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DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Role of AKT–regulated miRNAs in Non Small Cell Lung Cancer (NSCLC)

MAFALDA RAQUEL DA CONCEIÇÃO SANTOS

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professora Giuseppe Viglietto (Università degli studi Magna Graecia di Catanzaro) e sob co-orientação da Professora Paula Veríssimo (Universidade de Coimbra)

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2012

“O único lugar em que o sucesso vem

primeiro que o trabalho é no dicionário...”

“The only place where success comes

first than work is in the disctionary...”

Albert Einstein

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Abstract

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008 globally, accounting for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in 2008. Non-small cell lung cancer (NSCLC) is the most common form of lung cancer, being is divided into three major histological subtypes, adenocarcinoma, squamous cell carcinoma and large cell carcinoma.

Accumulating evidence shows that miRNAs are grossly deregulated in NSCLC. Recent studies have shown that not only can miRNAs be used to sub-classify NSCLCs but specific miRNA profiles may also predict prognosis and disease recurrence in early-stage NSCLCs.

miRNAs are single‑stranded RNAs (ssRNAs) of approximately 22 nucleotides in length that are generated from endogenous hairpin‑shaped transcripts. They have become known as key post-transcriptional regulators of gene expression and have revolutionized the comprehension of the post-transcriptional regulation of gene expression. miRNAs have a big impact on shaping transcriptomes and proteomes as they participate in the regulation of every cellular process investigated and changes in their expression are associated with many human pathologies, such as cancer. The over-expression of hsa-miR-196a, in particular, has been associated with different types of tumour, however there are some reports indicating that it can also act as a tumour suppressor. The role of this miRNA in tumorigenesis is not clear, and data in literature indicates that the effects may be cell-type specific.

Previously to the beginning of this thesis project hsa-miR-196a showed to be over-expressed in 29 samples of lung tumours, and this over-expression was also confirmed in cell lines derived from different lung cancer histotypes. NCI-H460 interfered for AKT1 and its infection control SCR also demonstrated a differential expression of this miRNA and highest levels of expression were found in NCI-H460 SCR. In this project, there were created four cell lines in order to evaluate the biological effect of hsa-miR-196a and to correlate it to the PI3K/AKT pathway.

There were chosen 3 predicted targets for hsa-miR-196a whose expression has proven to be inhibited by the PI3K/AKT pathway: FoxO1, FoxO3 and p27. Only Foxo1 and p27 show a reduced luciferase activity indicating the binding of the miRNA to the 3'UTR. mRNA expression levels all genes did not seem to be affected by the over-expression or knock-down of hsa-miR-196a1 but the protein levels of FoxO1 and p27 were reduced in cells expressing the hsa-miR-196a1 vector and increased in cells expressing the anti-miR-196a vector, indicating a inhibition of protein synthesis of these genes by the miRNA in study.

Using this cellular system is possible not only to see the effects of the miRNA in study but also to correlate the results obtained to the PI3K/AKT pathway.

The results obtained point to regulation of proliferation and migration by this miRNA as its over-expression promotes these processes and the knock-down of its expression has a negative effect on them. At least in part the observed effects are due to the differential regulation of genes inhibited by the activation of PI3K/AKT pathway.

Keywords: miRNA, miR-196a, PI3K/AKT, Proliferation, Migration, Tumour induction *in vivo*.

Resumo

O Cancro é a maior causa de morte em países desenvolvidos e a segunda em países em desenvolvimento. O cancro do pulmão, em específico, foi a forma de cancro mais comum diagnosticada assim como a maior causa de morte por cancro em pessoas do sexo masculino em 2008, contando com 13% (1,6 milhões) de casos totais e 18% (1,4 milhões) de mortes. O cancro do pulmão de não-pequenas células é a forma mais comum de cancro do pulmão, sendo dividido em três subtipos histológicos, adenocarcinoma, carcinoma de células escamosas e carcinoma de grandes células.

Um acúmulo de evidências revela que os miRNAs estão bastante desregulados em cancro do pulmão de não-pequenas células. Estudos recentes, mostram que os miRNAs podem ser utilizados para descriminar entre os diversos histótipos de cancro e que a caracterização de miRNAs expressos pode ser útil na previsão de prognósticos e recorrência em fases iniciais de cancro do pulmão de não-pequenas células.

Os miRNAs são RNAs de cadeia simples com aproximadamente 22 nucleótidos que são gerados de transcritos com forma de hairpin. São considerados reguladores pós-transcripcionais da expressão génica e têm revolucionado a compreensão da regulação pós-transcripcional, tendo um grande impacto em modulação de transcriptomas e proteomas, uma vez que influenciam vários processos celulares já investigados. Mudanças nos seus padrões de expressão estão associadas a patologias humanas, como o cancro.

A sobre-expressão do hsa-miR-19611, em particular, foi associada com diferentes tipos de cancro, contudo existem artigos que indicam que este miRNA pode actuar como um onco-supressor. O papel deste miRNA na tumorigénese não é claro e os dados presentes na literatura parecem ser específicos para cada linha celular.

Antes do início deste projecto, verificou-se a sobre-expressão do hsa-miR-196a1 em 29 amostras de tumores de pulmão e em linhas celulares de diversos histótipos de cancro do pulmão. NCI-H460 interferidas para AKT1 e o seu controlo, SCR, também demonstraram uma expressão diferencial deste miRNA e os níveis mais altos eram expressos em NCI-H460 SCR. Neste projecto foram criadas quatro linhas celulares de modo a avaliar o efeito biológico do hsa-miR-196a e relacioná-lo com a via PI3K/AKT.

Foram escolhidos 3 alvos previstos para o hsa-miR-196a1 cuja expressão é negativamente regulada pela via PI3K/AKT: FoxO1, FoxO3 and p27. Apenas FoxO1 e p27 mostraram uma reduzida actividade da luciferase, evidenciando a ligação do miR-196a aos seus 3'UTR. Os níveis de expressão de todos os mRNAs não se mostraram alterados, mas os níveis proteicos de FoxO1 e p27 mostraram-se reduzidos em células que expressam o vector contendo o hsa-miR-196a e aumentados em células que expressam o vector contendo o anti-miR-196a, indicando uma modulação da síntese proteica destes genes pelo miRNA em estudo.

Utilizando este sistema celular é possível observar os efeitos deste miRNA e também relacionar aos resultados obtidos à via PI3K /AKT. Os resultados obtidos apontam para uma regulação da proliferação e migração por este miRNA e a sua sobre – expressão promove estes processos e o seu knockdown tem um efeito negativo nos mesmos. Pelo menos parte dos efeitos observados são devidos à regulação diferencial dos genes inibidos pela activação da via PI3K/AKT,

Palavras-chave: miRNA, miR-196a, PI3K/AKT, Proliferação, Migração, Indução de tumores *in vivo*.

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Most relevant abbreviations and Symbols used

~ - Approximately

ºC – Celsius degrees

% - Percentage

ac-pre-miRNA – AGO-cleaved precursor miRNA

Ago – Argonaute

AKT – Serine-threonine protein kinase

bp – Base pairs

C. elegans - *Caenorhabditis elegans*

dsRNA - Double-stranded RNA

endo-siRNAs/ esiRNA - Endogenous small interfering RNA

EXP5 – Exportin 5

DGCR8 – DiGeorge syndrome critical region gene 8

g - Gram

GW182 – Glicine-tryptophan repeat-containing protein kinase of 182 kDa

GPCR – G protein-coupled receptor

kDa – kilo Daltons

µl – Microliter

µg – Microgram

mg – Milligram

ml – Millilitre

mM – Millimolar

M - Molar

miR-196a1 – hsa-miR-196a

miRNA – microRNAs

miRISC - miRNA-induced silencing complex

mRNA – Messenger RNA

nt – Nucleotides

P-bodies - Processing-bodies

PCR – Polymerase chain reaction

PI3K – Phosphoinositide 3-kinase

PIP2 - Phosphatidylinositol -4, 5-biphosphate

PIP3 – Phosphatidylinositol -3, 4, 5-triphosphate

piRNA - Piwi-interacting RNA

pre-miRNA – Precursor miRNA

pri-miRNAs – Primary miRNA

PolII – RNA polymerase II

PTEN - Phosphatase and tensin homolog

RBP - RNA-binding protein

RLC – RISC loading complex

RNA – Ribonucleic Acid

RNaseIII – RNA polymerase III

rpm – Rotations per minute

RTK – Tyrosine kinase receptor

shRNA – short hairpin RNA

snoRNA – small nucleolar RNA

ssRNAs - Single‑stranded RNA

TRBP – TAR RNA-binding protein

tRNA – Transfer RNA

UTR – Untranslated region

State of the art

I.1. microRNAs

The first small RNA, *lin-4*, was discovered in 1993 by *Lee, et al.* by genetic screens on *C. elegans*.

Small RNAs are involved in almost every biological process, including developmental timing, cell differentiation, proliferation and death, metabolic control, transposon silencing and antiviral defense.

The eukaryotic small RNAs are distinguished by their limited size (about 20 to 30 nucleotides) and have been subdivided in three classes: Piwi-interacting RNAs (piRNAs), endogenous small interfering RNAs (endo-siRNAs or esiRNAs) and microRNAs (miRNAs). The differences among them are based on their biogenesis mechanism and the subfamily of Argonaute (AGO) protein, Piwi or AGO, that they are associated with. However, there were found numerous non-canonical small RNAs which have blurred the boundaries between the classes.

piRNAs are 24 to 30 nucleotides (nt) in length, associate with Piwi-subfamily proteins and their biogenesis does not depend on Dicer. These molecules are abundant in germ cells and at least some of them are involved in transposon silencing through heterochromatin formation or RNA destabilization. On the other hand, esiRNAs which associate with AGO-subfamily proteins (similarly to miRNAs) are derived from long double - stranded RNAs (dsRNAs) and are dependent only on Dicer. They are also slightly shorter (~21 nt) than miRNAs. Some of the esiRNAs have been shown to function as post-transcriptional regulators targeting RNAs.

miRNAs are classified as single-stranded RNAs (ssRNAs) of approximately 22 nucleotides in length that are generated from endogenous hairpin-shaped transcripts, being the most understood small RNAs (Kim, *et al.*, 2009). They have become known as key post-transcriptional regulators of gene expression in metazoans and plants, and have revolutionized the comprehension of the post-transcriptional regulation of gene expression. It is becoming clear that miRNAs have a big impact on shaping transcriptomes and proteomes of eukaryotic organisms (Siomi and Siomi, 2010). In mammals, miRNAs are predicted to control the activity of approximately 60% of all protein-coding genes. Functional studies indicate that miRNAs participate in the regulation of almost every cellular process investigated so far, like modulation of gene expression, chromosome structure, innate immune defence, and that changes in their

expression are associated with many human pathologies, such as cancer (Cheloufi, *et al*., 2010; Krol, *et al*., 2010; Siomi and Siomi, 2010).

Generally, miRNAs inhibit protein synthesis either by repressing translation and/or by bringing about deadenylation and subsequent degradation of mRNA targets. More recently, however, some miRNAs were reported to activate mRNA translation. miRNAs function in the form of ribonucleoprotein complexes, miRISCs (miRNA-induced silencing complexes). Argonaute (AGO) and GW182 [glycine-tryptophan (GW) repeat-containing protein of 182 kDa] family proteins represent the best-characterized protein components. Components of miRISC (including miRNAs as well as AGO and GW182 proteins) and repressed mRNAs are enriched in processing bodies (P-bodies, also known as GW bodies), which are cytoplasmic structures thought to be involved in the storage or degradation of translationally repressed mRNAs.

miRNAs almost always interact with their mRNA targets via imperfect base-pairing of their 5' nucleotides 2–8, representing the seed region, with the 3'UTR (untraslated region) of target mRNA. Animal miRNAs may also target 5' UTR and coding regions of mRNAs and interestingly association of miRNAs with 5' UTR target sites appears to activate rather than repress translation. In addition, an A residue across position 1 of the miRNA and A or U across position 9 improve miRNA activity, although they do not need to base-pair with mRNA nucleotides. However, functional miRNA sites containing mismatches or even bulged nucleotides in the seed have also been identified.

