of lipid kinases that phosphorylate the 3-hydroxyl group of phosphoinositide (Cantley, 2002). After PI3K activation the kinase converts PIP2 (phosphatidylinositol 4,5-bisphosphate) into PIP3 (phosphatidylinositol 3,4,5-triphosphate), this conversion allows for AKT and PDK1 to be brought together near the inside of the cell membrane. AKT is the central molecule in the PI3K/AKT/mTOR pathway, activating and modulating numerous downstream targets involved in protein synthesis and cell growth, further activation of mTOR will increase cell proliferation by the inactivation of cell cycle inhibitors (Brunet et al., 1999; Diehl et al., 1998; Markman et al., 2010)

Due to the complexity of the PI3K/AKT/mTOR axis, its role in ovarian cancer is also very complex. Two main factors contribute to the complicated interactions between this pathway and carcinogenesis, first the diverse alterations found within the PI3K/AKT/mTOR pathway itself and second the variety of alterations in inputs into the pathway.

According with the TCGA analysis (Cancer Genome Atlas Research Network, 2008) mutations in PIK3CA and PTEN (Phosphatase and tensin homolog), a well-known tumor suppressor gene, are among the most common alterations present in ovarian cancer.

Figure 4

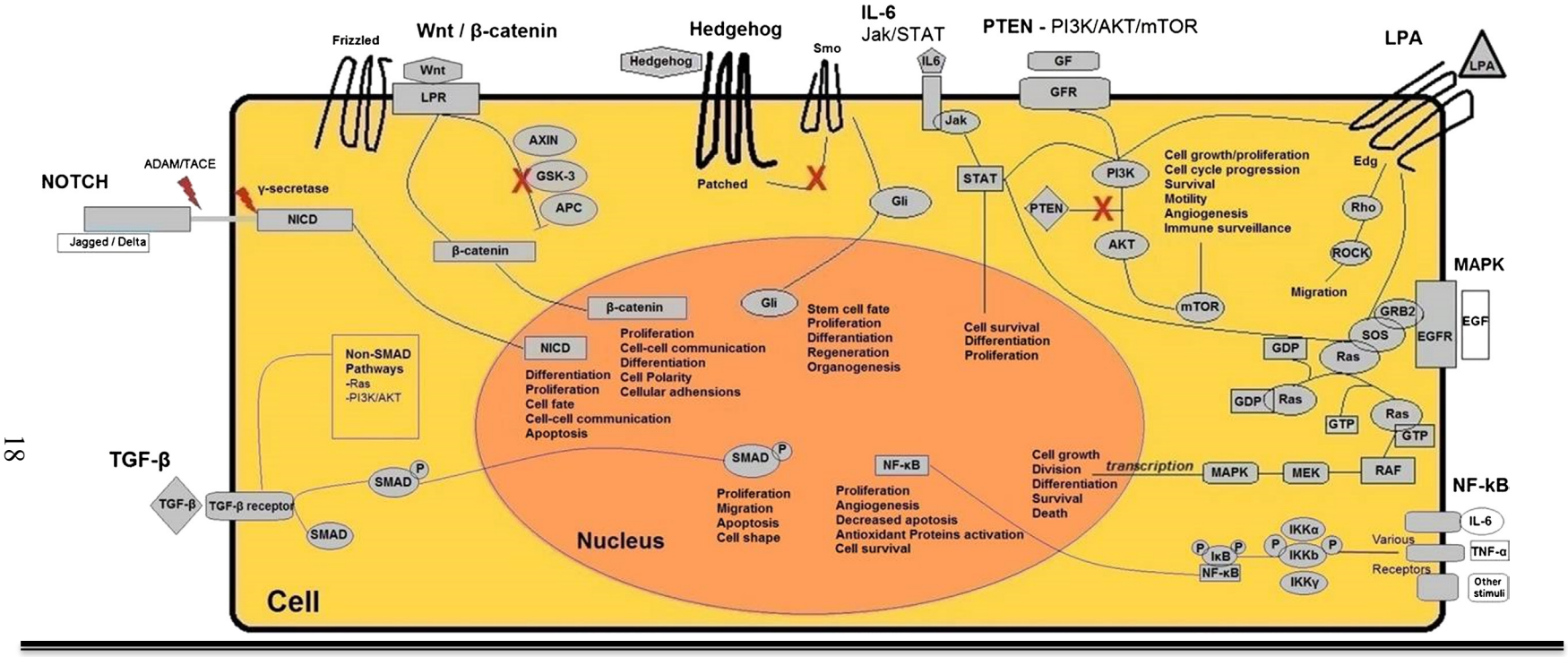


Figure 4. Simplified representation of the main signaling pathways implicated in ovarian cancer.

(Kotsopoulos et al., 2014)

MicroRNAs and Cancer

Since miRNAs are involved in the maintenance of virtually all the biological pathways it comes to no surprise that deregulation of miRNAs has been documented in several diseases including cancer (Figure 5).

By definition, a tumor-suppressor miRNA is a miRNA whose expression is downregulated in cancerous cells compare with its expression in healthy cells on the other hand, an oncomiR is a miRNA whose expression is upregulated in tumor cells compare with normal cells. However the denomination of a miRNA as a "tumor suppressor" or "oncomiR" is cancer specific, and a single miRNA can be tumor suppressor for one type of tumor or oncomiR for another type.

MiRNAs downregulation is a common hallmark of tumors (Lu et al., 2005), silencing of miRNAs is the consequence of alterations at different levels of miRNAs regulation. A compilation of the roles of microRNA in cancer is described in table 2.

Table 2. MicroRNAs and cancer

microRNA:	Expression in cancer:	Function	Mechanism of deregulation	Targets
Let-7a-2	Down in breast, lung, colon, ovarian and stomach cancer	Tumor suppressor	Repressed by MYC	KRAS, HMGA2, MYC, DICER, BCLXL, IMP-1, CDC34, IL6
miR-15/16	Down in CLL, prostate cancer, and pituitary adenomas	Tumor suppressor	Genomic loss, mutated, activated by p53	BCL2, COX2, CHECK1, CCNE1, CCND1, CCND2, BMI-1, FGF2, FGFR1, VEGF, VEGFR2, CDC25a
miR-29 family	Down in AML, CLL, lung, lymphoma, hepatocarcinoma, rhabdomyosarcoma	Tumor suppressor	Genomic loss, activated by p53, repressed by MYC	CDK6, MCL1, TCL1, DNMT1, DNMT3a, DNMT3b
miR-34 family	Down in colon, lung, breast, kidney, and bladder cancer	Tumor suppressor	Repressed by MYC	SIRT1, BCL2, NOTCH, HMGA2, MYC, MET, AXL. NANOG, SOX2, MYCN, SNAIL
miR-200 family	Down in aggressive breast and ovarian	Tumor suppressor	Repressed by ZEB1/2	EB1, ZEB2, BMI-1, SUZ-12, FN1, LEPR, CTNNB1, JAG1, MALM2, MALM3, p38 alpha
miR-26a	Down in liver cancer	Tumor suppressor	Repressed by MYC	CCND2, CCNE2
miR-155	Up in high risk CLL, AML, breast, lung, colon cancer, and lymphoma	OncomiR	Activated by NfKB	SOCS1, BACH1, MEIS1, ETS1, FOXO3A, hMSH2, hMSH6, hMLH1, SMAD5, WEE1, SHIP1, CEBPB
miR-21	Up in lung, breast, pancreas stomach, ovary prostate cancer, and CLL, AML, glioblastoma, myeloma	OncomiR	Activated by IL6, GF1alpha	PTEN, TPM1, PDCD4, SPRY1, TIMP3, RECK

Continued

microRNA:	Expression in cancer:	Function	Mechanism of deregulation	Targets
miR-221/-222	Up in invasive ductal carcinoma, lung cancer, hepatocellular carcinoma, papillary thyroid cancer	OncomiR	Activated by MET in lung cancer; repressed by ERalpha in breast; activated by PLZF in melanoma; activated by NF-Kb and cJun in prostate cancer and glioblastoma cells	p27(Kip1), p57(Kip2), PTEN, TIMP3, FOXO3A, ERalpha, KIT, TRSP1, DICER, APAF1, PUMA, PTPμ
miR-17/92	Up in lung, breast, colon cancers	OncomiR	Activated by E2F1 and MYC	PTEN, BIM, HIF1, PTPRO, p63, E2F2, E2F3, TSP-1, CTGF, p21(WAF1), JAK1, SMAD4, TGFbetaII, MnSOD, GPX2, TRXR2

Modified from (Di Leva and Croce, 2013)

Genetic alterations

Several studies have shown that miRNAs are located within fragile sites and cancer susceptibility loci (Calin et al., 2004; Lagana et al., 2010) however the same occurs to protein coding genes and there is no significance difference between the miRNAs genes and protein coding genes distribution in the genome. Statistically significant differences have been found when specific chromosomes were analyzed; chromosome 19 is an example of a chromosome with higher incidence of miRNAs in fragile sites (Lamy et al., 2006).

Mutations that affect the seed sequence of tumor suppressor miRNAs can potentially alter the regulation of their target mRNAs. Sequence variations such as SNPs (single nucleotide polymorphisms) in the 3'UTR and other gene regions have been reported to influence miRNA targeting in several cancer related pathways (Mishra et al., 2008), yet tumor specific mutations of miRNAs seem to be uncommon.

Epigenetic mechanisms

The expression of miRNAs can be influenced by epigenetic changes associated with cancer cells. MiRNA loci are associated with CpG islands giving strong evidence to their susceptibility to be regulated by DNA methylation (Weber et al., 2007). The fact that some miRNAs are able to regulate components of the epigenetic machinery suggests that there is a strong feedback loop between the miRNAs and epigenetic pathways (Sato et al., 2011).

Transcription factors

Deregulated activity of transcription factors can lead to abnormal miRNAs expression. Since most of the miRNAs are transcribed by RNA polymerase II, and miRNAs are often located within introns or in cluster units, a single transcription factor associated with Pol II could have the potential to activate or repress a variety of miRNAs genes (Jansson and Lund, 2012).

Biogenesis pathways

Alteration in the enzymes and cofactors involved in miRNAs biogenesis can affect the levels of mature mRNAs. Other mechanism that results in alterations of the regulation exert by miRNAs relay in their target mRNAs and include mutations in the miRNA target sites (Yu et al., 2007), alternative splicing (Sandberg et al., 2008), target site exclusion by secondary structures (Kedde et al., 2007) and competition for miRNAs binding sites by other RNAs (Seitz, 2009).

Figure 5

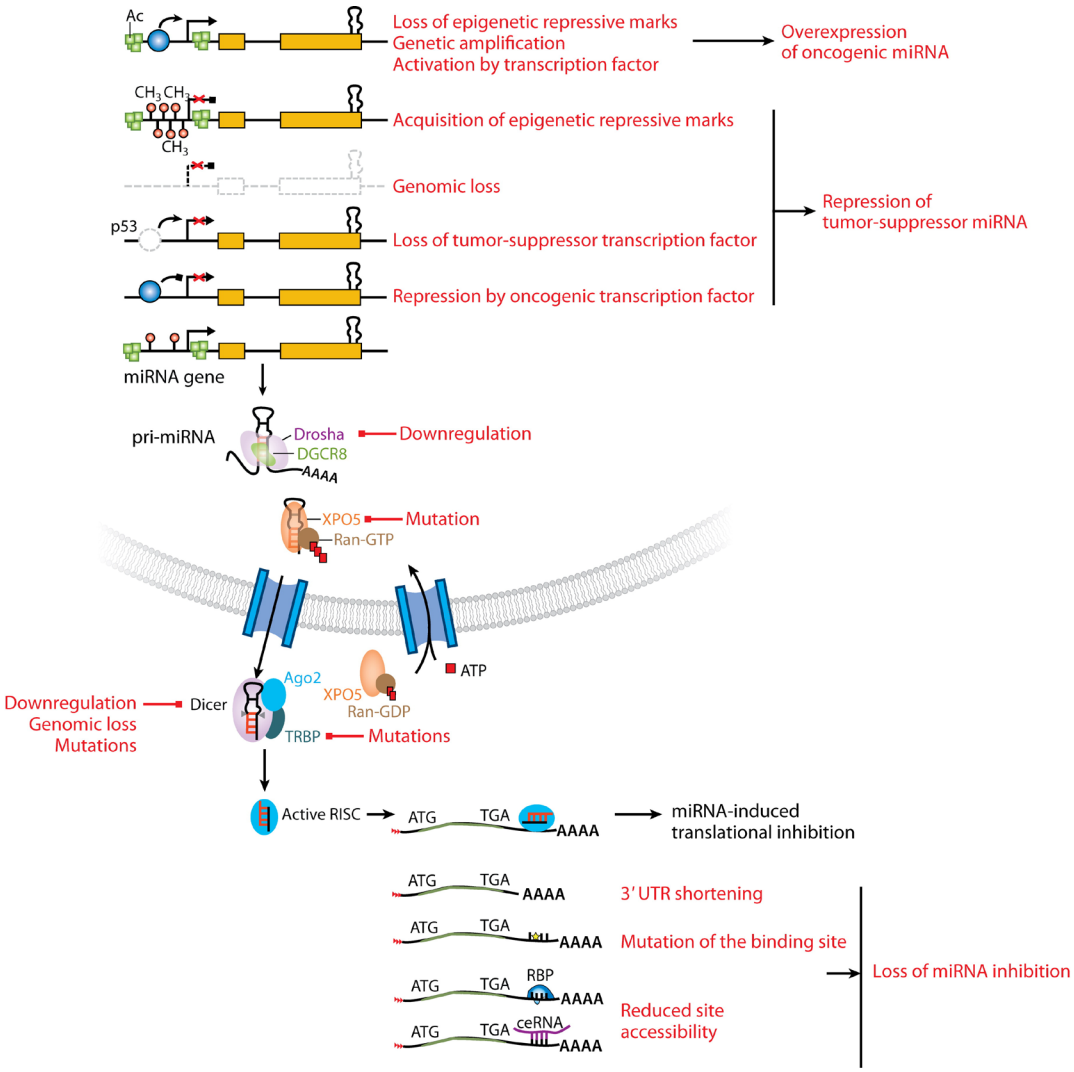


Figure 5. MicroRNAs dysregulation in cancer.

Processes commonly deregulated in cancer (shown on red) are linked with miRNA dysregulation in cancer (Di Leva et al., 2014).

The Cancer Genome Atlas Project (TCGA)

The Cancer Genome Atlas (TCGA) is a multidisciplinary project, whose mission is to accelerate the understanding of the molecular basis of cancer, through the application of cutting edge genome analysis technologies. One of the main goals of TCGA is to improve the ability of the medical community to diagnose, treat and prevent cancer. In order to achieve this goal, the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) developed a framework to explore the entire spectrum of genomic changes involved in more than 20 types of human cancer. TGCA chose the cancers for the study based on specific criteria that included: poor prognosis, overall public health impact, availability of human tumor, and matched normal tissue samples. Ovarian cancer was among the first three cancers analyzed by TCGA and the main findings were published in Nature 2011 (Cancer Genome Atlas Research Network, 2011). In summary, the ovarian cancer TCGA publication provided a large-scale of 489 HGSOC samples. Mutations on TP53 were the most common occurring in at least 96% of the cases; BRCA1 and BRCA2 were mutated in 22% of tumors. Other mutations were identified but the incidence was low (2-6%). Somatic copy number alterations (SCNA) were high compare with other tumors previously analyzed (Cancer Genome Atlas Research Network, 2008). The mutation spectrum marks identified in this work were clearly different from the ones reported for other ovarian cancer subtypes (Kuo et al., 2009; Nakayama et al., 2010), highlighting the importance of genomic analyses to help stratify subtypes and ultimately improved the ovarian cancer outcomes through personalized therapy. The TGCA project is a valuable source of information that

allows the scientific community to explore the genomic alterations present in different types of cancer and conduct hypothesis driven investigation as well as genome wide studies.

The present research work used the information of the TCGA ovarian cancer project as the starting point to carry out a genome-wide search for tumor suppressor microRNAs for ovarian cancer and try to understand their roles in the development, maintenance, and metastasis of ovarian cancer. From the bioinformatics analysis microRNAs with putative "tumor suppressor" activities, and the biological pathways targeted by those miRNAs were chosen to be validated using an *in vitro* model. Our comprehensive approach lead us to three microRNAs, miR-29a, miR-509-3p and miR-130b that have the ability to impact different biological pathways and act as tumor suppressors.

An overview of the current information on these three miRNAs and their targeted pathways is presented in following sections.

The miR-29 Family

The miR-29 family contains miR-29a, miR29b and miR29c. MiR-29b further includes two members, miR-29b-1 and miR-29b-2. MiR-29a and miR-29b-1 are 652 bases apart and are transcribed into the same primary transcript from a locus in chromosome 7q32. The other two members miR-29b-2 and miR-29c are located 507 bases apart from each other and are also transcribed together from a locus in chromosome

1q32 (Garzon et al., 2009). The sequences encoding the two miRNAs in each cluster are separated by < 1 kb (Mott et al., 2010). Since all the mature members of the 29 family share the same 5'-seed sequence it is consider that they redundantly regulate the same set of target genes. The biological function of miR-29 family members has been studied mainly in the context of cancer and other diseases such as fibrosis, cardiovascular injury, ischemia, and aneurism development. Aberrant expression of miR-29s has been reported in multiple cancers and seems to be involved in complex regulatory process affecting diverse biological pathways such as cell cycle, proliferation, senescence, differentiation, cell apoptosis, metastasis, epigenetic modulations etc. (Figure 6). The known functions of miR-29 family in cancer are shown in Table 3.

Figure 6

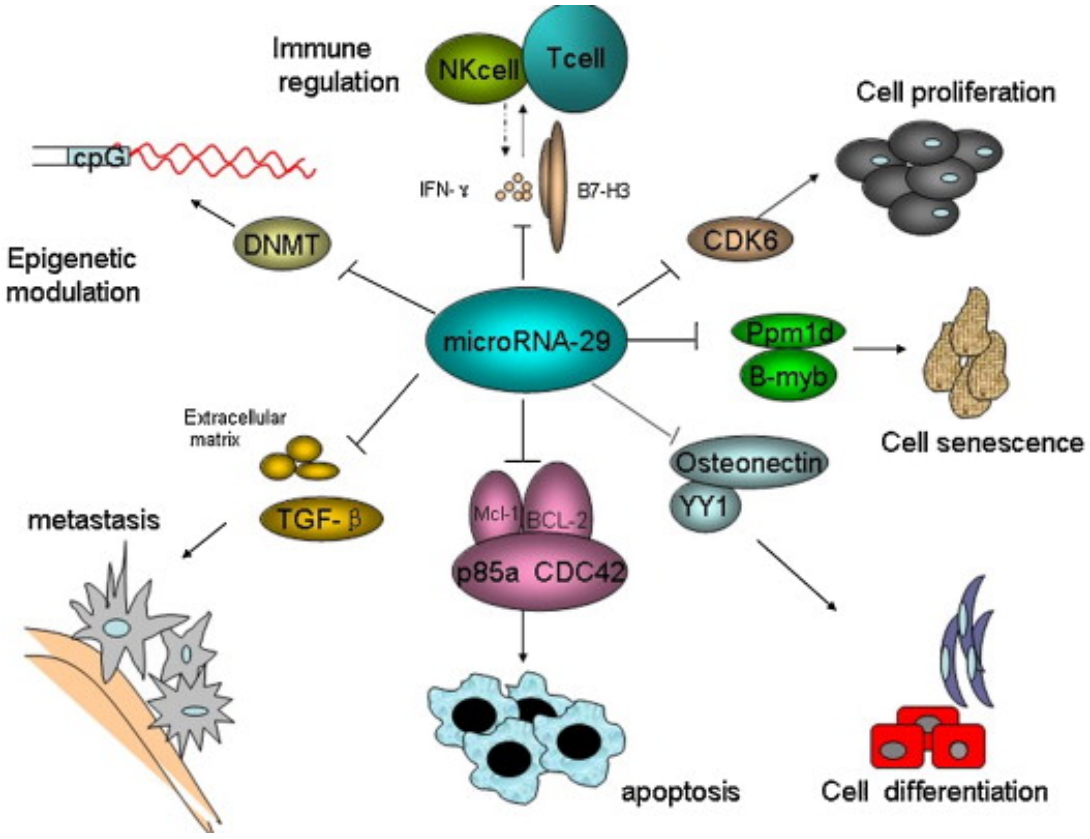


Figure 6. The regulatory mechanisms of the miR-29 family.

The miR-29 family can be an effective regulator of tumorigenesis and cancer progression by targeting key pathways (Wang et al., 2013).

Table 3. The function of miR-29s

Targets:	Functional process:	Cancer type:	Reference:
CDK6	Cell proliferation and cell cycle	Acute Myeloid leukemia, Mantle cell lymphoma Cervical carcinoma	(Garzon et al., 2009; Li et al., 2009) (Li, Hassan et al. 2009, Garzon, Heaphy et al. 2009)
Histone deacetylase, TGF-β3, NF-kB-YY1	Cell differentiation	Rhabdomyosarcoma	(Wang et al., 2008)
Mcl-1, Tcl-1 p85a, CDC42	Apoptosis	Acute Myeloid leukemia Hepatocellular carcinoma B-cell lymphocytic leukemia Cholangiocarcinoma Mixed cancer types	(Garzon et al., 2009; Garzon et al., 2009; Mott et al., 2007; Park et al., 2009; Pekarsky et al., 2006; Xiong et al., 2010)
Collagen laminins-1 Mmp2 Sparc TGF-β3	Metastasis	Nasopharyngeal carcinomas Endometrial carcinomas Breast cancer	(Castilla et al., 2011; Gebeshuber et al., 2009; Sengupta et al., 2008)

Continued

Targets:	Functional process:	Cancer type:	Reference:
DNMT3a DNMTb Sp1	DNA methylation	Lung cancer Epithelial mesothelioma Cutaneous melanoma Acute myeloid leukemia Hepatocellular carcinoma	(Braconi et al., 2011; Fabbri et al., 2007; Garzon et al., 2009; Nguyen et al., 2011; Pass et al., 2010)
INF-γ B7-H3	Immune regulation	Neuroblastoma Burkitt's lymphoma Adenocarcinoma	(Ma et al., 2011; Steiner et al., 2011; Xu et al., 2009) (Ma, Xu et al. 2011, Steiner, Thomas et al. 2011, Xu, Cheung et al. 2009)

Chromosome Xq27.3 microRNA cluster

The chromosome Xq27.3 microRNA cluster, also known as the miR-506 family, is composed of 15 microRNAs that share 8 different 5'-seed sequences (Figure 7). This microRNA cluster is considered to be an example of rapid evolution in primates, ranging from New-World monkeys to humans. Previous studies reported that the microRNAs from this cluster are preferentially expressed in the testis (Zhang et al., 2007).

The biological functions and pathways regulated by this cluster have not been well established. Recent studies have reported both tumor suppressor and oncogenic characteristics for different cluster members. In melanoma, the miR-506 cluster is overexpressed and Streicher and collaborators concluded that it is needed to induce melanocyte transformation also expression of miR-513, miR-506, miR-507 and miR-509 is critical to maintain the cancer phenotype (Streicher et al., 2012). In pediatric neuroblastoma, miR-506-3p is upregulated in differentiated cell lines, suggesting a role for this microRNA in neural differentiation and tumorigenesis (Zhao et al., 2014). In other neoplasias such as ovarian, cervical, gastric and renal cancer, members of the Xq27.3 cluster have shown tumor suppressor activities by regulating EMT, hedgehog, multi-drug resistance and cell migration and invasion pathways respectively (Shang et al., 2013; Wen et al., 2014; Yang et al., 2013; Zhai et al., 2012).

Figure 7

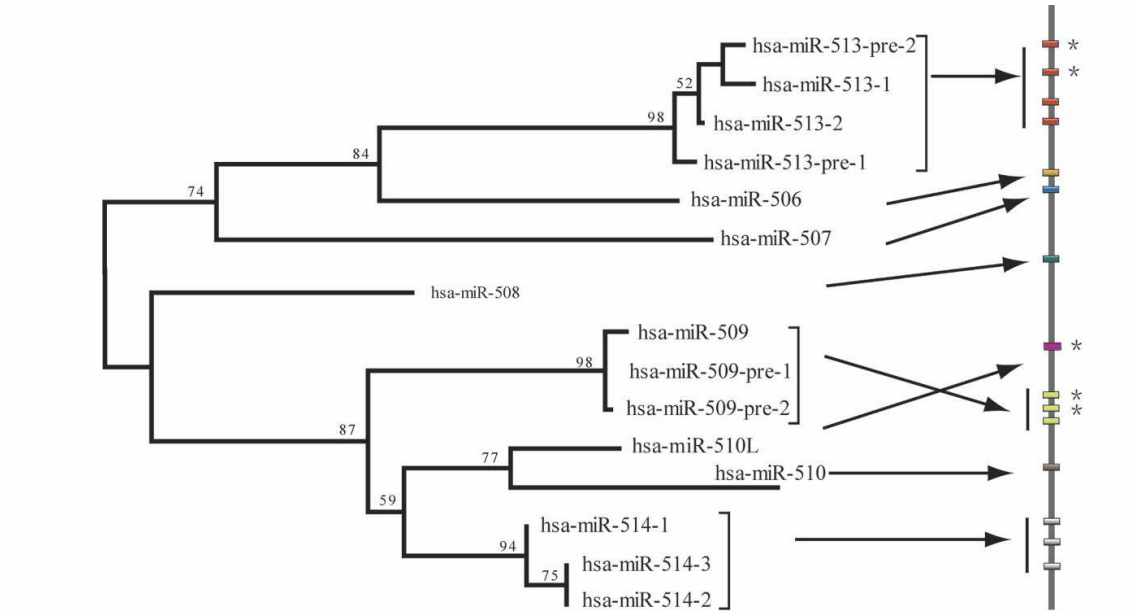


Figure 7. The phylogenetic relationships of the 15 X-linked miRNAs in human.

Modified from (Zhang et al., 2007)

Hsa-miR-130b

In humans, the miR-130 family includes 4 members: miR-130b and miR-301b, that are clustered together in chromosome 22, miR-130a in chromosome 11, and miR-301a encoded in chromosome 17. As family some of their biological functions include regulation of hypoxia response (Saito et al., 2011) and induction of pluripotent stem cells (iPSCs) (Pfaff et al., 2011).

In cancer some members have been described as oncomiRs, such is the case of miR-301 in breast cancer (Shi et al., 2011). Another member miR-130a has been involved in chemio-resistance/sensitization in ovarian cancer, however there are contradictory results that highlight the complexity of the role of this microRNA and others in cancer.

In the present work, I focused on studying the tumor suppressor potential of miR-130b in the context of ovarian cancer hence further detail of the known functions of miR-130b in tumorigensis will be discuss to understand this miRNAs in the full context of its fucntions.

In hematological malignancies, miR-130b act as oncomiR in both chronic myeloid leukemia (CML) (Suresh et al., 2011) and multiple myeloma (MM) (Tessel et al., 2011) by downregulating tumor suppressor activities of CNN3 and silencing the glucocorticoid receptor, respectively.

In hepato-cellular carcinoma, increased levels of miR-130b was found in cells with stem cell characteristics and this increase was correlated with chemoresistance, enhanced tumorigenicity, and higher rate of self-renewal (Ma et al., 2010).

Downregulation of tumor suppressors is another mechanism used by miR-130b to potentiate cancer. In gastric cancer miR-130b silences RUNX3, a known tumor suppressor, and enhances cell viability contributing to tumorigenesis (Lai et al., 2010).

On the other hand, miR-130b has the potential to act as a tumor suppressor in different cancer types. In pancreatic cancer, miR-130b-mediated silencing of the oncogene STAT3 suppressed proliferation and increased apoptosis and cell cycle arrest. In pituitary adenomas miR-130b is downregulated compared with normal pituitary tissue and overexpression of miR-130b caused G1 and G2 arrest in an *in vitro* model (Zhao et al., 2013).

MiR-130b is downregulated in colorectal cancer (CRC) and overexpression in an *in vitro* model silenced integrin β1 and impaired migration and invasion, suggesting that miR-130b has the potential to inhibit metastasis in CRC (Zhao et al., 2014).

MiR-130b was found to be downregulated in 4 different ovarian cancer chemoresistant cell lines, suggesting a possible role in chemosensitization (Sorrentino et al., 2008). However Sorrentino *et al*. did not describe a mechanism of action to explain their observations. Yang *et al*. also found miR-130b to be downregulated in chemoresistant cell lines as well as in patients, where the clinical stage negatively correlated with miR-130b expression, the lower the miR-130b expression the higher the clinical stage. MiR-130b was found to be hyper-methylated and treatment with demethylation compounds increased miR-130b expression as well as drug sensitivity *in vitro*. CSF-1 was confirmed to be a direct target of miR-130b and downregulation of

CSF-1 was proposed as a possible mechanism of action for miR-130b-mediated drug sensitization in ovarian cancer (Yang et al., 2012).

Epithelial to mesenchymal transition (EMT)

The epithelial-to-mesenchymal transition (EMT) program directs epithelial cells to convert into mesenchymal cells and is a pivotal pathway during embryo development. Epithelial and mesenchymal cells have unique characteristics specific to their functions. Epithelial cells form polarized sheets or layers tightly connected laterally via cellular junctions such as adherent junctions, desmosomes, and tight junctions. Epithelial cells are always anchored to an underlying basement membrane via hemidesmosomes, which allow them to maintain apical-basal polarity. Desmosomes and hemidesmosomes are further connected to the epithelial-specific cytokeratin filaments. On the other hand, mesenchymal cells are embedded inside the extracellular matrix (ECM) and rarely establish cell-to-cell contact (Boyer and Thiery, 1993).

Many intra- and extra-cellular factors can induce or potentiate EMT (Figure 8). Reduction of cell-to-cell adhesion due to transcriptional regulation of the proteins involved in making the junction is a key factor to initiate EMT. Transcriptional downregulation of E-cadherin, an important marker of the epithelial phenotype, has been observed in different types of cancer. The transcription factors involved in this phenomenon include the epidermal growth factor, insulin growth factor 1, interleukin, vascular endothelial growth factor, transforming growth factor ***β,*** notch, fibroblast growth factor, and Wnt/***β***-catenin signaling pathways (Barrallo-Gimeno and Nieto, 2005). Most of these signals exert their action on E-cadherin repression through the modulation of

SNAI1 and SNAI2 (Snail and Slug) and basic helix-loop-helix (E47 and TWIST) families, as well as two double-zinc finger and homeodomain (ZEB1 and ZEB2) factors (Moreno-Bueno et al., 2008).

Figure 8

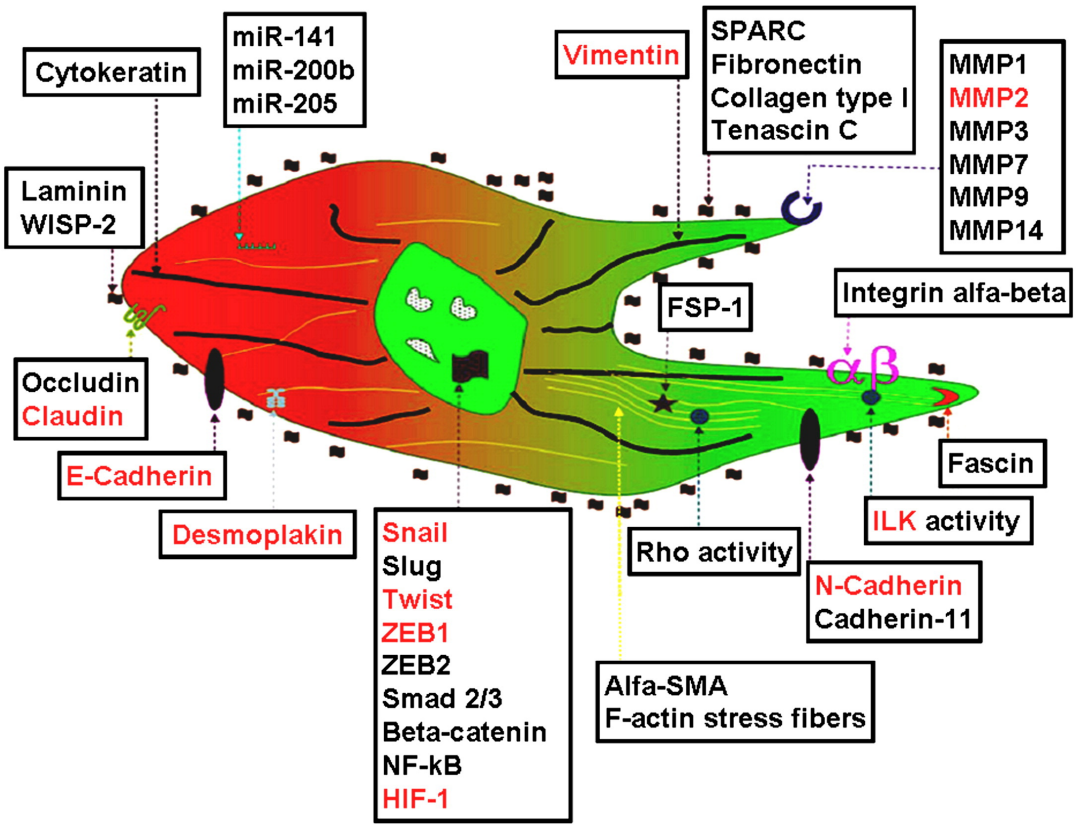


Figure 8. Schematic representation of EMT markers.

Upregulated factors implicated in cytoskeletal changes and the mesenchymal phenotype (green side) and repressed markers (red side) implicated in the maintenance of the epithelial phenotype (Mirantes et al., 2013).

Entering EMT is not necessarily an irreversible commitment and there are instances where cells can activate EMT and undergo the reverse process, mesenchymal to epithelial transformation (MET). The term epithelial-mesenchymal plasticity refers to the dynamic interchange between the epithelial and mesenchymal phenotypes (Thompson and Haviv, 2011). Interplay between these phenotypes has been linked with the metastatic process of carcinomas. Metastasis is responsible for more than 90% of cancer-associated mortality. For distant metastasis, primary tumor cells must detach, invade the blood/lymphatic vessels, seed at distant sites and colonize to macrometastases.

Completion of the metastatic process requires EMT to be induced but it should be turn off (or the MET activated) to allow the new tumor to be established (Figure 9). Two different groups recently added support to this concept. Tsai and co-workers induced EMT and tumor cells transit into the bloodstream by overexpressing Twist1, but metastasis did not form if the cells could not switch off Twist1, this result suggested that halting EMT to allow MET is required for completion of the metastatic process (Tsai et al., 2012).

Ocaña *et a*l. found that forced and continuous overexpression of Prrx1 (a newly characterized EMT inducing transcription factor) blocked the capacity of metastasis-competent cells to produce metastatic tumors, and that Prrx1 suppression is needed for MET activation (Ocaña et al., 2012).

Figure 9

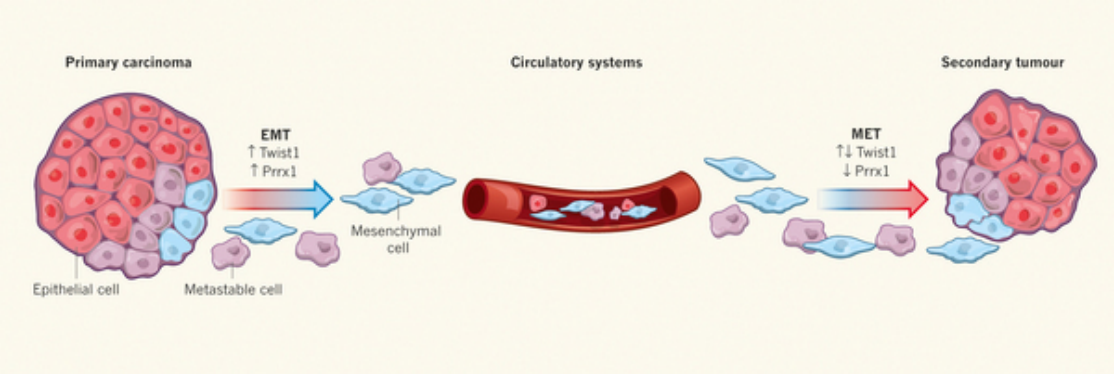


Figure 9. Cellular transition in cancer metastasis.

Dissemination of cells from a primary solid tumour is facilitated by the epithelial–mesenchymal transition (EMT). Sustained expression of EMT genes prevents the formation of secondary tumors, possibly by preventing the cells from undergoing the mesenchymal–epithelial transistion (MET) that is needed for them to revert to the epithelial state and form metastases (van Denderen and Thompson, 2013).

The p53 family

P53 is a critical tumor suppressor best known as "the guardian of the genome". The ability of p53 to suppress tumor development is thought to relate to its function as cellular-stress sentinel. When the cells receive stress signals such as DNA damage, oncogene activation or nutrient deprivation, p53 can promote cell cycle arrest and/or apoptosis to prevent the propagation of compromised cells (Vousden and Lu, 2002). P53 mainly acts as a transcription factor to regulate the expression of genes that contribute to its biological responses (Bieging and Attardi, 2012).

The biological pathways that are activated by p53 are cell-type dependent and the intensity of the activating signal can also influence the biological response. Cells with extensive DNA damage may enter senescence or become apoptotic, both of these responses will eliminate damaged cells from tissues. Cells with limited DNA damage may undergo a temporary cell cycle arrest, giving them time to repair the damage, and in such a case p53 can act as a survival factor (Jones et al., 2005).

The ability of p53 to respond to DNA damage is believed to be an ancient function, since it is shared by all members of the p53 family, including p53, p63 and p73. P63 and p73 are evolutionary older homologues of TP53. The 3 relatives share the same structural domains including, a terminal transactivator domain (TAD), a DNA binding domain (DBD), and a carboxyterminal oligomerization domain (OD) (Figure 10) (Yang et al., 1998). In primitive organisms, a p63/p73 ancestor protected germline integrity and this role is conserved in flies, worms and mice (Belyi et al., 2010; Rutkowski et al., 2010). Upon evolution each member specialized, with p53 being the key member for

tumor suppressor activities and p63 and p73 promoters of stratified epithelial development and nervous system functions.