Complementarity of the miRNA 3' half is quite relaxed, though it stabilizes the interaction, particularly when the seed matching is suboptimal. Generally, miRNA-mRNA duplexes contain mismatches and bulges in the central region (miRNA positions 10–12) that prevent endonucleolytic cleavage of mRNA by an RNAi mechanism. AU-rich sequence context and structural accessibility of the sites may improve their efficacy. Usually, multiple sites, either for the same or different miRNAs, are required for effective repression, and when the sites are close to each other, they tend to act cooperatively (Fabian, *et al*., 2010).

miRNAs direct RISC to repress translation of target mRNAs in diverse ways and stages. They can block translation initiation or elongation by promoting premature dissociation of ribosomes (Figure 1A) or act in cotranslational protein degradation (Figure 1B, however the putative protease is unknown. Argonaute proteins compete with eIF4E (eukaryotic translation initiation factor 4E), which is essential for cap-

dependent translation initiation, for binding to the cap structure (Figure 1C); recruit eIF6, preventing the large ribosomal subunit from joining the small subunit (Figure 1D); and prevent the formation of the closed loop mRNA configuration by an ill-defined mechanism that includes deadenylation (Figure 1E). miRNA can also mediate mRNA decay by triggering deadenylation and subsequent decapping of the mRNA target (Figure 1F) (Eulalio, *et al*., 2008; Filipowicz, *et al*., 2008).

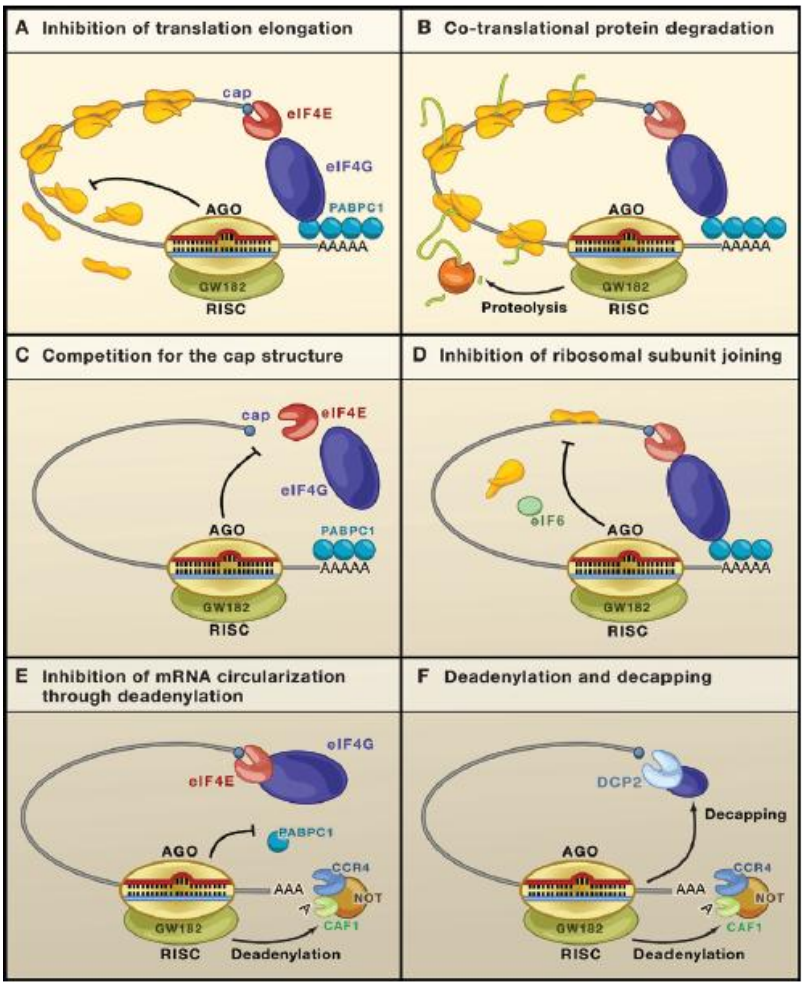


Figure 1 – Processes through which miRNAs can repress the translation of a mRNA. Source: Eulalio, *et al*., 2008).

All four human AGO proteins, AGO1–4, bind similarly to identical populations of small RNAs, suggesting that human AGO1–4 might not have significantly differentiated functions. On the other hand, the catalytic domain remains conserved and active only in AGO2 which is especially active in the embrionary phase (Cheloufi *et al*., 2010).

Some miRNAs have tissue-specific expression, modulating lineage-specific gene expression and cell cycle progression (Carleton, *et al*., 2007).

Nevertheless, they are themselves subject to sophisticated, tight and dynamic regulation at both the levels of miRNA metabolism and function, particularly during rapid developmental transition or changes in cellular environment. The numbers of individual miRNAs expressed in different organisms (~800 in humans) are comparable to those of transcription factors or RNA-binding proteins (RBPs), and many of them are expressed in a tissue or developmental stage-specific manner, thus contributing to cell-type-specific profiles of protein expression. Having the potential to target dozens or even hundreds of different mRNAs, individual miRNAs can coordinate or fine-tune the expression of proteins in a cell (Kim, *et al*,. 2009).

I. 1.1 microRNAs biogenesis

I. 1.1.1 microRNA transcription

miRNAs are manly located in intergenic regions, forming clusters, predicting a long transcript encompassing several coordinated expressed miRNAs. Generally miRNAs can be classified in three groups according to their genomic localization: exonic miRNAs in non-coding transcription units, intronic miRNAs in non-coding transcription units or intronic miRNAs in protein coding transcription units (Gregory and Shiekhattar, 2005; Kim, 2005).

In the canonical biogenesis process, microRNAs are processed from precursor molecules that are called Primary miRNA (pri-miRNAs) (Chong, *et al*., 2010; Krol, *et al*., 2010). pri-miRNAs are several kb long molecules with a stem of 33 bp, a terminal loop and flanking ssRNA segments. These molecules are transcribed by RNA polymerase II (Pol II) from specific transcripts of independent genes and they often produce more than one functional miRNA (Krol, *et al*., 2010; Siomi and Siomi, 2010). Transcription by Pol II allows the differential expression control of miRNAs and leaves specific structures such as 5’ cap structures and poly-A tails (Kim, 2005; Miyoshi, *et al.*, 2010). Promoters of some miRNAs although able to direct Pol II-dependent transcription, lack all typical promote elements normally required for transcription initiation (Gregory and Shiekhattar, 2005).

There are some non-canonical processes by which specific miRNAs can be processed. Mirtrons, which are pre-miRNA-like hairpins that are made by splicing and debranching of short hairpin introns are also transcribed by RNA polymerase II from

short introns in protein-coding genes. On the other hand, the minor group of microRNAs which is associated with Alu repeats and the miRNAs encoded by the major cluster in humans are transcribed by RNA polymerase III (Kim, *et al*., 2009; Winter, *et al*., 2009; Chong, *et al*., 2010; Siomi and Siomi, 2010). Both polymerases are regulated differently and recognize specific promoter and terminator elements, facilitating a wide variety of regulatory options (Winter, *et al*., 2009).

I. 1.1.2 Pri-miRNAs cleavage by Microprocessor complex

pri-miRNAs are substrate for a member of the RNA polymerase III enzyme family, Drosha, that acts in the nucleus.

In humans, Drosha associates with its cofactor through the amine domains, the DiGeorge syndrome critical region gene 8 (DGCR8) protein to form the Microprocessor complex (Kim, *et al*., 2009). Drosha has two RNase III domains and one dsRNA binding domain (Kim, 2005). DGCR8 interacts with pri‑miRNAs through the ssRNA segments and the stem of ~33 bp and orients the catalytic core of Drosha to cleave it asymmetrically ~11 bp away from the ssRNA–dsRNA junction, leaving a stem loop structure – the precursor-miRNAs (pre-miRNAs). Pre-miRNAs, are molecules with approximately 70 nt, a 22 nt hairpin stem, a 3' overhang of approximately 2 nt and a terminal loop (Gregory and Shiekhattar, 2005; Winter, *et al*., 2009; Siomi and Siomi, 2010).

Recent studies show that intronic miRNA processing by Drosha might take place after the transcript is tied to the early spliceosome complex, but before the intron is excised and the first process does not influence the second. Cropping and splicing of pri-miRNAs might be highly coordinated co‑transcriptional processes. The nuclear exosome (which comprises 3′→5′ exonucleases) and XRN2 (a 5′→3′ exonuclease) also associate with the intronic miRNA hairpin region and promote the degradation of the fragments (Kim, *et al*., 2009; Winter, *et al*., 2009). Drosha can also cleave other mRNAs that contain long hairpins from protein -coding genes, non-coding genes or structural RNAs, although it is not as efficient as for miRNAs. One example is the second exon of the DGCR8 mRNA, meaning it regulates its own cofactor, negatively (Kim, *et al*., 2009; Chong, *et al*., 2010). When the miRNA hairpin is located in the exonic region, Drosha processing can destabilize the transcript and reduces protein synthesis from it.

Multiple non-canonical pathways can feed pre-miRNAs into the miRNA pathway through Drosha-independent processes (Kim, *et al*., 2009). The biogenesis of a subset of microRNAs, mirtrons, has been found to depend on the splicing machinery rather than the Microprocessor. These small RNAs are embedded in introns of protein coding genes in flies and mammals. In humans the only real mirtron found so far is mir-877. After splicing, the branch point of the lariat-shaped intron is resolved and the debranched intron forms a hairpin structure that resembles pre-miRNA. With conventional mirtron loci, the resultant small RNAs begin and end precisely with splice donor and splice acceptor sites, being both ends of the pre-miRNA generated by the splicing reaction. However, in certain mirtron-like loci the small RNA-generating hairpin resides at one end of the intron, leaving and extended tail at either the 5′ or 3′ end which consequently requires exonucleolytic trimming to become a substrate for nuclear export. The majority of Drosha-independent miRNAs appear to be derived from long introns of protein-coding mRNAs, such as miR-342 or from independent transcriptional units, such as miR-320. This suggests that release of the pre-miRNA stem–loop intermediate requires a processing step rather than splicing alone. These Drosha-independent miRNAs tended to form these pre-miRNAs structures with extended stems of up to 65 nucleotides in length, instead of the 33 nucleotides of pre-miRNAs originated from the canonical process (Kim, *et al*., 2009; Winter, *et al*., 2009; Chong, *et al*., 2010; Westholm and Lai, 2011).

Some small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), and endogenous short hairpin RNAs (shRNAs) are also processed into miRNA-like molecules independently of the Microprocessor complex. Deep sequencing efforts also reveal that endo-siRNA loci produce a distinct fraction of small RNAs that seem to load onto AGO1, and therefore might constitute functional miRNAs (Miyoshi, *et al.*, 2010; Siomi and Siomi, 2010).

I. 1.1.3 Transport to the cytoplasm

The structure of pre-miRNAs, with marked signals of processing by Drosha is essential to their transport and further processing. The transport of these molecules to the cytoplasm is mediated by exportin 5 (EXP5), a member of the nuclear transport receptor family, that was mostly known as a transport factor for tRNAs, in a RAN-GTP-dependent manner. EXP5 recognizes the dsRNA stem longer than 14 bp along with a

short 3′ overhang (1–8 nt) (Gregory and Shiekhattar, 2005; Kim, 2005; Krol, *et al*., 2010).

Interaction with EXP5 may stabilize pre-miRNAs, avoiding their degradation (Kim, 2005; Winter, *et al*., 2009).

I.1.1.4 Processing by Dicer

Once in the cytoplasm, the pre-miRNAs are cleaved near the terminal loop by Dicer, which measures ~22 nt from the pre-existing terminus of the pre-miRNA, releasing a miRNA/miRNA* duplex.

Dicer is highly conserved and present in almost all eukaryotic organisms and in some of them multiple Dicer isotypes with different roles have been found. Dicer is constituted by two RNase III domains, one dsRNA binding domain, a N' terminal with a Dead box RNA helicase domain, a DUF283 domain and a PAZ domain (also present in AGO) that binds to the protruding end of small RNAs (Gregory and Shiekhattar, 2005; Kim, 2005). Human Dicer interacts with TRBP (TAR RNA-binding protein) and PACT (also known as PRKRA) which are not required for processing but they seem to contribute to the stability of miRNAs. Their biochemical roles remain to be established. TRBP functions as a negative regulator of dsRNA-dependent protein kinase (PKR), whereas PACT stimulates it, but it is not clear at present, whether there is any functional connection between the miRNA pathway and PKR signaling (Kim, 2005; Kim, *et al*., 2009; Krol, *et al*., 2010).

Not all miRNAs require to be processed by Dicer. Although AGO2 does not need its catalytic domain active to exert its function, this domain is essential in erythropoiesis once pre-miR-451 has an unusual structure and cannot be recognized by Dicer. miR-451 is rendered active by AGO which cleaves its stem loop. This process is common to other miRNAs (Cheloufi *et al*., 2010; Miyoshi, *et al*., 2010).

For miRNAs that have a high degree of complementarity along the hairpin stem, an additional endonucleolytic cleavage step occurs before Dicer mediated cleavage. The slicer activity of AGO2 cleaves the 3' arm of the hairpin in the middle to generate a nicked hairpin. This precursor, called AGO-cleaved precursor miRNA (ac-pre-miRNA) facilitates subsequent strand dissociation and miRISC activation (Winter, *et al*., 2009).

In some cell lines, Drosha deficiency has a stronger impact than that of Dicer defiency, resulting therefore in different impacts in the canonical biogenesis pathway (Chong, *et al*., 2010).

I.1.1.5 The miRISC complex

In humans, Dicer, TRBP (and/or PACT) and AGO proteins form a RISC loading complex (RLC). The miRNA duplex is released from Dicer after cleavage and the stable end of the RNA duplex is bound to TRBP in the RLC, whereas the other end interacts with the AGO protein. After processing by Dicer, the duplex needs to be unwinded, however a universal helicase responsible for this process has not been identified yet. This means that different helicases may regulate different miRNAs and that they may not be generally required as in the case of ac-pre-miRNAs (Winter, *et al*., 2009).

The mechanistic details of strand selection and RISC assembly have not been well delineated in humans. One strand of the RNA duplex remains in AGO as a mature miRNA (the guide strand or miRNA), to form the miRNA-containing RNA induced silencing complex (miRISC), whereas the other strand (the passenger strand or miRNA*) is thought to be degraded, but recent deep-sequencing efforts indicate that a large number of miRNA*s are not degraded and rather associate with AGO1 or AGO2. In most miRNA precursors the strand with thermodynamically relatively unstable base pairs at the 5′ end typically survives, for example, a GU pair compared with a GC pair. Once this is not a stringent process, some pre-miRNAs produce miRNAs from both strands at comparable frequencies. Although strand selection based on 5' thermodynamic stability still contributes greatly to the sorting process, relative expression levels of the miRNA/miRNA* strands vary widely among tissues, indicating that strand selection factors other than sequence features might exist and that miRNA processing pathways are more complex than currently recognized (Kim, *et al*., 2009; Winter, *et al*., 2009; Siomi and Siomi, 2010). For example, the phosphorylated 5' end of the miRNA guide strand seems to be critical for the interaction with the AGO proteins (Miyoshi, *et al.,* 2010).

The ends of miRNAs are often heterogeneous owing to either the addition or deletion of 1–2 nt. Sequence variations are found at both 5′ and 3′ ends where it pairs with target mRNAs and induce their translational repression or deadenylation and degradation. In some cases, miRNA repression can be reversed and the miRISC can

even activate expression of the target. How the miRISC represses or activates the expression of specific genes appears to be context-dependent and is the subject of ongoing debate (Miyoshi, *et al.*, 2010).

The mechanisms of the variations of the 5′ end are unknown, but they might be explained by imprecise or alternative processing by RNase III domains. Changes in the 5′ terminus result in shifts of the seed sequences (2–7 nt from the 5′ end), which alter the target specificity of the miRNA.

Paralogues often have identical sequences at seed sequence. The seed sequence is composed of 6 nucleotides and is crucial in base pairing with the target mRNA, the paralogues are thought to act redundantly. 3' sequences of miRNAs also contribute to target binding and because the expression patterns of these sister miRNAs are often different from each other, members of the same seed family might have distinct roles *in vivo.* The 3′ end often contains untemplated nucleotides (mostly uracil and adenine), which must be added after processing by unknown terminal uridyl/adenyl transferases. Deletions of the 5′- and 3′-end nucleotides are also often observed, which are probably due to exonucleolytic activities (Kim, *et al*., 2009).

In Figure 2 we can observe most the steps of miRNA biogenesis that have been described so far.

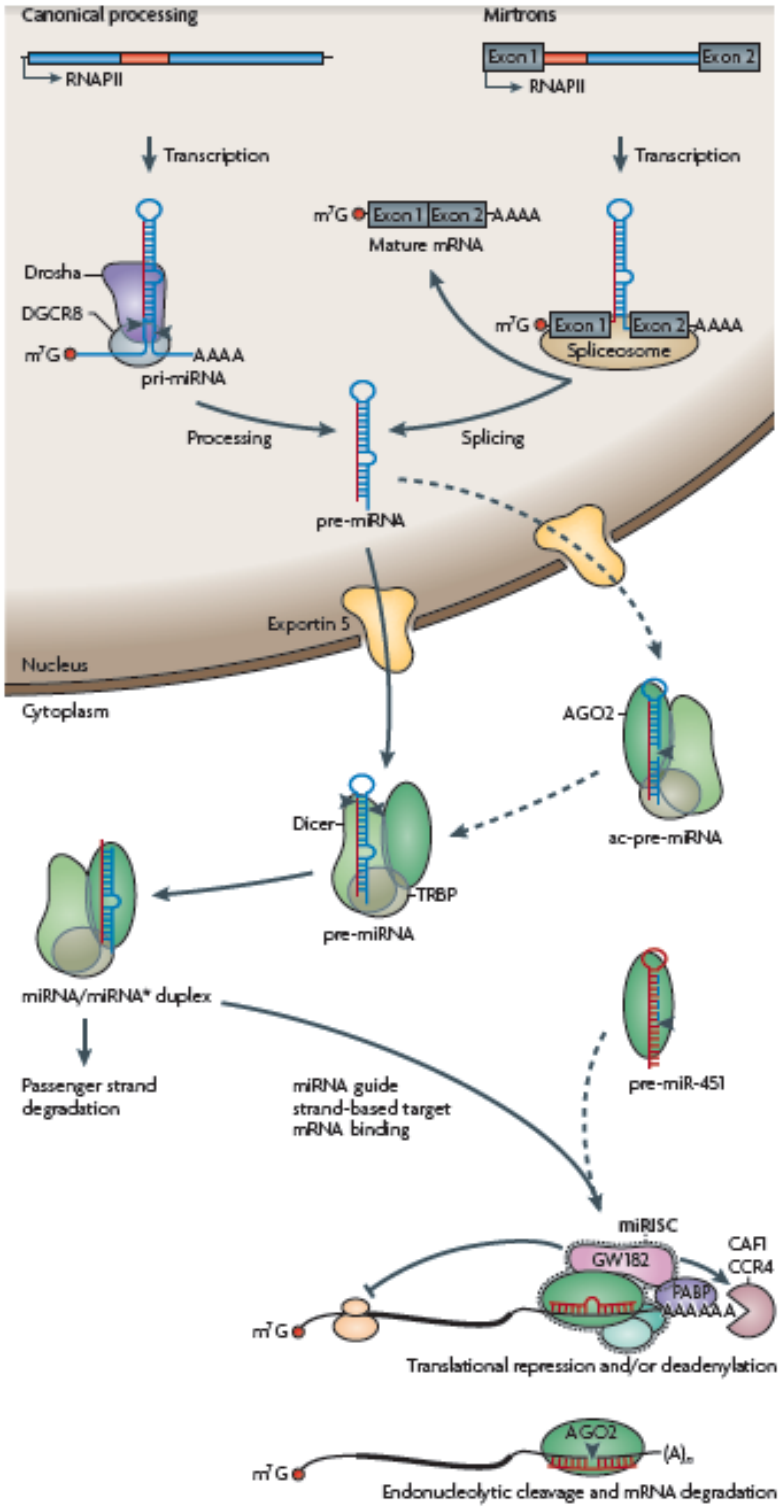


Figure 2 miRNA biogenesis trough cananical and non-canonical pathways. Source: Krol, *et al.*, 2010.

I.1.1.6. Re-import of miRNAs into the nucleus

Some mature miRNAs are mainly localized in the nucleus, such as miR-29b. These miRNAs have a hexanucleotidic terminal motif, a transferable nuclear localization element. The nuclear localization of a fraction of the subcellular AGO pool

in human cells is affected by RAN-GTP shuttle and Importin-8. Evidence is accumulating that miRNAs can regulate gene expression in the nucleus at the transcription level (Winter, *et al*., 2009).

I.1.2. Regulation of miRNA biogenesis

miRNAs are associated with most of the cellular processes, therefore its deregulation is often associated with human diseases, such as cancer. Deregulation of miRNA expression can result from impaired processing, copy number alteration of miRNA encoding loci or methylation of miRNA promoter region (Carleton, *et al.*, 2007).

Transcription of miRNA genes is a major point of regulation in miRNA biogenesis, being regulated similarly to protein-coding genes. There are many Pol II-associated transcription factors that are involved in transcriptional control of miRNA genes. Some miRNAs that are involved in the cell cycle progress and apoptosis are under the control of tumour‑suppressive or oncogenic transcription factors (Kim, *et al*., 2009; Krol, *et al*., 2010).

Control of gene expression by autoregulatory feedback loops is a regulatory mechanism that is particularly important during cell fate determination and development. miRNAs are uniquely suited to participate in feedback circuits due to their potential to directly base-pair with and repress mRNAs that encode factors involved in the biogenesis or function of the same miRNAs. Indeed, many examples have been described of miRNAs regulating their own transcription through single-negative or double-negative (or positive) feedback loops with specific transcription factors. This coordinated expression might serve to prevent misexpression of cell-type-specific miRNAs and to confer robustness to the underlying gene expression programs during development (Krol, *et al*., 2010; Siomi and Siomi, 2010). Some miRNAs are convergently transcribed from both DNA strands of a single locus, giving rise to two miRNAs with distinct seed sequences, therefore regulating different targets. miRNAs in the same genomic cluster can be transcribed and regulated independently (Winter, *et al*., 2009).

Epigenetic control is also important to miRNA gene regulation. For example, the miR-203 locus frequently undergoes DNA methylation in T-cell lymphoma but not in normal T lymphocyte.