The p63 and p73 genes are expressed as multiple protein isoforms. Alternative transcription start sites result in the generation of TA isoforms that contain a N-terminal exon encoding a p53 like TAD domain, and ΔN (dN) isoforms that lack this domain (Figure 10). The ΔNp63 and ΔNp73 are unable to transactivate the canonical p53 target promoters and can act as dominant negatives for the TA isoforms. Thus maintaining a delicate balance of all the isoforms is critical to preserve homeostasis (Murray-Zmijewski et al., 2006).

Figure 10

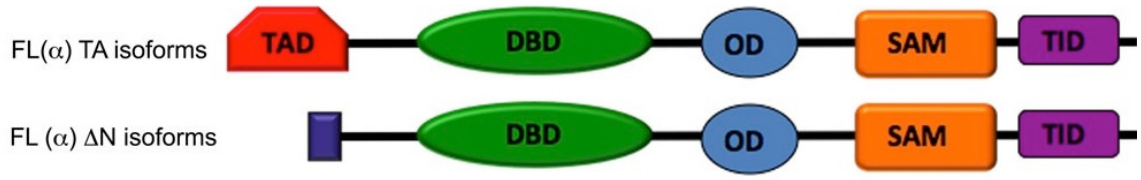


Figure 10. Structural features of the p53 family proteins.

Schematic representation of the protein modular structure of the p53 family members. Modified from (Candi et al., 2014).

TP53 and TP63 in cancer development

Dysregulation in p53 signaling pathways is believed to be required for the development of most cancers. TP53 is the most commonly mutated gene in human cancers (Figure 11) (Kandoth et al., 2013). The spectrum of mutation spans trough every region of the protein (Leroy et al., 2013), in some cases frameshifts or nonsense mutations result in loss of p53 protein expression. However most of tumor-associated alterations result in missense mutations causing the substitution of a single amino acid in the p53 protein. These point mutations occur throughout the protein, but usually cluster within the DNA binding domain. The mutations generally lead to a loss of p53 wild type function and since p53 acts as a tetramer, the mutant proteins can also act as dominant negative inhibitors of p53. P53 mutant proteins can also acquire novel functions and the term "gain of function" (GOF) has been coined to describe this phenomenon. The GOF phenotype by mutant p53 is supported by clinical data showing that patients carrying a p53 missense mutation, leading to expression of a mutant protein in the germline show a significant earlier onset than patients with loss of p53 expression (Bougeard et al., 2008; Zerdoumi et al., 2013).

TP53 mutants are often considered to be equivalent but evidence suggests that different mutants show specific profiles with respect to loss of wild type p53 function, their abilities to act as dominant negative inhibitors, and their GOF phenotypes (Halevy et al., 1990; Petitjean et al., 2007). The large number of different p53 mutations complicates the elucidation of their functions and adds another layer of complexity by

tissue specificity, the expression patterns of p53 targets, and protein interactions. Mutant p53s have been classified into two groups 1) contact mutants which include mutants that prevent wild type transcriptional activity without affecting the conformation of the p53 protein, and 2) conformational mutants which comprise of mutations that disrupt the three-dimensional structure of the protein (Solomon et al., 2012). Shown below is the distribution of mutations in p53 across a panel of cancers analyzed by TCGA.

Projects like TCGA represent an important tool to first describe all the mutations present in a given cancer and then increase our understanding of how the mutations affect tumorigenesis, patient outcome and drug responses.

Figure 11

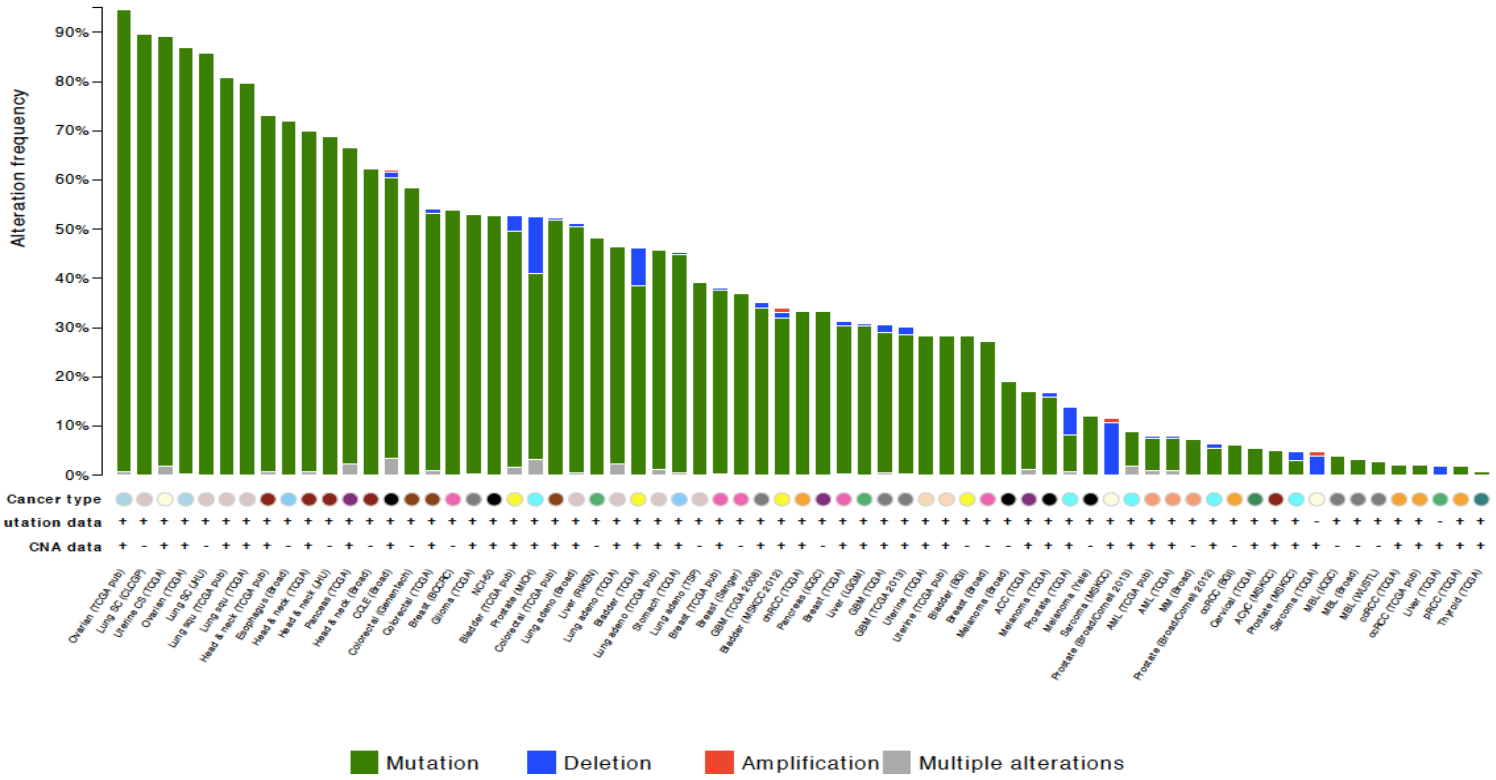


Figure 11. Cross-cancer alteration summary for p53.

TP53 alterations across the cancer types analyzed by TCGA (Cerami et al., 2012).

In contrast with the high rate of p53 mutation in cancers, p63 is rarely mutated in human cancers, which is unusual for a protein with tumor suppressor functions (Melino et al., 2002; Melino, 2011). However, overexpression due to chromosomal amplifications is common in several cancers (Figure 12) including basal cell and squamous cell carcinomas of the head and neck (Candi et al., 2007), basal-like breast tumors, prostate and bladder carcinomas, and recently in primary and metastatic melanoma, where p63 overexpression was correlated with poor prognosis (Matin et al., 2013). When p63 is expressed, ΔN isoforms are the most widely expressed in tumors. Generally TAp63 is involved in protection against cancer metastasis and in preserving the integrity of female germ cells (Melino, 2011; Su et al., 2010). ΔNp63 is considered to act more as an oncogene, since its normal functions include the maintenance of the proliferative potential of stem cells, suggesting that ΔNp63 contributes to the proliferative potential of cancer stem cells (Pignon et al., 2013; Senoo et al., 2007). The biological impact of ΔNp63 and TAp63 is cancer type dependent and genetic studies in mouse models have provided conflicting results on the role of p63 isoforms in tumorigenesis.

Figure 12

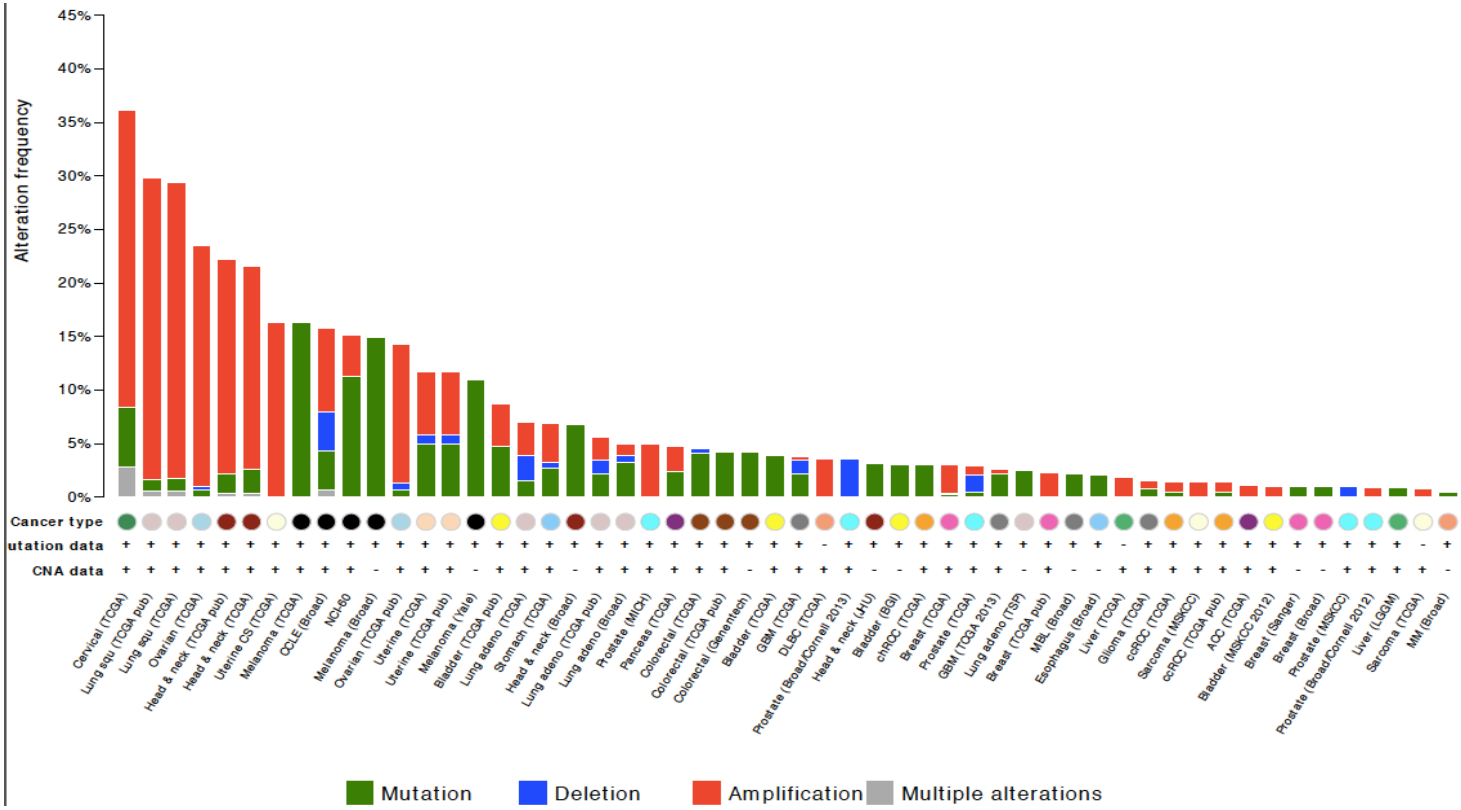


Figure 12. Cross-cancer alteration summary for p63.

TP63 alterations across the cancer types analyzed by TCGA (Cerami et al., 2012).

Aside from the canonical pathways activated by the p53 family to suppress tumorigenesis, recent evidence suggests that all the members are able to activate key genes of the autophagy pathway. A global genomic profiling analysis revealed a number of p53 targets that are core regulator of different steps of the autophagic process. Activation of autophagy seems to play an important role in p53 tumor suppressor functions since silencing of ATG5, a core gene of the pathway, impairs the ability of p53 to activate apoptosis and suppress oncogenic transformation. The other two members of the family also induced most of the autophagy genes identified in this study; under p53 null background p63 and p73 were able to compensate for the loss of p53 (Kenzelmann Broz et al., 2013).

Autophagy

Autophagy (from the Greek "auto" self and "phagos" eat) is an evolutionary conserved catabolic process involved in the delivery and lysosomal degradation of long-lived intracellular components such as proteins, nucleic acids, and organelles.

Autophagy is a multistage process. On the first stage cytoplasmic cargo is surrounded by a double membrane to form phagophores, in the next step the phagophores closed to form the autophagosomes, which then move towards the microtubule-organizing center where lysosomes are concentrated. Following this autolysosomes are formed in two different ways: by direct fusion of an autophagosome with a lysosome or in the course of consecutive fusion of an autophagosome first with an endosome and then with a lysosome (Eskelinen, 2005).

Autophagy is a process that helps to maintain cellular homeostasis through the recycling of proteins and organelles, and its level of activation differs from cell to cell and also at different times during differentiation (Mizushima et al., 2004) Terminally differentiated cells have higher autophagy levels compare with cells undergoing proliferation where the autophagy pathway is inhibited by Cdk1. The process is activated under diverse circumstances such as starvation conditions, oxidative stress (Eng et al., 2010), early stages of embryogenesis (Tsukamoto et al., 2008), degradation of one of the parental mitochondria after fertilization (Al Rawi et al., 2012), and during the transition from fetal to neonatal stage (Kuma et al., 2004).

Molecular mechanism

The autophagy process is divided in different mechanistic steps including induction, cargo selection, vesicle formation, autophagosome-vacuole fusion, breakdown of the cargo and release of the degradation products back into the cytosol (He and Klionsky, 2009). A number of genes involved in those processes have been identified using the *Sccharomyces cerevisae* model (the *S. cerevisae* nomenclature is standard and will be use thereafter). Each step requires a distinct set of proteins; most of them are part of the ATG (autophagy-related genes) group (Klionsky et al., 2003).

Due to the potential involvement of the autophagy pathway in miR-130b-mediated tumor suppression, the molecular mechanism involved in autophagy activation will be briefly discuss in the following section.

Basal levels of autophagy are generally low, as a result very efficient induction mechanisms are needed. A central autophagy inhibitor pathway is the serine/threonine protein kinase TOR that phosphorylates and inactivates the initiation proteins ULK1 (Unc-51-like-kinase 1) and ULK2 (Unc-51-like-kinase 2) whose main function is to phosphorylate ATG13. Inhibition of mTOR by starvation will result in ULK1 and ULK2 activation; site-specific phosphorylation of ATG13 by ULK1 and ULK2 which bypasses the inhibitory effects of mTOR.

Autophagosomes are assembled at the PAS (phagophore assembly site) by the addition of new membranes the form vesicles to contain the cargo. This is the most complicated step of autophagy. The nucleation and assembly of the initial phagophore membrane require the class III phosphatidylinositol 3-kinase (PI3K) complex: PI3K, Vps34 (vacuolar protein sorting 34), p150, Atg14, and Beclin1 (Ishihara et al., 2001; Kihara et al., 2001; Liang et al., 1999; Sun et al., 2008). The function of Beclin1 is regulated by the anti-apoptotic protein Bcl2 that binds and sequesters Beclin1 under nutrient rich conditions, dissociation of the Beclin1-Bcl2 complex is required for autophagy induction. The PtdIns3K along with the Atg18, Atg20, Atg21 and Atg24 proteins recruit two ubiquitin like (Ub1) conjugated systems Atg12-Atg5-Atg16 and Atg8-PE (Phosphatidylethanolamine) (Suzuki et al., 2001; Suzuki et al., 2007), which play a central role in the membrane elongation and expansion of the autophagosome. Atg8 (LC3B in mammals) controls the size of the autophagosome (Xie et al., 2008) and its lipidation is used to monitor autophagy induction.

After autophagosome formation is completed, Atg8 is cleaved from PE by Atg4 and released back to the cytosol (Kirisako et al., 2000). In mammals the autophagosome-lysosome fusion is not well characterized but it is known that the lysosomal membrane proteins LAMP-2 and the small GTPase Rab7 proteins are requiredFigure 13 (Jager et al., 2004; Tanaka et al., 2000)

Multiple biological pathways are also part of the autophagy machinery, including the secretory pathway (Ishihara et al., 2001), the endocytic pathway (Meiling-Wesse et al., 2005) and the cytoskeletal network (Kochl et al., 2006). In a similar way several signaling pathways are involved in autophagy regulation including TOR (Noda and Ohsumi, 1998), Ras/PKA (Schmelzle et al., 2004), insulin/growth factor (Stokoe et al., 1997), energy sensing (Liang et al., 2007) and stress response (Yorimitsu et al., 2006).

Autophagy and cancer

Disruption of autophagy affects homeostasis and it has been linked to the development of a variety of diseases including cancer (Cadwell et al., 2008; Kroemer and White, 2010; Mathew et al., 2007; Rosenfeldt and Ryan, 2009).

The initial connection between autophagy and cancer came from the observation that BECN1 is monoallelically deleted in approximately 50% of breast, ovarian and prostate cancers (Aita et al., 1999; Liang et al., 1999). Further studies in a mouse model proved that $Becn1^{+/-}$ mice are viable but prone to develop lymphomas, liver and lung cancer (Qu et al., 2003; Yue et al., 2003).

On the other hand, there is evidence that suggests that autophagy can promote tumor cell survival (Degenhardt et al., 2006), it can be upregulated or suppress by cancer therapies and can be either pro-survival or pro-death for malignant cells (Levy and Thorburn, 2011; Wilkinson and Ryan, 2010).

Autophagy as a tumor suppressor pathway

Normal cells require the presence of the autophagy pathway to maintain their integrity and genomic stability (Ryan, 2011) thus loss of autophagy will disrupt homeostasis and prime the cells for tumor development. Deletion and mutations of autophagy related genes have been associated with a number of human cancers (Table 4). Some of the mechanisms that may contribute to the tumor suppressor effect of autophagy include: removal of damaged protein and organelles such as mitochondria, the reduction of reactive oxygen species (ROS) and DNA damage, the reduction of double strand breaks and polyploidy nuclei (Karantza-Wadsworth et al., 2007; Mathew et al., 2007). Autophagy is also needed for the establishment of oncogene-induced senescence and since senescence is use as a mechanism to stall tumor development, this might be another process in which autophagy can help to suppress tumor development (Krizhanovsky et al., 2008).

Figure 13

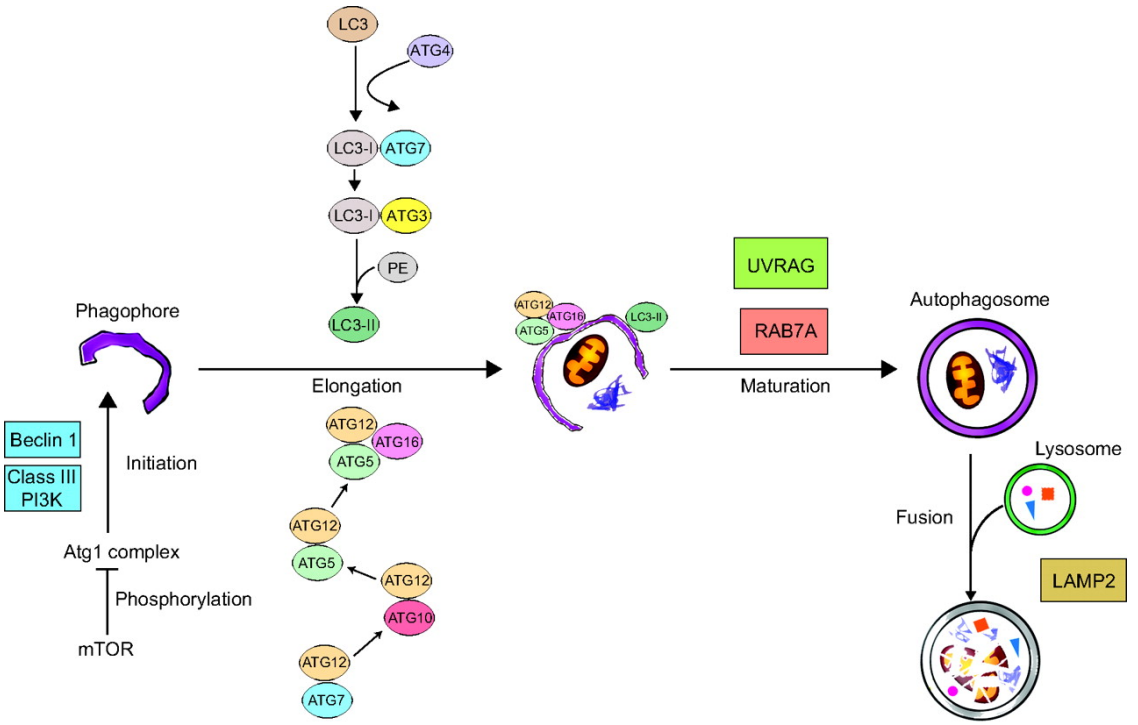


Figure 13. The pathways controlling autophagy.

mTOR is a master regulator of autophagy: it is a bioenergetic sensor and inhibits the ATG1 protein complex by phosphorylating it. In the initiation phase, the Beclin-1–VPS34 complex converts PtdIns into PtdIns(3)*P*, which then recruits two separate ubiquitin-like conjugation systems, resulting in the formation of the ATG12–ATG5–ATG16L and LC3–PtdEtn complexes. This process results in the formation of a vesicle surrounded by a double membrane – referred to as an autophagosome – which engulfs cytosolic contents, such as proteins and organelles. Autophagosomes ultimately fuse with lysosomes to form new organelles, termed autolysosomes, within which the cargo of the autophagosome is degraded by the acidic hydrolases provided by the lysosome. Maturation of autophagosomes and fusion with lysosomes is mediated by UVRAG, RAB7A and LAMP2. Modified from (Liu and Ryan, 2012).

Table 4. Autophagy related genes and cancer

Gene name:	Stage of autophagy:	Type of human cancers:	Type of mutation in cancer	References
BECN1	Initiation	Breast, ovarian and prostate	Monoallelic deficiency	(Aita et al., 1999)
UVRAG	Initiation	Colorectal and gastric	Monoallelic deficiency	(Goi et al., 2003; Ionov et al., 2004; Kim et al., 2008)
SH3GLB1 (bif-1)	Initiation	Gastric and prostate	Decreased expression	(Takahashi et al., 2007) (Takahashi, Coppola et al. 2007)
ATG2B, ATG5, ATG9B, ATG12	Elongation	Gastric and colorectal	Frameshift mutation	(Kang et al., 2009)
RAB7A	Fusion	Leukemia	Gene rearrangement and deletion	(Kutsenko et al., 2002)

Autophagy as an oncogenic pathway

During the initial phases of tumor formation, malignant cells experience hypoxia and lack of nutrients due to deficient blood supply (Harris, 2002). These conditions result in limited proliferation and cancer cells can enter a dormant stage, it is during this stage that they rely on autophagy to survive (Lu et al., 2008). Other studies suggest that autophagy is also required for cancer cell survival in established tumors, especially in the areas with low oxygen and nutrients (Mathew et al., 2007). Inhibition of the autophagy pathway in cancer cells with impaired apoptosis can lead to necrosis both *in vitro* and *in vivo* models (Degenhardt et al., 2006).

Deletion of FIP200, an essential regulator of autophagy, has inhibitory effects on oncogenic driven tumorigenesis, however it is possible that this effect occurs through a function of FIP200 that is independent of autophagy (Wei et al., 2011). *In vivo* studies using mice with mosaic deletions of ATG5 or ATG7 failed to developed adenocarcinomas and metastasis, indicating that more developed cancers might require autophagy to progress (Takamura et al., 2011).

Because autophagy seems to have opposing roles in cancer development and progression the current scientific data suggest that autophagy can inhibit tumor initiation but promotes tumor establishment and progression (Figure 14).

Figure 14

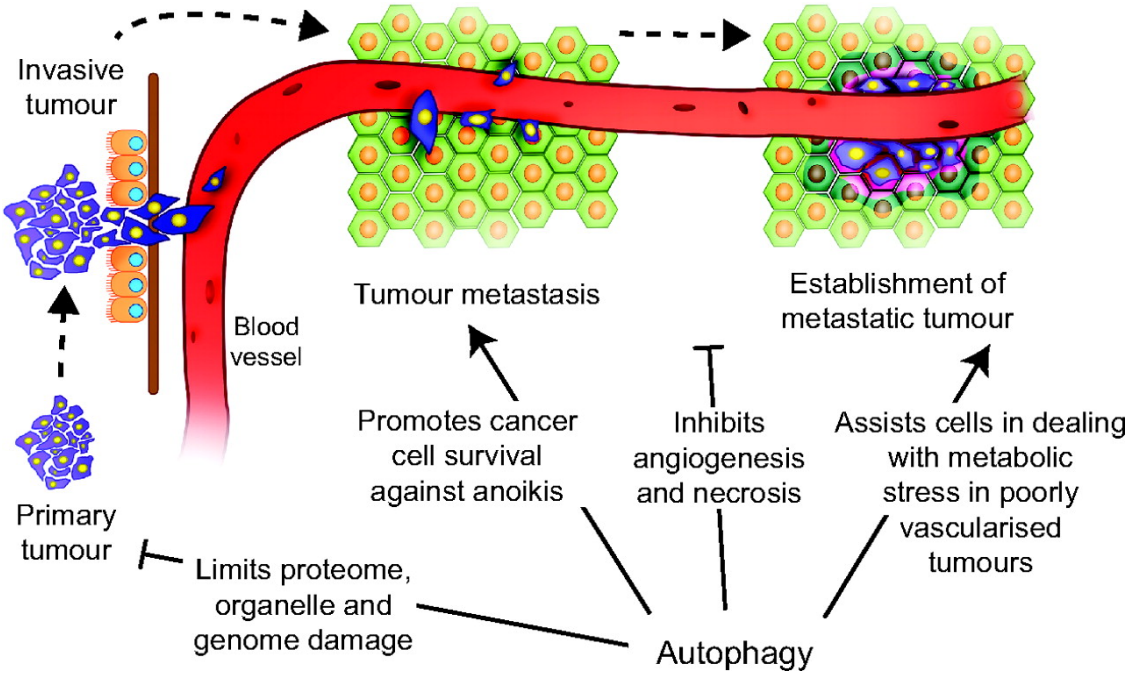


Figure 14. Autophagy and cancer.

(Liu and Ryan, 2012)

Autophagy and ovarian cancer

Deregulation of the autophagy pathway may play an important role in the pathogenesis, dormancy, and chemoresistance of ovarian cancer. Among the genes and proteins that have been found altered in ovarian cancer and that have a connection with autophagy 3 main groups have been identified: 1) the tumor suppressors PTEN, ARH1 and p53, 2) the components of the autophagy machinery LC3, Beclin-1 and DRAM, 3) the growth factor and nutrient sensor signaling pathways, including PI3-k/Akt/mTOR and Ras/Raf/ERK pathways.

Poorly differentiated and highly malignant ovarian cancer cells express low levels of LC3 compare with the LC3 levels found in benign hyperplastic and borderline tumors (Shen et al., 2008). P53 mutant proteins can also affect autophagy signaling, due to their inability to bind to Bcl2 and Bcl-XL, these anti-apoptotic proteins display a dominant negative function by inhibiting Beclin-1 (He and Levine, 2010).

Beclin-1 was the first tumor suppressor that showed the connection between autophagy and carcinogenesis. Monoallelic deletion of Beclin-1 is found in more than 50% of sporadic ovarian cancers (Liang et al., 1999) and downregulation of Beclin-1 has been found in ovarian cancer compared to benign lesions (Shen et al., 2008).

The following chapters will describe the findings on the phenotypes and biological pathways impacted by hsa-miR-29a, hsa-miR-509-3p and hsa-miR-130b in the context of p53 wild-type and mutant ovarian cancer cell lines, and the possible clinical implications and future applications for selected miRNAs in cancer therapy based on the underlying genetic mutations determined through whole exome or whole genome sequencing.

CHAPTER 2: MATERIALS AND METHODS

Cell culture.

The ovarian cancer cell line OVCAR8 was provided by NCI and the HEYA8 cell line was a kind gift from Dr. Sood (MD Anderson). HEYA8 and OVCAR8 cell lines were maintained in RPMI media (Life Technologies) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals) and 1% of penicillin/streptomycin. All the cell lines were incubated at 37 °C with 90% humidity and 5% CO_2.

MicroRNA mimics.

MicroRNA mimics, inhibitors and negative controls were purchased from Life Technologies. RNA was resuspended with RNase free water to make 50 μM working stocks.

siRNAs.

The following siRNAs were used to silencing the expression of their target genes, p53: HSC.RNAI.N000546.12.2 from IDT, p21: J-003471-12 from Dharmacon, BIM: Bim siRNA I #6461 from cell signaling. All the siRNAs were resuspended in siRNA 1X buffer (Dharmacon) and stored at -20 °C.

Transient transfections.

Cells were seeded in six-well plates (6 X 10^4 cells per well) or 10 cm dishes (3.6 x 10^5 cells) 24 h. before transfection. Transfections of mimics/inhibitors/siRNAs were carried out using between 10 to 20 nM of mimics/inhibitors/siRNAs and lipofectamine (2 μL to 12 μL per complex) in OptiMEM serum free media following manufactures

instructions (life technologies). 24 h after transfection the media was replaced by complete media and the cells were harvested at 24, 48, 72, 96 and 120 h after transfection.

Proliferation assays, drug treatments and expression vectors

Cell proliferation was measured using CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS, Promega). Briefly 2500-3000 cells/well were seeded on 96 well plates 24 h before transfection. Transient transfections were done as describe above and proliferation was assayed by adding 20 μL of MTS reagent in each well, the plate was further incubated (37 °C with 90% humidity and 5% CO_2) for 2-4 h.Absorbance was read at 490 nm.

For Cisplatin and ABT-737 treatments cells were transfected as described above, and media was replaced after 24 h. with media containing different concentrations of Cisplatin (Sigma) (0-7.5 mg/mL) or ABT-737 (3 μM/5 μM) and proliferation was assayed as previously described.

Expression vectors for p63 isoforms were a kind gift from Dr. Elsa Flores (MD Anderson); the empty vector pCDN3.1 was used as control for overexpression experiments.

Total RNA extraction

Total RNA from cellular pellets was isolated using the miRNeasy mini kit (Qiagen) following manufactures instructions. Purity and RNA concentration was measured using a ND-100 nanodrop spectrophotometer.

cDNA synthesis

Reverse transcription of mRNAs was done using the applied biosystems kit (N808-0234); 30 ng of total RNA was used for every 20 μL reaction. Reverse transcription for miRNAs was carried out using the applied biosystems kit (4566596) following manufactures recommendations. In general 10 ng of total RNA was used for each 10 μL of Taq-Man RT reaction.

qPCR SYBR Green and Taq Man

Quantitative polymerase chain reaction (q-PCR) was performed using the Sybr green reagent from Applied Biosystems (4367659) or Kappa technologies (KM4103). For microRNAs q-PCRs were done using Taq Man probes for each specific miR and the Taq-Man 2X Universal PCR master mix (Applied Biosystems 4440047).

Protein extraction and quantification

Total proteins were extracted from cellular pellets using protein extraction buffer (triton X-100 1%, NaCl 150 mM, Tris 25 mM, pH 7.6). Cellular pellets were resuspended with 50-80 μL of cold buffer with protease inhibitors (Complete mini Roche) vortex and incubated on ice for 30 min. followed by centrifugation at 8,000 rmp for 5 min. The supernatant was recovered and stored at -80 °C for further quantification.

Proteins were quantified using the Bio-Rad kit (500-0114). Reagent A and S were mixed in a ratio of 50:1 and 25 μL of the mix were put in each well of a 96 (flat bottom) well plate. Protein samples were diluted 1:5 using protein extraction buffer and 5 μL were added to an individual well, three technical replicates were done by sample, 200 μL of reagent B were added per well and the plate incubated 15 min at room temperature (r.t).

A series of know standards (Bio-Rad 500-0207) were used to make a standard curve. The colorimetric reaction was read using a plate reader at 590 nm. After quantification proteins were stored at -80°C until needed.

Western blot

Electrophoresis

Proteins were separated using the XCell SureLock™ Mini-Cell Electrophoresis System from Invitrogen and the Nupage-NOVEX Bis-Tris 4-12% gels, MOPS buffer, antioxidant and loading sample buffer from the same company. 20-50 μg of protein were used per sample, and the electrophoresis was run for an average of 2 h.

Transference

After electrophoresis proteins were transfered to a Nitrocellulose (Whatman 8565499) or PVD membrane (Invitrogen LC 2005) using the XCell II blot module (Invitrogen EI9061).

Immunodetection

After transference the membranes were blocked during 1h at room temperature with 5% non-fat milk dissolved on TBS-T (50 mM Tris-HCl, 150 mM NaCl, 1% Tween 20, pH 7.5). Primary antibodies were added at the concentrations suggested by manufactures and the membranes incubated at 4 °C over night. The next day the membranes were washed three times (10 min. each) with TBS-T and then incubated with

the corresponding secondary antibody (anti-rabbit 1:10000 and anti-mouse 1:5000) for 2 h. Membranes were washed three times (10 min. each) with TBS-T and equal volumes of chemioluminescence reagent (ECL plus Thermo Scientific 32132) were added, after five-min incubation, membranes were wrapped and taken to the dark room to be exposed to X-ray films.

Autophagic flux

To measure the autophagic flux the amount of LC3BI to LC3BII conversion was determined by western blot. Briefly, the cells were transfected with miR-130b as described above, 72 h after transfection, cells were treated with 10 nM of the lysosomal inhibitor bafilomycine for 2 h. After the drug treatment, cells were collected and western blots were carried out as previously described.

Cell viability analysis by flow cytometry

Cell viability analyses were done using the propidium ioide-Annexin V standard protocol. Briefly the cells were harvested and washed with Annexin binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$) twice, and then the cellular pellets were resuspended in 100 µL of Annexin binding buffer and incubated with Annexin V antibody (2.5 µg/mL) for 15 min. 10 µL of PI (50 µg/mL) were added just before the samples were loaded into the cytometer.

CHAPTER 3: INTEGRATED ANALYSES OF MICRORNAS IN HIGH-GRADE SEROUS OVARIAN CARCINOMA

PLoS One has published a modified version of this chapter as a peer-reviewed paper.

Full citation: Creighton CJ, Hernandez-Herrera A, Jacobsen A, Levine DA, Mankoo P, Schultz N, Du Y, Zhang Y, Larsson E, Sheridan R, Xiao W, Spellman PT, Getz G, Wheeler DA, Perou CM, Gibbs RA, Sander C, Hayes DN, Gunaratne PH; Cancer Genome Atlas Research Network. Integrated analyses of microRNAs demonstrate their widespread influence on gene expression in high-grade serous ovarian carcinoma. PLoS One. 2012; 7(3).

Introduction.

In this work, we used the first dataset from the TCGA ovarian cancer project, consisting of 489 samples, and established an integration platform for mRNA and miRNA expression data that allowed us to stratified the samples and predict patient outcomes based on their miRNA-expression signature. Our analysis helped us to identified the putative mRNA:miRNA functional pairs and the biological pathways targeted by those interactions. Based on the bioinformatics predictions, we carried out functional analysis and validated the regulation exerted by miR-29a in a set of putative targets as well as the tumor suppression and drug sensitization effect of miR-29a overexpression in ovarian cancer cell lines.

Results

MicroRNA expression can be indicative of clinical outcome in ovarian cancer patients

TCGA data can be used as a resource to find signatures that can predict clinical outcomes such as overall and disease free survival. A transcriptional signature predictive of overall survival in ovarian cancer has been described before (Cancer Genome Atlas Research Network, 2008). In this study we carried out a similar analysis to define a miRNA signature that has prognostic significance and is predictive of clinical outcomes. In a training subset of 228 ovarian tumors (with outcome data, TCGA batches 9-15), 34 human miRNAs were individually correlated with the time of death (Figure 15, left). Each of the 253 validation samples (batches 17-24) was assigned a prognostic score, reflecting the similarity between its expression profile and the prognostic miRNA signature pattern; the signature showed statistically significant associations with survival (Figure 15, right). Previously (Shih et al., 2011) three miRNAs were associated with outcome in ovarian cancer; two of these miRNAs, miR-337 and miR-410, were also significant ($P<0.05$) in our training dataset; our analysis suggested that miRNAs expression patterns can complement gene expression patterns in predicting survival.