Processing is another regulation point is especially important in defining the spatiotemporal pattern of miRNA expression. There is an unknown number of molecules that can stimulate or inhibit processing of certain miRNAs by Drosha, although the mechanisms are not clear yet. Recent works have identified a battery of proteins that regulate miRNAs processing (either positively or negatively) by interacting with Drosha, Dicer or by binding to miRNAs. Some auxiliary factors may function to promote the fidelity, specificity, and/ or activity of Drosha such as p68 (DDX5) and p72 (DDX17) helicases, as well as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Siomi and Siomi, 2010). KH-type splicing regulatory protein (KSRP) serves as a component of both Drosha and Dicer complexes and regulates the biogenesis of a subset of miRNAs, optimizing the positioning and or recruitment of both the miRNA precursor processing complexes through protein–protein interactions. KSRP binds with high affinity to the terminal loop (TL) of the target miRNA precursors and promotes their maturation, remaining attached to the TL even during nucleo-cytoplasmic transition (Trabucchi, *et al*., 2009).

Drosha and Dicer usually operate in complexes with double-stranded RBP partners, such as DGCR8 and TRBP in mammals. The levels and activity of all these proteins are subject to regulation that affects the accumulation of miRNAs. For example, DGCR8 has a stabilizing effect on Drosha through the interaction with its middle domain, whereas Drosha controls DGCR8 as described before. The Drosha to DGCR8 ratio is important, as a threefold excess of DGCR8 dramatically inhibits Drosha processing activity in vitro. Binding of an haem group to DGCR8 promotes its dimerization and facilitates pri-miRNA processing (Winter, *et al*., 2009; Krol, *et al*., 2010).

On the other hand, a decrease in TRBP leads to Dicer destabilization and pre-miRNA processing defects. In human carcinomas, mutations causing diminished TRPB expression impair Dicer function. TRBP is stabilized through serine phosphorylation, catalysed by mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (eRK). Dicer mRNA is targeted by let-7, forming a negative feedback loop influencing miRNA biogenesis, both physiologically and in cancer. Dicer’s amino-terminal helicase domain may have an autoregulatory function as its removal, or interaction with TRBP

stimulates catalytic activity of human Dicer. There is also evidence that proteolysis may play a role in modulating Dicer activity or even changing its specificity towards DNA (Krol, *et al*., 2010; Siomi and Siomi, 2010).

The position of Drosha and Dicer cleavage determines the identity of 5'-terminal and/or 3'- terminal miRNA nucleotides. Notably, processing of some precursors by these enzymes is not regular and generates miRNA isoforms with different termini. Heterogeneity at the 5′ end in particular can have important functional consequences, as it affects the seed region consequently, changing the identity of targeted mRNAs. In addition, identity of the 5′-terminal nucleotide may also directly affect the efficiency of miRNA loading into miRISC, independent of the duplex end stability.

Generally, most miRNA genes produce one dominant miRNA species. However, the ratio of miRNA to miRNA* can vary in different tissues or developmental stages, which probably depends on specific properties of the pre-miRNA or miRNA duplex, or on the activity of different accessory processing factors. Moreover, the ratio might be modulated by the availability of mRNA targets as a result of enhanced destabilization of either miRNA or miRNA* occurring in the absence of respective complementary mRNAs (Krol, *et al*., 2010).

Although the final synthesis rate of a miRNA can, in principle, be controlled at any step of miRNA biogenesis, from transcription to mature miRNA turn over, recent findings have uncovered a significant role for post-transcriptional mechanisms in the regulation of miRNA biogenesis and activity (Siomi and Siomi, 2010). Post-transcriptional regulation can be achieved through several processes and is unclear how many of such regulatory factors exist. It is plausible that nuclear RNA-binding proteins, influence miRNA processing through specific interactions with a subset of pri‑miRNAs. The LIN-28–let-7 regulatory system is a good example, once it is highly conserved in evolution and has an important role in maintaining the pluripotency of eSCs, and also in development and oncogenesis. If LIN-28 is expressed it represses the expression of mature let-7, thus contributing to a staminal phenotype and to the expression of several oncogenes, including MYC and K-RAS.

Turnover of miRNAs is an unexplored area compared to biogenesis regulation, yet several examples of accelerated or regulated miRNAs turnover are known. RNA decay enzymes might target not only mature miRNAs but also their precursors

(pri-miRNAs and pre-miRNAs). Once bound to AGO proteins, mature miRNAs seem to be more stable than average mRNAs, however, certain miRNAs might be degraded much more rapidly than other miRNAs, which suggests that nucleases recognize miRNAs' sequences. In mammals, these nucleases are still unknown (Kim, *et al.*, 2009). miRNA stability can be modulated by viral infection, although the mechanism remains unknown. Notably, some miRNAs turnover in neurons seems to be activity dependent, suggesting that active miRNAs metabolism may be important for neuronal function. The rapid turnover of miRNAs might be required for regulation of the newly synthesized mRNAs that are expressed in neurons, in response to their activation.

Arsenite-resistance protein 2 (ARS2), a component of the nuclear cap-binding complex, interacts with Drosha and is required for pri-miRNA stability and processing in mammals. It seems to influence the processing of a subset of pri-miRNAs, having a regulatory rather than constitutive function. Several splicing factors also function as miRNAs processing regulators, independently from their role in splicing sometimes acting at both the Drosha and Dicer cleavage steps (Krol, *et al.*, 2010; Siomi and Siomi, 2010).

RNA editing is another possible way of regulating miRNA biogenesis. The alteration of adenines to inosines in dsRNA segments, a process that is mediated by adenine deaminases (ADARs), has been observed in many pri- or pre-miRNAs. Since the modified pri-miRNAs or pre-miRNAs become poor substrates of Drosha and Dicer, editing of the precursor can interfere with miRNA processing. Editing can also change the target specificity of the miRNA if it occurs in miRNA seed sequences. This process is responsible as well for preventing the export of pre-miRNAs and for the degradation of some by nucleases. Nevertheless, editing of some miRNAs can enhance Drosha processing, underlying tissue-specific expression (Winter, *et al*., 2009).

I.1.3. Regulation of miRNA function

miRNAs effector pathway may be regulated at different levels. (Winter, *et al*, 2009).

They can control patterns of gene expression that occur during cell cycle progression, so the cell must have mechanisms that control their activity and it is possible that their stability and expression are altered during cell cycle. Controlling cell cycle, miRNAs act as buffers to limit the accumulation of many gene products that

impact cell cycle progression under a variety of complexes. Coordinate regulation of many targets may also allow for rapid reversal of miRNA –induced cell cycle regulation upon changes in miRNA synthesis, stability or location.

miRNAs can also be controlled after biogenesis, especially at the level of miRISC components such as AGO proteins and GW182.

Recent findings point to some distinct functions of individual AGOs in vertebrates. Different mammalian AGO proteins seem to differ in their potency to repress protein synthesis when binded to mRNAs. Hence, the relative abundance of individual AGO proteins may affect in a different manner the strength of miRNA repression in particular cells or tissues.

miRNAs may differ on their intrinsic ability to be loaded into miRISC complex, therefore changes in cellular concentration of AGO proteins might have quantitative and qualitative effects on a range of miRNAs associated with miRISC.

Several mechanisms that regulate AGO2 levels in mammalian cells have been described, including stabilization by the chaperone heat shock protein 90 (HSP90), modulating effects of protein modifications, such as polyubiquitylation promoted by TRIM-NHl, hydroxylation of Pro700 by type I collagen prolyl-4-hydroxylase C-P4H(I), phosphorylation on Ser387 in response to stress and mediated by the MAPK/p38 kinase signalling pathway. The last two modifications increase the localization of AGO2 to P-bodies. The number and size of P-bodies is larger in proliferating cells compared to quiescent ones (Carleton, *et al*., 2007; Winter, *et al*., 2009; Krol, *et al*., 2010).

On the other hand, TRIM32 enhance some miRNAs activity without changing their levels, being still unknown how TRIM32 enhance miRISC activity and whether it is able to bind RNA, either directly or through additional proteins. The specificity for only selected miRNAs could possibly arise from the recognition of unique features of the miRNA/mRNA duplex or selective enrichment of TRIM binding sites in the vicinity of sites recognized by a specific miRNA. Other TRIMs that polyubiquitylate AGO attenuate repression by miRNAs by proteasomal degradation of AGO2 (Krol, *et al*., 2010).

There are three GW182 paralogues in mammals (trinucleotide repeat-containing proteins TNRC6A, TNRC6B and TNRC6C) with multiple transcription start or splice variants. GW182 has an effector role on the repressive function of miRISC, as it interacts with and acts downstream of AGO, but little is yet known about their regulation. There is evidence that these proteins undergo modifications that modulate

their stability and function and they can also interact with many additional factors. Some of these factors have roles in translational repression or mRNA decay and some represent components of P-bodies, but so far the interaction mechanisms for most of the accessory proteins remains unknown. Moreover, their C-terminal segment, contains DUF RRM domains as well as sequences C-proximal to RRM as a minimal protein fragment, which effectively causes both translational repression and mRNA destabilization when tethered to the mRNA. Thereby, disruption of the GW182-AGO interaction, by point mutations or a peptide competing with GW182 for AGO binding, abrogates miRNA-mediated repression (Fabian, *et al.*, 2010).

Many miRISC-interacting proteins belong to the family of DexD/H RNA helicases, which catalyze activities for miRISC assembly, binding to and dissociation from mRNA targets, or miRISC disassembly and turnover. Another group of factors attenuating or enhancing the effect of miRNAs are RBPs. Their association with miRISC components is often RNA-dependent, suggesting that they do not directly interact with miRISCs, but rather are recruited to the same targeted mRNA. The same RBP can, depending on the mRNA or cellular context, either prevent or activate miRISC repression. Not only could RBPs modulate miRISC effects, but miRISCs could also activate or repress RBP function by either competing for binding sites or blocking RBP activity by binding to them.

The nuclear import receptor importin 8 (IMP8) associates with all four human AGO proteins independently of RNA, and localizes to the nucleus and P-bodies. IMP8 is required for efficient binding of AGO2 to a large set of target mRNAs.

miRNAs have been found to act not only as repressors but also act as activators of translation. Under conditions of serum starvation (or general growth arrest, or at the G0 stage) the AGO2-miRISC complex has been shown to switch from a translational repressor to an activator. This switch required fragile X-related protein 1 (FXR1).

Considerable effort has been put into establishing whether components of miRISCs are associated with particular cellular structures and whether miRNA repression can be regulated at this level. P-bodies and stress granules have emerged as being potentially relevant to miRNA repression, as have multivesicular bodies (MVBs). Compartmentalization can control access to binding partners, concentrate factors that act together or temporarily segregate pathway components away from the rest of the cellular environment.

MVBs are specialized late-endosomal compartments, which contribute to miRNA function. Blocking MVB formation inhibits miRNA silencing, whereas blocking MVB turnover stimulates repression by miRNAs. The depletion of some endosomal sorting complex (ESCRT) factors leads to an increase in GW182 protein levels and an impaired loading of miRISC with small RNAs, suggesting that selective removal of GW182 from the miRISC, and its transit to MVBs, is important for the efficient formation of new miRISCs. Components of the miRISC loading complex had been found to be associated with membranous fractions in previous and mammalian AGO2 was initially characterized as a Golgi-associated or endoplasmic reticulum-associated protein (Fabian, *et al*., 2010; Krol, *et al*., 2010).

miRNAs can be transferred from cell to cell by exossomes or through immune synapses and can repress target genes in recipient cells.

Although miRNA-mediated repression is considered to be a cytoplasmic event, substantial amounts of AGO2 and miRNAs have been found in the nuclei of different mammalian cell lines, and human GW182 protein TNRC6B has been shown to shuttle between the cytoplasm and the nucleus. It is yet unclear whether the nucleocytoplasmic shuttling of miRISC components is important for their cytoplasmic functions, or if it indicates that miRISCs have nuclear targets or perform nuclear function or functions unrelated to the established roles of miRNAs. Nevertheless miRNAs may play a role in transcriptional silencing in mammalian cells.