Figure 15

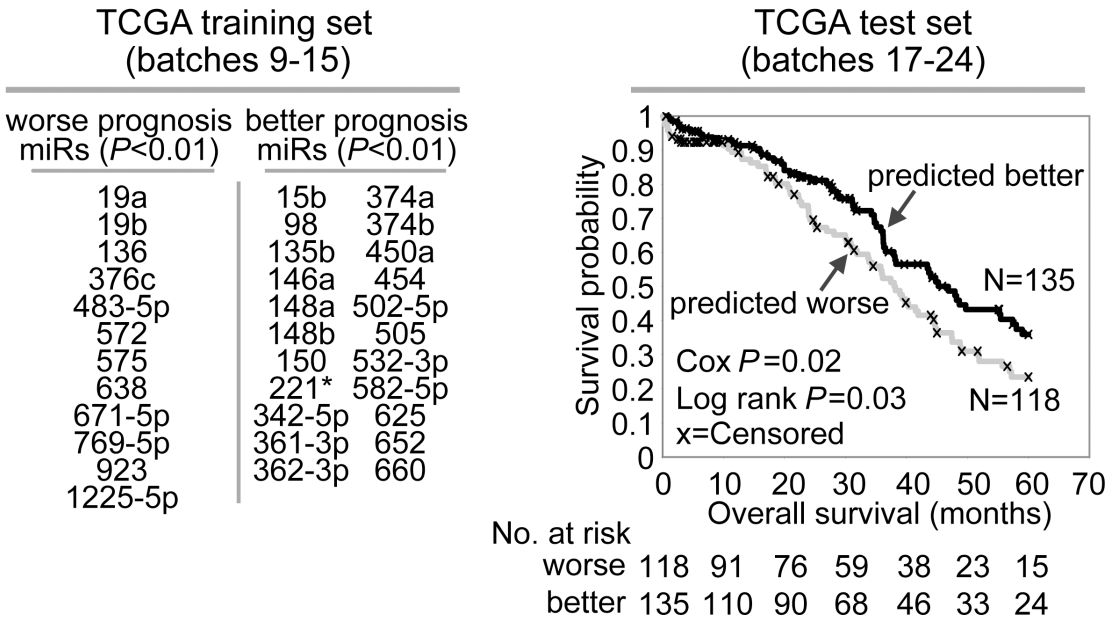


Figure 15. MiRNAs correlate with patient survival.

Using a training dataset of TCGA miRNA expression profiles, a prognostic miRNA signature was defined and then applied to a test dataset, each tumor being assigned a score measuring how well the tumor's expression patterns reflected those of the signature. Kaplan-Meier analysis (right) compares time to death for ovarian cancer patients showing higher risk (prognostic score > 0) versus lower risk (prognostic score < 0).

We predicted that miRNAs that are associated with better prognosis may possess tumor suppressor activities. MiR-148a, one of our better prognosis miRNAs is able to inhibit proliferation in ovarian cancer cells (Zhou et al., 2012). With an aim toward uncovering new candidates for therapeutic targeting, we overexpressed miR-26b *in vitro*, which was correlated with better prognosis (Figure 16). Interestingly, miR-26b was able to inhibit the proliferation of the ovarian cancer cell line HEYA8 but had no effect in the ovarian cancer cell line OVCAR8 (Figure 17), suggesting that the phenotypic impact of miRNAs maybe sensitive to the genetic background of the tumor. Cell line-specific effects of miRNAs have been reported for several cancer models including ovarian cancer (Creighton et al., 2010) and it is believe to be the result of genotypic differences among the cell line models of a given disease.

MicroRNAs and their predicted mRNA targets tend to be anti-correlated within ovarian tumors

A key to studying miRNAs is the identification of their mRNA targets. While miRNA targeting predictions made *in silico* may have high rates of both false positives and negatives, we hypothesized that considering correlations between gene and miRNA expression across a large number of tumor samples and looking for significantly anti-correlated miRNA:mRNA pairs could strengthened the miRNA:mRNA functional pairs predictions. To this end, all the possible miRNA:mRNA correlations across the 489 TCGA ovarian tumors were computed, then we sorted a total of 191 x 8547 miRNA:mRNA pairs by low to high correlations and found that among the most anti-

correlated pairs, there was a high enrichment for predicted miRNA:mRNA targeting interaction by the miRanda algorithm (Figure 18) (John et al., 2004), where no such enrichment was observed for the positively correlated pairs. In addition to validating the public target prediction tools, this finding indicated that thousands of miRNA:mRNA targeting interactions are active in ovarian cancer and maybe playing an important role in the biology of the ovarian cancer tumors.

To better understand the possible roles that the miRNA:mRNA functional pairs are playing in the biology of ovarian cancer, we constructed a matrix of correlation coefficients for all the miRNA:mRNA pairs (35 miRNAs X 1760 mRNAs) with the strongest negative correlation (regression coefficient <0.07), thereby grouping miRNAs when they are negatively correlated with the same genes and vice versa, posteriorly the gene dendrogram was cut to extract six clusters, each of which was enriched for genes corresponding to different biological pathways including, p53 signaling pathway, the Wnt/Hedgehog pathway, a cluster with cell adhesion genes, two clusters corresponding to immune response and a cluster of cell cycle related genes (Figure 19). These clusters are indicative of the biological pathways being regulated by the miRNAs in ovarian cancer and represent a useful tool to carry out functional validations for specific miRNA:mRNA interactions.

Figure 16

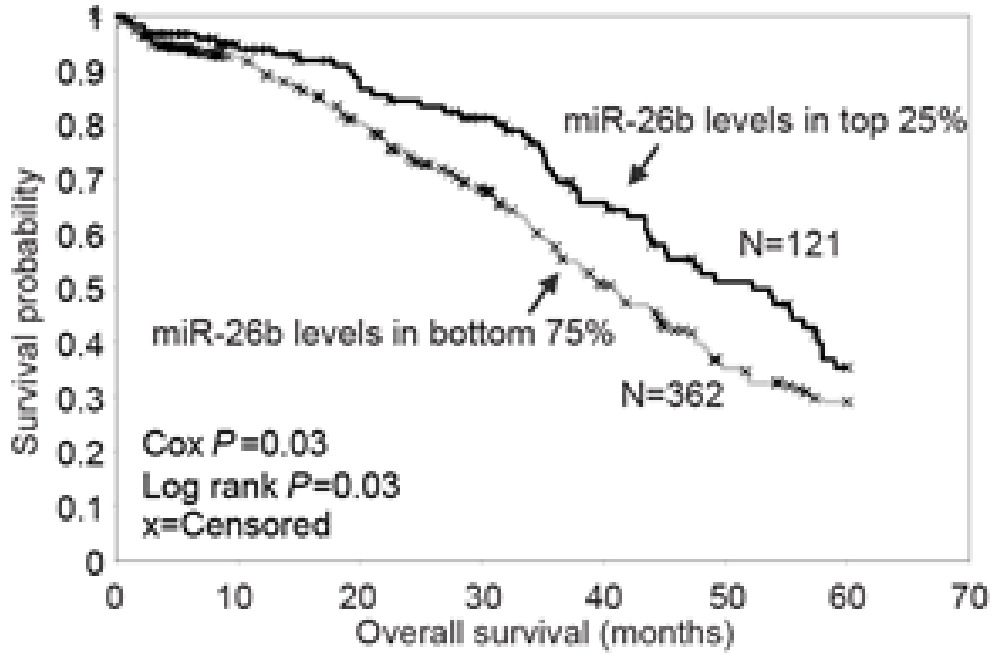


Figure 16. MiR-26b expression associates with longer survival of ovarian cancer patients.

Kaplan-Meier analysis evaluating survival time for patient with higher versus lower levels of miR-26b ($P<0.05$).

Figure 17

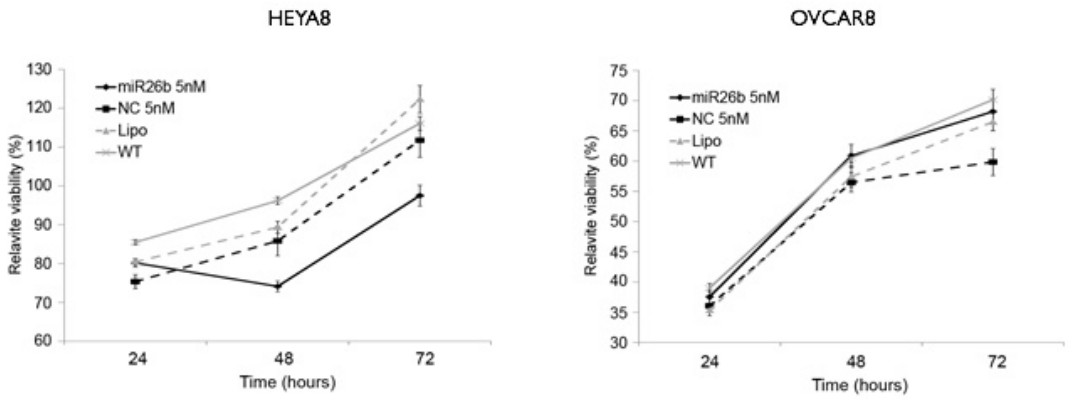


Figure 17. MiR-26b impacts HEYA8 cell viability but has no effect on OVCAR8.

Overexpression of miR-26b inhibits the proliferation of ovarian cancer cells HEYA8 (left) but has no effect on the OVCAR8 cell line (right), (lipo, lipofectamine-treated no miRNA; $P<0.001$ two sided t test miR-26b vs each of the controls at both 48 and 72 h).

Figure 18

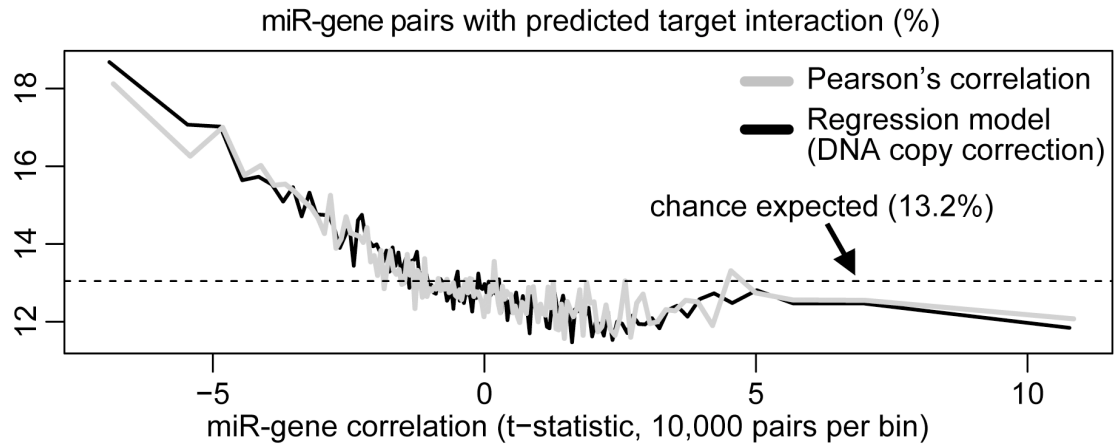


Figure 18. MiRNAs and their predicted targets tend to be anti-correlated.

Scatter plot showing mean correlation and fraction of predicted target interactions (total number of pairs represented: 191 miRNAs X 8547 genes). Dashed line corresponds to chance expected baseline fraction (13.2 %) of predicted target interactions.

Figure 19

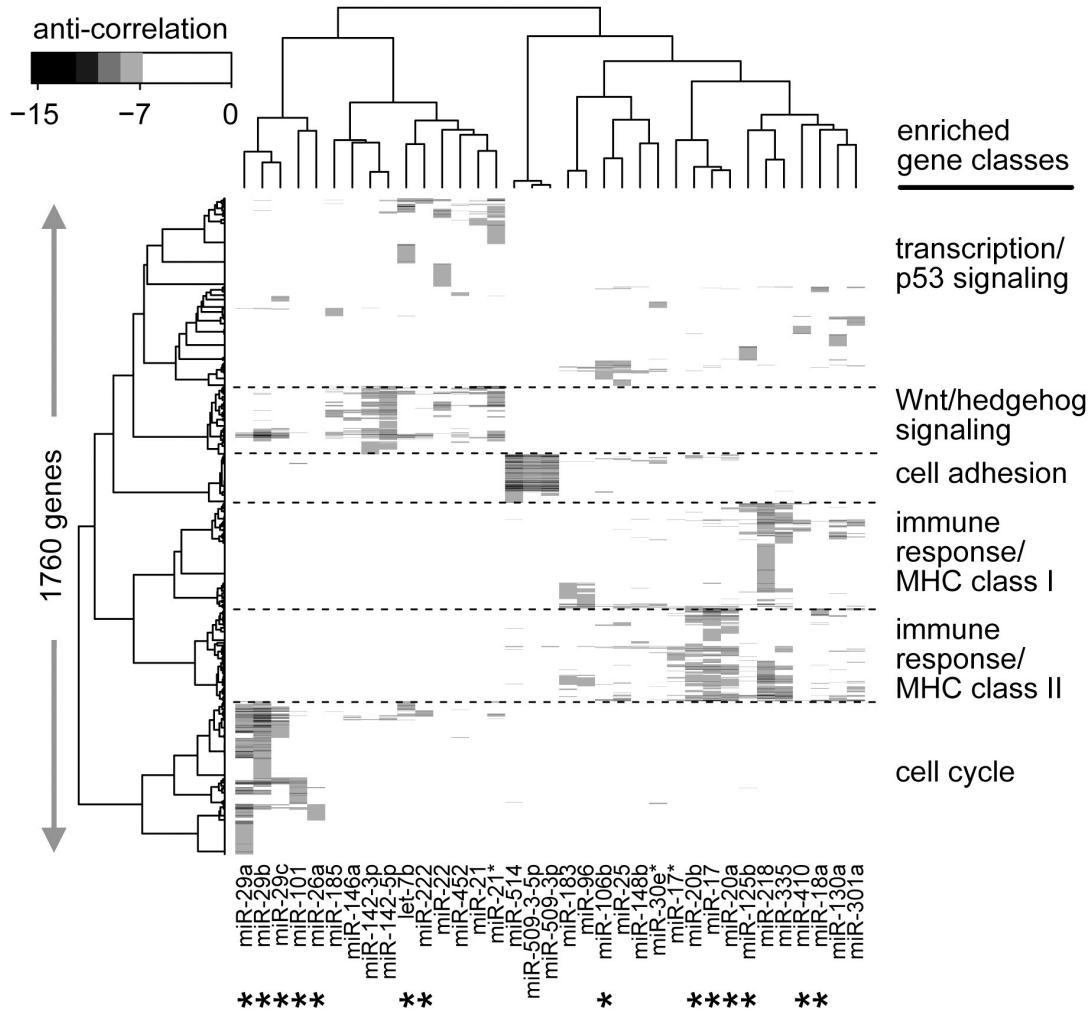


Figure 19. Anti-correlated miRNAs:mRNAs pairs cluster in specific biological pathways.

Hierarchical clustering matrix of correlation coefficients for all miRNA:mRNA pairs having a strong negative correlation. For each gene cluster, enriched gene classes are indicated (*), Significant anti-enrichment for predicted targets within miRNA:mRNA correlations (P<0.001, one-sided Spearman's rank).

MiR-29a impacts anti-correlated target genes and ovarian cancer cell viability

The previous results indicate widespread effects of miRNAs on gene expression in ovarian cancer, however all the putative miRNA:mRNA interactions remain to be validated. There are a number of ways to select a candidate miRNA for functional studies, using any of the results of our integrated analyses.

We focused our attention on the miR-29 family due to its strong anti-correlation with a significant number of cell cycle related genes (Figure 19). It has been previously demonstrated that members of the miR-29 family can act as tumor suppressors in different cancers by regulating several pathways including, cell proliferation and methylation (Table 3). Top anti-correlated genes of miR-29a in ovarian cancer included the DNA methyltransferases (DNMT), DNMT3A and DNMT3B (Figure 20), suggesting a similar role for miR-29 in high-grade serous ovarian cancer.

Genes anti-correlated with miR-29a were enriched for miR-29a targets as predicted by sequence analysis (using the bioinformatics platforms TargetScan (Friedman et al., 2009) or miRanda (John et al., 2004) (Figure 20). However many *in silico* predicted targets did not show the anticipated anti-correlated patterns, suggesting that by using the expression data to enriched the *in silico* analysis we can reduce the rate of false positives in the target prediction generated by the algorithms. Another piece of supporting evidence in favor of miR-29a activity, is a correlation-based sequence motif analysis that found that miR-29a seed sequence complement was the top enriched motif present in the 3'UTRs of mRNAs anti-correlated with miR-29a expression (Figure 21).

Our analysis also showed enrichment for non-canonical miR-29a seed motifs, with a bulge in position 3 of the 5'-seed sequence of miR-29a, suggesting that target prediction methods based solely on perfect base pairing at the 5'-seed region, could miss a substantial fraction of functional miRNA target interactions.

To test the effect of miR-29a in its putative targets, the ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with miR-29a mimics or scrambled negative control (Figure 22) and the expression levels of the putative targets: DNMT3A, DNMT3B, CDC6, CBX1, MYBL2 and TIMELESS (four of which were predicted direct targets) were analyzed by q-PCR.

Overexpression of miR-29a in the p53 wild type cell lines reduced the expression of 6 of the 7 targets tested (Figure 23), which demonstrates that the anti-correlation between miR-29a and the predicted targets is relevant in both cancer cell lines and the human tumor samples. Only one of the genes tested, SAE1, showed anti-correlations but not functional repression. On the other hand, miR-29a did not have a statistical significant effect on its putative targets when it was upregulated in the p53 mutant cell line OVCAR8 indicating that the silencing impact of miR-29a in that set of target genes may be dependent on p53 status.

While miR-29a expression was not associated with survival, overexpression of miR-29a impacted cell proliferation in the ovarian cancer cell lines, HEYA8 and OVCAR8, and had some sensitization effect to the chemotherapeutic agent cisplatin (Figure 24). Collectively these results suggest that miR-29a may be playing an important role in keeping the homeostasis in the ovarian epithelial tissue.

Figure 20

Figure 20. 3'UTRs of genes anti-correlated with miR-29a are enriched for miR-29a binding sites.

Correlation coefficients of all the miR-29a:mRNAs interactions. The 3'UTRs of anti-correlated mRNAs are enriched for miR-29a binding sites. Predicted targets are indicated.

Figure 21

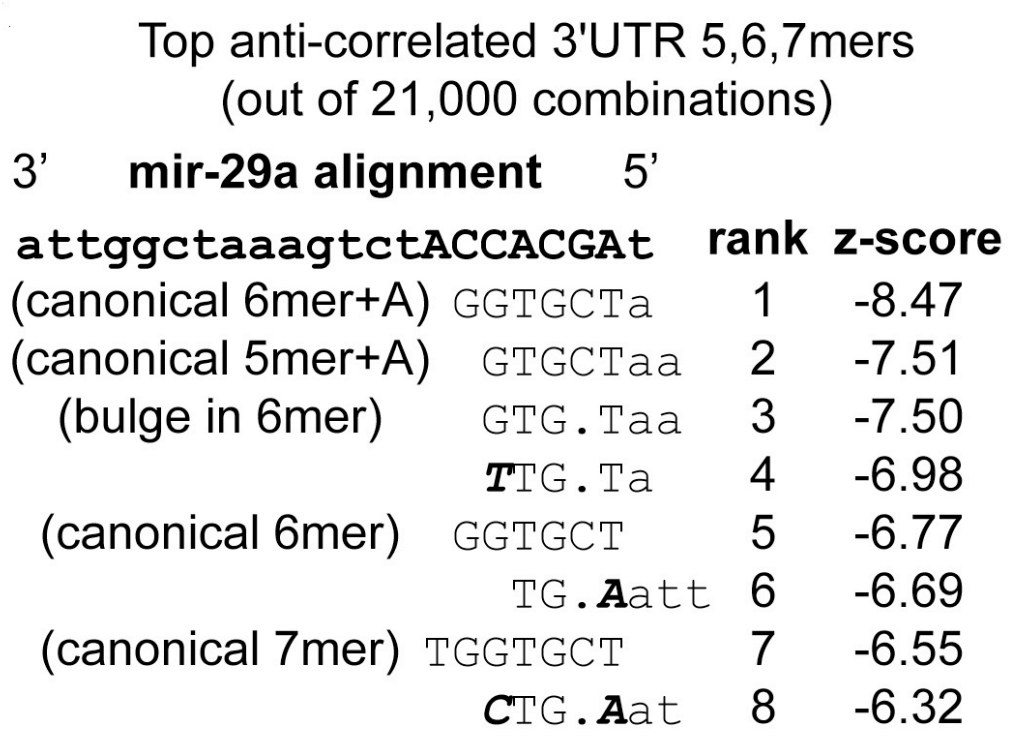


Figure 21. Complementary sequences to miR-29a seed region were enriched in the 3'UTRs of mRNAs anti-correlated with miR-29a.

Figure 22

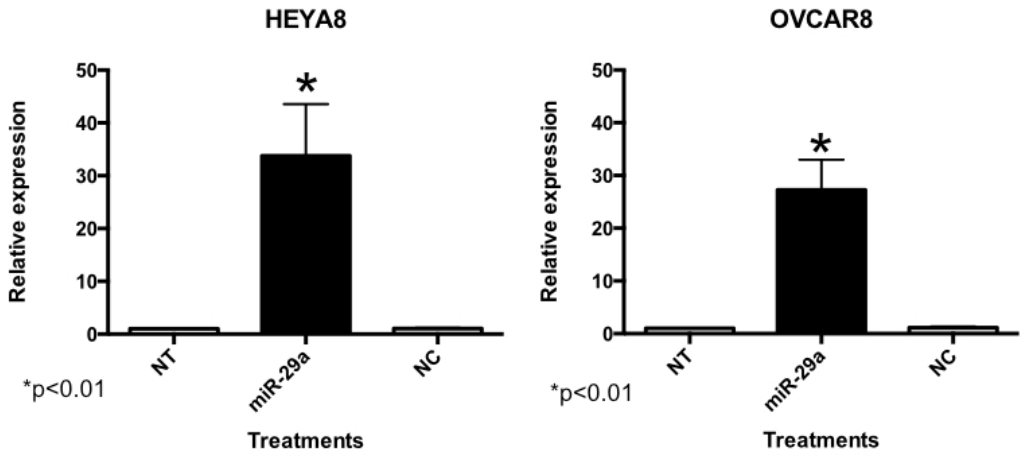


Figure 22. MiR-29a overexpression after transient transfection.

HEYA8 and OVCAR8 cell lines were transiently transfected with miR-29a mimics or scrambled negative control (NC) and the transfection efficiency was assayed by measuring the mature miR using taq-man probes.

Figure 23

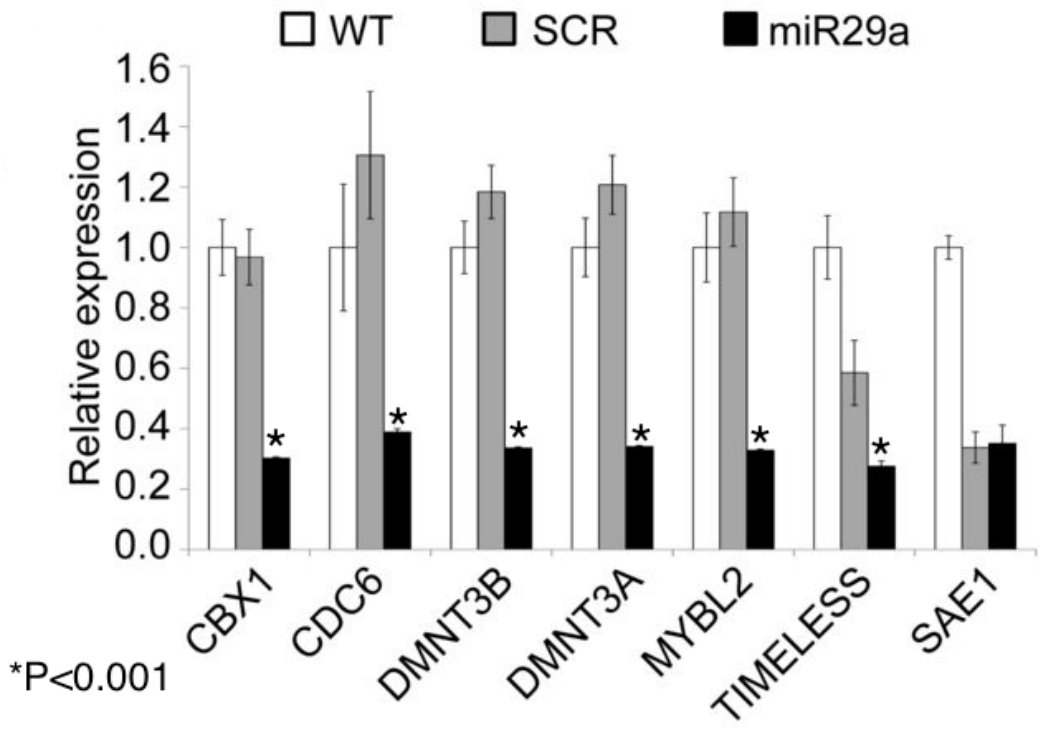


Figure 23. MiR-29a downregulates the expression of predicted target genes.

Q-PCR analysis showing the effect of miR-29a overexpression on the mRNA levels of anti-correlated gene targets in HEYA8 ovarian cancer cells (WT, untreated; SCR, scrambled control; two sided t-test $P<0.05$, miR29a vs SCR and miR-29a vs WT, each comparation except for SAE1).

Figure 24

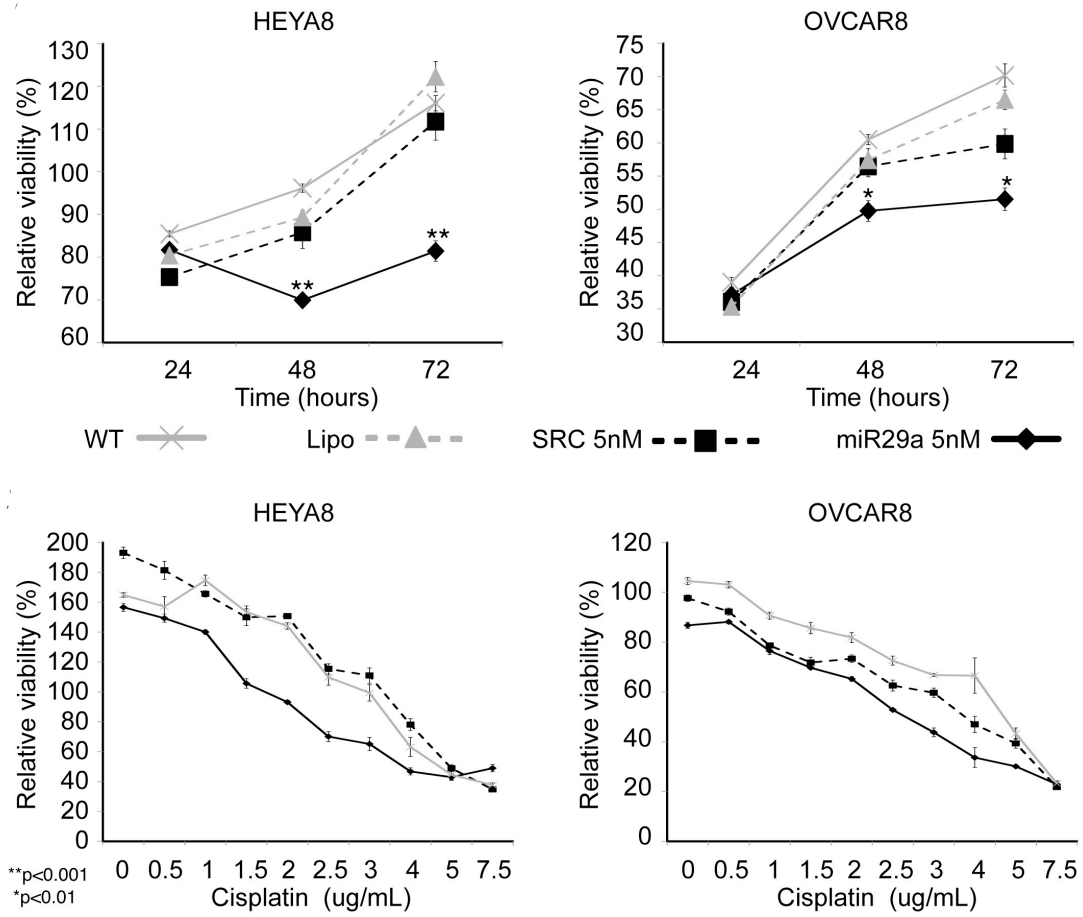


Figure 24. Effect of miR-29a in ovarian cancer cells proliferation and drug sensitization.

Overexpression of miR-29a inhibits the proliferation of ovarian cancer cells HEYA8 (top left) and OVCAR8 (top right)(lipo, lipofectamine-treated no miRNA; P<0.001 two sided t test miR-29a vs each of the controls at both 48 and 72 h). Effect of miR-29a on proliferation of HEYA8 (bottom left) and OVCAR8 (bottom right) under a range of concentrations of cisplatin treatment (proliferation was assayed at 72h post-transfection, cisplatin treatments were added at 24 h post-transfection).

Discussions and Conclusions

Molecular profiling is a powerful tool that can be use to improve the classification of patients and gain new insights into the heterogeneity within a disease. In this study, we see extensive diversity of miRNA patterns within high-grade serous ovarian cancer, suggestive of disease molecular subtypes and patient outcomes differences.

Numerous studies have validated miRNA targets regulation thru the use of *in vitro* cell models, however due to the limitations of the cell line models often is hard to determine if the interactions observed *in vitro* are also valid and truly relevant to human tumors. The integration of miRNA and mRNA expression data within the same large number of tumors allows us to define miRNA:mRNA correlations that are indicative of miRNA targeting. The observed enrichment of *in silico* predicted miRNA targets within anti-correlated miRNA:mRNA pairs helps to increase our confidence in the *in silico* prediction tools and allowed us to prioritize those predictions that appear to be most relevant to the biology of ovarian cancer. Our integrated analyses lead us to the identification of miR-29a, previously showing tumor suppressive effects in other cancers, as a potential tumor suppressor miRNA in ovarian cancer. This study provided the platform to identify and explore functional miRNA:mRNA interactions using the TCGA dataset and to discover novel candidate targets for cancer therapy in ovarian cancer as well as other cancers.Based on this work and the work of others, a US patent application was filed for "microRNA-29a,b,c as tumor suppressor and sensitizing agents for chemotherapy" (US 2012/0251619A1, Publication Date October 4th 2012. I am named on this patent as a contributor).

CHAPTER 4: ROLE OF MIR-509-3P IN OVARIAN CANCER MIGRATION/INVASION AND CLINICAL OUTCOME

Contributions from this chapter are part of a manuscript that was submitted to Nature Communications on June 2014.

Full reference: **microRNA 509-3p attenuates migration and invasion in ovarian cancer cells by targeting the YAP1/ECM axis**

A. Gordon Robertson[1], Yinghong Pan[2], Lykke Pedersen[3#], Emilia Lim[1#], Anadulce Hernandez-Herrera[2#], Amy C Rowat[4], Clara Chan[4], Andy Chu[1], Yunfei Wen[5], Xinna Zhang[5,6], Sagar L. Patil[2], Upal Basu-Roy[8], Alka Mansukhani[8], Payal Sipahimalani[1], Reanne Bowlby[1], Denise Brooks[1], Nina Thiessen[1], Yussanne Ma[1], Richard A. Moore[1], Jacquie E. Schein[1], Andrew J. Mungall[1], Chad V. Pecot[10], Anil K. Sood[5,6,7], Steven J.M. Jones[1] and Marco A. Marra[1**], Preethi Gunaratne[2,10**]

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Introduction

In this work we reanalyzed 289 TCGA ovarian tumors using messenger RNA sequencing (mRNA-seq) and 475 TCGA ovarian tumors using microRNA sequencing (miRNA-seq). Here we used sequencing data from 289 of the same TCGA ovarian tumor analyzed trough microarrays followed up in the previous chapter. Because sequencing technologies allow us to determine levels of mRNA and microRNAs with higher

sensitivity and specificity, compared with microarray data, we expected to find new insights into miR:mRNA networks regulating ovarian cancer through these datasets. In addition to the advantages listed above two features that are critical for the microRNA:mRNA target interaction can be ascertained with greater accuracy from RNA-seq and microRNA-seq data as compared to microarray-based profiling. Sequencing allowed us to differentiate between pri-mRNAs, pre-miRNAs, and mature miRNAs. Mutation in the 5'-seed region can also be detected by microRNA-seq. A large fraction of genes in out genome generate multiple splicing isoforms, some of them include changes in the 3'UTR region. Alternative usage of 3'UTR can affect the availability of microRNA binding sites. In this chapter I present my work on miR-509-3p uncovered through the integrated analyses of miRNA:mRNA gene pairs from the sequence based HGSOC transcriptome.

Results

Mir-509-3p is a clinically significant microRNA that is strongly anti-correlated with mRNA transcripts enriched for components of the extra cellular matrix

The work of Emilia Lim on 289 TCGA ovarian tumors that were analyzed using miR-seq and mRNA-seq is shown in

Figure 25. MiRNA:mRNAs anti-correlated pairs were identified by calculating Spearman correlation (r) between the abundance profiles for transcript isoforms and for 5p and 3p processed miRNA strands and thresholding at a q-value FDR (false discovery rate) < 0.05. This analysis returned 225,203 positive and negative correlations between miRNA-isoform pairs, and 50,713 significantly anti-correlated pairs that were

functionally linked by target predictions. Interactions with coefficients in the range of r< -0.3 were dominated by miR-9 and the miR-29 family, previously reported by (Creighton et al., 2012), and unexpectedly, by a ~100-kb miRNA cluster in Xq27.3 (Figure 25, top). 94% of the most strongly anti-correlated (r< -0.5) putative miRNA:mRNA pairs consisted of miR-506, miR-508, miR-509, miR-513 and miR-514 (Figure 25, bottom left). For the 250 targeted genes with the strongest anti-correlations to members of the Xq27.3 miR-cluster, enriched GO process, function and cellular locations highlighted the extracellular matrix (ECM), and enriched KEGG pathways included ECM-receptor interactions (FDR=6e-8) and Focal adhesion (FDR=0.02) (Figure 25, bottom, right).

miR-509-3p positively correlates with survival and is localized to tumor cells

To determine the relation between miR-506 family members and clinical outcome, we carried out *in-situ* hybridization for miR-509-3p to probe and independent set of 157 HGSOC samples from a tissue microarray. Each individual tumor sample was scanned and quantitative scores were determined in a blinded experiment using CellProfiler 2.0 software as previously described (Pecot et al., 2013). The scores were used to determine whether miR-509-3p expression levels were associated with the clinical outcome in HGSOC. Consistent with results from the sequencing data, overall survival (OS) was favorable for samples with higher miR-509-3p expression levels; extending the sequencing results. Progression-free survival was also favorable in tumors with higher expression (Figure 26 a and b). Representative pictures demonstrate high

versus low miR-509-3p expression within tumor samples (Figure 26 c and d). In addition miR-509-3p was mainly localized within the tumor cells.

Figure 25

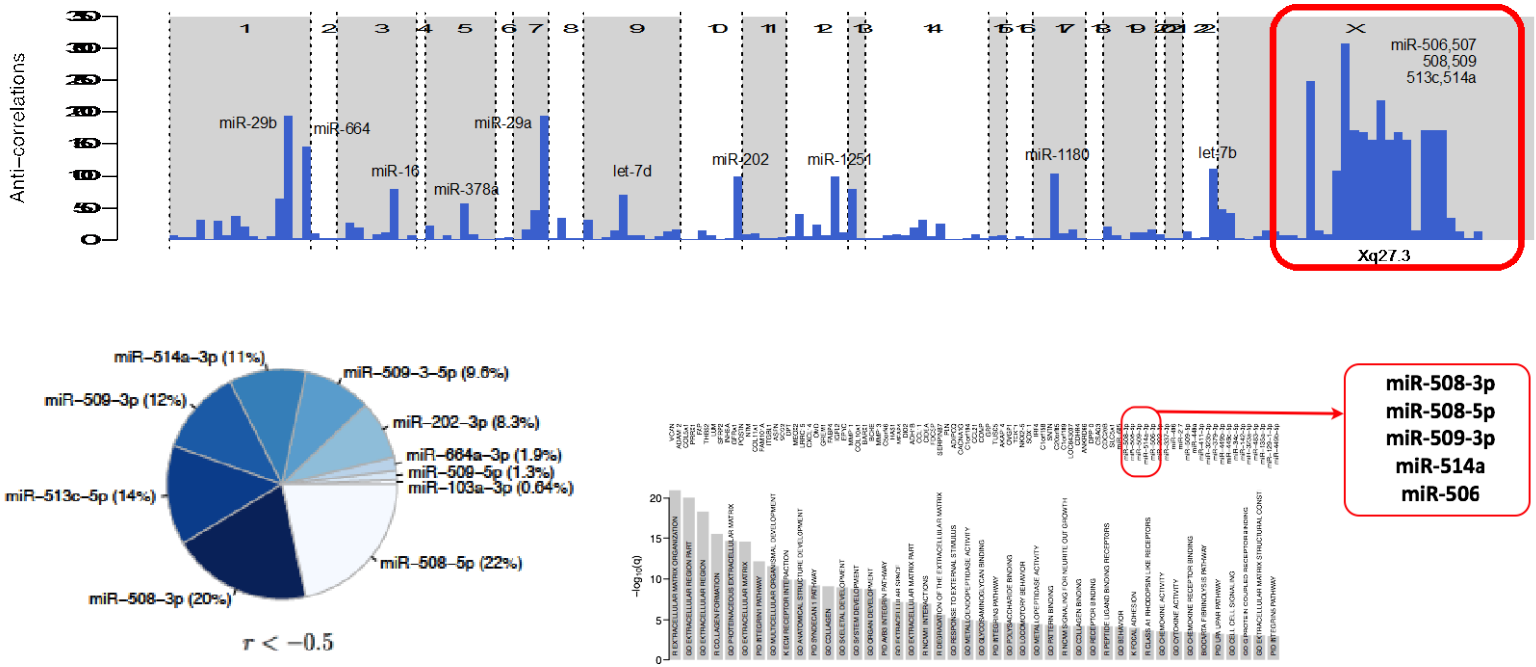


Figure 25. MiRNAs significantly anti-correlated with expressed mRNAs that were predicted targets.

A schematic transcriptome-wide view of the number of significant anti-correlations per miRNA, for Spearman coefficients less than -0.3. MiRNAs are ordered by their relative positions along chromosomes (top). MiR-gene interactions with Spearman correlation coefficients less than -0.5 are dominated by Xq27.3-miRNAs (bottom left). Most enriched pathways that anti-correlate with miRNAs from the Xq27.3 cluster (bottom right).

Figure 26

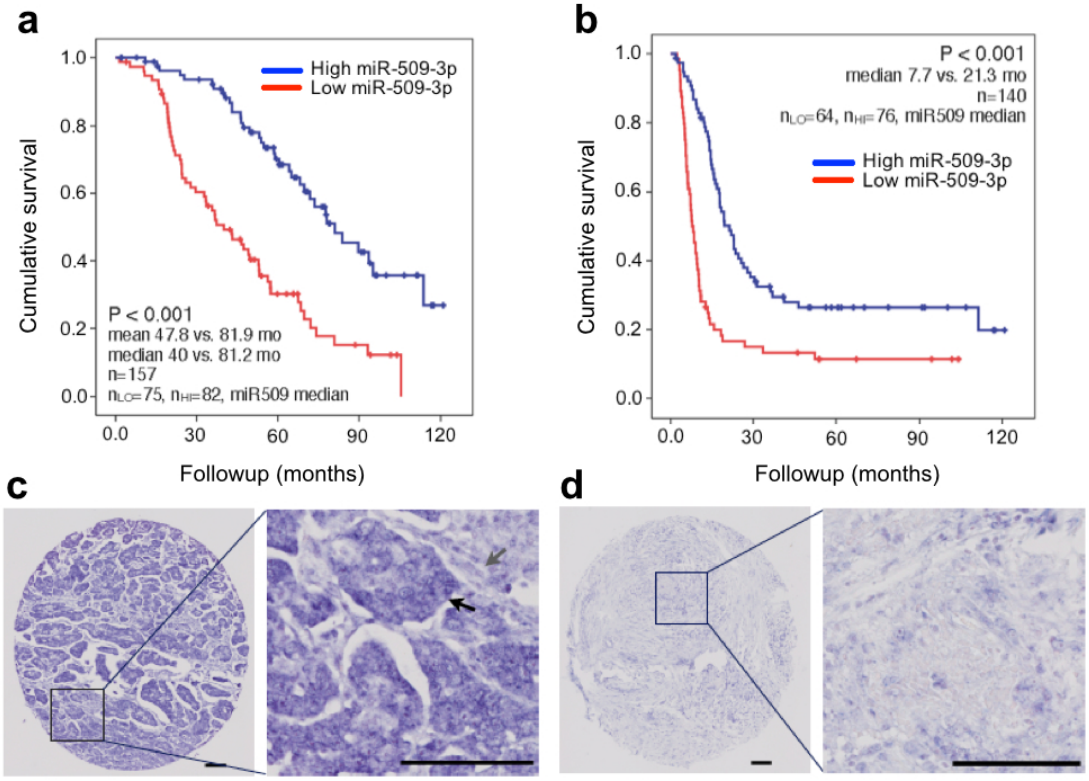


Figure 26. MiR-509-3p *in situ* hybridization in serous carcinoma samples.

Kaplan-Meier plots for overall survival (n=157) (a) and progression-free time (n=140) (b) for sample groups separated by the median miR-509-3p ISH score. Representative pictures from samples with high (291) miR-509-3 score (c) and low (0) score (d). Black and gray arrows indicate tumor and stromal cells respectively. Scale bars = 100 μm.

MiR-509-3p Inhibits proliferation of ovarian cancer cell lines

Our previous result showed that high miR-509-3p expression correlates with better clinical outcome, suggesting that miR-509-3p is playing a tumor suppressor role in ovarian cancer, because of that I decided to analyze the effect of miR-509-3p overexpression on ovarian cancer cell proliferation using the HEYA8 and OVACR8 cell lines. The ovarian cancer cells were transiently transfected with miR-509-3p mimics or scrambled negative control (NC) and proliferation was assayed using the MTS reagent. Proliferation was reduced by 20% and 30% at 72 and 96 hours respectively after transfection in HEYA8. In the p53 mutant cell line OVCAR8, increased miR-509-3p reduced the relative proliferation by 20% at 48 hours and by 40% at 72, 96 and 120 hours compared with the proliferation of the cells treated with NC (Figure 27).

MiR-509-3p influences transcript levels of ECM and EMT genes

Our previous results suggested that miR-509-3p is playing and important role in the regulation of ovarian cancer proliferation and patients' clinical outcomes. In order to identify downstream targets of miR-509-3p, we relied in an integrated analysis in which miR-509-3p putative targets were overlapped with its most anti-correlated genes from other ovarian cancer signatures previously identified (Anastassiou et al., 2011; Bignotti et al., 2007; Tothill et al., 2008; Zhang et al., 2013). This analysis identified eleven genes dominated by ECM members consisting of the collagens *COL11A1*, *COL5A1*, *COL5A2*, *COL3A1*, and *COL1A1*; the cytoskeleton-associated actin alpha 2 (*ACTA2*); genes encoding ECM glycoproteins *FN1*, *SPARC*, and *THBS2*; the endothelin receptor gene

EDNRA; and the homeobox transcription factor *PRRX1*. The EMT regulator *SNAI2* (Slug) was also included since it was significant in three of the four signatures used.

We then assessed how miR-509-3p mimics influenced transcripts levels from the 11-gene invasive signature and predicted EMT targets, in the ovarian cancer cell lines HEYA8 (p53 wild type) and OVCAR8 (p53 mutant). The cell lines were transiently transfected with miR-509-3p mimics or scrambled negative control, and the efficiency of the transfection was assayed by measuring the amount of mature miRNAs present on the cells (Figure 28). The expression levels of putative targets was analyzed by q-PCR. *ACTA2*, *COL1A1* and *COL3A1* were downregulated in response to miR-509-3p mimics in both cell lines (Figure 29 and Figure 30). In HEYA8, *COL5A1* was downregulated in response to miR-509-3p mimics while ECM genes *SPARC* and *FN1* were upregulated in response to miR-509-3p mimics in both cell lines. We also evaluated the effect of miR-509-3p in the expression of the EMT regulators TWIST and SNAI2 (SLUG), and the mesenchymal marker VIM. TWIST was downregulated at all three time points in both cell lines, and more significantly in HEYA8 than in OVCAR8. SNAI2 (SLUG) was up-regulated at all three time points in OVCAR8 and at 72 h in HEYA8, with changes more significant in OVCAR8 than in HEYA8. VIM expression did not change in HEYA8 and was upregulated in the later time points in OVCAR8 (Figure 29 and Figure 30).

Figure 27

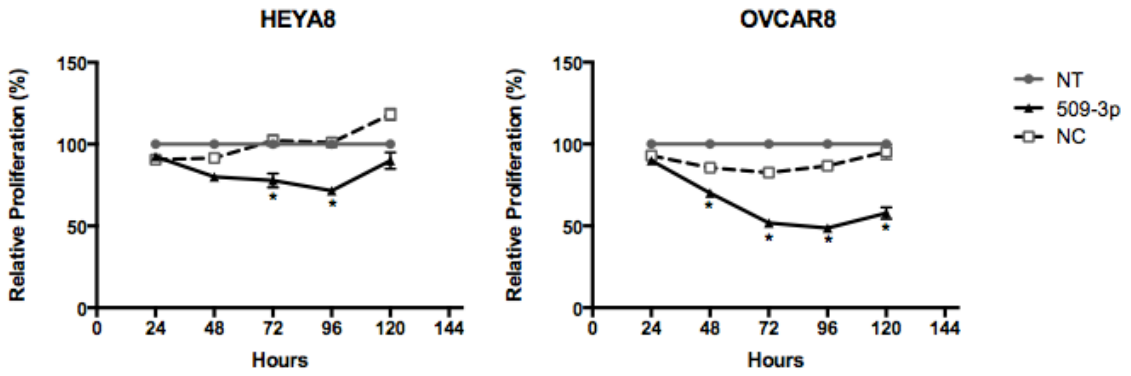


Figure 27. MiR-509-3p inhibits proliferation of ovarian cancer cell lines.

Ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with miR-509-3p and proliferation was assayed by MTS. Error bars show standard deviation of three biological replicates. * P<0.001.

Figure 28

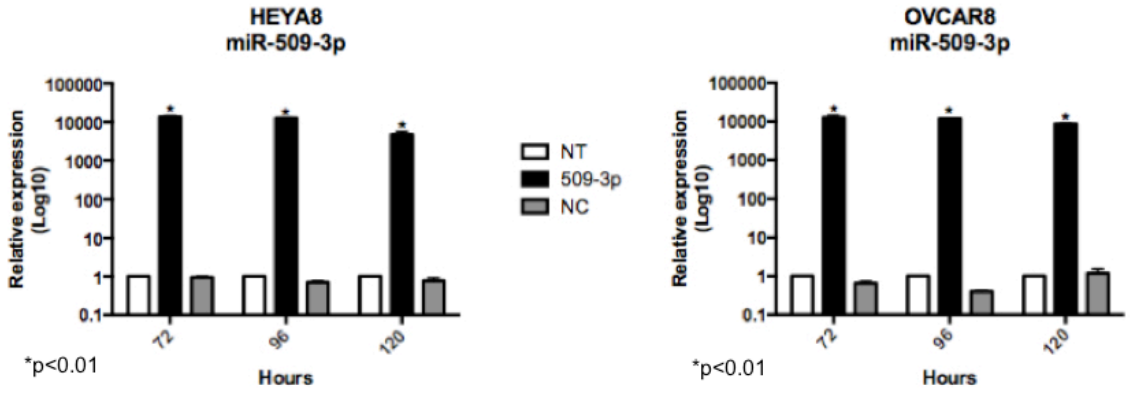


Figure 28. miR-509 overexpression.

HEYA8 and OVCAR8 cell lines were transiently transfected with miR-509-3p mimics or scrambled negative control (NC) and the relative expression of miR-509-3p was measured using taq-man q-PCR at different time points after transfection. Relative expression was calculated using non-treated cells (NT) as reference sample.

Figure 29. Effect of miR-509-3p in the expression of EMT/ECM related genes in HEYA8.

HEYA8 cells were transfected with miR-509-3p mimics or scrambled negative controls (NC) an their impact in the mRNA levels of the gene signature was assayed by q-PCR at different times after transfection. Relative expression was calculated normalizing against non-treated parental cells (NT). Error bars show standard deviation of three biological replicates. * $P<0.01$.

Figure 29

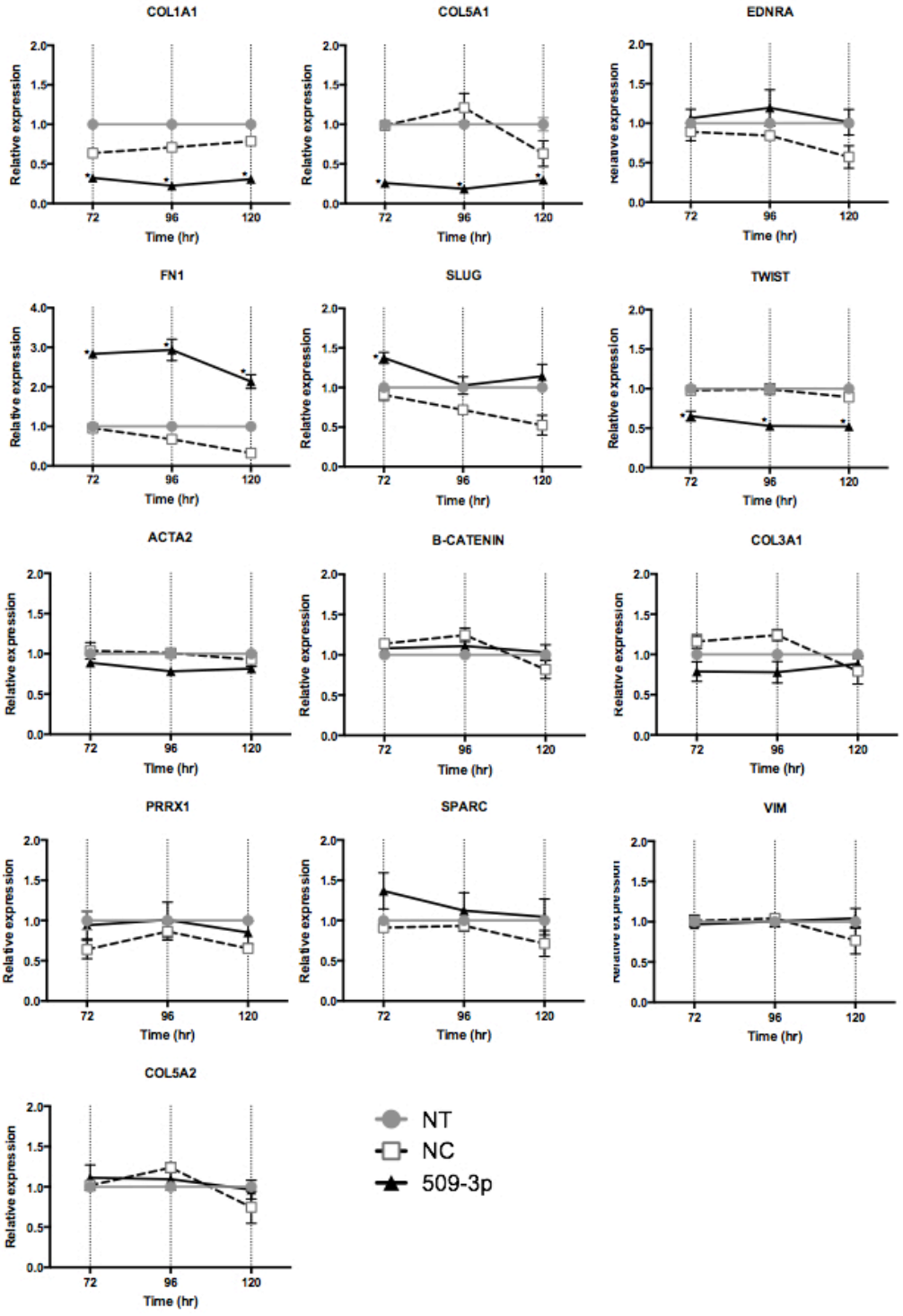
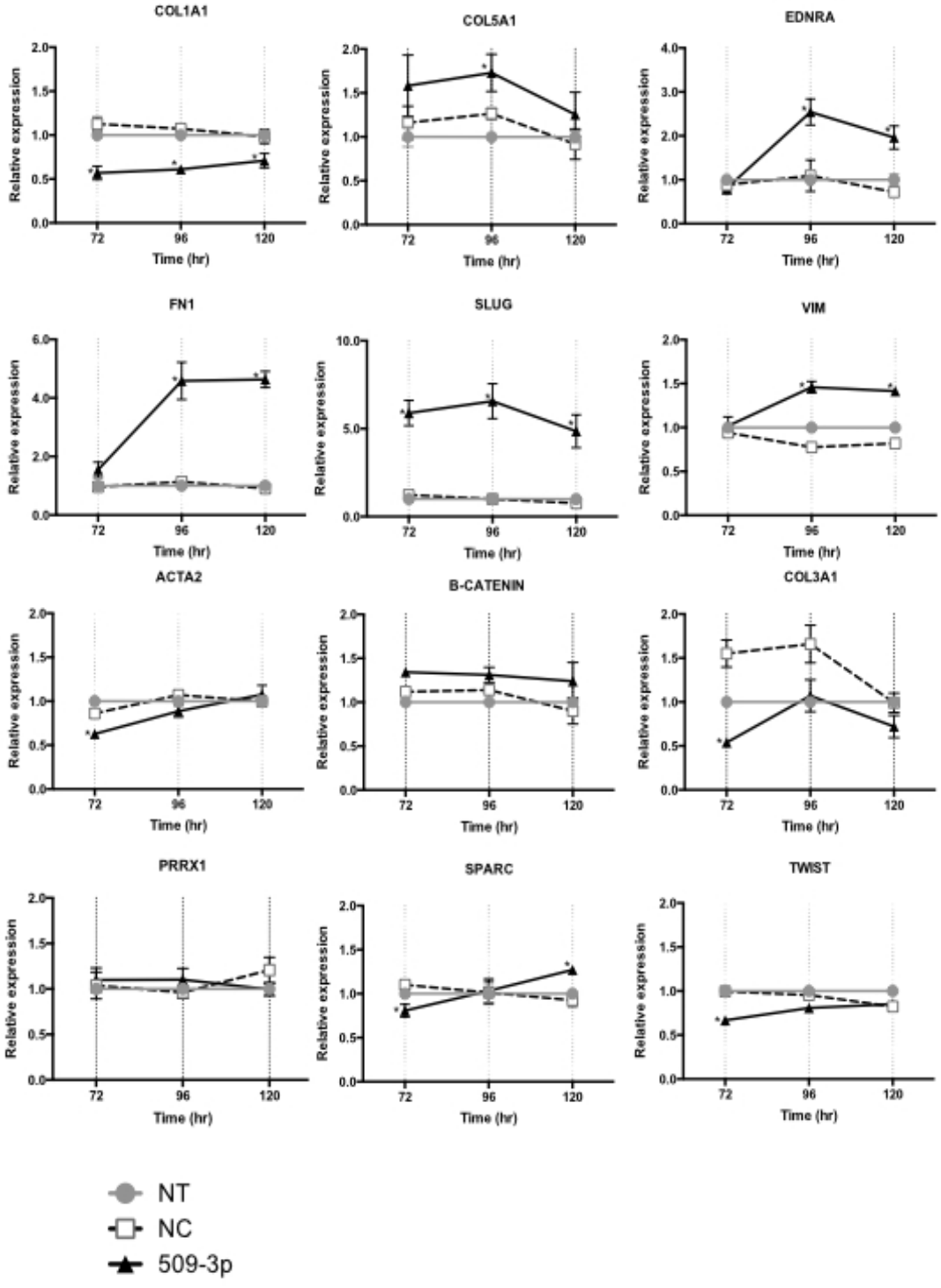

COL1A1
COL5A1
EDNRA
FN1
SLUG
TWIST
ACTA2
B-CATENIN
COL3A1
PRRX1
SPARC
VIM
COL5A2
Relative expression
Time (hr)
72
96
120
NT
NC
509-3p

Figure 30. Effect of miR-509-3p in the expression of EMT/ECM related genes in OVCAR8.

OVCAR8 cells were transfected with miR-509-3p mimics or scrambled negative controls (NC) an their impact in the mRNA levels of the gene signature was assayed by q-PCR at different times after transfection. Relative expression was calculated normalizing against non-treated parental cells (NT). Error bars show standard deviation of three biological replicates. * $P<0.01$.

Figure 30


COL1A1
COL5A1
EDNRA
FN1
SLUG
VIM
ACTA2
B-CATENIN
COL3A1
PRRX1
SPARC
TWIST
Relative expression
Time (hr)
72
96
120
NT
NC
509-3p

We also analyzed the impact of miR-506 family members, miR-508-3p, miR-508-5p, miR-509-3p and miR-509-5p overexpression in the protein levels of the signature genes and other EMT related genes. MiR-509-3p that previously showed tumor suppressor phenotypes in our experiments did not have a strong silencing effect on the protein levels of its putative targets. For the HEYA8 cells, FN1 protein levels showed to be upregulated in congruency with the q-PCR results, SLUG protein levels also correlated with the q-PCR results showing an upregulation at earlier time points and a slight downregulation at the latest time point. VIMENTIN mRNA levels did not change however we found the protein levels to be upregulated (Figure 31, left). In the case of the OVCAR8 cells, FN1, SLUG and VIMENTIN were upregulated at the protein levels, which correlated with the q-PCR results (Figure 31, right). MiR-508-3p, miR-508-5p and miR-509-3p showed some downregulation of vimentin in the HEYA8 but had no effect on the other proteins tested. MiR-508-3p upregulated the expression of SLUG in the OVCAR8 cell line while miR-509-5p downregulated it (Figure 32). The targets analyzed here were anti-correlated with the miR-506 family members in the gene signatures and we expected them to be downregulated upon overexpression of miR-508-3p, miR-508-5p, miR-509-3p and miR-509-5p, however, that was generally not the case.

Figure 31

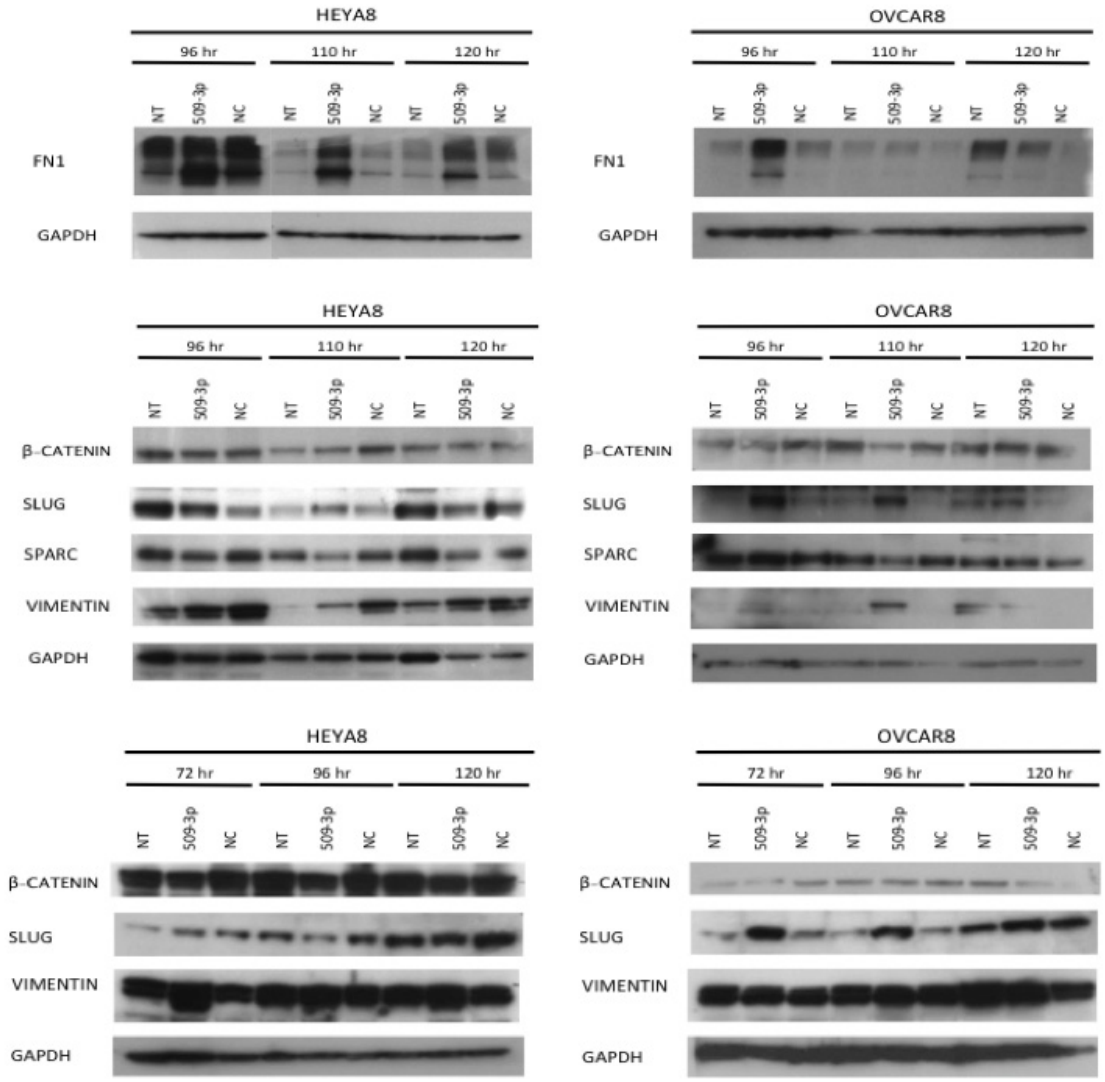


Figure 31. Effect of miR-509-3p in the expression of EMT/ECM related proteins.

HEYA8 and OVCAR8 cell lines were transfected with miR-509-3p mimics or scrambled negative controls and their impact in EMT/ECM protein levels was assayed by Western blot at different times after transfection. Representative images from 3 biological replicates. NT= untreated parental cells; NC= scrambled negative control treated cells.

Figure 32

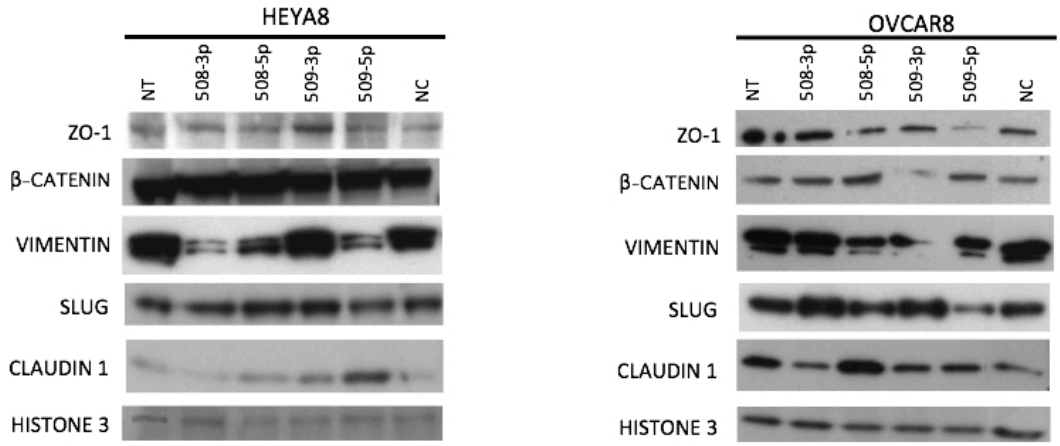


Figure 32. Effect of miRNAs from the Xq27.3 cluster in the expression of EMT/ECM related proteins.

HEYA8 and OVCAR8 cell lines were transiently transfected with miRNA mimics or scrambled negative control (NC) and EMT proteins we assayed by western blot.

Discussions and Conclusions

In this chapter, we analyzed the impact of a second candidate tumor suppressor miR-509-3p and clinical outcomes in HGSOC and cell proliferation in ovarian cancel cell lines. I also studied the effect of miR-509-3p, and other miR-506 family members on the regulation of an EMT/ECM gene signature derived from integrated microRNA-seq and RNA-seq data from 289 of the 489 TCGA HGSOC tumors. The sequencing data highlight functionally-correlated miRNA-target gene pairs that influence chromatin modifiers, EMT, and the ECM. The integrated analysis suggested that hsa-miR-508, miR-509, and miR-514, all members of a microRNA cluster located in chromosome Xq27.3 may have significant roles in the pathophysiology of HGSOC tumors.

Members of the miRNA cluster have been previously reported to be associated with clinical stage and survival in ovarian cancer patients (Eitan et al., 2009; Lee et al., 2009; Yu et al., 2013).

In-situ hybridization analyses carried out in an independent ovarian cancer tumor array showed that miR-509-3p expression correlates with both overall survival and progression free survival, suggesting that miR-509-3p may drive tumor suppressor programs in ovarian cancer. Overexpression of miR-509-3p in ovarian cancer cell lines HEYA8 and OVCAR8 was able to inhibit proliferation in both cell lines. Similar tumor suppressor activities have been reported in renal cancer, in which cluster members are downregulated, and their overexpression can reduce proliferation and migration while inducing apoptosis (Hidaka et al., 2012; Zhai et al., 2012; Zhang et al., 2013).

Predicted targets of miR-509-3p are enriched for ECM proteins and EMT drivers TWIST and SNAI2. We found that miR-509-3p significantly reduced mRNA levels of ECM related genes, including COL1A1, COL3A1, COL5A1, ACTA2 and TWIST, however other putative targets such as SNAI2, COL5A2, EDNRA, FN1 and SPARC, were upregulated upon miR-509-3p overexpression. This phenomenon where putative targets are upregulated instead of downregulated by their cognate miRNAs has been previously reported, even for cases in which the miRNA:mRNA interaction has been validated. This observation maybe the result of a complex feedback loop interactions between miRNAs and other targets and/or downstream effectors (Tang et al., 2010). At the protein level, most of the proteins tested, with the exception of SLUG and SPARC on HEYA8, were upregulated and correlate well with the corresponding mRNA levels measured by q-PCR.

In summary with the use of sequencing data and integrated analyses, we were able to identify the miR-506 family as an important microRNA involved in regulating the complex interaction between chromatin modifiers, EMT, and ECM processes in the ovarian cancer dataset from TCGA. We were also able to identify miR-509-3p as a microRNA with the potential to be developed as tumor suppressor due to its ability to inhibit ovarian cancer cell proliferation. We also confirmed in an independent cohort of HGSOC patients that higher levels of miR-509-3p expression correlate with good prognosis.

CHAPTER 5: ESTABLISHING MIR-130B AS A TUMOR SUPPRESSOR OF EPITHELIAL OVARIAN CANCER (EOC)

Introduction

Downregulation of miR-130b has been observed in chemoresistant ovarian cancer cell lines (Sorrentino et al., 2008). Also an inverse correlation between miR-130b expression and clinical stage has been also reported for ovarian cancer samples (Yang et al., 2012). Unpublished data from our group showed miR-130b reduces tumor burden in a xenograft mouse model, however the molecular mechanisms underlying miR-130b tumor suppressor activities remain unknown. Understanding such mechanisms in the context of p53 wild type (WT) and p53 mutant ovarian cancer cell line models is the focus of this chapter.

Results

Hsa-miR-130b induces apoptosis in the ovarian cancer cell lines HEYA8 and OVCAR8

Our first step in understanding the tumor suppressor activities of miR-130b in ovarian cancer was to analyze its impact on cell viability. HEYA8 p53 wild type (WT) and OVCAR8 p53 mutant cell lines were transiently transfected with miR-130b mimics or scrambled negative control (NC). The percentage of live, apoptotic and necrotic cells was analyzed using propidium iodide (PI) and AnnexinV staining followed by FACS. Transfection efficiency was determined by measuring the amount of mature miR-130b

present in the cells by TaqMan microRNA q-PCR assay (Figure 33). Both HEYA8 and OVCAR8 showed a 10% reduction on cell viability and a 6 to 12 increase in the amount of apoptotic cells compared to non-treated cells. The cell viability of non-treated cells and NC-treated cells was not statistically significant (Figure 34).

Figure 33

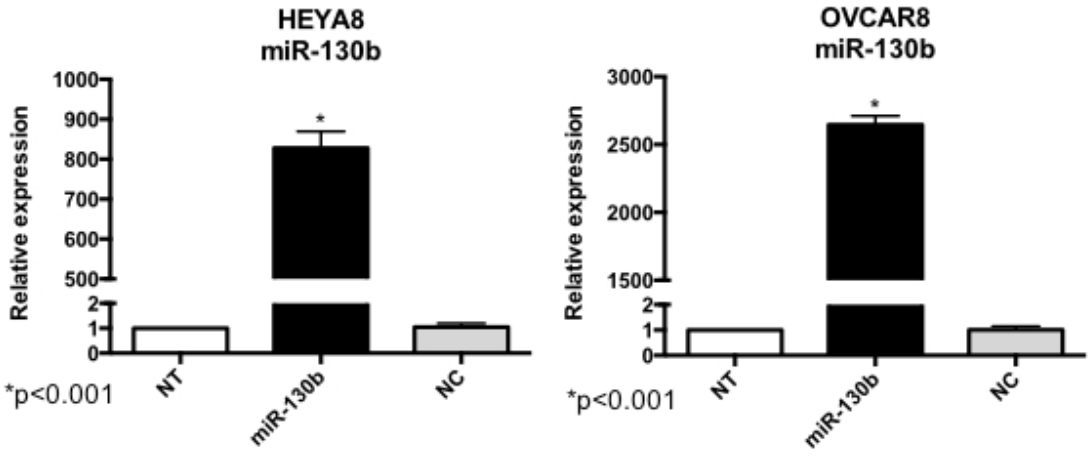


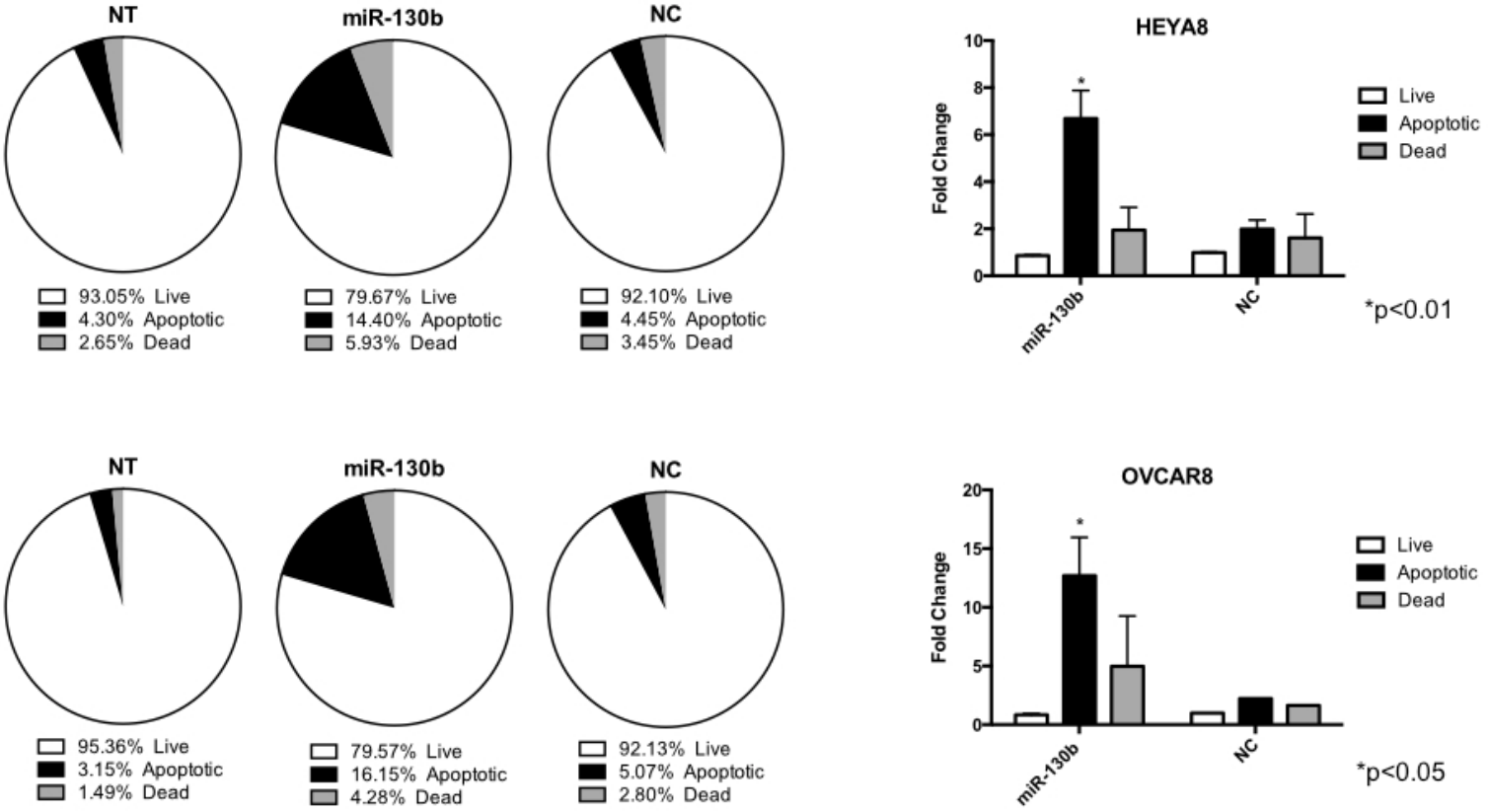
Figure 33. MiR-130b overexpression.

The ovarian cancer cell lines were transiently transfected with miR-130b mimics or scrambled negative control (NC) and the amount of mature miR-130b was measured by Taq-Man q-PCR 72 hours post-transfection.

Figure 34. MiR-130b induces apoptosis in HEYA8 and OVCAR8 cell lines.

Ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with miR-130b mimics or scrambled negative control (NC), 120 hours after transfection the cells were stained with PI and Annexin V and cell viability was assayed by FACS (left panels). Fold changes in the percentage of life, apoptotic and necrotic cells were calculated using the percentage of untreated cells as baseline (right panels). (FACS experiments were done with the help of Sesha Duvvuri).

Figure 34


NT
miR-130b
NC
93.05% Live
4.30% Apoptotic
2.65% Dead
79.67% Live
14.40% Apoptotic
5.93% Dead
92.10% Live
4.45% Apoptotic
3.45% Dead
HEYA8
Fold Change
Live
Apoptotic
Dead
miR-130b
NC
*p<0.01
NT
miR-130b
NC
95.36% Live
3.15% Apoptotic
1.49% Dead
79.57% Live
16.15% Apoptotic
4.28% Dead
92.13% Live
5.07% Apoptotic
2.80% Dead
OVCAR8
Fold Change
Live
Apoptotic
Dead
miR-130b
NC
*p<0.05

The induction of apoptosis by miR-130b is congruent with the potential tumor-suppressor activities of this miRNA in ovarian cancer. The fact that miR-130b was able to induce apoptosis in both p53 WT and p53 mutant genetic backgrounds was encouraging since most 96 % of the patients diagnosed with HGSOC present with tumors carrying mutations in p53. Identification of molecular pathways activated by miR-130b under the different genetic backgrounds and downstream effectors used by miR-130b to overcome p53 mutations was the next step in understanding the tumor suppressor mechanism driven by miR-130b.

MiR-130b activates p53/p21 axis and enhances TAp63 expression in the p53 WT ovarian cancer cell line HEYA8

I hypothesized that the apoptosis induction after miR-130b upregulation was the result of the activation of the canonical p53 pathway, since the HEYA8 cell line is p53 WT. HEYA8 cells were transiently transfected with miR-130b mimics or scrambled negative control and 72 h after transfection a series of p53 related genes and proteins were assayed by q-PCR and western blot respectively. MiR-130b was able to induce the transcription of p53, p21, MDM2 and PTEN. The tumor suppressor RB1 showed some upregualtion but was not statistically significant (Figure 35, top right).

The protein levels of known tumor suppressors p53, TAp63 and p21 were upregulated along with p27. P27 is a cycling dependent kinase inhibitor that blocks the activity of CDK2 and CDK4 complexes and controls the progression of cell cycle at G1. CDK4 protein levels were downregulated while surprisingly CDK6 was upregulated. The

pro-apoptotic protein BIM was upregulated whereas PUMA was slightly downregulated (Figure 35, left).

The upregulation of p53 upon miR-130b treatment explains the activation of downstream targets such as p21 and BIM allowing cells to enter apoptosis.