I.1.4. Competing endogenous RNA (ceRNA)

This new theory states that all types of RNA transcripts communicate through a new ‘‘language’’ mediated by miRNA-binding sites (‘‘miRNA response elements’’ – MREs).

miRNAs bind to sequences with partial complementarity on target RNA transcripts, called microRNA response elements (MREs) being able to function in a combinatorial manner if a mRNA transcript harbors numerous MREs. Furthermore, each miRNA may repress up to hundreds of transcripts, and thus, it is estimated that microRNAs regulate a large proportion of the transcriptome. Once pseudogenes associated with one gene are widely conserved, it is possible that miRNAs can also target them, and as a matter of fact their expression displays tissue specificity and can be activated or silenced in specific pathological conditions, such as cancer. Therefore,

pseudogenes can act as miRNA competitors, competing with their ancestral protein-coding genes for the same pool of miRNAs through sets of conserved MREs.

Notably, microRNAs also regulate long noncoding RNAs (lncRNAs), which have been linked to epigenetic mechanisms, as shown in a recent global analysis of Argonaute (AGO)-bound transcripts.

The consequence of competition for microRNAs is observed as a decrease in microRNA detection and thus an impairment of microRNA activity. On the basis of this hypothesis, MREs can be viewed as the letters of an ‘‘RNA language’’ by which transcripts can actively communicate to each other to regulate their respective expression levels. It is predicted that this ‘‘RNA language’’ can be used to functionalize the entire mRNA dimension through the identification of crosstalking ceRNAs, as well as ceRNA networks. This also challenges the notion that a protein-coding gene must be translated into a protein to exert function. Moreover, the noncoding function of mRNA may or may not be consistent with the coding function, thereby creating built-in regulatory loops, functional complexity, and diversification in both physiological and pathological conditions.

Overall, ceRNA networks effectiveness depend on the identity, concentration, and subcellular distribution of the RNA and the miRNA species that are present in a tissue, developmental, or pathological context. The effectiveness of a ceRNA would depend on the number of miRNAs that it can ‘‘sponge’’. This, in turn, would depend on the ceRNA’s accessibility to miRNA molecules, which is influenced by its subcellular localization and its interaction with RBPs.

Although two MREs may be predicted to bind the same microRNA, their specific nucleotide composition may be partially different, and the effectiveness of each MRE to bind a miRNA is critical for ceRNA function, so miRNAs are predicted not to exert the same degree of repression on all of them (Salmena, *et al*., 2011).

I.2. Tumourigenesis

Tumourigenesis is the process of tumour development from a normal cell. Malignancy is the outcome of two successive events: cell transformation and tumour progression. Transformation could be defined as the autonomous proliferation of an immortal cell clone, whereas progression is the process which leads the immortal transformed clone to invasion and metastasis. Transformation involves a stepwise accumulation of genetic alterations, mainly chromosomal translocations and gene mutations, resulting in altered protein activity and/or expression of oncogenes and/or tumour suppressor genes which confer growth advantages to cancer cells leading to immortalization. Progression proceeds through continuing selection of the most advantageous clones from a genetically unstable population of transformed cells.

Acquisition of malignancy is the result of tumour progression rather than transformation. During tumour progression, cancer cells lose their original shape and polarity and become more independent from neighboring tissues. Finally, they acquire the capacity to invade nearby tissues and metastasize to distant locations through the blood or lymphatic system.

The tumour microenvironment plays an essential role in tumour progression as it is responsible in maintaining its differentiation state and in favoring the proliferation of cancer cells (Giannakakis, *et al*., 2007).

Pathological analyses of a number of organs reveal lesions that appear to represent intermediate steps in a process through which cells evolve progressively from normalcy via a series of pre-malignant states into invasive cancers.

These observations have been rendered more concrete by a large body of work indicating that the genomes of tumour cells are invariably altered at multiple sites, having suffered disruption through lesions as subtle as point mutations and as obvious as changes in chromosome complement.

Taken together observation of human cancers and animal models argue that tumour development proceeds via a succession of genetic changes, each conferring one or another type of growth advantage leads to the progressive conversion on normal human cells to cancer cells (Hanahan and Weinberg, 2000).

However more recent studies have defined tumours as a group of cells which may exhibit different phenotypes and different differentiation stages. A sub-population

of cells shows both cancer properties and stem cell characteristics were named Cancer Stem Cells (CSC). CSCs might arise through neoplastic changes initiated in normal self-renewing stem cells or downstream progenitors with limited or no selfrenewal. Initial events in stem cells could cause expansion of the stem cell pool and/or expansion of downstream progenitors. Secondary events are more likely to occur in these expanded pools of target cells; thus, it is possible that transformation is initiated in a normal stem cell, but the final steps occur in downstream progenitors. The reactivation of a self-renewal program is a central feature of the oncogenic transformation of progenitors because these short-lived cells will otherwise die or undergo terminal differentiation before enough mutations occur for full neoplastic transformation. CSCs themselves might acquire additional genetic or epigenetic changes during tumour progression, and non-CSCs can acquire CSC-like properties through processes such as Epithelial–mesenchymal transition (EMT), leading to the evolution of tumour phenotype and intratumour heterogeneity (Wang, 2010).

The hallmarks of cancer (Figure 3) are distinctive and complementary capabilities that enable allow cancer cells to survive, proliferate and disseminate growth, providing a solid foundation for understanding the biology of cancer. When they were first described, there were 6 fundamental characteristics considered: sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis, inducing angiogenesis and resisting cell death.

In 2010 *Hanahan and Weinberg* suggested four other characteristics considered to be essential to most tumour induction and progression. Two of them, deregulating cellular genetics and avoiding immune destruction, are considered as emerging hallmarks because neither capability is yet generalized and fully validated. Additionally, two consequential characteristics of neoplasia facilitate acquisition of both core and emerging hallmarks: genomic instability endow cancer cells with genetic alterations that drive tumour progression; inflammation by innate immune cells can result in their inadvertent support of multiple hallmark capabilities, thereby manifesting the now widely appreciated tumour-promoting consequences of inflammatory responses (Hanahan and Weinberg, 2010).

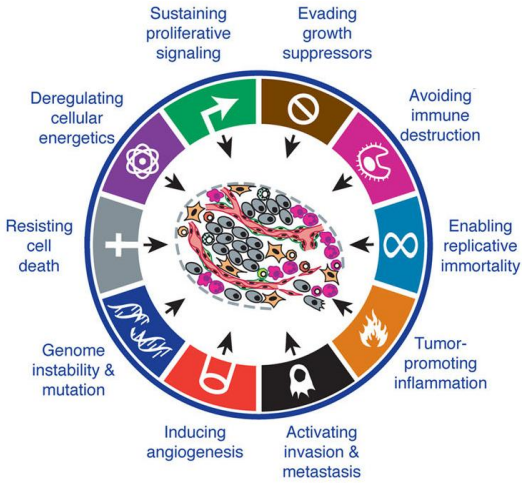


Figure 3 - The hallmarks of cancer. Source: Hanahan and Weinberg, 2010.

Arguably the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation signaling in a number of alternative ways. They may produce growth factor ligands themselves, to which they can respond via the expression of cognate receptors, resulting in autocrine proliferative stimulation. Alternatively, cancer cells may send signals to stimulate normal cells within the supporting tumour-associated stroma, which reciprocate by supplying the cancer cells with various growth factors. Receptor signaling can also be deregulated by elevating the levels of receptor proteins displayed at the cancer cell surface, rendering such cells hyperresponsive to otherwise-limiting amounts of growth factor ligand; the same outcome can result from structural alterations in the receptor molecules that facilitate ligand-independent firing.

Growth factor independence may also derive from the constitutive activation of components of signaling pathways operating downstream of these receptors, obviating the need to stimulate these pathways by ligand-mediated receptor activation.

Somatic mutations that activate additional downstream pathways and disruptions of negative-feedback mechanisms that attenuate proliferative signaling also contribute largely to the maintenance of this characteristic.

In addition to the hallmark capability of inducing and sustaining positively acting growth-stimulatory signals, cancer cells must also circumvent powerful programs that negatively regulate cell proliferation. Many of these programs depend on the actions of tumour suppressor genes such as RB or TP53 that regulate cell cycle progression and apoptosis. The evasion of mechanisms of contact inhibition and corruption of the TGF-β pathway are also noteworthy to promote malignancy.

I. State of the Art

Programmed cell death by apoptosis serves as a natural barrier to cancer development. Elucidation of the signaling circuitry governing the apoptotic program has revealed how apoptosis is triggered in response to various physiologic stresses that cancer cells experience during the course of tumourigenesis or as a result of anticancer therapy. Notable among the apoptosis-inducing stresses are signaling imbalances resulting from elevated levels of oncogene signaling and DNA damage associated with hyperproliferation. Apoptosis is attenuated in those tumours that succeed in progressing to states of high-grade malignancy and resistance to therapy. In contrast to apoptosis, necrosis, has pro-inflammatory and tumour-promoting potential, as necrotic cell death releases pro-inflammatory signals into the surrounding tissue microenvironment.

The immortalization of cells that proceed to form tumours has been attributed to their ability to maintain telomeric DNA at lengths sufficient to avoid triggering senescence or apoptosis, achieved most commonly by upregulating expression of telomerase or, less frequently, via an alternative recombination-based telomere maintenance mechanism. Hence, telomere shortening has come to be viewed as a clocking device that determines the limited replicative potential of normal cells and thus one that must be overcome by cancer cells. Moreover, cell senescence is emerging as a protective barrier to neoplastic expansion that can be triggered by various proliferation-associated abnormalities, including high levels of oncogenic signaling and, apparently, subcritical shortening of telomeres.

During tumour progression, an ‘‘angiogenic switch’’ is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths. The angiogenic switch is governed by countervailing factors that either induce or oppose angiogenesis. Some of these angiogenic regulators are signaling proteins that bind to stimulatory or inhibitory cell surface receptors displayed by vascular endothelial cells. The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively.

Tumour neovasculature is marked by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, microhemorrhaging, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis. Not all tumours maintain the same level of activation of angiogenesis, thereby resulting in different levels of vascularization.

I. State of the Art

The capacity to invade and metastatize to other tissues is essential to tumour progression. The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps. This depiction envisions a succession of cell-biologic changes, beginning with loss of cell-cell and cell-matrix adhesion, migration or local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumours (colonization). In some types of cancer, the primary tumour may release systemic suppressor factors that render micrometastases dormant. Micrometastases may lack some hallmark capabilities necessary for vigorous growth, such as the ability to activate angiogenesis.

Invasion seems to be facilitated by inflammatory cells that assemble at the boundaries of tumours, producing the extracellular matrix-degrading enzymes and other factors that enable invasive growth (Hanahan and Weinberg, 2010).

Tumour cells have a metabolism that is remarkably different from that in normal differentiated cells. Transformed cells take up and metabolize nutrients such as glucose and glutamine at high levels that support anabolic growth. In contrast to normal differentiated cells that rely primarily on mitochondrial oxidative phosphorylation to generate energy needed for cellular processes, most cancer cells instead depend on aerobic glycolysis, a phenomenon termed ‘‘the Warburg effect’’. The metabolic alterations and adaptations of cancer cells create a phenotype that is essential for tumour cell growth and survival, altering flux along key metabolic pathways such as glycolysis and glutaminolysis. Indeed there is mounting evidence for the therapeutic potential of targeting cancer metabolic reprogramming (Garcia-Cao, *et al*., 2012).