MiR-130b induced the p53 family member, TAp63, which is not present in the untreated cells. The TA isoform of p63 is considered to be a strong tumor suppressor that can also activate p21 and BIM and induce apoptosis. The function of both p53 and TAp63 can be antagonized by the deltaN (ΔN) p63 isoforms, that lacks the transactivator domain and can act as dominant negative for p53 and the TAp63. ΔNp63 was expressed on the untreated cells and the protein levels were reduced by miR-130b overexpression.

Upregulation of tumor suppressor genes by miR-130b is p53-dependent

To test the dependency of miR-130b effects on the presence of p53, I silenced p53 expression using siRNA. HEYA8 cells were transiently co-transfected with miR-130b or scramble negative control and a siRNA targeting p53. Q-PCR and Western blots were done to confirm the efficacy of p53 silencing (Figure 36, left top). Silencing of p53 abrogated the upregulation of p53 and p21 driven by miR-130b (Figure 36). The upregulation of other genes such as CDK6, p27, PTEN and RB1 was not affected by the absence of p53 suggesting that miR-130b uses p53 independent pathways to induce the expression of those genes (Figure 36 left and Figure 37).

Figure 35. MiR-130b upregulates the p53 canonical pathway

The p53 WT cell line HEYA8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and genes/proteins from the p53 pathway were analyzed by q-PCR (right top) and Western blot (left and right bottom). P63 Western blots were done by Avinashnarayan Venkatanarayan at Dr. Elsa Flores lab, MD Anderson.

Figure 35

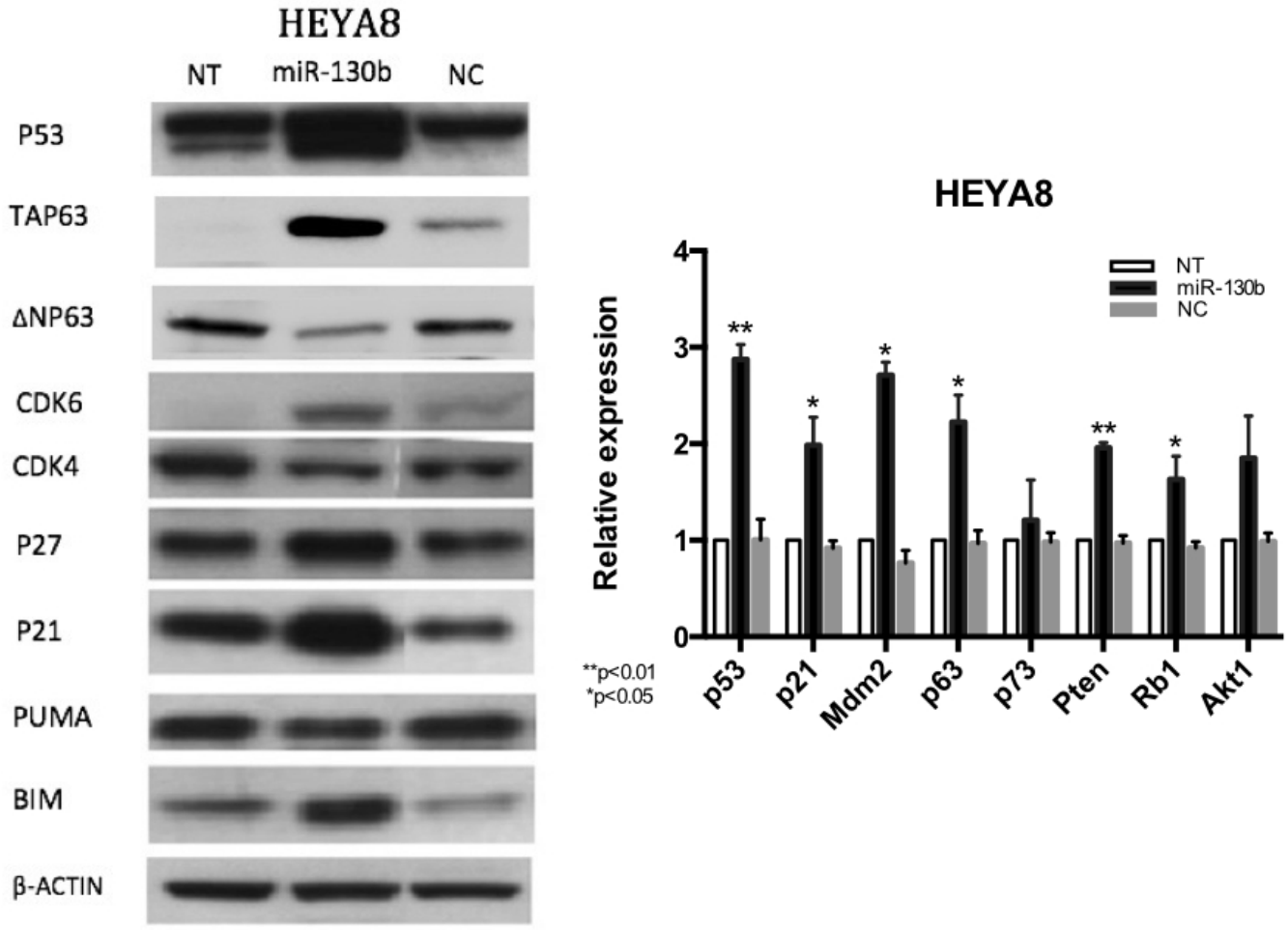

HEYA8
NT
miR-130b
NC
P53
TAP63
ΔNP63
CDK6
CDK4
P27
P21
PUMA
BIM
β-ACTIN
HEYA8
Relative expression
NT
miR-130b
NC
**p<0.01
*p<0.05
p53
p21
Mdm2
p63
p73
Pten
Rb1
Akt1

Figure 36

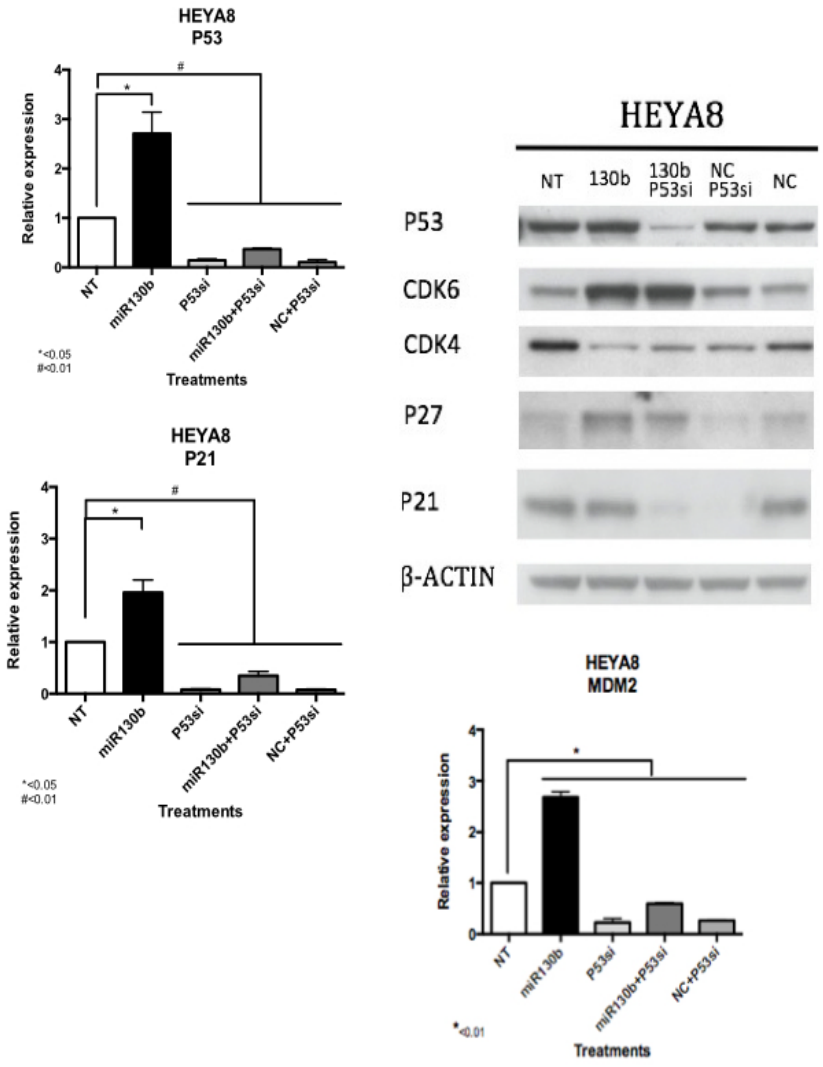


Figure 36. Effect of p53 abrogation on miR-130b regulated genes.

HEYA8 cells were transiently transfected with miR-130b mimic or scrambled negative control (NC) and siRNA against p53. The efficacy of p53 silencing was assayed by q-PCR (left top) and Western blot (right). The effect of p53 abrogation on the expression of miR-130b regulated genes was measured by q-PCR and Western blot.

Figure 37

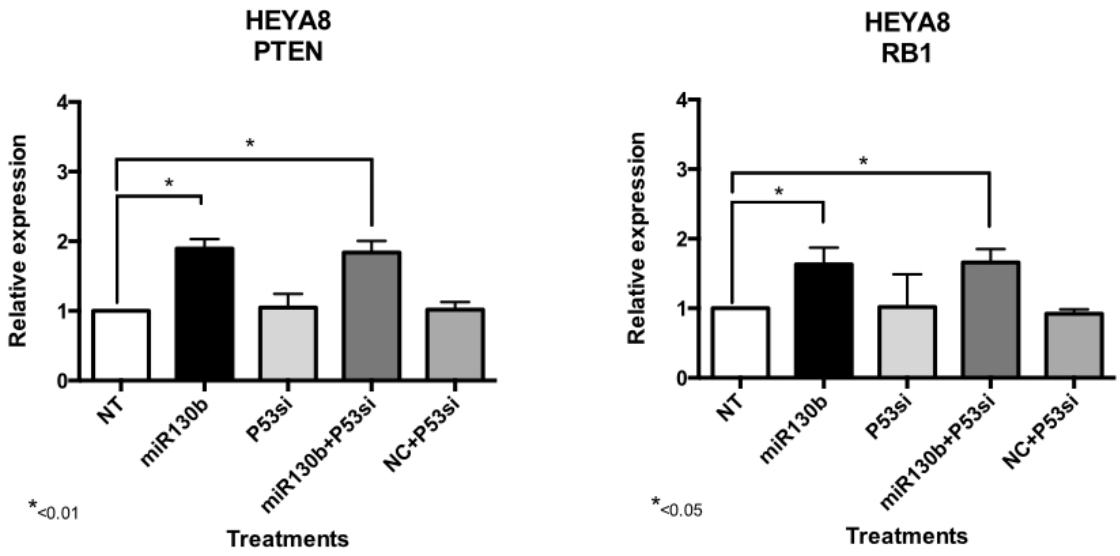


Figure 37. p53 independent miR-130b upregulated genes.

HEYA8 cells were transiently transfected with miR-130b mimic or scrambled negative control (NC) and siRNA against p53. The effect of p53 abrogation on the expression of miR-130b regulated genes was measured by q-PCR.

In order to further understand the p53-dependence of miR-130b regulated genes, I preceded to silence p21, which is a direct downstream target of p53, and a key gene for p53 driven tumor suppressor activities. Silencing of p21 inhibited the ability of miR-130b to induced p53 and BIM. Co-transfection of miR-130b and p21 siRNA partially rescued p21 from the siRNA silencing (Figure 38) On the other hand, silencing of BIM had no effect on the upregulation of p21 and p53 by miR-130b (Figure 39).

In the p53 mutant ovarian cancer cell line OVCAR8, miR-130b overcomes p53 mutation by activating TAp63 and inducing cell death

Overexpression of miR-130b on the OVCAR8 cell line induced about 10% of apoptotic cell death. In order to identify the miR-130b downstream targets behind the apoptotic phenotype I transiently transfected miR-130b on the OVCAR8 cell line and looked for gene regulation in the p53 canonical pathway. As expected, due to the mutation of p53 in this cell line, the expression levels of p53, CDK4, CDK6, p27, p21 and PUMA were not affected by miR-130b. Only three genes were induced by miR-130b at transcriptional level, MDM2, PTEN and TP63 (Figure 40, top right). At the protein level miR-130b strongly induced BIM and the TAp63 (Figure 40, left). The tumor suppressor activities of TAp63 are well known, part of which are by activating common targets of p53 such as p21, however in this model upregulation of TAp63 seems to be acting in a different pathway since most of the p53 downstream targets did not change, except for MDM2. Upregulation of TP63 is dependent on the presence of mutant p53,

silencing of mutant p53 abrogated this effect, while the effect on PTEN and MDM2 seems to be p53 independent (Figure 41).

Although the pro-apoptotic protein BIM was significantly upregulated by miR-130b our previous results showed that apoptosis is not dramatically increased in the OVCAR8 cell line (Figure 34). It has been demonstrated before that aside from its pro-apoptotic functions BIM can also act as the gatekeeper between the processes of apoptosis and autophagy (Delgado and Tesfaigzi, 2013).

Figure 38

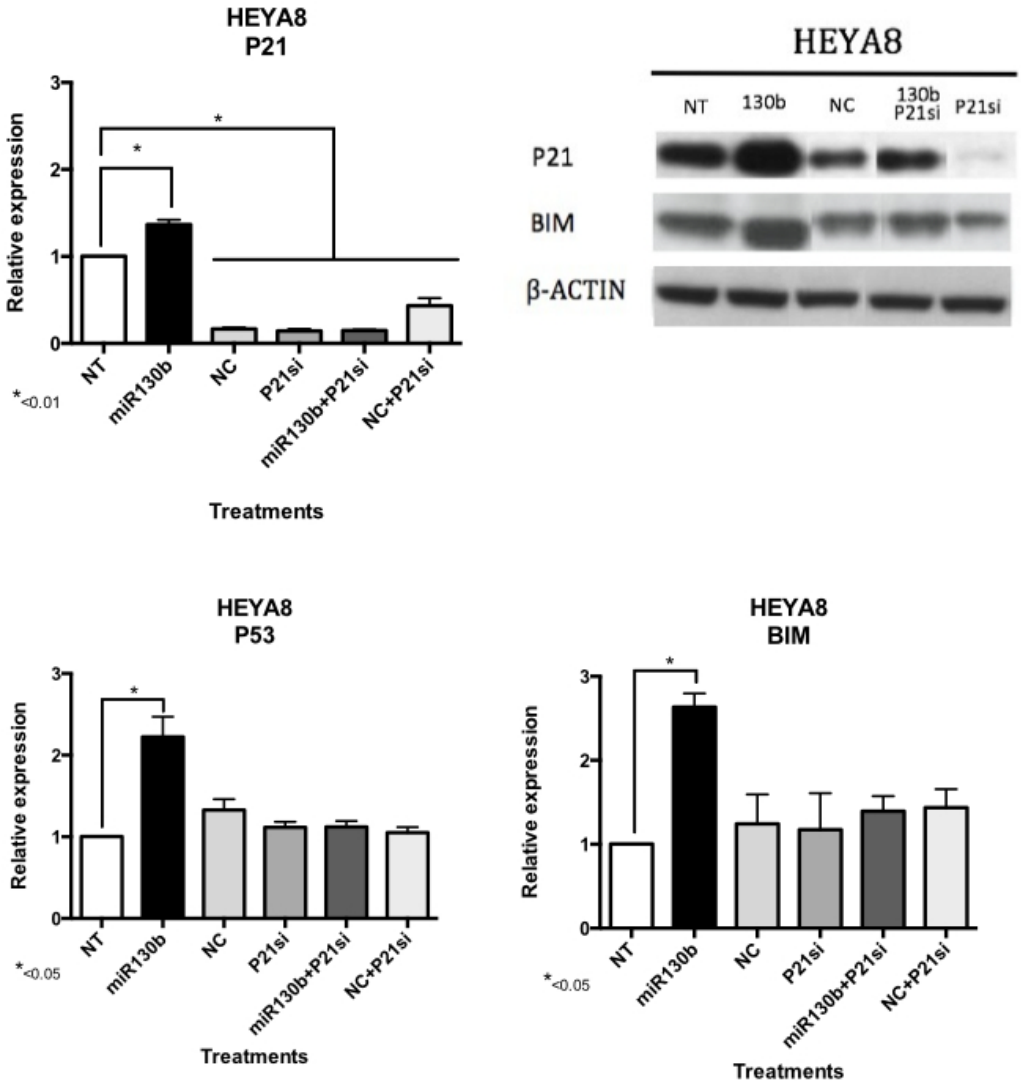


Figure 38. Effect of p21 abrogation on miR-130b regulated genes.

HEYA8 cells were transiently transfected with miR-130b mimic or scrambled negative control (NC) and siRNA against p21. The efficacy of p21 silencing was assayed by q-PCR (left top) and Western blot (right top). The effect of p21 abrogation on p53 and BIM was assayed by q-PCR and Western blot respectively.

Figure 39

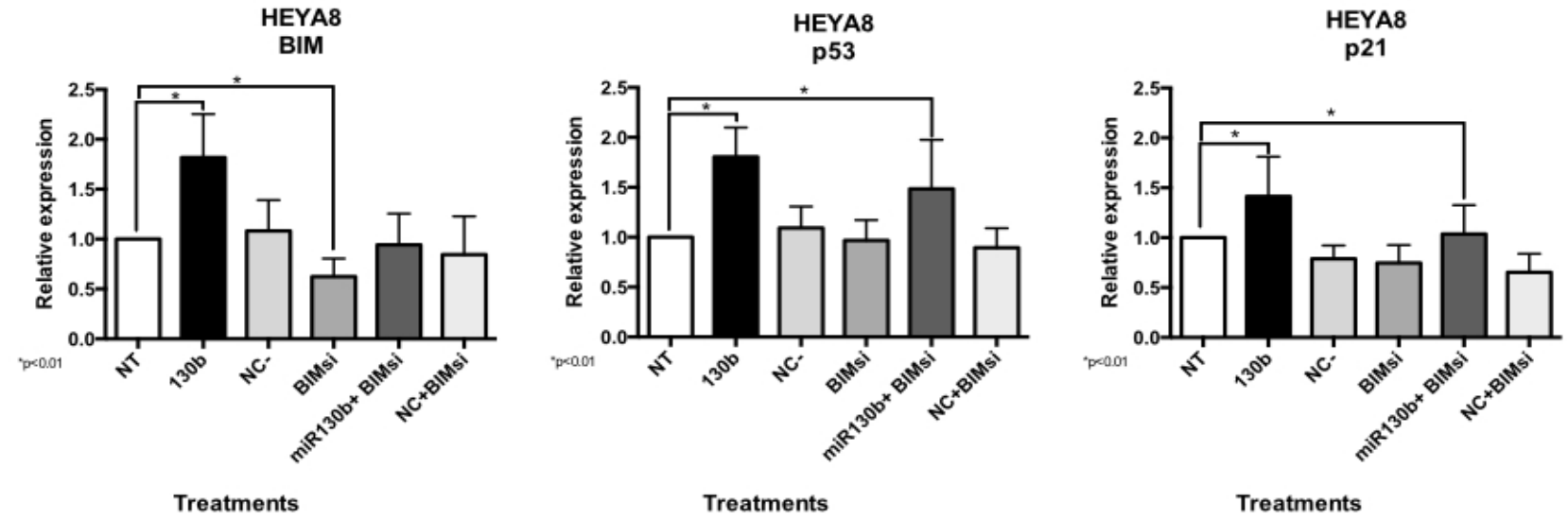


Figure 39. Effect of BIM abrogation on miR-130b regulated genes.

HEYA8 cells were transiently transfected with miR-130b mimic or scrambled negative control (NC) and siRNA against BIM. The efficacy of BIM silencing was assayed by q-PCR. The effect of BIM abrogation on the expression of miR-130b regulated genes was measured by q-PCR.

Figure 40. MiR-130b upregulates TAp63 and BIM in OVCAR8.

The p53 mutant cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and genes/proteins from the p53 pathway were analyzed by q-PCR (right top) and Western blot (left and right bottom). P63 Western blots were done by Avinashnarayan Venkatanarayan at Dr. Elsa Flores lab, MD Anderson

Figure 40

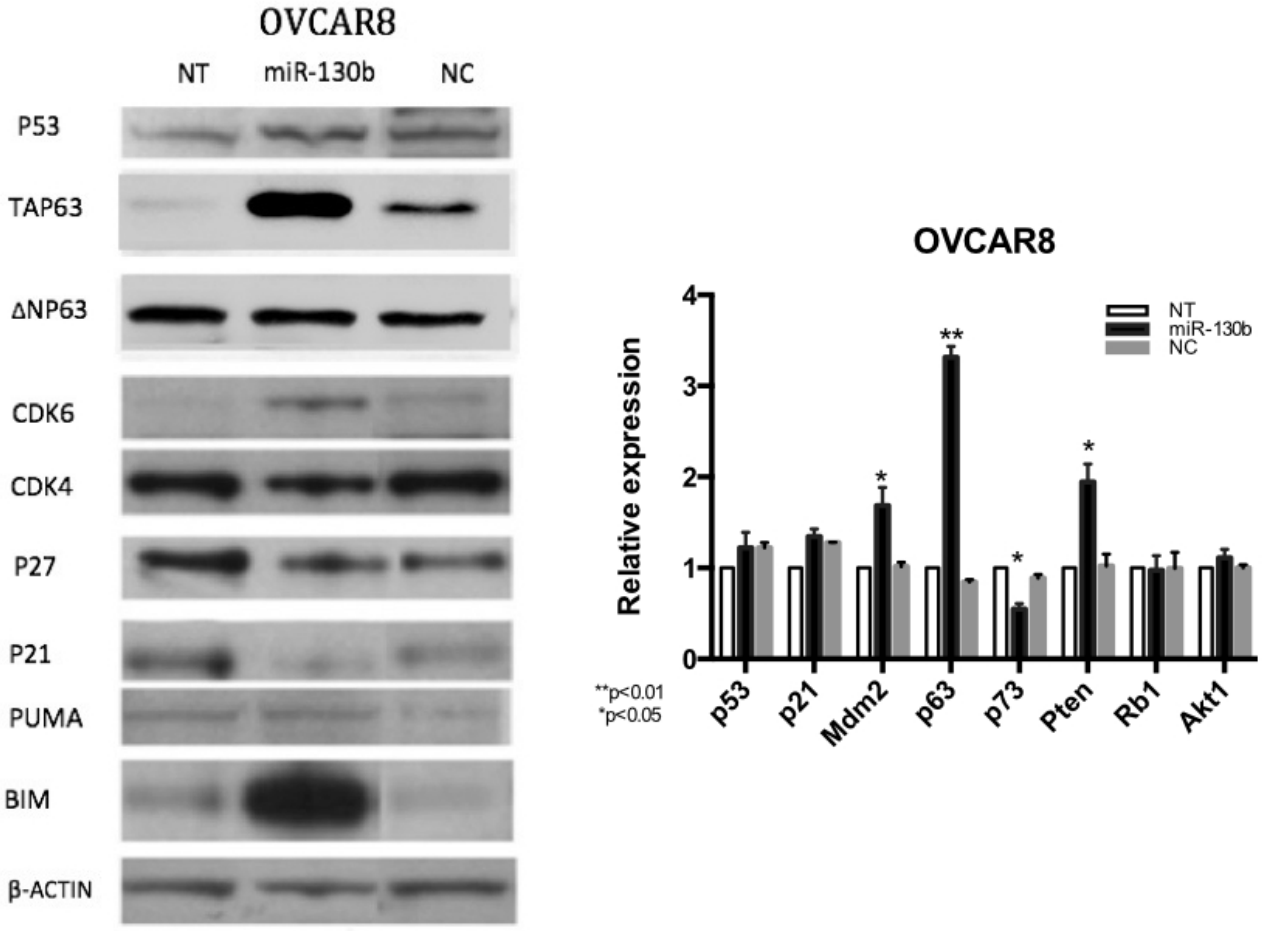
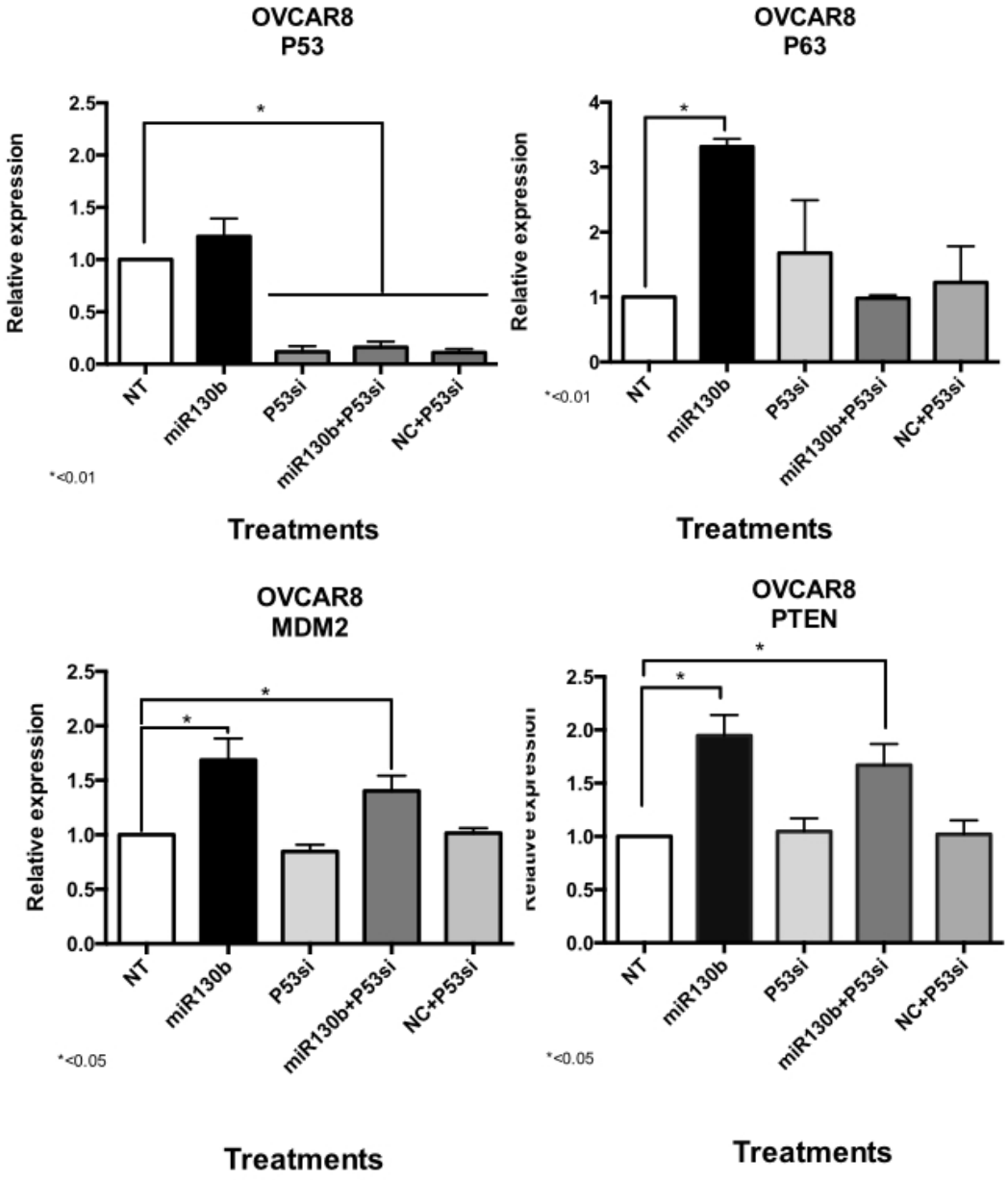

OVCAR8
NT
miR-130b
NC
P53
TAP63
ΔNP63
CDK6
CDK4
P27
P21
PUMA
BIM
β-ACTIN
OVCAR8
Relative expression
NT
miR-130b
NC
**p<0.01
*p<0.05
p53
p21
Mdm2
p63
p73
Pten
Rb1
Akt1

Figure 41. Effect of p53 mutant silencing on miR-130b induced genes.

OVCAR8 cells were transiently transfected with miR-130b mimic or scrambled negative control (NC) and siRNA against p53. The efficacy of p53 silencing was assayed by q-PCR (left top). The effect of p53 abrogation on the expression of miR-130b regulated genes was measured by q-PCR.

Figure 41


OVCAR8
P53
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
NT
miR130b
P53si
miR130b+P53si
NC+P53si
*<0.01
Treatments
OVCAR8
P63
Relative expression
4
3
2
1
0
*
*<0.01
NT
miR130b
P53si
miR130b+P53si
NC+P53si
Treatments
OVCAR8
MDM2
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
NT
miR130b
P53si
miR130b+P53si
NC+P53si
*<0.05
Treatments
OVCAR8
PTEN
*
*
2.5
2.0
1.5
1.0
0.5
0.0
NT
miR130b
P53si
miR130b+P53si
NC+P53si
*<0.05
Treatments

MiR-130b activates the autophagy pathway on the OVCAR8 cell line but not on HEYA8

The autophagy pathway has been described as both pro-cancer and anti-cancer and its role in ovarian cancer is not well understood.

Upregulation of p63 has been previously linked to activation of the autophagy pathway (Kenzelmann Broz et al., 2013), so I hypothesized that miR-130b was able to induce autophagy related genes thru the activation of TAp63 and BIM to push the cells from autophagy to cell death.

The OVCAR8 cell line was transiently transfected with miR-130b mimics and a set of 13 autophagy-related genes was analyzed by q-PCR. MiR-130b was able to induce the expression of core genes of the autophagy pathway: ULK1, ATG5, which are consider fundamental for autophagy, LC3B, the marker for active autophagy, and GABARALPL1 and L2, essential genes for later stages in autophagosome maturation (Figure 42). Other genes tested showed no change (Figure 43), yet it is possible that those genes are responsive to miR-130b at different time points or at protein levels. The same set of genes was tested on the HEYA8 cell line, but just LC3B and ATG5 showed some upregulation upon miR-130b overexpression (Figure 44).

Figure 42

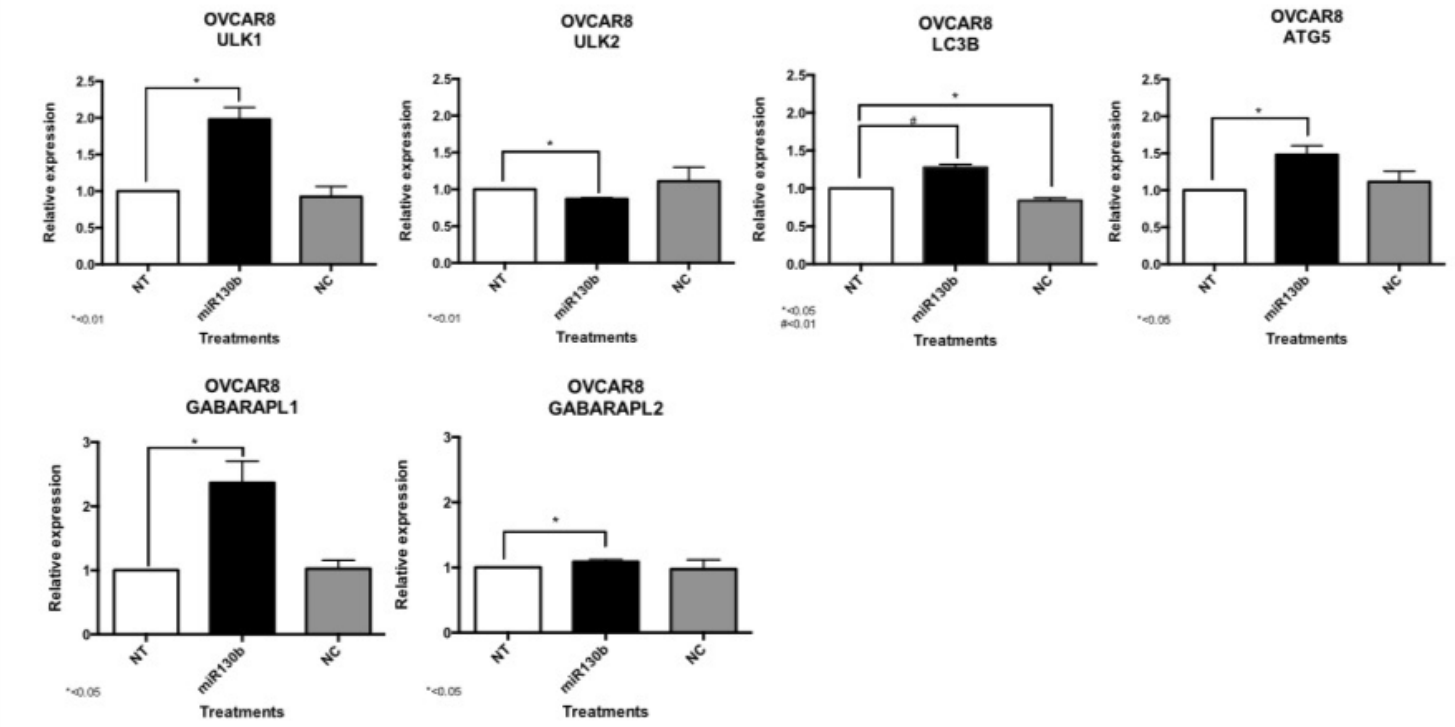


Figure 42. MiR-130b upregulates core autophagy genes in the OVCAR8 cell line.

The OVCAR8 cell line was transiently transfected with miR-130b mimics or scramble negative control (NC) and the expression of core autophagy genes was analyzed by q-PCR 72 hours post-transfection**.**

Figure 43

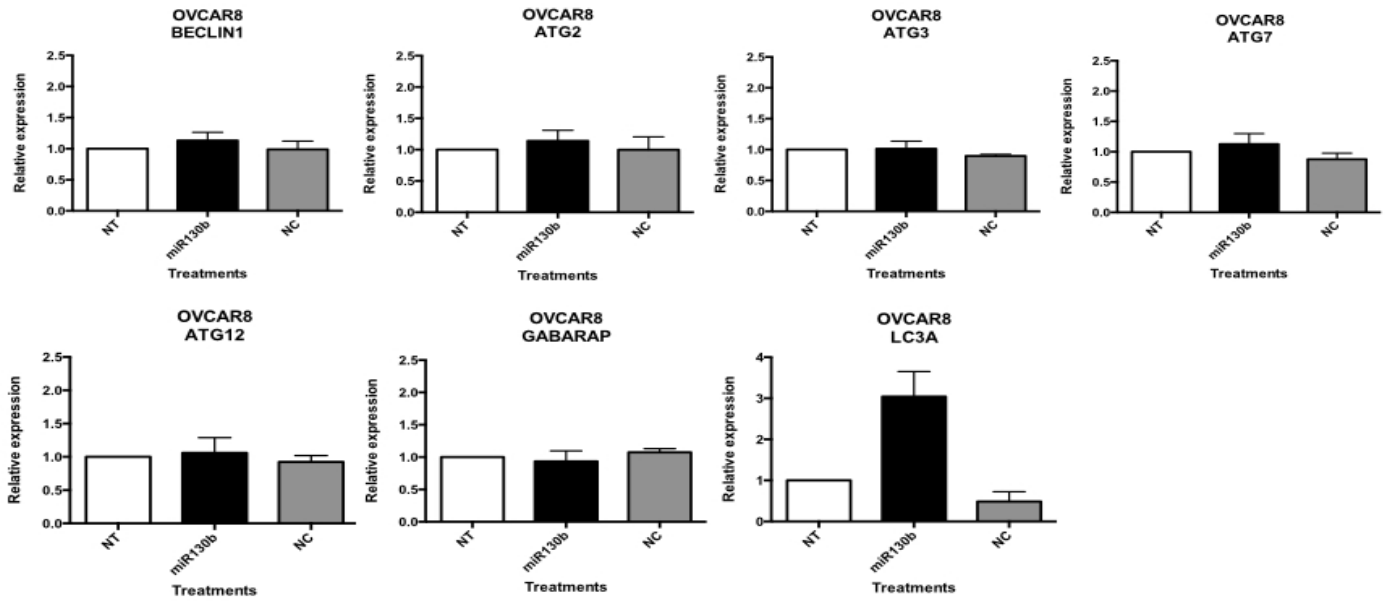


Figure 43. Autophagy-related genes not affected by miR-130b overexpression.

The OVCAR8 cell line was transiently transfected with miR-130b mimics or scramble negative control (NC) and the expression of autophagy genes was analyzed by q-PCR 72 hours post-transfection

Figure 44

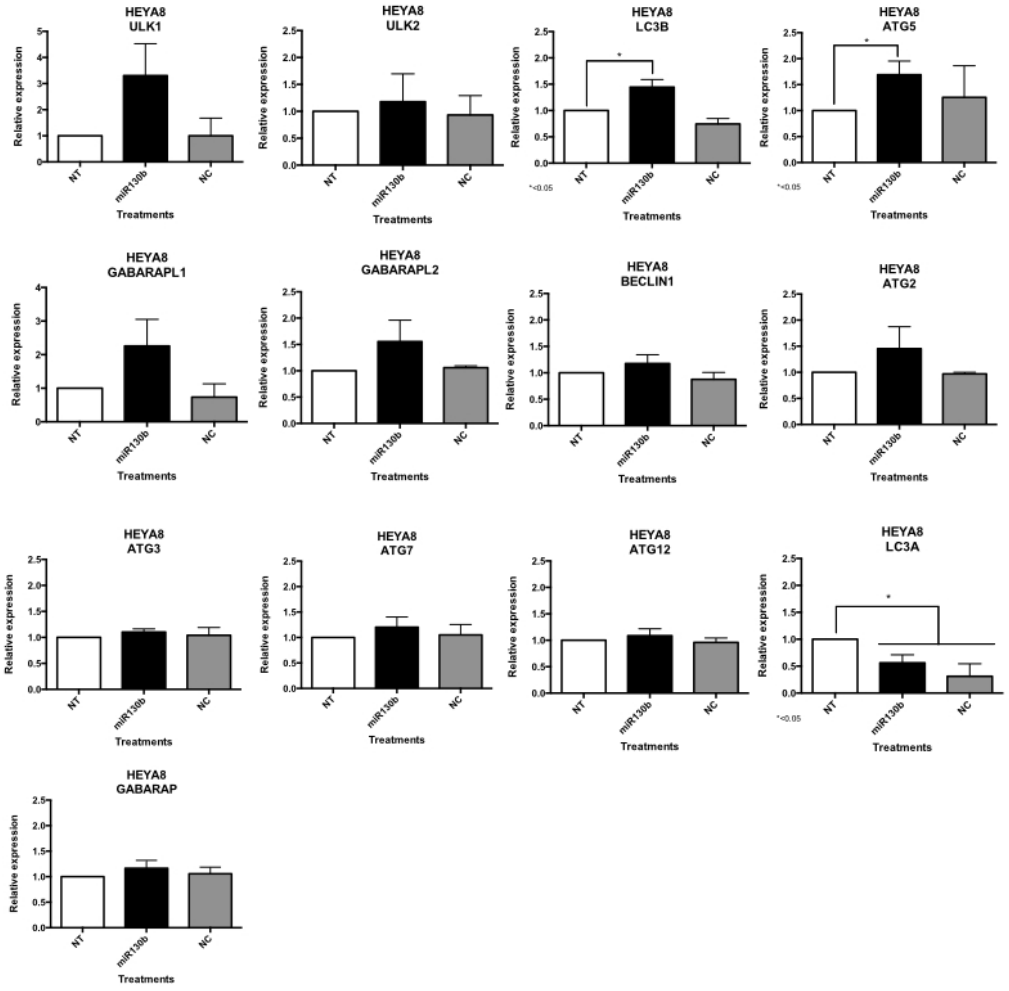


Figure 44. Effect of miR-130b overexpression in autophagy-related genes in the HEYA8 cell line.

The HEYA8 cell line was transiently transfected with miR-130b mimics or scramble negative control (NC) and the expression of core autophagy genes was analyzed by q-PCR 72 hours post-transfection.

Induction of Autophagy-related genes by miR-130b is p53 mutant-dependent and BIM-independent

After analyzing the effect of miR-130b on the expression of autophagy-related genes in p53 WT and mutant genetic backgrounds it seems that miR-130b requires the presence of mutant p53 in order to be able to induce the autophagy-related genes, to test this hypothesis I silenced mutant p53 using siRNA in co-transfection with miR-130b and analyzed the expression of the autophagy-related genes that showed greater upregulation in the previous experiment. Upon p53 silencing miR-130b was not able to induced any of the genes tested: ULK1, LC3B, ATG5 and GABRAPL1 (Figure 45). Due to the important role that BIM plays in in autophagy and the fact that miR-130b was able to significantly upregulate BIM expression, I decided to test the dependency of the autophagy related genes upregulation on BIM. Silencing of BIM did not abrogate the miR-130b driven induction of autophagy genes (Figure 46).

Figure 45. The upregulation of autophagy-related genes by miR-130b is p53 mutant-dependent.

The p53 mutant ovarian cancer cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and siRNA against mutant p53. The efficacy of p53 silencing was assayed by q-PCR (left). The effect of p53 abrogation on the autophagy related genes was assayed by q-PCR.

Figure 45

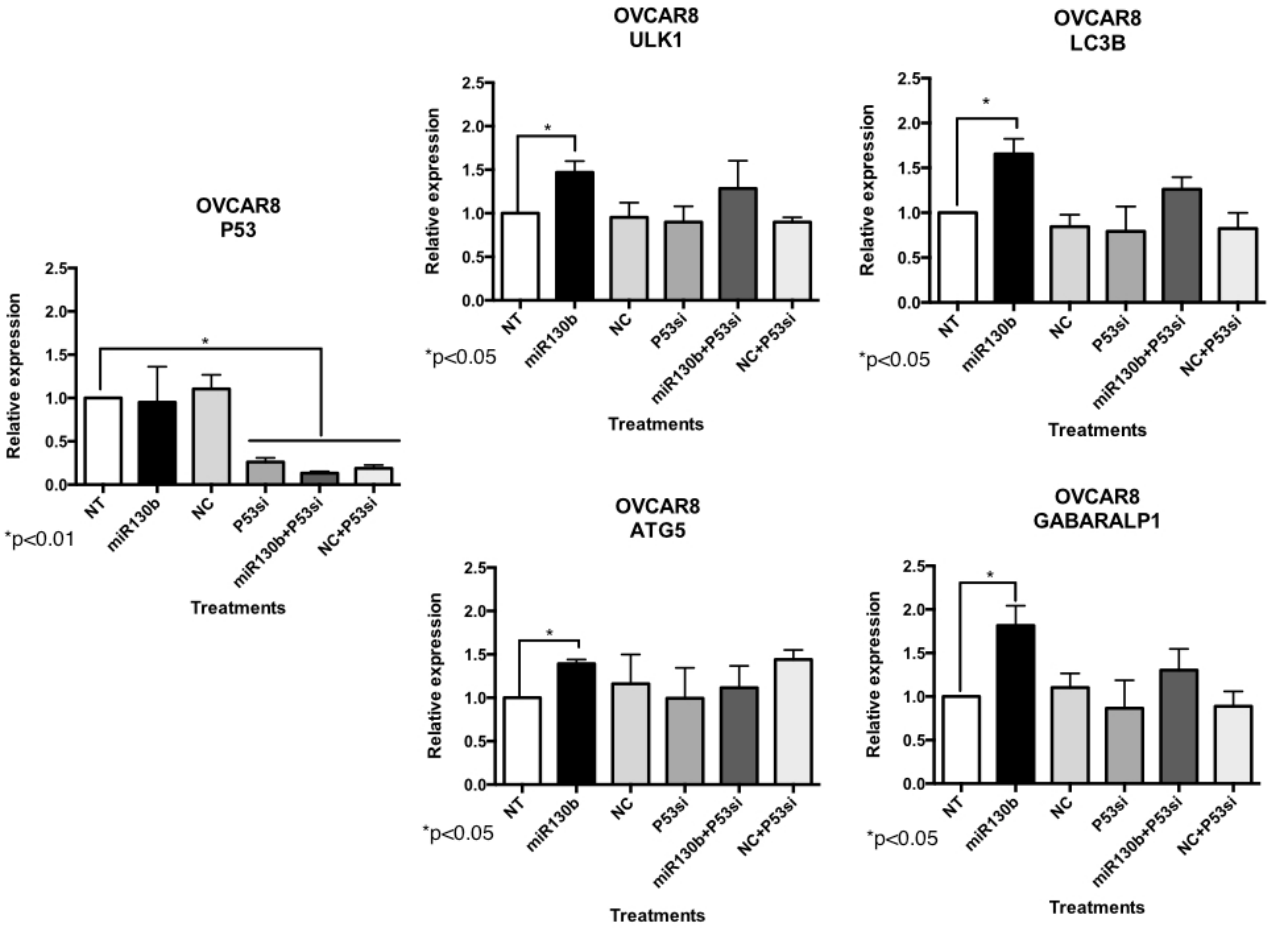
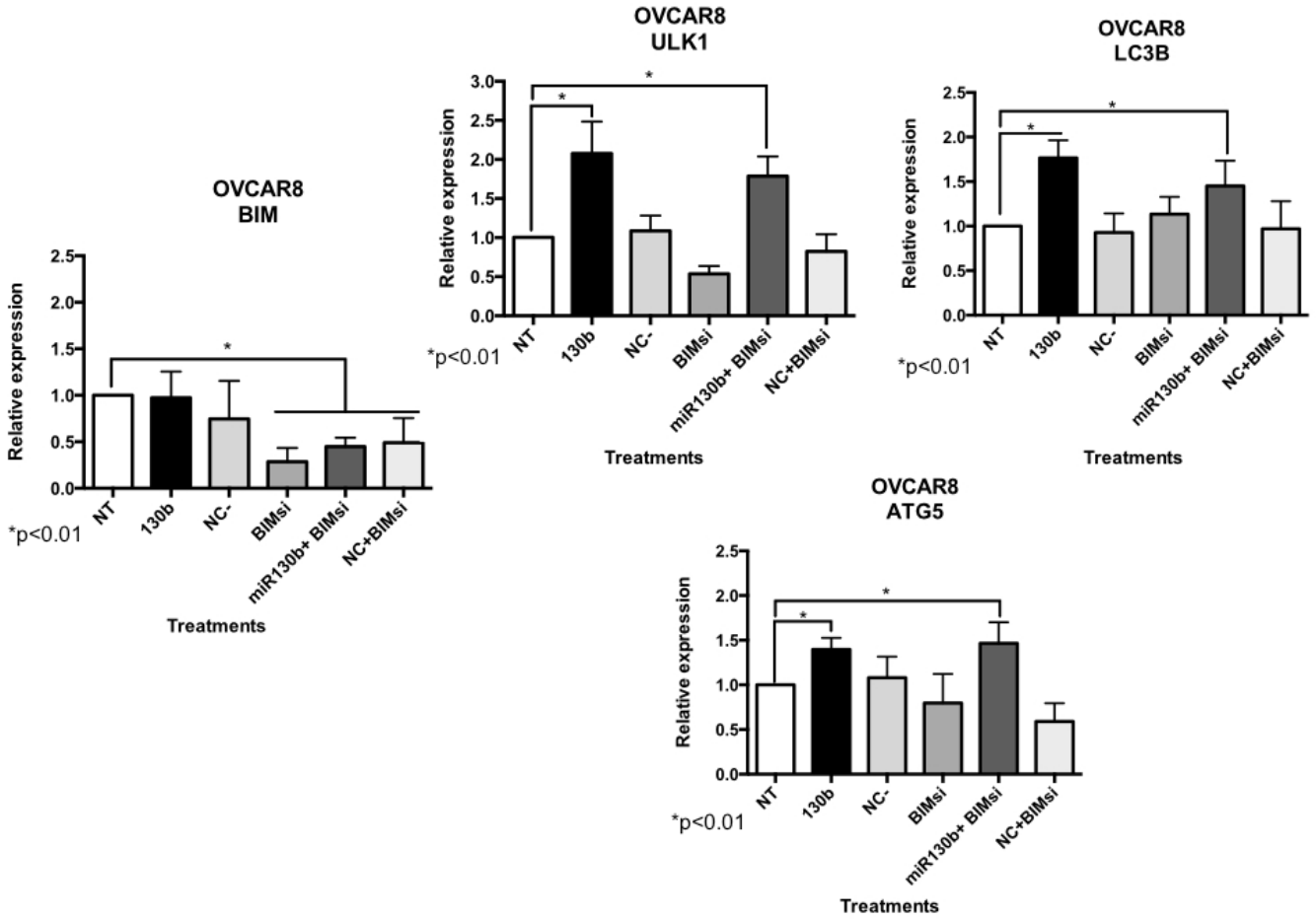

OVCAR8
P53
Relative expression
*p<0.01
NT
miR130b
NC
P53si
miR130b+P53si
NC+P53si
Treatments
OVCAR8
ULK1
Relative expression
*p<0.05
Treatments
OVCAR8
LC3B
Relative expression
*p<0.05
Treatments
OVCAR8
ATG5
Relative expression
*p<0.05
Treatments
OVCAR8
GABARALP1
Relative expression
*p<0.05
Treatments

Figure 46. Upregulation of autophagy genes is BIM independent.

The p53 mutant ovarian cancer cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and siRNA against BIM. The efficacy of BIM silencing was assayed by q-PCR (left). The effect of BIM abrogation on the autophagy related genes was assayed by q-PCR.

Figure 46


OVCAR8
BIM
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*p<0.01
NT
130b
NC-
BIMsi
miR130b+ BIMsi
NC+BIMsi
Treatments
OVCAR8
ULK1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*p<0.01
Treatments
OVCAR8
LC3B
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*p<0.01
Treatments
OVCAR8
ATG5
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*p<0.01
Treatments

MiR-130b induces autophagic flux in the p53 mutant ovarian cancer cell line OVCAR8 but not in the p53 WT cell line HEYA8

Activation of the autophagy pathway requires the coordination between a complex network of genes. Regulation of the pathway occurs at transcriptional and post-transcriptional level. Induction of autophagy related genes does not necessary mean that the pathway is active. One of the standard measures for autophagy is the autophagic flux assay, in which a lysosomal inhibitor is used to make the autophagosomes to accumulate and the amount of LC3B II protein (present on a the autophagosomes membrane) is used as a direct indicator of active autophagy. The use of lysosomal inhibitors can activate other pathways and cause non-autophagic cell death, and the standardization of the right concentration and time of treatment needs to be done for each cell line. Choroquine and bafilomycin A are widely use to test autophagic flux and I tested both in a concentration curve using 5, 10 and 20 nM treatments for 4 hours before harvesting the cells to do protein extraction and LC3B II Western blot (Figure 47).

During the titration process it is important to choose a concentration that can induce the accumulation of LC3BII but that at the same time does not compromise the integrity of the cells. Bafilomycin at 10 nM was effective for both cell lines and was the combination used for the experiments to follow.

Figure 47

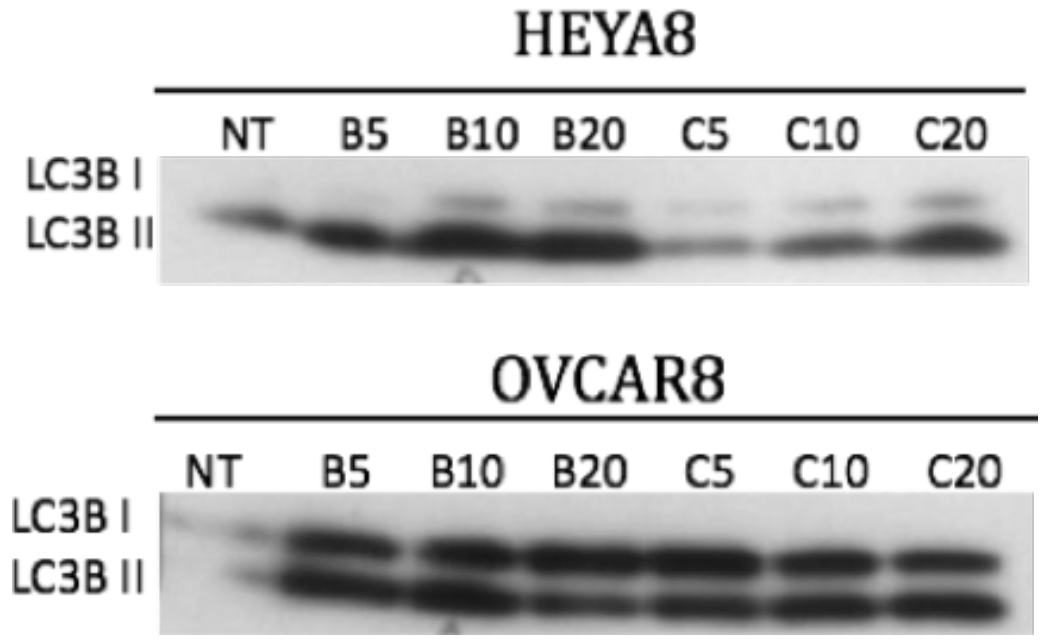


Figure 47. Titration of lysosomal inhibitors.

Ovarian cancer cells HEYA8 and OVCAR8 were incubated during 4 hours with media containing Bafilomycin (B) and Chloroquine (C) at different concentrations, 5,10 and 20 nM, then cells were harvested and proteins extracted to measure the amounts of LC3BII protein by Western blot.

Accumulation of LC3B II was observed on OVCAR8 upon miR-130b treatment, indicating that miR-130b can induce active autophagy on the cells (Figure 48). An increase of LC3B I was also observed in the cells treated with miR-130b implying not only an increased rate of LC3B I to LC3B II conversion but and increment in the total amount of LC3B I. The HEYA8 cells showed no difference in the amount of LC3B II after miR-130b upregulation (Figure 49).

Figure 48

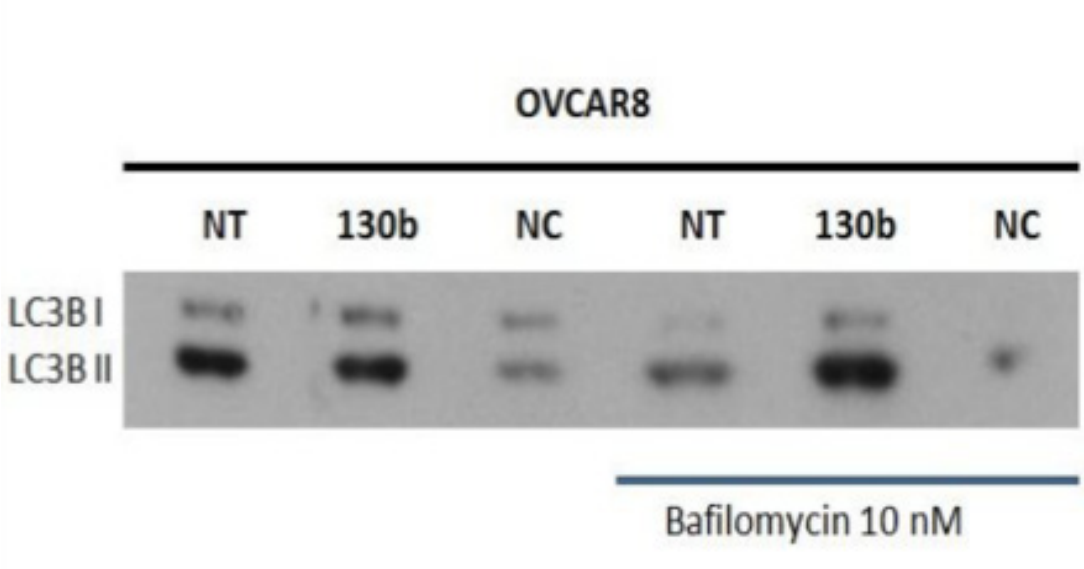


Figure 48. MiR-130b induces autophagic flux in the p53 mutant OVCAR8 cell line.

The ovarian cancer cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative control (NC), 72 hours post-transfection the cells were incubated for 4 hours with complete media or media containing 10 nM of bafilomycin. Posteriorly proteins were extracted and the accumulation of LC3B II protein was measured by western blot.

Figure 49

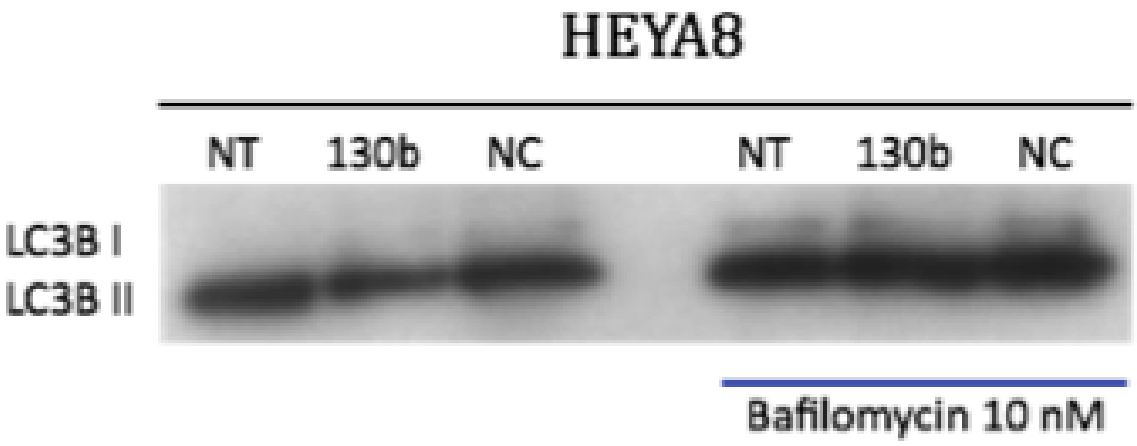


Figure 49. MiR-130b has no impact on autophagic flux in the p53 WT HEYA8 cell line.

The ovarian cancer cell line HEYA8 was transiently transfected with miR-130b mimics or scrambled negative control (NC), 72 hours post-transfection the cells were incubated for 4 hours with complete media or media containing 10 nM of bafilomycin. Posteriorly proteins were extracted and the accumulation of LC3B II protein was measured by western blot.

p63 isoforms impact cell viability of ovarian cancer cell lines HEYA8 and OVCAR8

The fact that miR-130b is able to induce TAp63 in both p53 WT HEYA8 and mutant OVCAR8 cell lines suggests that TAp63 is an important downstream target of miR-130b in our model. Previously it has been shown that miR-130b is a direct target of TAp63 (Su et al., 2010) so it is possible that there is a feed-forward loop in which miR-130b upregulates TAp63 expression and TAp63 feeds backs by upregulating miR-130b. To test this hypothesis first I reanalyzed the expression of TAp63 and ΔNp63 isoforms on cells overexpressing miR-130b.

In the HEYA8 cell line miR-130b increased the mRNA expression of TA by 2.5 fold change and no effect was observed on ΔNp63 mRNA levels (Figure 50, top), this result correlates with the previous western blot (Figure 35), where TAp63 is not present in the untreated cells (NT) but is strongly expressed on the miR-130 treated cells whereas ΔNp63 was present on the untreated cells (NT) and went down upon miR-130b treatment.

The OVCAR8 cell line showed upregulation at the transcriptional level of both TAp63 and ΔNp63 isoforms (Figure 50, bottom), however from the previous western blot we knew that the protein levels of TAp63 significantly went up but ΔNp63 remained unchanged after miR-130b treatment (Figure 40).

Figure 50

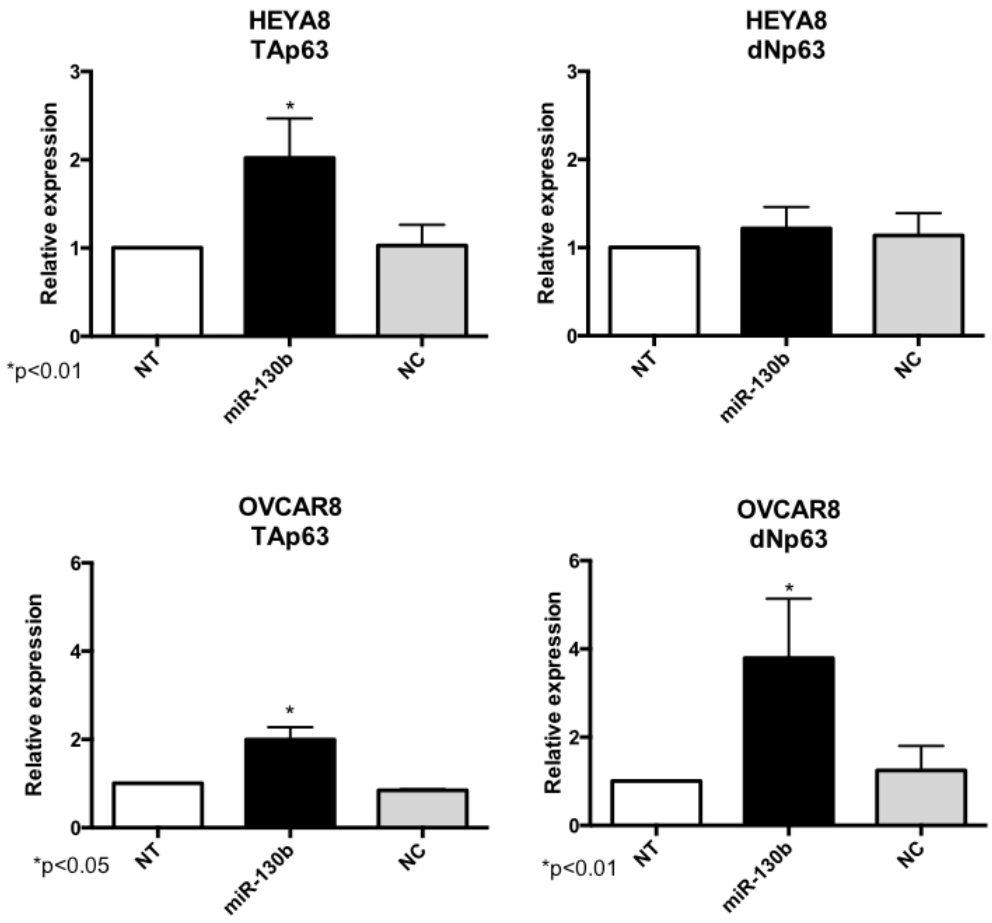


Figure 50. Effect of miR-130b on p63 mRNA levels.

The ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with miR-130b mimics or scrambled negative control (NC) and the expression levels of TAp63 and ΔNp63 were measured by q-PCR.

To test the other side of the hypothesis I overexpressed TAp63 and ΔNp63 and then measured the amount of primary and mature miR-130b present on the cells. Efficiency of p63 upregulation was measured by q-PCR. Upregulation of TAp63 led to downregulation of the ΔNp63 isoform in both of the cell lines (Figure 51); surprisingly ΔNp63 overexpression increased significantly the mRNA levels of TAp63 on the HEYA8 and had a similar trend on the OVCAR8 (Figure 52)

The levels of pri-miR-130b in both of the cell lines remained unchanged after TAp63 and ΔNp63 overexpression (Figure 53). Mature miR-130b was increased by 2.5 and 3.5 fold change by TAp63 in HEYA8 and OVACR8 respectively, whereas ΔNp63 had just a marginal effect on HEYA8 and had no effect on OVCAR8 (Figure 54).

Figure 51

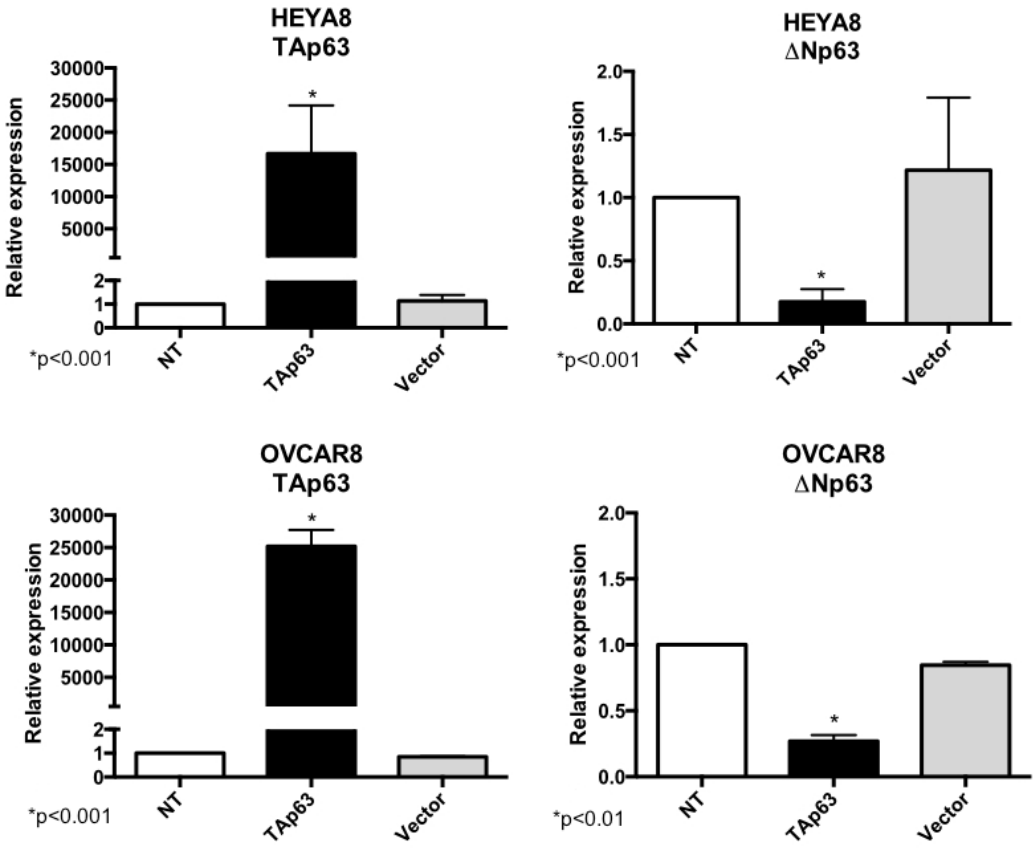


Figure 51. Overexpression of TAp63 and its effect on ΔNp63 expression.

The ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with TAp63 expression vector or pCDNA3.1 empty vector (vector) and the expression levels of TAp63 and ΔNp63 were measured by q-PCR.

Figure 52

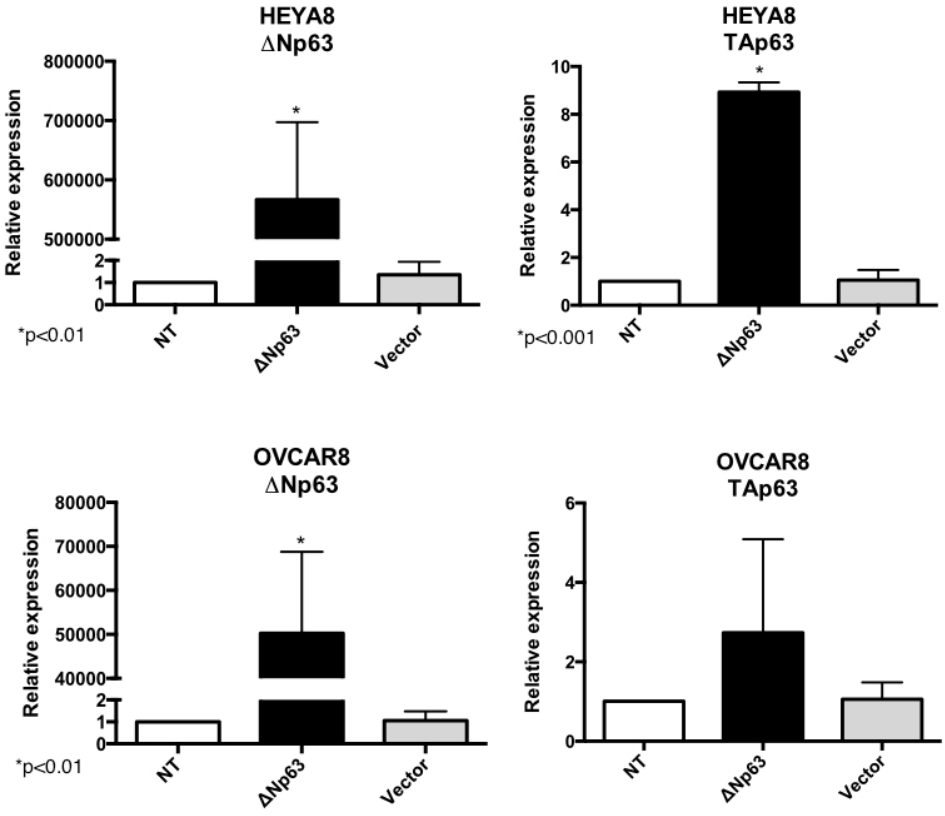


Figure 52. Overexpression of ΔNp63 and its effect on TAp63 expression.

The ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with ΔNp63 expression vector or pCDNA3.1 empty vector (vector) and the expression levels of TAp63 and ΔNp63 were measured by q-PCR.

Figure 53

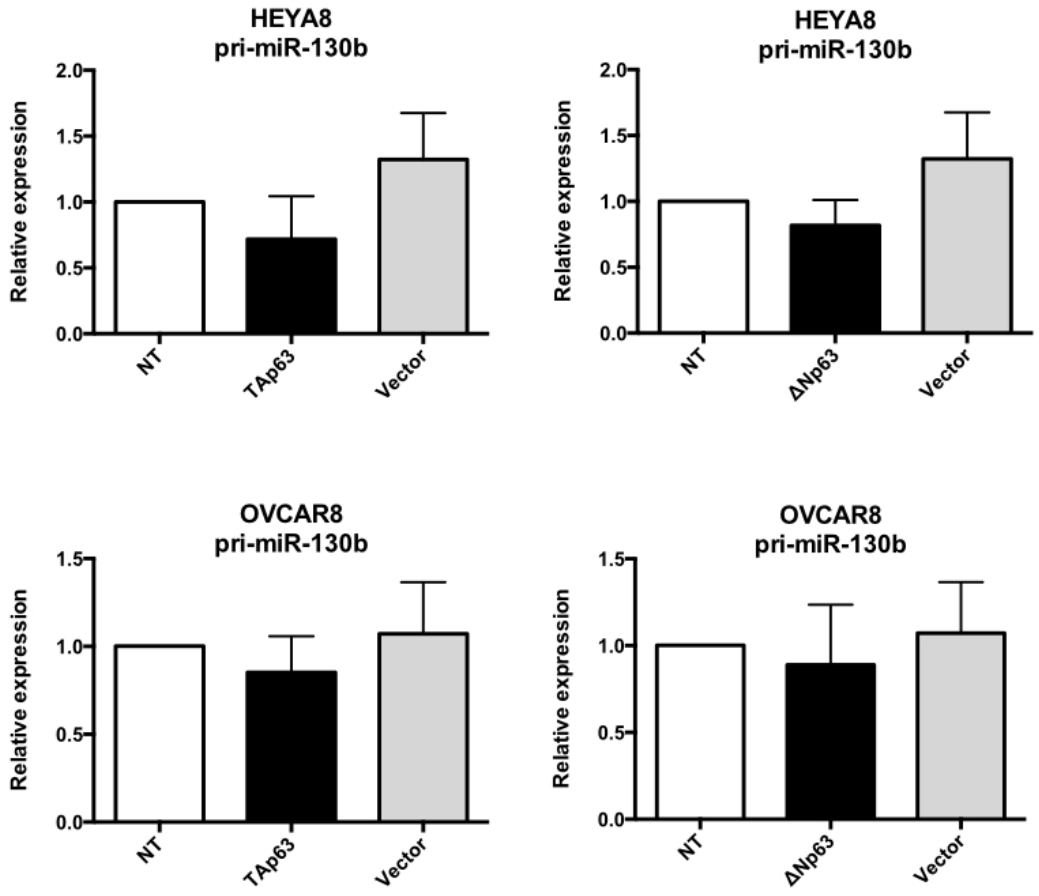


Figure 53. Effect of TAp63 and ΔNp63 overexpression on pri-miR-130b levels.

The ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with TAp63 or ΔNp63 expression vectors or pCDNA3.1 empty vector (vector) and the expression levels of primary miR-130b were measured by q-PCR.

Figure 54

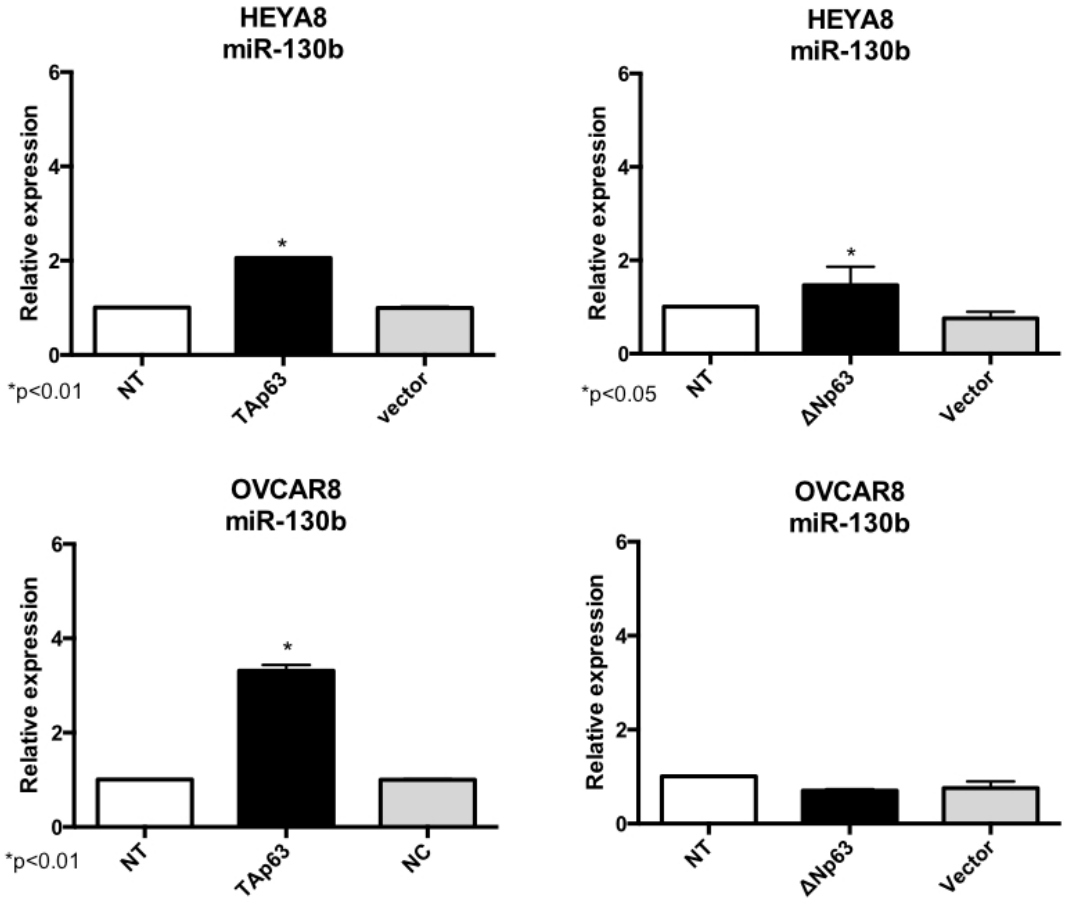


Figure 54. Effect of TAp63 and ΔNp63 overexpression on miR-130b.

The ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with TAp63 or ΔNp63 expression vectors or pCDNA3.1 empty vector (vector) and the expression levels of miR-130b were measured by q-PCR.

Despite the fact that in my model it was not possible to established the presence of a feed forward loop between p63 and miR-130b, it is clear that p63 is a key downstream target of miR-130 activities by virtue of published data from (Su et al., 2010) and our experiments that showed induction of TAp63 in OVCAR8 and HEYA8 and downregulation of ΔNp63 in HEYA8. One of the characteristics of the mechanisms of miRNAs actions is their ability to fine-tune the expression of potentially hundreds of targets across different gene networks. Thus, the phenotypes induced by miRNAs are not driven by a single gene, however miRNAs can guide us in the identification of critical promoters and novel and unexpected divers of oncogenic pathways that can be manipulated by other methods, such as gene therapy or small molecules to produce the desired effects.