I.2.1. miRNAs and tumourigenesis

Studies in the past few years have established miRNAs as important molecules in the pathogenesis of various solid tumours, suggesting their possible role and utilization in cancer screening, treatment and prediction of outcome (Torres, *et al.*, 2011).

miRNAs are frequently located at genomic regions involved in cancers and their regulatory function, such as chromosomes hot-spots, and are able to modulate oncogenic molecular networks by activating oncogenes and inactivating tumour suppressor genes, as can be seen in Figure 4 (Gregory and Shiekhattar, 2005). These alterations contribute to the loss of cell cycle control and increased proliferative capacity, characteristics of transformed cells. They can also contribute to the less differentiated state of tumours and can act simultaneously as apoptosis and checkpoint regulators directly or indirectly, regulating different targets.

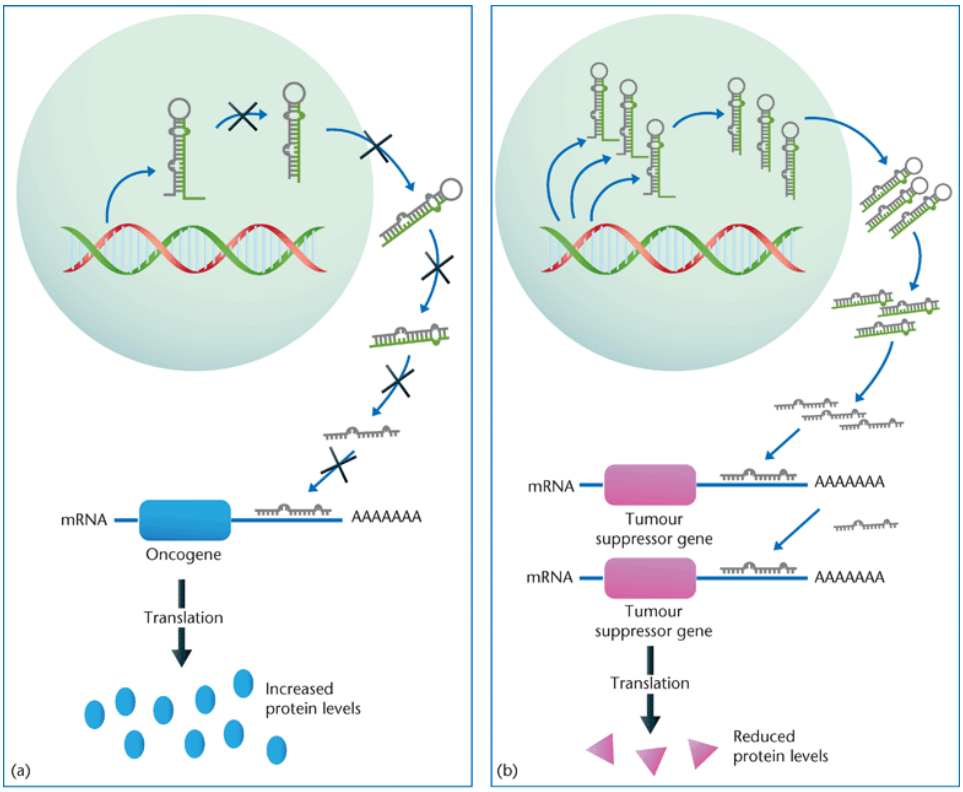


Figure 4 - miRNA involvement in oncogenic transformation. (a) miRNAs act as tumour suppressors when they target oncogenes. Any deregulation in their processing may lead to the non regulation of these targets and thereby to the increased expression of oncogenes, increasing the levels of their respective proteins. (b) miRNAs act as oncogenes when they target tumour suppressor genes due to copy number alteration and other processes. Like this, miRNAs inhibit their expression and the levels of the respective proteins are negatively modulated. Source: Ferracin and Negrini, 2012.

I. State of the Art

Altered miRNA expression leads to altered regulation of target mRNAs which is important in tumourigenesis. The impact of miRNA sequence variation is a result of cell type–specific expression levels of both miRNAs and their targets: cell type–specific transcriptomes. In addition, it is important to note that tumourigenesis-related alterations in a cell's transcriptome may also affect the regulatory capacity of miRNAs to the extent that a miRNA variant allele may have a protective effect for disease, as well as predict poor prognosis (Christensen, *et al*., 2010).

Tumours may exhibit global decrease in levels of mature miRNAs due to failure of Drosha. Global repression of miRNAs processing can promote tumourigenesis and cell cycle entry in part by relieving constraints on the activity of known oncogenes. Deletion of certain miRNAs loci in different tumour types and correlation of miRNA deletion with poor clinical prognosis has led to the prediction that miRNAs act as tumour suppressors (Carleton, *et al*., 2007; Siomi and Siomi, 2010). An interesting fact is that in patients with cancer, the loss of Drosha expression is correlated with the body mass index (BMI), decreasing with the increase of this factor. This is also associated with a greater invasion and metastatic potential and a worse prognosis. However data present in literature is inconsistent.

Proteins p53, p63 and p73 regulate processing components of miRNAs including Drosha and Dicer through specific miRNAs (Torres, *et al*., 2011).

Low amounts of Dicer1 mRNA are associated with a poor prognosis in lung and advanced breast cancer (Siomi and Siomi, 2010; Bahubeshi, *et al*., 2011).

The expression of Dicer1 and Drosha affects differently diverse tumours and different tumour stages. This means that there is a tissue specific effect associated with aberrant expression patterns of miRNA pathway genes and that the effects of an aberration in the miRNA processing pathway on cancer initiation and progression are complex. Non-consistent pattern emerge with conflicting data arising from different studies. Nevertheless, it is clear that the miRNA processing pathway plays a crucial role in the initiation and progression of many cancer types.

Many miRNAs sequence aberrations found in tumours alter the secondary structure without affecting processing and reveal the structural flexibility of the Microprocessor complex (Winter, *et al*., 2009). Even though a minority of miRNAs may be deregulated in a wide range of tumours, most of them are cancer-type specific. In solid tumours, it appears as the most common miRNA event is gain of expression,

whereas loss of expression in cancer is a more limited event and more tissue specific (Volinia, *et al.*, 2006).

miRNA expression patterns appear to be more reliable than mRNA expression patterns and possess a greater resolution analysis: they can successfully identify different subtypes and predict the biologic and clinical behavior of a cancer type.

Plasma miRNAs have already been referred to as potential biomarkers for non-invasive diagnostics and prognosis, being possibly able to regulate expression in other cells (Figure 5). There have also been described miRNAs contained in exosomes in other human fluids, such as saliva or urine, which have an altered concentration in diverse human pathologies and thus can be useful in detecting and distinguishing different diseases in a non-invasive way. miRNAs can also associate with other biomolecules, such as lipids, to achieve improved nuclease resistance. This suggests that some cell-free miRNAs carry regulatory signals between widely separated organs to regulate functions.

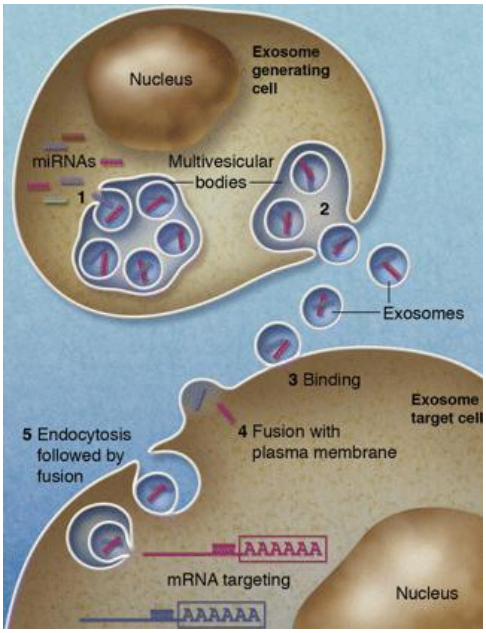


Figure 5 - miRNAs may be transported by exossomes from cell to cell. Source: Stoorvogel, 2012.

miRNAs and their processing machinery could be possible important targets for therapy. The delivery of miRNAs to selected tumour sites may require novel delivery systems such as the polyethylenimine-based nanoparticles that were recently reported to be effective carriers in a mouse model of colon cancer (Bahubeshi, *et al*., 2011; Liu, *et al*., 2012; Pant, *et al*., 2012).

I.3. PI3K/AKT Pathway

Since its discovery in the 1980s, the family of lipid kinases termed phosphoinositide 3- kinases (PI3Ks) has been found to play key regulatory roles in many cellular processes including cell survival, proliferation and differentiation. PI3Ks have been divided into three classes according to their structural characteristics and substrate specificity. The most commonly studied are the class I enzymes that are activated directly by cell surface receptors. Class I PI3Ks are further divided into class IA enzymes, activated by receptors of tyrosine-kinases (RTKs), G protein-coupled receptors (GPCRs) and certain oncogenes such as the small G protein Ras, and class IB enzymes, regulated exclusively by GPCRs (Liu, *et al*., 2009).

PI3K catalyzes the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. PIP3 in turn contributes to the recruitment and activation of a wide range of downstream targets, including the serine-threonine protein kinase AKT multi-gene family (AKT-1, AKT-2 and AKT-3, also known as protein kinase B – PKB). The PI3K-AKT signaling pathway (Figure 6) regulates many normal cellular processes including cell proliferation, survival, growth, and motility processes that are critical for tumourigenesis. Indeed, the role of this pathway in oncogenesis has been extensively investigated and altered expression or mutation of many components of this pathway have been implicated in human cancer (Luo, *et al.*, 2003; McCubrey, *et al*., 2008).

Both growth factor RTKs and GPCRs can activate PI3K signaling, however the first has been better described and has a clear role in human pathologies. The binding of Insulin-like growth factor 1 (IGF-1) to its receptor triggers the activation of several intracellular kinases, including PI3K (Stitt, *et al*., 2004).

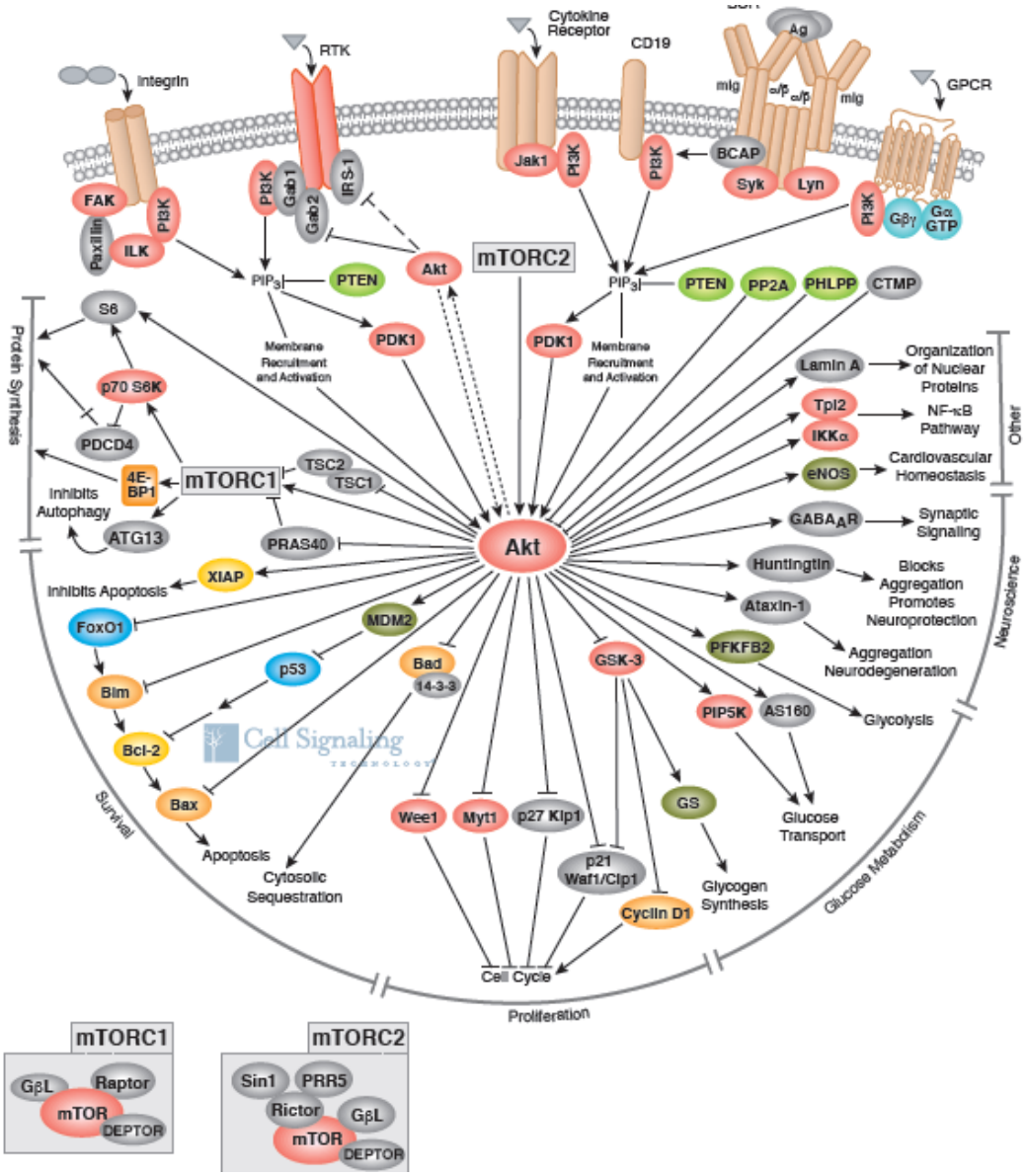


Figure 6 - The PI3K-AKT pathway. Source: [5]