In order to explore the hypothesis that TAp63, which is not expressed in HEYA8 or OVCAR8, is a critical effector of miR-130b-mediated tumor suppression and ΔNp63, which is expressed robustly in both HEYA8 and OVCAR8 cell lines and downregulated in HEYA8 upon miR-130b treatment, is oncogenic. I used expression vectors to overexpress TAp63 and ΔNp63 isoforms in both HEYA8 and OVCAR8 cell lines and assayed cell proliferation by MTS every day for 5 days.

Surprisingly both isoforms TAp63 and ΔNp63 reduced the cell proliferation in a dose and time-dependent manner in both of cell lines. The effect of TA was stronger in the p53 WT background (HEYA8) and the higher concentration (10 ng) reduced the proliferation by 70-80% in all the time points. The lower concentration (5 ng) had a statistical significant effect on the earlier time points from 24 to 72 hours where

proliferation was inhibited by 40-60 % however the cells recovered and by the final reading at 120 h. their proliferation was close to the one showed by the non-treated and negative control treated cells (Figure 55, top left). Overexpression of ΔNp63 (10 ng)reduced the proliferation of HEYA8 by 60 % in the earlier time points from 24 to 72 hours and by 25 % in the 96 and 120 hours. At a lower concentration (5 ng) ΔNp63 reduced the proliferation by 60 % and 40% at 24 and 48 hours respectively at the later times there was not difference between the cells overexpressing ΔNp63 and the non-treated and control vector treated cells (Figure 55, bottom left).

Overexpression of TAp63 and ΔNp63 also impacted the proliferation of the p53 mutant OVCAR8 cell line, although the proliferation inhibition was less than in HEYA8, the trend was the same. TAp63 at the higher concentration (10 ng) reduced cell proliferation between 40% and 60% in all the time points. At lower TAp63 concentration (5 ng) proliferation was reduced between 20% and 35 % depending of the time point, with the later time points showing the least reduction (Figure 56, top left). Overexpression of ΔNp63 reduced the proliferation by 20-50% at the high concentration (10 ng) and in the lower concentration (5 ng) just the 48 hours time point showed a reduction in proliferation of 20%, none of the other time points had a statically significant reduction (Figure 56, bottom left). Based on these results I decided to use the higher concentration of TAp63 and ΔNp63 and the 48 hours time point to analyze cell viability by FACS.

In the HEYA8 cell line cell viability dropped from ~90% to ~77% and from ~90% to ~81% upon TAp63 and ΔNp63 overexpression respectively compared with the

untreated and empty vector treated cells. The percentage of apoptotic and necrotic cells in the cells overexpressing TAp63 and ΔNp63 increased by ~ 2 fold change compare with the percentages present on untreated and negative control treated cells (Figure 55, right). For the OVCAR8 cell line, viability was reduced about 30% by both TA63 and ΔNp63 and the percentage of apoptotic and necrotic cells increased by 7-10 fold change (Figure 56, right).

Figure 55. P63 impacts HEYA8 cell proliferation and viability.

The p53 WT ovarian cancer cell line HEYA8 was transiently transfected with 5 ng or 10 ng of TAp63 or ΔNp63 expression vectors or control vector and proliferation was measured by MTS during 5 days. The relative proliferation was calculated using the values of the non-treated cell as baseline and the backbone vector was used as control (left panels). For the cell viability experiments cells were transfected with 100 ng of TAp63 or ΔNp63 expression vectors or empty vector (vector) and 48 hours post-transfection stained with PI and AnnexinV. (FACS experiments were done with the help of Sesha Duvvuri).

Figure 55

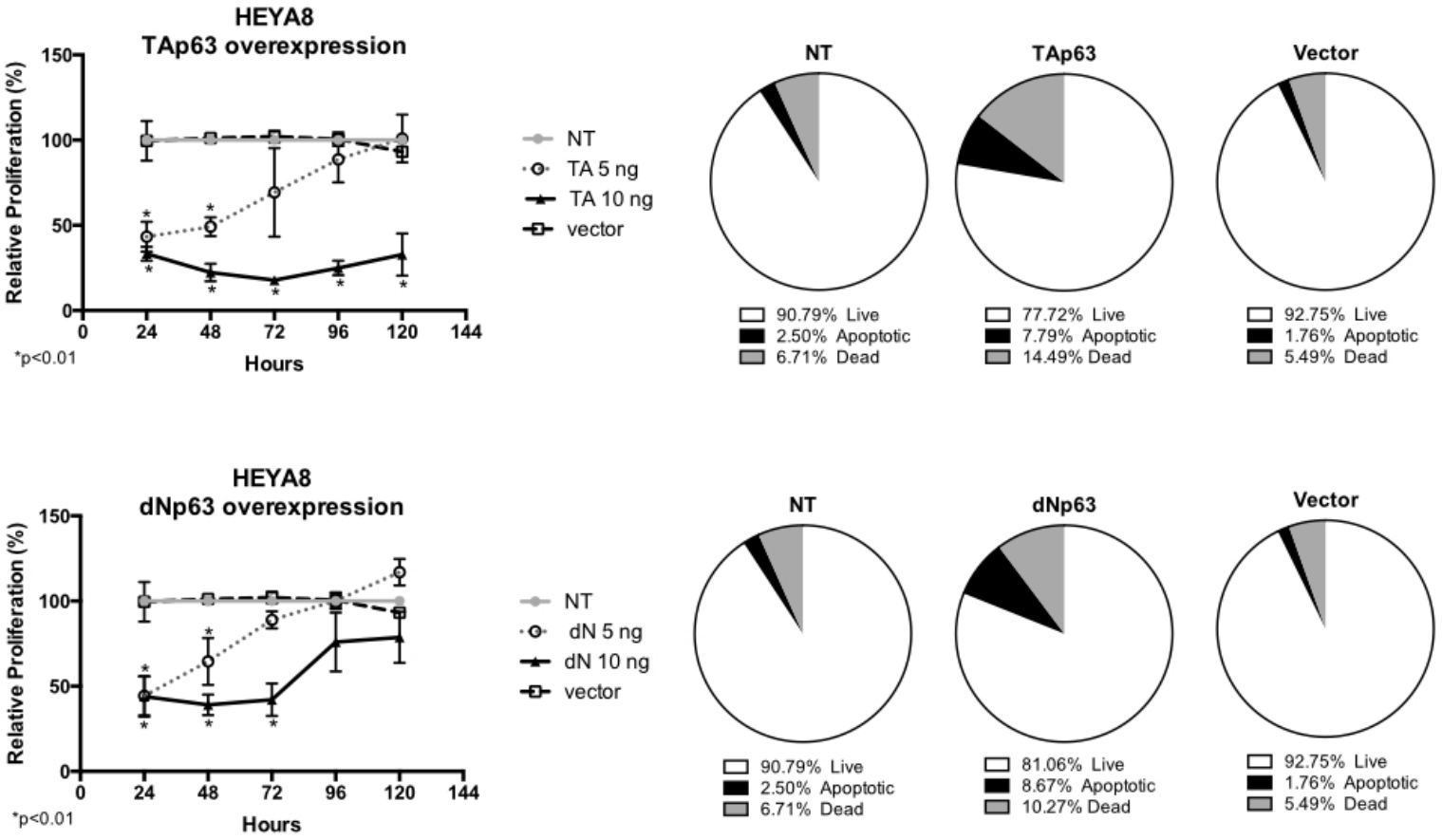
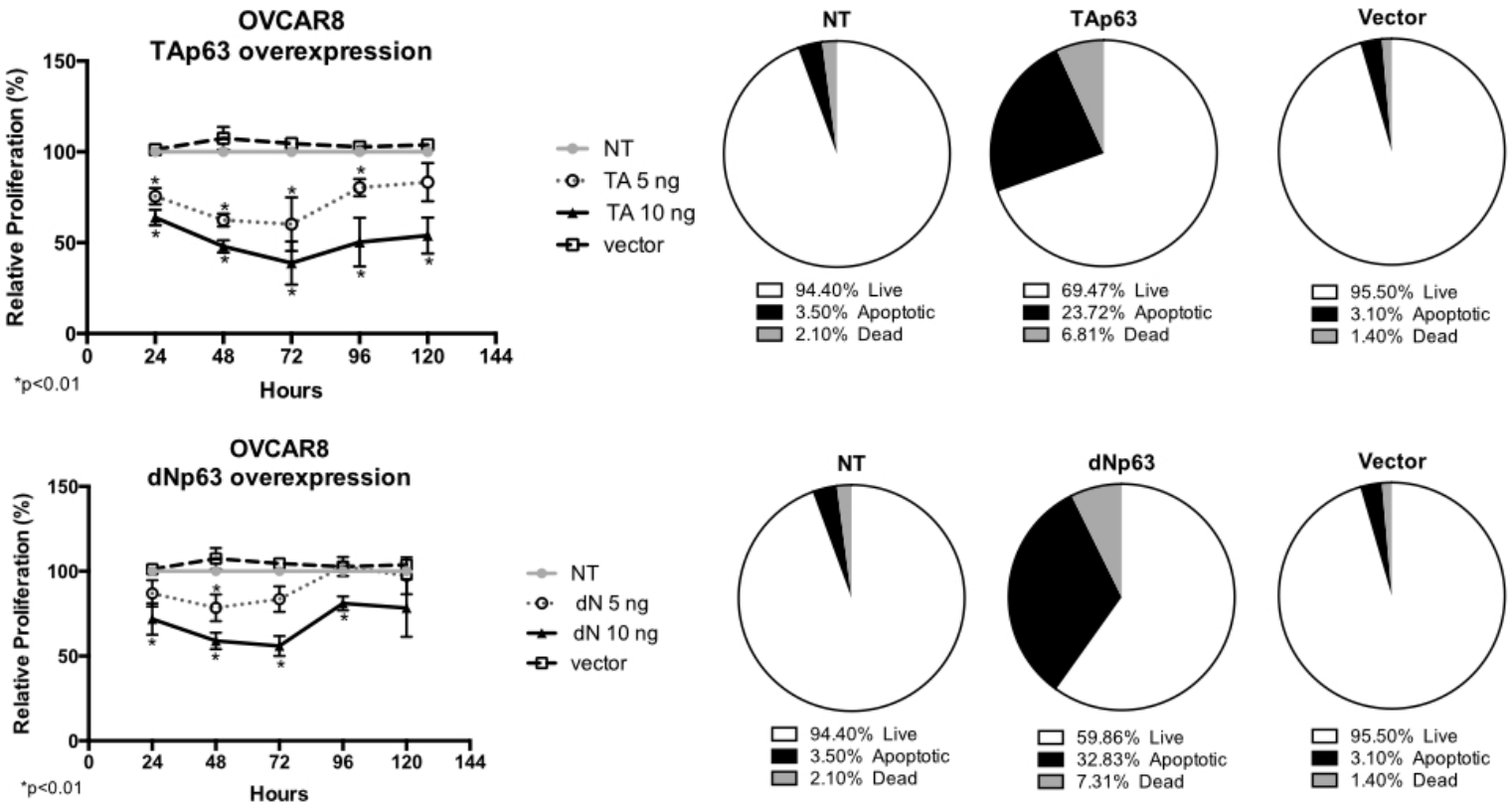

HEYA8
TAp63 overexpression
Relative Proliferation (%)
150
100
50
0
0
24
48
72
96
120
144
Hours
*p<0.01
NT
TA 5 ng
TA 10 ng
vector
NT
TAp63
Vector
90.79% Live
2.50% Apoptotic
6.71% Dead
77.72% Live
7.79% Apoptotic
14.49% Dead
92.75% Live
1.76% Apoptotic
5.49% Dead
HEYA8
dNp63 overexpression
Relative Proliferation (%)
150
100
50
0
0
24
48
72
96
120
144
Hours
*p<0.01
NT
dN 5 ng
dN 10 ng
vector
NT
dNp63
Vector
90.79% Live
2.50% Apoptotic
6.71% Dead
81.06% Live
8.67% Apoptotic
10.27% Dead
92.75% Live
1.76% Apoptotic
5.49% Dead

Figure 56. P63 impacts OVCAR8 cell proliferation and viability.

The p53 mutant ovarian cancer cell line OVCAR8 was transiently transfected with 5 ng or 10 ng of TAp63 or ΔNp63 expression vectors or control vector and proliferation was measured by MTS during 5 days. The relative proliferation was calculated using the values of the non-treated cell as baseline and the backbone vector was used as control (left panels). For the Cell viability experiments cells were transfected with 100 ng of TAp63 or ΔNp63 expression vectors or empty vector (vector) and 48 hours post-transfection stained with PI and AnnexinV. (FACS experiments were done with the help of Sesha Duvvuri).

Figure 56


OVCAR8
TAp63 overexpression
Relative Proliferation (%)
150
100
50
0
0
24
48
72
96
120
144
Hours
*p<0.01
NT
TA 5 ng
TA 10 ng
vector
NT
94.40% Live
3.50% Apoptotic
2.10% Dead
TAp63
69.47% Live
23.72% Apoptotic
6.81% Dead
Vector
95.50% Live
3.10% Apoptotic
1.40% Dead
OVCAR8
dNp63 overexpression
Relative Proliferation (%)
150
100
50
0
0
24
48
72
96
120
144
Hours
*p<0.01
NT
dN 5 ng
dN 10 ng
vector
NT
94.40% Live
3.50% Apoptotic
2.10% Dead
dNp63
59.86% Live
32.83% Apoptotic
7.31% Dead
Vector
95.50% Live
3.10% Apoptotic
1.40% Dead

MiR-130b sensitizes HEYA8 to cisplatin

Once I have established that miR-130b is able to induce apoptosis in ovarian cancer cell lines with different p53 genetic background and uncovered the molecular mechanism involved in this pro-apoptotic function, I wanted to explore the possible role of miR-130b on drug sensitization. Cisplatin (CDDP) is commonly used as the first line of treatment for ovarian cancer, for this reason it was the drug of choice for the sensitization experiment.

The ovarian cancer cell lines HEYA8 and OVCAR8 were transiently transfected with miR-130b and 24 hours after the transfection the media was replaced with fresh media containing 5 μg/mL of CDDP, 120 hours after drug treatment cell viability was assayed using FACS.

In the HEYA8 cell line the CDDP treatment alone or in combination with scrambled negative control showed a 50% reduction in cell viability, miR-130b in combination with CDDP further reduced the percentage of live cells to 33% and increased the ratio of apoptotic and dead cells by 3 fold and 1.5 fold change compared with the CDDP treatment (Figure 57).

Figure 57

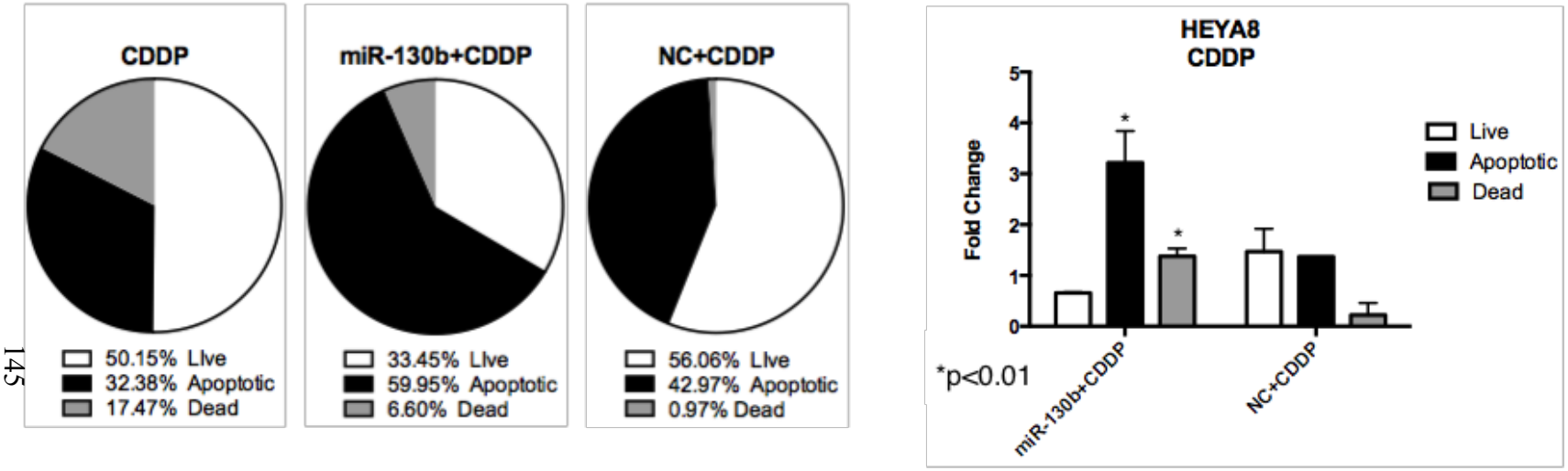


Figure 57. MiR-130b sensitizes HEYA8 cells to cisplatin.

The ovarian cancer cell line HEYA8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and 24 hours after transfection the media was replaced with media containing 5 μg/mL of CDDP. Cell viability was analyzed by FACS. (FACS experiments were done with the help of Sesha Duvvuri).

Data from our collaboration with Dr. Esther Chang has shown that with the use of the nanoliposomal delivery system (scL) patented by Dr. Chang and SynerGene Therapeutics Inc. it is possible to introduce miR-130b to the cells in concentrations that allow more CDDP sensitization than the one observed in my experiments, in which the amount of transfected miR is limited by the toxicity of the delivery method (lipofection). Dr. Chang shared with us material from her *in vitro* miR-130b + CDDP experiments to measure the effects of this treatment on the expression of the genes that I found to be regulated by miR-130b on my previous experiments.

The use of the liposome delivery considerably increased the amount of miR-130b present on the cells that went from hundred-folds (Figure 33) to the thousand-folds (Figure 58, left). A general trend can be observed on the genes belonging to the p53 canonical pathway, the combination of miR-130b and CDDP seems to have a synergistic effect on the upregulation of the genes however most of the changes were not statistical significant (Figure 58). On the other hand genes from the autophagy pathway showed more dramatic changes and were upregulated by miR-130b and their expression further increased by the miR-130b-CDDP treatment (Figure 59).

Figure 58. Effect of scL-miR-130b+CDDP combination treatment in the p53 pathway.

HEYA8 cells were transfected with 60 nM of miR-130b mimics or scrambled negative control (NC) using the nanoliposomal delivery system (scL). For the CDDP treatments, the transfection media was replaced by media containing 3 μM of CDDP. Samples were collected at different time points after transfection and gene expression was analyzed by q-PCR.

Figure 58

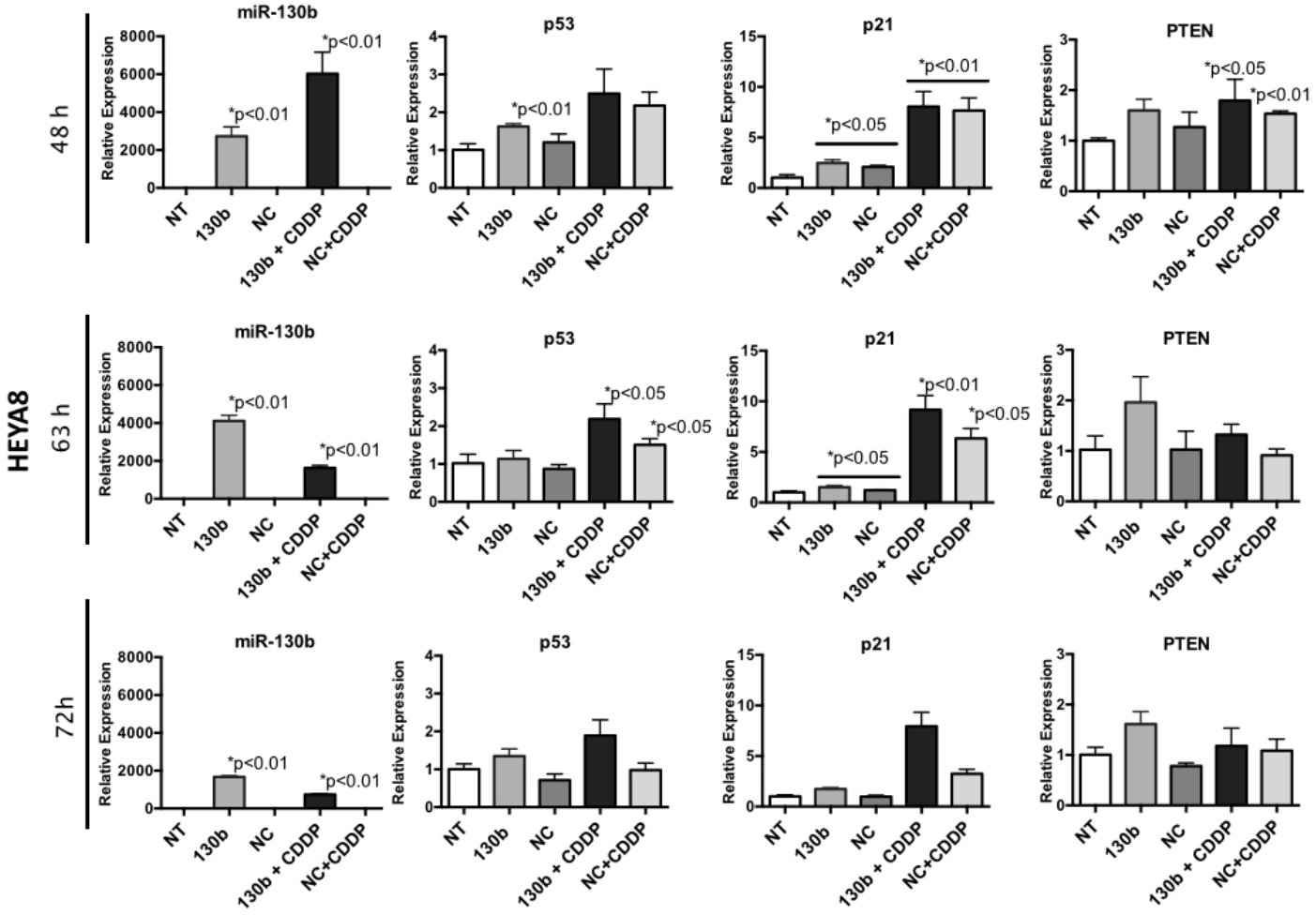
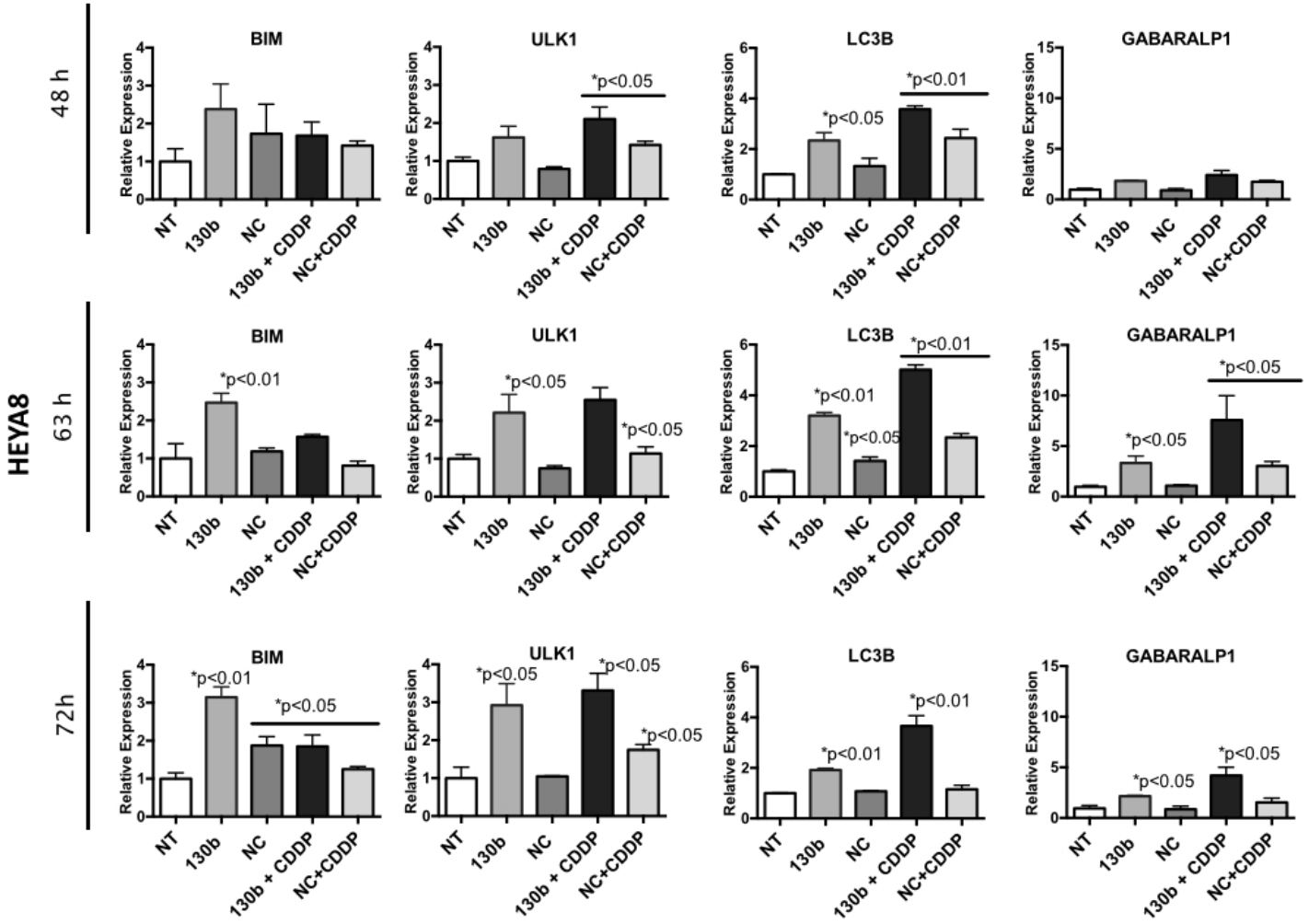

HEYA8
48 h
63 h
72h
miR-130b
p53
p21
PTEN
Relative Expression
*p<0.01
*p<0.05
NT
130b
NC
130b + CDDP
NC+CDDP

Figure 59. Effect of scL-miR-130b+CDDP combination treatment in the autophagy pathway.

HEYA8 cells were transfected with 60 nM of miR-130b mimics or scrambled negative control (NC) using the nanoliposomal delivery system (scL). For the CDDP treatments, the transfection media was replaced by media containing 3 μM of CDDP. Samples were collected at different time points after transfection and gene expression was analyzed by q-PCR.

Figure 59


HEYA8
48 h
63 h
72h
BIM
ULK1
LC3B
GABARALP1
Relative Expression
NT
130b
NC
130b + CDDP
NC+CDDP
*p<0.05
*p<0.01

In the OVCAR8 cell line the miR-130b + CDDP combination did not result in drug sensitization, all the treatments, NT, miR-130b and NC, responded similarly to CDDP and the higher percentage of apoptotic cells was observed in the NC+CDDP treatment indicating a non-specific effect (Figure 60).

I also analyzed samples from the *in vitro* experiments carried out by Dr. Chang using scL nanoliposomal delivery of miR-130b in combination with CDDP. Only one gene, PTEN, showed an increase in expression following scL-miR-130b treatment that was further increased by the scL-miR-130b + CDPP treatment. None of the other genes tested belonging to the p53 pathway (Figure 61) or the autophagy pathway were responsive to miR-130b or the combinational treatment (Figure 62). According with Dr. Chang results scL-miR-130b in combination with CDDP produced a strong phenotypic impact that killed about 50 % of the cells, 24 hours after treatment, and it is possible that by the time these samples were collected (between 48 and 74 hours after treatment) most of the cells were already dying which will make difficult to observed the molecular effect of miR-130b treatment.

Figure 60

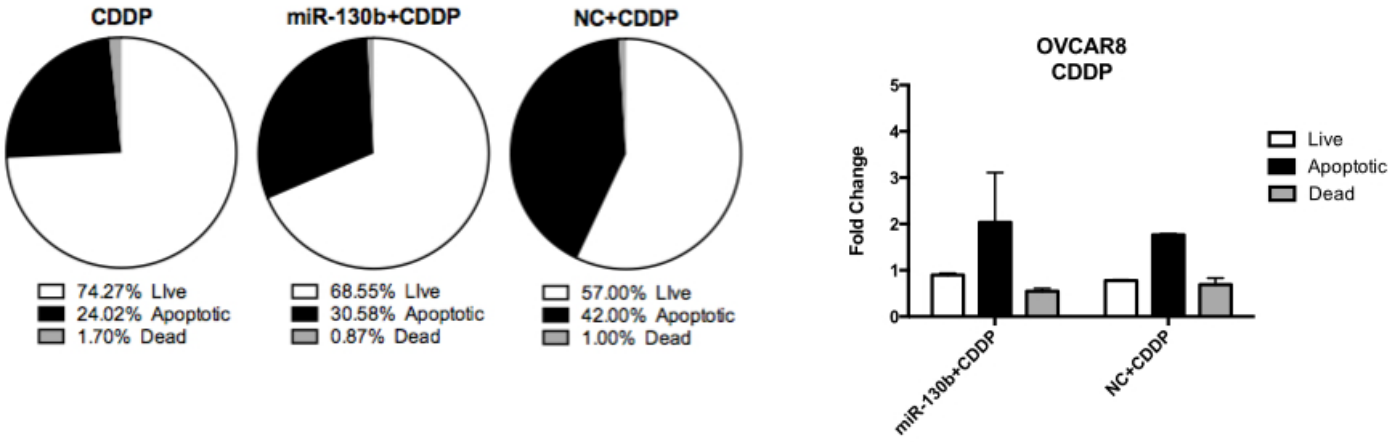


Figure 60. Effect of miR-130b on cisplatin sensitization in OVCAR8.

The ovarian cancer cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and 24 hours after transfection the media was replaced with media containing 5 µg/mL of CDDP. Cell viability was analyzed by FACS. (FACS experiments were done with the help of Sesha Duvvuri).

Figure 61 Effect of scL-miR-130b+CDDP combination treatment in the p53 pathway.

OVCAR8 cells were transfected with 60 nM of miR-130b mimics or scrambled negative control (NC) using the nanoliposomal delivery system (scL). For the CDDP treatments, the transfection media was replaced by media containing 3 μM of CDDP. Samples were collected at different time points after transfection and gene expression was analyzed by q-PCR.

Figure 61

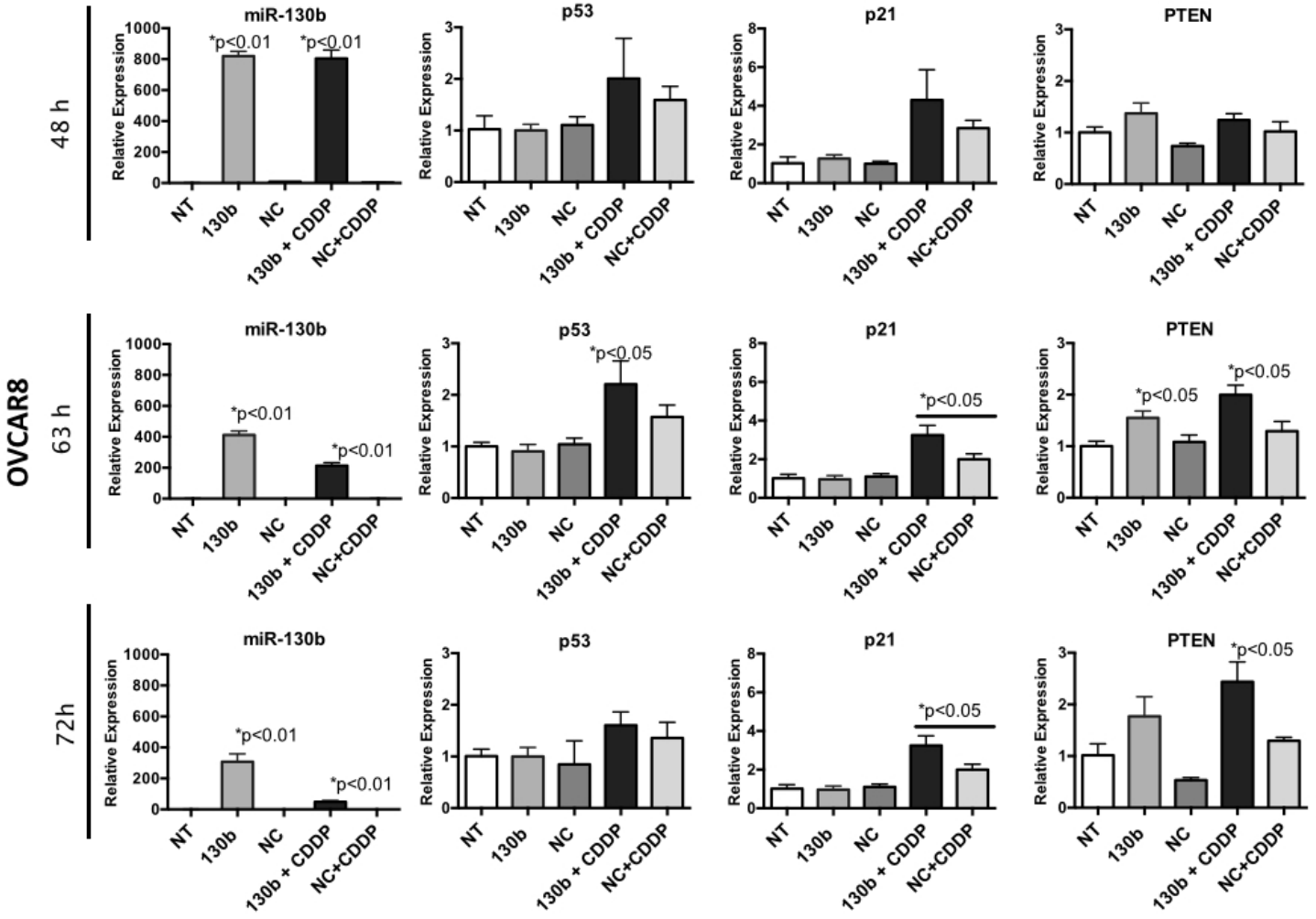
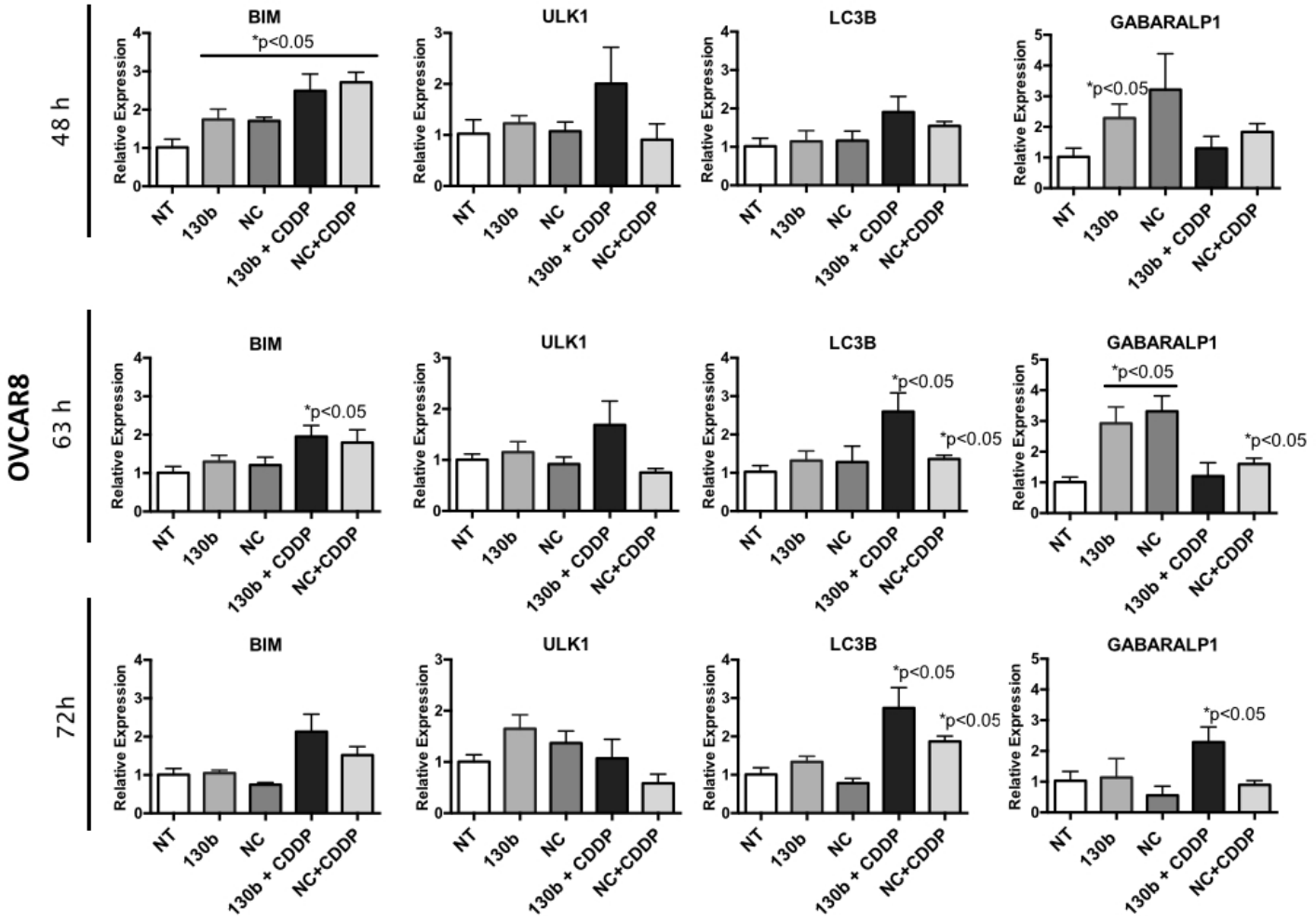

OVCAR8
48 h
63 h
72h
miR-130b
p53
p21
PTEN
Relative Expression
*p<0.01
*p<0.05
NT
130b
NC
130b + CDDP
NC+CDDP

Figure 62. Effect of scL-miR-130b+CDDP combination treatment in the autophagy pathway.