Growth factor RTKs engage the class-Ia PI3K, which is a heterodimer comprised of the p85 regulatory and p110 catalytic subunits. The p85 regulatory subunit contains Src-homology 2 (SH2) domains, that recognize phosphorylated (p) Y (pY) residues, an iSH2 domain (a rigid tether for p110), a conserved domain related to sequences present in the Break Point Cluster Region (BCR) gene and a SH3 domain which is found in proteins that interact with other proteins and mediate assembly of specific protein complexes via binding to proline-rich motifs. Specific phospho-tyrosine residues on the activated receptor or on associated adaptor proteins bind the SH2 domains of p85 and recruit the enzyme to the membrane.

The 110-kDA PI3K Class 1A catalytic subunit contains a p85 binding domain, a Ras binding domain, a kinase domain and an helical domain. Cytokine stimulation often

creates a PI3K binding site on the cytokine receptor. The p85 subunit SH2 domain associates with this site. The p85 subunit is then phosphorylated leading to activation of the p110 catalytic subunit. This often occurs at the inner leaflet of the cytoplasmic membrane, although there are important roles of PI3K in the nuclear membranes which have been registered (McCubrey, *et al*., 2008; Liu, *et al*., 2009).

In the membrane, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) converting PIP2 to PIP3. Subsequently, PIP3 recruits other downstream molecules, site particularly the serine-threonine kinases AKT and PDK1 via binding to their pleckstrin homology (PH) domains and creates a lipid binding for them in the membrane (Stitt, *et al*., 2004).

At the membrane, AKT is partially activated through phosphorylation in its activation loop by PDK1. Depending on the AKT isoform, PDK1 can phosphorylate AKT on T308/T309/T305. A second kinase, mostly mTORC2, phosphorylates AKT on a S regulatory residue (S473/S474/S472) in the C terminus of AKT, resulting in its full activation. AKT in turn has been postulated to phosphorylate over 9000 proteins, controlling cell proliferation, survival, growth, and other processes. The PH domain leucine-rich repeat protein phosphatase (PHLPP) dephosphorylates S473 on AKT inducing apoptosis and inhibiting tumour growth (Luo, *et al*., 2003; McCubrey, *et al*., 2008; Bozulic and Hemmings, 2009; Liu, *et al*., 2009).

The levels of PIP3 in the cell are strictly regulated and several lipid phosphatases act to rapidly remove it. Of particular interest is the 3'- phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten), which converts PIP3 back to PIP2, preventing AKT activation and has become known as a major tumour suppressor in different tissues. Two other phosphatases, SH2 domain-containing inositol 5'phosphatase (SHIP)-1 and SHIP-2, also dephosphorilate PIP3 to PIP2. Mutations in these phosphatases, which eliminate their activity, can lead to tumour progression. Consequently, the genes encoding these phosphatases are referred to as anti-oncogenes or tumour suppressor genes (Luo, *et al*., 2003; McCubrey, *et al*., 2008).

Aberrant activation of the PI3K-AKT pathway has been widely implicated in many cancers. Gene amplification of p110 occurs in some cases of human ovarian cancer, and amplification of AKT is found in ovarian, breast, and colon cancer.

Hyperactivation of the PI3K-AKT pathway allows cancer cells to improve proliferation and survival and is now evident that is also coinvolved in cell hypertrophy,

which are important processes for neoplastic progression (Stitt, *et al*., 2004; McCubrey, *et al*., 2008).

The PI3K-AKT pathway is a key regulator of cell survival through multiple downstream targets. The FOXO proteins, a subgroup of the Forkhead family of transcription factors are known to mediate apoptosis by activating the transcription of proapoptotic genes such as FasL and Bim, and to regulate genes involved in metabolism and cell cycle progression. Phosphorylation of the FOXO transcription factors on multiple sites by AKT leads to citoplasmic retention of phosphorylated FOXO proteins and inhibition of their transcriptional functions (Luo, *et al*., 2003; Stitt, *et al*., 2004). Similarly, AKT can phosphorylate the proapoptotic Bcl-2 family member Bad, causing its sequestration from the mitochondrial membrane.

There may be a reciprocal regulation between the PI3K-AKT pathway and the tumour suppressor protein p53, since PTEN is a transcriptional target of p53 and AKT can promote p53 degradation by phosphorylating and activating its negative regulator Mdm2. Consequently, there is a major inhibition of apoptosis.

The PI3K-AKT pathway, in parallel to the Ras/MAPK pathway, contributes to the regulation of cell cycle progression, particularly at the G1/S transition. The kinase GSK3 phosphorylates and promotes the degradation of cyclin D1 and Myc, two proteins that drive S phase entry. AKT phosphorylates and inhibits GSK3, thereby contributing to the stabilization of cyclin D1 and Myc. The FOXO transcription factors repress cyclin D1 expression while enhancing expression of the cell cycle inhibitors $p27^{kip1}$ and p130Rb2 and are themselves inactivated by AKT-mediated phosphorylation, conferring a proliferative vantage to cancer cells.

In response to both nutrient availability and mitogenic growth factors, the mammalian target of rapamycin (mTOR) can control cell growth. In mammalian cells, mTOR exists in at least two complexes of distinct structure, physiology and substrate specificity. Rapamycin-sensitive mTOR complex 1 (mTORC1) is defined by the presence of Raptor (Regulatory associated protein of mTOR). Activation of mTORC1 involves PI3-kinase-dependent activation of AKT and tuberous sclerosis (TSC) protein phosphorylation. 4E-BP1, p70S6K and PRAS40 are all substrates of mTORC1. On the contrary, Rictor (rapamycin-insensitive companion of mTOR), mSin1 and Protor mark the mTOR complex 2 (mTORC2), which is active in a PKCα-dependent manner. The main substrate of mTORC2 described to date is AKT. Thus, mTOR in the complex

mTORC2 acts as an AKT activator, whilst complex mTORC1 receives stimulatory input from AKT (Bozulic and Hemmings, 2009).

mTORC1 stimulate protein translation by activating the 70 kDa ribosomal S6 kinase (S6K1) and inhibiting the elongation-initiation factor 4E binding protein-1 (4E-BP1). Integration of a variety of signals (mitogens, growth factors, and hormones) by mTOR assures cell cycle entry only if nutrients and energy are sufficient for cell duplication. Consequently, mTOR controls multiple steps involved in protein synthesis, but importantly enhances production of key molecules such as c-Myc, cyclin D1, p27Kip1, and retinoblastoma protein (pRb). mTOR also controls the translation of hypoxia-inducible transcription factor-1a (HIF-1a) mRNA. HIF-1a upregulation leads to an increased expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Moreover, HIF-1a regulates the glycolytic pathway by controlling the expression of glucose-sensing molecules including glucose transporter (Glut) 1 and Glut3 (Luo, *et al*., 2003).

AKT-mediated regulation of mTOR activity is a complex multi-step phenomenon. AKT inhibits tuberous sclerosis 2 (TSC2) function through direct phosphorylation. TSC2 is a GTPase-activating protein (GAP) that functions in association with the putative tuberous sclerosis 1 (TSC1) to inactivate the small GTPase protein Rheb (Ras homolog enriched in brain). TSC2 phosphorylation by AKT represses GAP activity of the TSC1/TSC2 complex, allowing Rheb to accumulate in a GTP-bound state. Rheb-GTP then activates, the protein kinase activity of mTOR when complexed with Raptor protein and mLST8, a member of the Lethal-with-Sec-Thirteen gene family. The mTOR/Raptor/mLST8 complex (mTORC1) is sensitive to rapamycin and inhibits AKT via a negative feedback loop which involves, at least in part, p70S6K (Foster, *et al*., 2010; Goncharova, *et al*., 2011).

The relationship between AKT and mTOR is further complicated by the existence of the mTORC2, which displays rapamycinin sensitive activity. The mTORC2 complex directly phosphorylates AKT on S473 in vitro and facilitates T308 phosphorylation. AKT and mTOR are linked to each other via ill-defined positive and negative regulatory circuits, which restrain their simultaneous hyperactivation through a mechanism involving p70S6K and PI3K. Assuming that the equilibrium exists between these two complexes within the cell, when the mTORC1 complex is formed, it could antagonize the formation of the mTORC2 complex and reduce AKT activity. Thus, at

least in principle, inhibition of the mTORC1 complex could result in AKT hyperactivation.

Furthermore, this PI3K-AKT-TSC-mTOR branch is emerging as a critical contributor to the oncogenic properties of the PI3K-AKT pathway.

PI3K may contribute to other aspects of tumourigenesis in both AKT-dependent and -independent manners. PIP3 regulates the small GTPase Rac by activating a subset of its GTP-GDP exchange factors. Since Rac controls rearrangement of the actin cytoskeleton and cell motility and is important for Ras-mediated transformation, PI3K might activate both Rac- and Ras-dependent invasion events that contribute to metastasis. In addition, the PI3K-AKT pathway may also contribute to tumour angiogenesis.

Components of the PI3K-AKT pathway present promising targets for therapeutic intervention for several reasons. This pathway inhibits many "tumour suppressor-like" proteins such as the FOXO transcription factors, Bad, GSK3 that negatively regulate cell survival, proliferation, and growth. Blocking this pathway could therefore simultaneously inhibit the proliferation of tumour cells and sensitize them toward apoptosis. Second, many components in the PI3K-AKT pathway are kinases, one of the most "druggable" classes of intracellular targets, ideal for the development of small molecule inhibitors. Third, as hyperactivation of the PI3K-AKT pathway is found in a wide range of tumours, drugs inhibiting this pathway are likely to have broad applications for treating different types of cancer.

Because only a subset of the cellular processes regulated by the PI3K-AKT pathway are involved in tumourigenesis, the choice of drug targets must take into account the adverse effects resulting from the inhibition of other PI3K-AKT-dependent cellular processes. For example, insulin's effects on metabolism are mediated through the PI3K-AKT pathway and inhibitors are therefore likely to perturb glucose homeostasis.

A reasonable therapeutic index would depend on tumours being more sensitive to inhibitors of this pathway than normal tissues. The prevalence of a hyperactivated PI3K-AKT pathway in human cancers indicates this may indeed be the case, and a partial inhibition of this pathway might be sufficient to inhibit tumour growth while sparing normal cells (Luo, *et al.*, 2003).