OVCAR8 cells were transfected with 60 nM of miR-130b mimics or scrambled negative control (NC) using the nanoliposomal delivery system (scL). For the CDDP treatments, the transfection media was replaced by media containing 3 μM of CDDP. Samples were collected at different time points after transfection and gene expression was analyzed by q-PCR.

Figure 62


OVCAR8
48 h
63 h
72h
BIM
ULK1
LC3B
GABARALP1
Relative Expression
*p<0.05
NT
130b
NC
130b + CDDP
NC+CDDP

MiR-130b sensitize ovarian cancer cells to the BH3 mimetic ABT-737

Overexpression of miR-130b significantly induced BIM protein levels in both HEYA8 and OVCAR8 cell lines, this result led me to the conclusion that BIM could be playing and important role in the tumor suppressor activities driven by miR-130b and that a stronger upregulation of BIM could potentially have a greater phonotypical impact in ovarian cancer. To test this hypothesis I used the small molecule ABT-737 to treat the ovarian cancer cell lines and assayed proliferation by MTS and cell viability by FACS.

ABT-737 is a small molecule inhibitor that mimics the function of BH3-only proteins (like BIM and BAD) because it binds to Bcl-xL and Bcl-2 with great affinity and disrupts Bcl-2/Bax associations thereby inducing apoptosis (Kang and Reynolds, 2009). It has been also shown that ABT-737 can activate other pro-apoptotic pathways such as JNK and promote autophagy, however the mechanisms behind these functions are not well understood (Konopleva et al., 2012). In ovarian cancer cell line models treatment with ABT-737 as a single agent is not effective in inhibiting cell viability, however it was found to sensitize the cells to carboplatin and other platinum based compounds (Simonin et al., 2013; Witham et al., 2007).

In the HEYA8 cell line MTS experiments with ABT-737 did not show any impact on proliferation when used alone, combination of miR-130b and ABT-737 reduced proliferation by 20% to 40 % at the 96 and 120 h time points respectively (Figure 63).

Figure 63

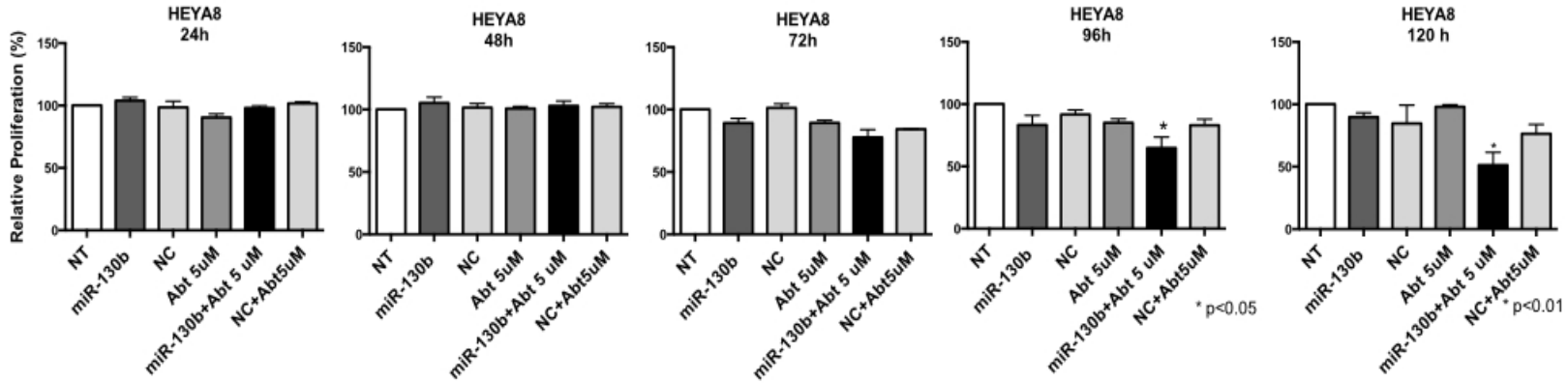


Figure 63. Effect of miR-130b-ABT-737 treatment on HEYA8 cell proliferation.

The ovarian cancer cell line HEYA8 was transiently transfected with miR-130b mimics or scrambled negative controls (NC) and 24 hours after transfection the media was replaced with media containing 5 μM of ABT-737. Cell proliferation was assayed by MTS for 5 days.

Cell viability was assayed by FACS 120 h after treatment with 3 μM and 5 μM of ABT-737. For the lower concentration the miR-130b-ABT-737 combination reduced the percentage of live cells from 81% on the NC-ABT-737 treated cells to 43%, and increased the ratio of apoptotic and dead cells by 3 fold with respect to the NC-ABT-737 treatment. There was no statistical difference between the ABT-737 and the NC-ABT-737 treatments.

In the higher ABT-737 concentration miR-130b was also able to sensitize the cells, however the difference between the miR treatment and the controls was not as dramatic as in the lower concentration experiments. Nevertheless cell viability was reduced by 35% and the percentage of apoptotic cells increased by 2 fold with respect to the NC-ABT-737 5 μM treatment (Figure 64). Similarly to the HEYA8 cell line OVCAR8 was not responsive to the ABT-737 treatment as single therapy, but the OVCAR8 cell line was more sensitive to the combinational therapy, showing up to 60 % reduction on cell proliferation assayed by MTS experiments (Figure 65). In the cell viability analysis the combinational therapy miR-130b-ABT-737 3 μM reduced the percentage of live cells by 78% compared with 23% cell viability reduction by the NC-ABT-737 3 μM treatment. Apoptotic and dead cells were increased by 2.5 and 5 fold compared with ABT-737 3 μM and NC-ABT-737 3 μM treatments.

In the higher concentration miR-130b was also able to sensitize to ABT however the difference between the treatments was not as dramatic as in the lower concentration experiment. Cell viability was reduced by 40% in the combinational therapy compare with the other treatments and apoptosis and cell death increased 2- and 3- fold (Figure 66).

Figure 64. MiR-130b sensitizes HEYA8 to ABT-737.

The ovarian cancer cell line HEYA8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and 24 hours after transfection the media was replaced with media containing 3 μM or 5 μM of ABT-737. Cell viability was analyzed by FACS. (FACS experiments were done with the help of Sesha Duvvuri).

Figure 64

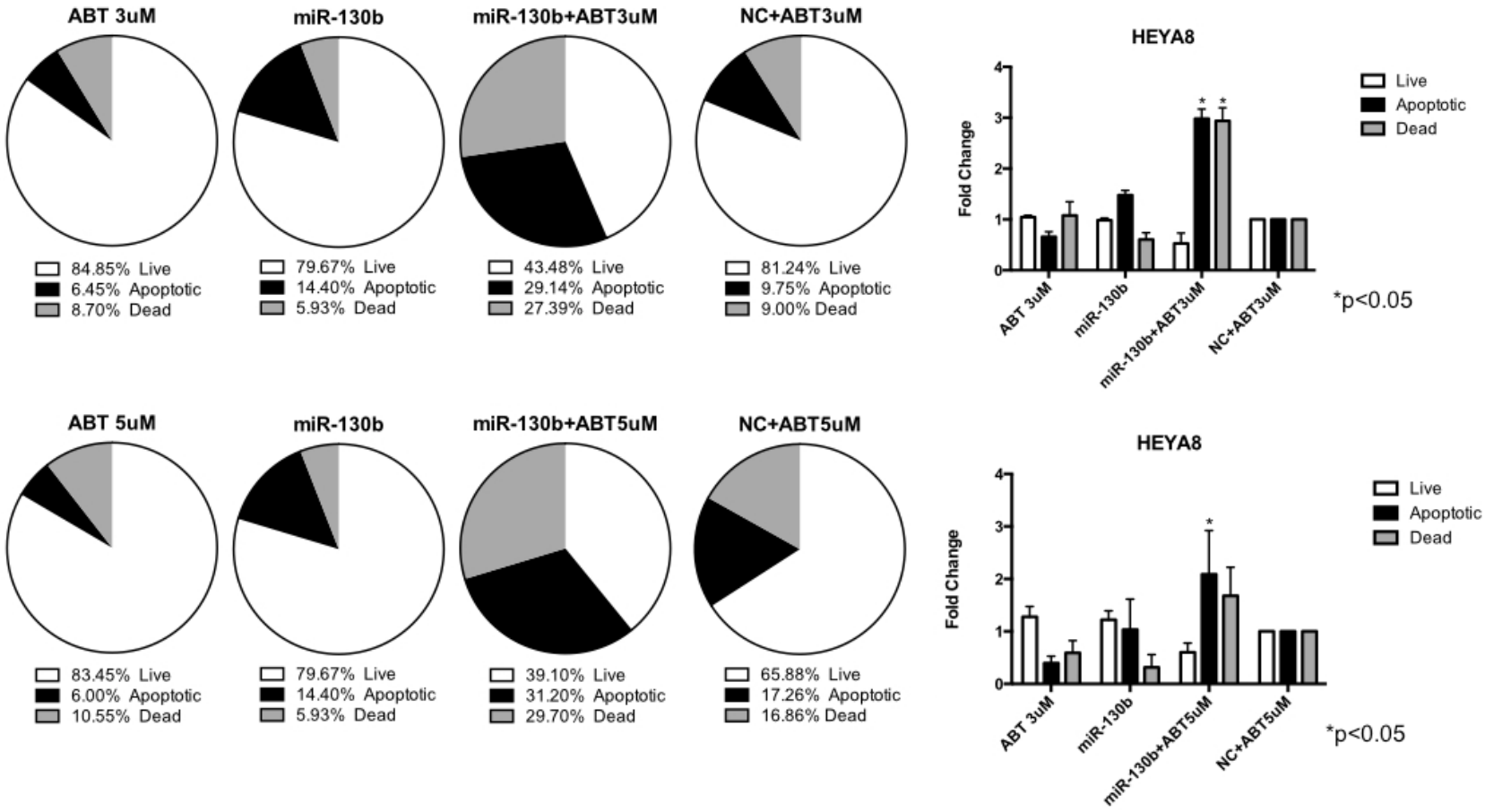

ABT 3uM
84.85% Live
6.45% Apoptotic
8.70% Dead
miR-130b
79.67% Live
14.40% Apoptotic
5.93% Dead
miR-130b+ABT3uM
43.48% Live
29.14% Apoptotic
27.39% Dead
NC+ABT3uM
81.24% Live
9.75% Apoptotic
9.00% Dead
HEYA8
Fold Change
Live
Apoptotic
Dead
ABT 3uM
miR-130b
miR-130b+ABT3uM
NC+ABT3uM
*p<0.05
ABT 5uM
83.45% Live
6.00% Apoptotic
10.55% Dead
miR-130b
79.67% Live
14.40% Apoptotic
5.93% Dead
miR-130b+ABT5uM
39.10% Live
31.20% Apoptotic
29.70% Dead
NC+ABT5uM
65.88% Live
17.26% Apoptotic
16.86% Dead
HEYA8
Fold Change
Live
Apoptotic
Dead
ABT 3uM
miR-130b
miR-130b+ABT5uM
NC+ABT5uM
*p<0.05

Figure 65

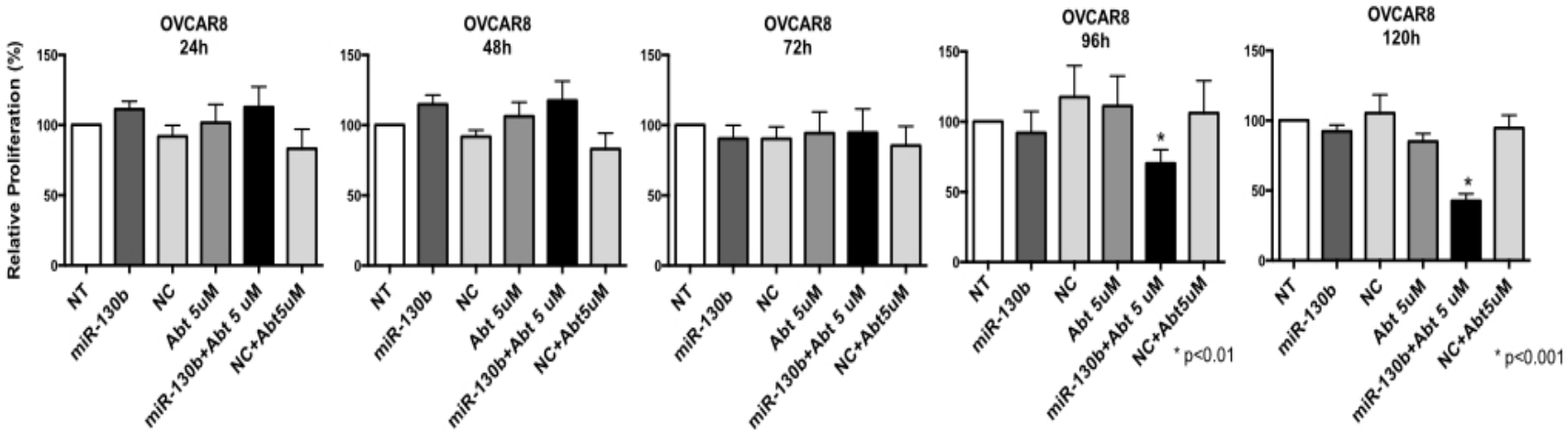


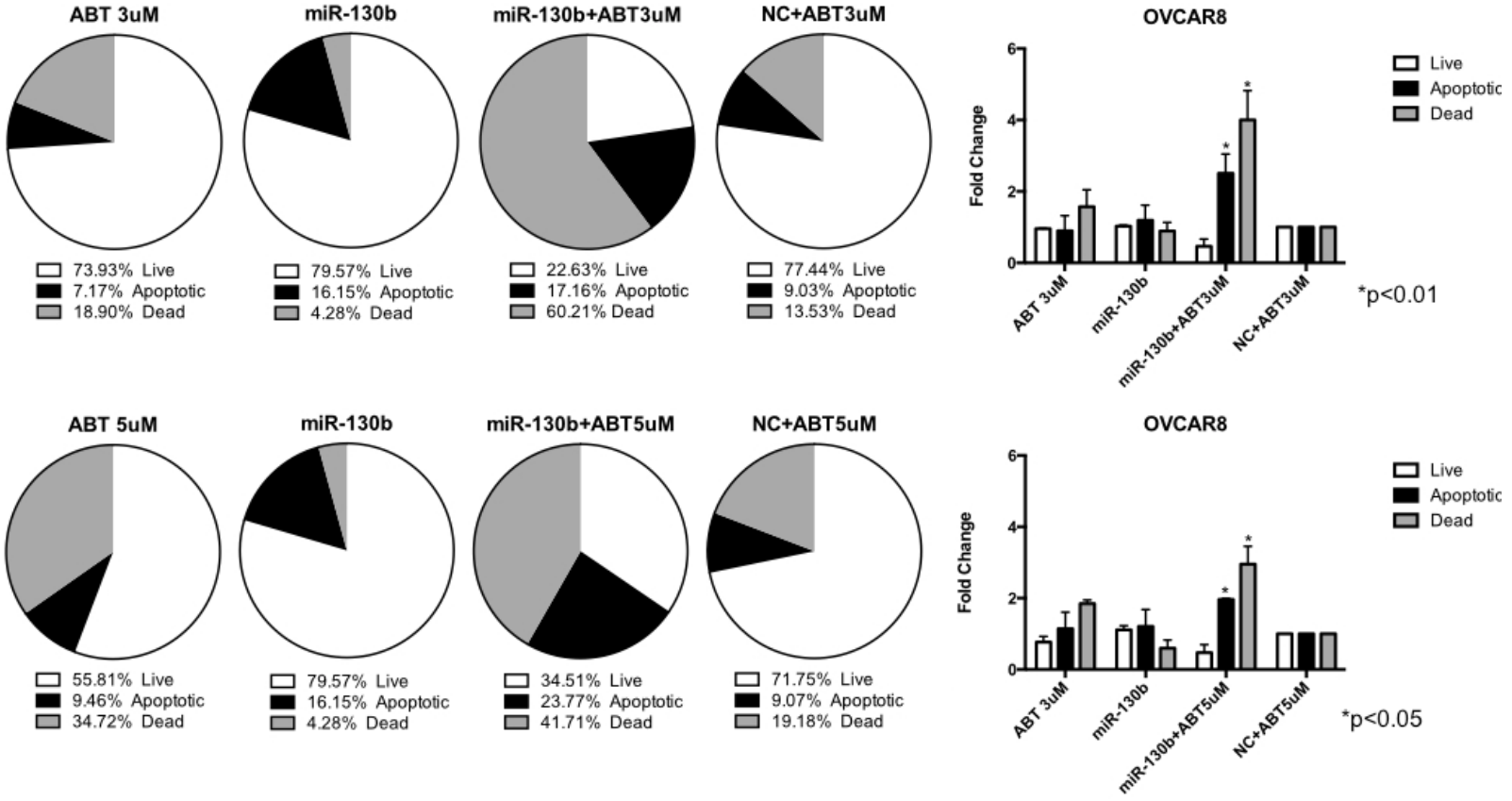
Figure 65. Effect of miR-130b-ABT-737 treatment on OVCAR8 cell proliferation.

The ovarian cancer cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative controls (NC) and 24 hours after transfection the media was replaced with media containing 5 μM of ABT-737. Cell proliferation was assayed by MTS for 5 days.

Figure 66. MiR-130b sensitizes OVCAR8 to ABT-737.

The ovarian cancer cell line OVCAR8 was transiently transfected with miR-130b mimics or scrambled negative control (NC) and 24 hours after transfection the media was replaced with media containing 3 μM or 5 μM of ABT-737. Cell viability was analyzed by FACS, (FACS experiments were done with the help of Sesha Duvvuri).

Figure 66


ABT 3uM
73.93% Live
7.17% Apoptotic
18.90% Dead
miR-130b
79.57% Live
16.15% Apoptotic
4.28% Dead
miR-130b+ABT3uM
22.63% Live
17.16% Apoptotic
60.21% Dead
NC+ABT3uM
77.44% Live
9.03% Apoptotic
13.53% Dead
OVCAR8
Fold Change
Live
Apoptotic
Dead
ABT 3uM
miR-130b
miR-130b+ABT3uM
NC+ABT3uM
*p<0.01
ABT 5uM
55.81% Live
9.46% Apoptotic
34.72% Dead
miR-130b
79.57% Live
16.15% Apoptotic
4.28% Dead
miR-130b+ABT5uM
34.51% Live
23.77% Apoptotic
41.71% Dead
NC+ABT5uM
71.75% Live
9.07% Apoptotic
19.18% Dead
OVCAR8
Fold Change
Live
Apoptotic
Dead
ABT 3uM
miR-130b
miR-130b+ABT5uM
NC+ABT5uM
*p<0.05

Discussions and Conclusions

In this chapter I explored the tumor suppressor mechanisms of miR-130b using as model ovarian cancer cell lines with different p53 genotypes, p53 WT HEYA8 and p53 mutant OVCAR8. Studies of the function of miRNAs under different p53 genotypes are especially important for ovarian cancer since most of the tumors present mutations in this gene (Cancer Genome Atlas Research Network, 2011).

Induction of apoptosis was a common effect of miR-130b upregulation in both cell lines, however due to the difference in the p53 status I expected to find diverse molecular mechanisms behind this phenotype. In p53 WT HEYA8 miR-130b significantly upregulates p53 and its downstream targets p21 and BIM and more moderately other known tumor suppressors such as RB1, PTEN and p27.

In the p53 mutant OVCAR8 cell line miR-130b had no effect on the expression of p53 or p21, however BIM was highly upregulated. The most striking effect is the induction of TAp63, which is typically expressed at negligible levels in both cell lines. This is a significant finding because p63 a family member of p53 has been shown to be able to compensate for loss or mutant p53 (Kenzelmann Broz et al., 2013). Our data suggests that miR-130b driven upregulation of TAp63 is a critical step in the activation of tumor suppressor programs that are used by this miRNA to impact cell viability. On the other hand downregulation of ΔNp63 by miR-130b in HEAY8 suggests that ΔNp63 may be playing an oncogenic role in this cell line, and upregulation of TAp63 leads to the downregulation of ΔNp63. This complex regulation among p63 isoforms has been previously described (Lu, 2010).

Previous reports have shown that miR-130b is a direct target of TAp63 and in our model miR-130b drives TAp63 upregulation, so I hypothesized that miR-130b and TAp63 are part of a feed forward loop in which miR-130b upregulates TAp63 and TAp63 feeds back by upregulating miR-130b, however the levels of pre-miR-130b were not induced after forced expression of TAp63 in HEYA8 and OVCAR8, suggesting that TAp63 does not induce the transcription of miR-130b. In the HEYA8 cell line the mature miR-130b levels were modestly increased (2 fold) by forced expression of TAp63 whereas in the OVCAR8 mature miR-130b levels were slightly upregulated by TAp63 (2.5 fold). There is previous evidence to indicate that mature miRNA levels can also be regulated at the pre-miRNA processing stage (Davis and Hata, 2009). It is possible that TAp63 and/or ΔNp63 play similar roles regulating miRNA biogenesis and/or stability in fact each of those isoforms have been shown to impact the levels of miRNAs globally by regulating Dicer and DGCR8 respectively (Chakravarti et al., 2014; Su et al., 2010).

Induction of BIM is the second most significant effect in miR-130b treated cells. Previous reports have shown that TAp63 can activate the mitochondrial apoptosis pathway by directly transactivating pro-apoptotic Blc-2 family members including BIM and BAX (Gressner et al., 2005), aside from their pro-apoptotic functions BIM and p63 have been linked to autophagy induction (Delgado and Tesfaigzi, 2013; Huang et al., 2012; Huang et al., 2012) and when tested in the OVCAR8 cell line miR-130b was able to induce autophagy core genes as well as to increase autophagic flux, although induction of the genes seems to be BIM independent. The role of autophagy in cancer is controversial, since it can act as survival strategy especially to promote chemotherapy

resistance and it can be tumor suppressive depending on the cellular context (Levy and Thorburn, 2011; Wilkinson and Ryan, 2010). In other studies of ovarian cancer, induction of autophagy has been linked to cisplatin and FTY720 resistance (Wang and Wu, 2014; Zhang et al., 2010) but other reports indicate that constant induction of autophagy leads to increased apoptosis and cell death (Levy and Thorburn, 2011). In our model of ovarian cancer further investigation is needed to determine if the autophagy induction is pro- or anti-cancer.

The autophagy pathway was not induced by miR-130b in the HEYA8 cell line, suggesting that the phenotype is dependent on the genetic background. Autophagy induction is known to be reduced by mutations in the oncogene BRAF (Armstrong et al., 2011) and the HEYA8 cell line has been reported to carry mutations in BRAF (Domcke et al., 2013).

Due to their ability to regulate hundreds of genes miRNAs can have wide-spread effects on gene networks to strongly impact biological processes in ovarian and other cancers. However this feature makes difficult to easily identify critical downstream targets, and understand all the connections and interactions behind miRNAs actions could be complicated. At the same time miRNAs can reveal new druggable pathways and targets and themselves have potential to be develop in the future as therapeutics agents for ovarian and other cancers.

In this study the expression of TAp63 and BIM was enhanced by miR-130b in both p53 WT and mutant cell lines, suggesting that they are playing a crucial role in the tumor suppressor activities driven by miR-130b. I decided to test these targets using

different approaches, an expression vector to overexpress p63 and BH3 mimetics. Overexpression of TAp63 reduced cell proliferation and increased apoptosis in both HEYA8 and OVCAR8, this result is congruent with other reports that have shown that TAp63 is a potent tumor suppressor (Su et al., 2013), this is to our knowledge the first time that TAp63 tumor suppressor activities are reported in ovarian cancer. Surprisingly although ΔNp63 was downregulated in response to miR-130b it is also able to induce apoptosis and reduced the proliferation of ovarian cancer cells. The ΔNp63 isoform is generally classified as an oncogenic, due to its ability to act as a dominant negative on p53, TAp63 and TAp73 (Murray-Zmijewski et al., 2006). There are reports in which ΔNp63 is described as tumor suppressor through the activation of ATM and phosphorylation of p53 to avoid malignant transformation of skin cells (Craig et al., 2010). ΔNp63 has been shown to be able to upregulate miRNAs such as miR-205 that targets EMT genes to attenuate migration and invasion in prostate cancer (Tucci et al., 2012) and activation of the tumor suppressor caspase 1 in osteosarcoma (Celardo et al., 2013).

In the p53 mutant OVCAR8 the impact of ΔNp63 on apoptosis induction was greater than the one induced by TAp63. Both of the cell lines showed a significant reduction on cell proliferation and subtle apoptosis induction, suggesting that TAp63 and ΔNp63 maybe inducing apoptosis and cell cycle arrest.

Most of the patients with ovarian cancer will develop tumors that are resistant to current drug therapies (Bast and Markman, 2010), therefore treatments that can sensitize tumors can be of great use to increase the response and reduce secondary effects. I tested

the effect of miR-130b overexpression on sensitizing the ovarian cancer cell lines to 2 different drugs, cisplatin (CDDP) and the BH3 mimetic ABT-737. MiR-130b was able to sensitize HEYA8 to CDDP however the effect was modest. The OVCAR8 cell line did not respond to the miR-130b-CDDP treatment. By contrast, miR-130b in combination with the BH3 mimetic ABT-737 showed efficiency in inducing apoptosis in both cell lines.

Upregulation of BIM was a common to both HEAY8 and OVCAR8 upon miR-130b treatment, suggesting than BIM is a critical downstream effector of miR-130b regardless of p53 status. The small molecule ABT-737 is a BH3 mimetic that mimics the function of proteins from the BH3 only family, including BIM. Ovarian cancer cells are not responsive to ABT-737, however it has been reported that ABT-737 can sensitize ovarian cancer to carboplatin and other platinum compounds (Jain et al., 2014; Simonin et al., 2013). The combination therapy miR-130b-ABT-737 had a strong effect inducing apoptosis in both cell lines, with greater apoptosis and cell death on OVCAR8.

Taking together this results suggest that miR-130b has potential as tumor suppressor miRNA. Research from our collaborators has shown promising results on animal models (Dr. Esther Chang, unpublished data). The molecular pathways activated by miR-130b are context dependent, however miR-130b is able to induce apoptosis in both p53 WT and mutant ovarian cancer cell lines, and seems to have a strong connection with p53 family members, specially TAp63, to act as tumor suppressor, and to be able to sensitize to the small molecule ABT-737.

The use of miRNAss is still controversial due to possible off-target effects, and the technical challenges for efficient RNA delivery, but efforts to bring miRNAs to the clinic are being made and currently the first potential therapeutic miRNA, miR-34 is undergoing clinical phase I (Bader, 2012). In collaboration with Dr. Esther Chang and SynerGene Therapeutics Inc., the FDA approved nanoparticle, that is been currently used to delivery p53 in a phase 2 clinical trial (Senzer et al., 2013), has been used to deliver miR-130b in ovarian cancer cell lines and *in vivo* models in combination with cisplatin with promising results. We envision to use this delivery system to translate the therapeutic benefits of miR-130 to the clinic, and to expand the currently p53 based gene therapy to include TAp63.

Despite of the challenges intrinsic to miRNAs, they can still be a powerful tool in the discovery of key pathways or genes that can be targeted by conventional therapies. In this study I described the tumor suppressor and drug sensitization activities of miR-130b, and uncovered TAp63 and ΔNp63 isoforms as a potent tumor suppressors for ovarian cancer regardless of p53 status. I also proved that miR-130b can be used to sensitize cancer cell to cisplatin and Bh3 only mimetics, through these discoveries I have increased our general understanding on the pathways used by mR-130b in ovarian cancer and its potential as tumor suppressor microRNA

CHAPTER 6: GENERAL CONCLUSION

Ovarian cancer is one of the most lethal gynecological malignancies and its heterogeneity represents a challenge for the scientific and medical communities as we try to understand the biology of ovarian cancer development to better diagnose and treat patients.

The data sets from the TCGA project are a valuable source of genomic data and through the different bioinformatics and validation analysis the scientific community is helping to reach the goal of the project to improve the ability to diagnose, treat and prevent cancer.

The present work shed light into the biological pathways and molecular mechanisms regulated by 3 ”tumor suppressor” microRNAs, miR-29a, miR-509-3p and miR-130b in ovarian cancer cells with different p53 genotypes. These miRNAs proved to be able to regulate biological pathways that are critical for cancer development, maintenance and metastasis, such as methylation, p53, EMT and drug sensitization. Regardless of the controversy behind the use of miRNAs as therapeutic agents and the intrinsic problems of RNA delivery the study of miRNAs contributes toward a better understanding of the biology of cancer, and are powerful tools to identify pathways and genes that can be targeted by conventional therapies. The contributions of this work provide a precedent for the study of miRNAs functions and targeted pathways in cancer using the TCGA datasets, as well as 3 putative tumor suppressor miRNAs with potential for clinical applications.

FUTURE DIRECTIONS

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancies. The high mortality rate has been attributed to multiple factors including the fact that more than 75% of patients present with advanced stage of the disease at the time of diagnosis and secondly the high rate of relapse with chemoresistant tumors, because of that understanding the mechanism of metastasis and chemotherapy resistant is crucial to develop new therapeutic strategies.

The process of ovarian cancer metastasis can be divided in 4 general stages (Shield et al., 2009):

1) Detachment of malignant cell from the primary tumor,
2) Dissemination of the cells throughout the abdominal cavity
3) Adhesion and Disaggregation
4) Invasion

During the dissemination stage the cells have the tendency to form spheroid-like structures, which are resistant to chemotherapy (L'Esperance et al., 2008; Makhija et al., 1999) and express stem cell markers (Condello et al., 2014). Understanding the biology of spheroid formation in EOC can contribute to the identification of new druggable targets to treat metastatic and chemo resistant disease.

For my thesis work I have identified miR-29a, miR-509-3p and miR-130b as strong tumor suppressors of ovarian cancer. Focusing much of my work on miR-130b I also uncovered the p53 family member TAp63 and its downstream target BIM as critical downstream effectors of miR-130b-mediated tumor suppression. In order to extend this

work to address the two most important factors hindering progress in the successful treatment of ovarian cancer I see two key directions for future studies. Firstly, to address the issue of chemotherapy resistance a full understanding of the genes and pathways downstream of miR-130b/TAp63/BIM is necessary to identify new druggable targets and new therapeutic modalities. Secondly, a better understanding of the impact of miR-130b on migration and invasion and therefore metastatic spread of ovarian cancer warrants functional studies using the 3D models of ovarian cancer that I assisted in establishing in our laboratory (Figure 67).

Identification of critical effectors of miR-130b/TAp63 axis in preventing spheroid formation

In the 2D model miR-130b induced the expression of TAp63 and when TAp63 expression was further increased with the use of an expression vector it inhibited cell proliferation and increased apoptosis in HEYA8 p53 WT and OVCAR8 p53 mutant cell lines. The molecular mechanisms downstream TAp63 are cell-dependent (Su et al., 2013) and to my knowledge they have not been described in the context of ovarian cancer. I hypothesized that TAp63 will inhibit spheroid formation by inducing apoptosis and cell cycle arrest as well as other molecular pathways that may be specific for each p53 genetic background. The discovery of alternative molecular pathways that can efficiently inhibit tumor progression in p53 mutant conditions is critical to design new therapeutic strategies for ovarian cancer patients, since 96% of the patients present with mutations or loss of p53 (Cancer Genome Atlas Research Network, 2011), and current drugs largely rely in p53 to exert their functions (Offer et al., 2002). I propose to overexpress miR-

130b/TAp63 in HEYA8 and OVCAR8 cells and then form spheroids with those cells and measure spheroid morphology. I expect miR-130b/TAp63 to inhibit the formation of spheroids and to activate downstream molecular pathways in the OVCAR8 p53 mutant cells that can lead us to new druggable targets. Analyses of the signaling pathways and networks activated by miR-130b/TAp63 can be done using RPPA (reverse phase protein arrays).

Role of miR-130b induced Autophagy in EOC spheroids drug response

Cancer spheroids are more chemoresistance than their 2D counterparts (Makhija et al., 1999). MiR-130b sensitized HEYA8 to cisplatin and HEYA8 and OVCAR8 to the BH3 mimetic ABT-737. MiR-130b also induced the activation of the autophagy program, whose upregulation has been linked to chemoresistance but it has also been described as tumor suppressor pathway(Levy and Thorburn, 2011; Zhang et al., 2010). I propose to use siRNAs against ATG5 and ULK1 (core genes of the autophagic program) in co-transfection with miR-130b in HEYA8 and OVCAR8 cells and then form spheroids and treat them with cisplatin or ABT-737 to measure spheroid formation and cell viability by annexinV staining. I expect the Spheroids to be more resistant to both drugs than the 2D cultures and a titration experiment needs to be done for the drugs before starting the combination of treatments. I also expect miR-130b to induce autophagy, if autophagy is playing a tumor protective role, the treatments with miR-130b + ATG5/ULK1 siRNA + drug should show inhibition of spheroid formation and/or reduction of cell viability compare with the miR-130b + drug treatment, on the other hand if the autophagy pathway is acting as tumor suppressive the treatments with miR-130b + ATG5/ULK1

siRNA + drug should show spheroid formation and/or cell viability levels close to the ones present in the drug alone treatments.

MiR-130b as an inhibitor of adhesion/migration of ovarian cancer spheroids

During the process of EOC metastasis the spheroids can attach and spread by interacting with multiple ECM proteins associated with the mesothelium and underlying basement including, integrins, fibronectin, and collagens (Burleson et al., 2004; Burleson et al., 2004; Burleson et al., 2006). Work form our lab has shown that miR-130b can impact migration and invasion of ovarian cancer cells and other research groups have shown that miR-130b can regulate negatively regulate ZEB1, a driver of the EMT program (Dong et al., 2013). In pancreatic cancer miR-130b inhibits invasion by targeting STAT3 (Zhao et al., 2013); in colorectal cancer Zhao et al demonstrated that miR-130b inhibits migration by downregulation of integrin β1 (Zhao et al., 2014) I want to explore the role of miR-130b in the process of spheroid attachment and migration in the HEYA8 and OVCAR8 cell lines using the mesothelial clearance assay, a technique that mimics the interaction between the spheroid and the mesothelial layer, mesothelial cells expressing GFP are used and the area of cells displaced by the spheroids can be measured by microscopy (Iwanicki et al., 2011). I propose to overexpress miR-130b in the cell lines and form spheroids that will be use for the mesothelial clearance assay. I expect the HEYA8 and OVCA8 spheroids overexpressing miR-130b to have a reduce rate of mesothelial clearance compare with the control spheroids. At the molecular level I expect to find key EMT genes such as TWIST, ZEB1 and SNAI1, which play important roles in the process of spheroid formation in ovarian cancer (Davidowitz et al., 2014) to

be downregulated in the spheroids overexpressing miR-130b. With this set of experiments I expect to increase our understanding of the molecular pathways underlying the metastatic mechanism in ovarian cancer and to find new druggable target downstream of the miR-130b/TAp63 axis, that can be develop for the treatment of chemoresistant metastatic ovarian and other cancers.

Figure 67

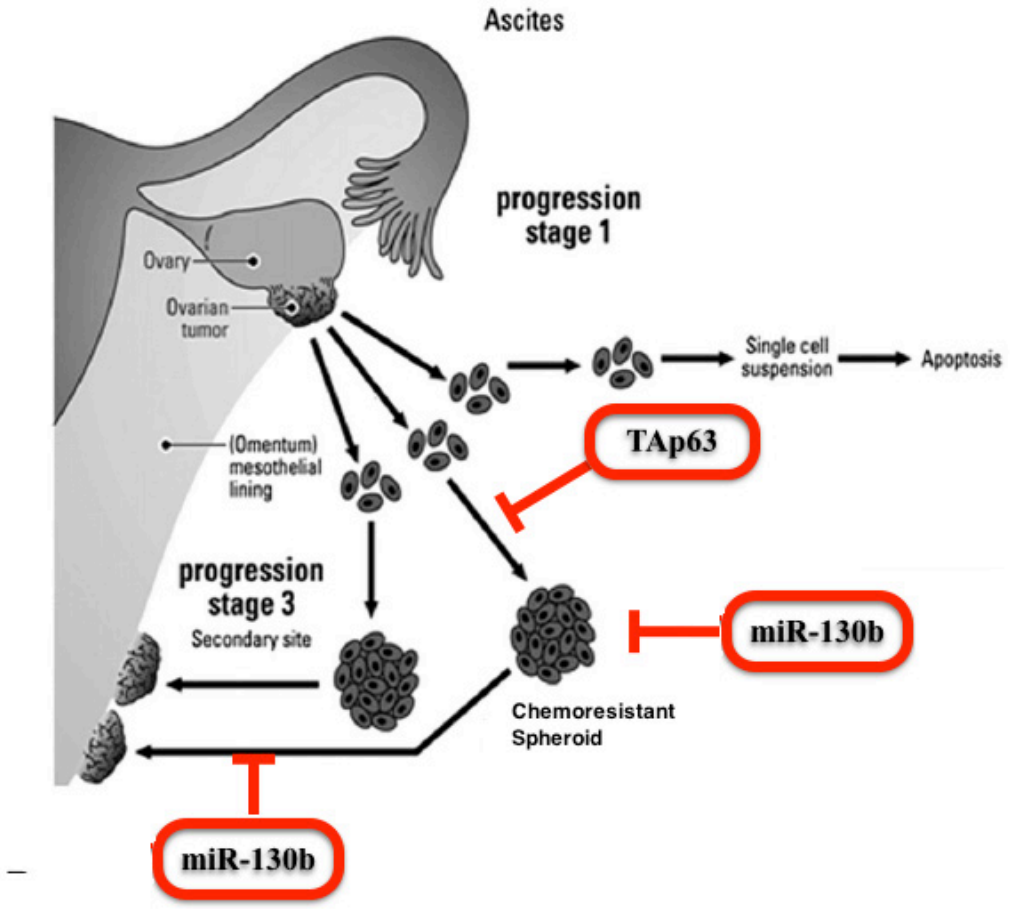


Figure 67. Model of Ovarian cancer progression showing the steps target by TAp63, miR-130b and miR-29a.

Adapted from (Ahmed et al., 2007)

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