Very recent studies showed PTEN as a key node for the control of obesity and tumourigenesis. Mice overexpressing PTEN exhibit an unexpected cancer-resistant and

unique metabolic state, which is the outcome of the ability of PTEN to regulate metabolism at multiple levels both from the cytosol and from the nucleus. On the one hand, these mice exhibit increased oxygen consumption and energy expenditure. On the other hand, mice and cells are less prone to transformation and cancer development. Thus, the features observed resemble an ‘‘anti-Warburg state’’, in which less glucose is taken up but is more efficiently directed to the mitochondrial tricarboxylic acid cycle. Moreover, PTEN elevations induce mitochondrial biogenesis and thus increased mitochondrial ATP production. Thus PTEN couples mitochondrial function and dynamics to cellular metabolism through PI3K-dependent and PI3K-independent mechanisms.

PTEN elevation hence represents a potentially attractive therapeutic approach that could both prevent cancer development and increase energy expenditure to oppose fat accumulation and obesity (Garcia-Cao, *et al*., 2012).

I.5. Lung Cancer

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets. Breast cancer in females and lung cancer in males are the most frequently diagnosed cancers and the leading cause of cancer death for each sex in both economically developed and developing countries, except lung cancer is preceded by prostate cancer as the most frequent cancer among males in economically developed countries.

Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008 globally. Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in 2008 (Jemal, *et al*., 2011).

The observed variations in lung cancer rates and trends across countries or between males and females within each country largely reflect differences in the stage and degree of the tobacco consume. Smoking accounts for 80% of the worldwide lung

cancer burden in males and at least 50% of the burden in females. Male lung cancer death rates are decreasing in most Western countries, where the tobacco epidemic peaked by the middle of the last century. In contrast, lung cancer rates are increasing in several countries in Asia and Africa, where the epidemic has been established more recently and smoking prevalence continues to either increase or show signs of stability.

Generally, lung cancer trends among females lag behind males because females started smoking in large numbers several decades later than males (Jemal, *et al*., 2011).

Based on pathologic features, lung cancers are classified into non-small cell lung cancer (NSCLC), which accounts for about 85% of cases, and small cell lung cancer (SCLC) that accounts for approximately 15% of cases. NSCLC is divided into three major histologic subtypes, adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma (a poorly differentiated subtype usually diagnosed by exclusion of the other three types of lung cancer). SCC used to be the most common histologic subtype of NSCLC, however, in recent years there has been a significant increase in the proportion of adenocarcinoma cases with a corresponding decline in proportion of SCC cases. On the one hand, squamous and small cell tumours are thought to derive mainly from epithelial cells that line the larger airways. On the other hand, adenocarcinomas and large cell carcinomas, are thought to derive from epithelial cells that line the peripheral small airways (Garber, *et al*., 2001; Kotsakis, *et al*., 2010).

Patients with lung cancer present with progressive shortness of breath, cough, chest pain/oppression, hoarseness or loss of voice. Pneumonia (often recidivant) is the presenting feature in many patients. Relative to other forms of non small cell lung cancer, adenocarcinoma is more often asymptomatic, being more frequently identified in screening studies or as an incidental radiologic finding. Patients with small SCLC often present symptoms referable to distant metastases (Travis, *et al*., 1999).

NSCLC cases cases frequently diagnosed at advanced stages, resulting in an overall 5-year survival rates are: 56% for stage I, 34% for stage II, 10% for stage III, and 2% for stage IV. Conversely, the 5-year survival rate in stage I NSCLC patients who have received effective treatments can be as high as 83%. Therefore, finding NSCLC earlier may reduce the mortality (Clements, *et al.,* 2010; Shen, *et al*., 2011).

Patients with nonsmall cell lung tumours are treated differently from those with small cell tumours. The pathological distinction between small SCLC and NSCLC is, therefore, very important.

There is relative consensus among pathologyists on the diagnosis of SCLC. These tumours progress along a typical clinical course that is characterized by an excellent initial response to chemotherapy and is often associated with several months of complete regression. This short-term regression is followed by recurrence, development of chemo-resistance, and finally death caused by systemic dissemination.

In contrast, the morphological subtyping of NSCLC is more difficult and far less reliable in predicting patient outcome. Approximately 50% of patients die from metastatic disease even after complete surgical removal of the primary tumour. The initial tumour pathologic diagnosis is based on small bronchoscopic biopsy specimens and may change when surgically removed specimens are re-examined.

Lung tumour heterogeneity is well documented and is reflected in the morphological classification of mixed tumours such as adenosquamous carcinoma or combined SCLC containing both small cell and non-small cell components (Garber, *et al.*, 2001).

The diagnostic tests available to date are mainly invasive, thereby it is important to develop a reliable non-invasive strategy, taking advantage of recent developments in molecular genetics for diagnosis of early stage NSCLC.

Numerous tumour-specific molecular alterations have been identified in plasma/serum and shown the potential as biomarkers in patients with lung cancers. However, none of the tested markers thus far had sufficiently achieved the required characteristics for diagnosis of NSCLC. miRNAs present in plasma possess considerable resistance to the enzymatic cleavage of RNase A and a panel of plasma miRNAs would provide potential biomarkers that could differentiate NSCLC patients from healthy individuals and among them (adenocarcinoma and squamous cell carcinoma (Lin, *et al*., 2010; Shen, *et al*., 2011).

I.4.1. miRNAs in lung cancer

There are several reports that show the co-involvement of miRNAs in different stages of lung cancer, and in this subsection are presented some examples of how miRNAs have been found to regulate mechanisms linked to lung cancer and especially to non-small cell lung cancer (NSCLC) .

Accumulating evidence shows that miRNAs are grossly deregulated in NSCLC. Recent studies have shown that not only can miRNAs be used to sub-classify NSCLCs but specific miRNA profiles may also predict prognosis and disease recurrence in early-

stage NSCLCs (Lin, *et al.*, 2010). An inherited single nucleotidic polymorphism (SNP) in Drosha's gene (RNASEN) has been shown to be associated with a differential prognosis and overall survival in patients with this malignancy (Rotunno, *et al.*, 2010).

There are a number of miRNAs that have already been successfully associated with lung cancer. For example, the let-7 family is a cluster of miRNAs whose genes map to different chromosomal regions that are frequently deleted in lung cancer. Thereby, its down-regulation is associated with poor prognosis, especially in NSCLC patients, and a single nucleotide polymorphism in let-7 complementary site 6 of the K-RAS mRNA 3'-UTR is significantly associated with increased risk for NSCLC among moderate smokers.

All members of the miR-17-29 cluster cooperate with c-Myc to accelerate tumour development and help promote tumour neovascularisation. The miR-17-92 cluster is overexpressed in small-cell lung cancer (Lin, *et al*., 2010; Wang, *et al*., 2010).

miRNAs may also be involved in chemo-sensitivity/-resistance determination, suggesting the possibility that manipulating miRNAs may be potentially useful to modulate the cancer chemoresistance. As a matter of example, miR-221 and miR-222 contribute to lung cancer resistance to TRAIL (Tumour necrosis factor-Related Apoptosis Inducing Ligand) therapy by downregulating the tumour suppressors PTEN and TIMP3.

Epidermal growth factor receptor (EGFR) is one of the most common proto-oncogenes in lung cancer and the expression of some miRNAs is altered and directly linked to this pathway. For example, miR-21 is upregulated under conditions in which EGFR signalling is activated, especially in the context of EGFR-activating mutations, and is suggested to be related to lung carcinogenesis in non-smokers (Lin, *et al*., 2010).

The miRNAs predicted to have a major oncogenic potential in lung cancer, and that are associated with a worse prognosis are the following: miR-17-92, miR-21, miR-27, miR-93, miR-106, miR-128, miR-155, miR-181, miR-196, miR-199, miR-210, miR-214 and miR-301. Wang *et al*. (2010), also identified some miRNAs that are down-regulated in the lungs of rats exposed to cigarette smoke, those being: let-7, miR-10, miR-26, miR-30, miR-34, miR-99, miR-122, miR-123, miR-124, miR-125, miR-140, miR-145, miR-146, miR-191, miR-192, miR-219, miR-222 and miR-223.

Furthermore, the stoichiometry of miRNA machinery and RISC depends on histologic subtype of lung carcinoma and varies along the atypical adenomatous hyperplasia-bronchoalveolar carcinomas (AAH-BAC), adenocarcinoma sequence. Dicer

is up-regulated in AAH and BAC and down-regulated in areas of invasion and in advanced adenocarcinoma. Higher Dicer level in SCLC is found when compared with adenocarcinoma.

In conclusion, miRNAs can regulate autocrine/paracrine loops, anti-apoptotic genes, genes that promote tumour vasculature development, cell cycle related genes, metastasis related genes and chemoresistance genes in lung tissue, contributing grately to the initiation and progression of lung cancer (Wang, *et al*., 2010).

I.5. hsa-miR-196a

In a generic manner, miR-196 seems to be conserved only in vertebrates. miR-196 is encoded at 3 paralogous locations in the A, B, and C mammalian HOX clusters, with miR196a1 in an intergenic region at the HOXB cluster on chromosome 17q21-q22, miR196a2 at the HOXC cluster on 12q13, and miR196b at the HOXA cluster on 7p15-p14.2 [1,2,3]. HOX proteins are major transcription factors that play a crucial role during embryogenesis, organogenesis and diverse oncogenic malignancies.

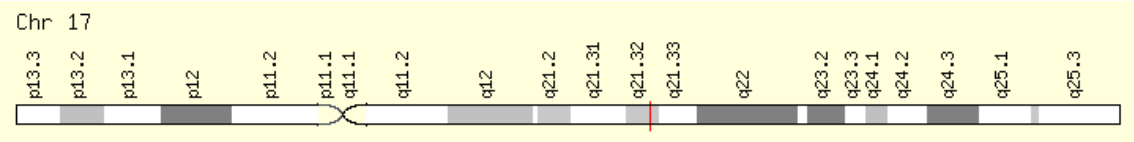


Figure 7 - Cytogenetic localization of hsa-miR-196a1. Source: [6]

miR-196 family members may be influenced by regulatory controls imposed on the HOX cluster, but this regulation by control elements is not simply shared with the nearest HOX gene, as they can target HOX genes. For example, miR-196a1 targets HOXb8 3' UTR with a high complementarity level. The perfect complementarity between miR-196a1 and the HOXb8 3' UTR suggests that miR-196a1 can mediate degradation rather than translational repression of HOXb8 mRNA (Mansfield, *et al*., 2004). These results indicate a miRNA-mediated regulation of HOX gene expression during vertebrate embryogenesis (Schimanski, *et al*., 2009).

A variety of reports identified miR-196a as a potential biomarker, which was associated with multiple kinds of malignant tumours. According to this data, this miRNA has been reported to be highly expressed in human breast cancer and might be

related to the survival of patients with pancreatic adenocarcinoma. Additionally, miR-196a was believed to participate in the regulation of determinant oncogenes during leukaemogenesis (Dou, *et al*., 2010).

miR-196a present in the plasma has also been indicated as an effective early marker for patients with pancreatic carcinoma (Liu, *et al*., 2012).

Recently, many studies research the association between rs11614913 single-nucleotide polymorphism (SNP) in hsa-miR-196a2* and cancer susceptibility, such as breast cancer, lung cancer, hepatocellular carcinoma, gastric cancer, esophageal cancer and glioma. This SNP has also been indicated as a prognostic biomarker for non-small cell lung cancer, if the mature miRNA* is considered, once there was not a change in the precursor levels of both forms (Hu, *et al*., 2008). These results were found to be true also for breast cancer. The SNP referred before affects the maturation of the pre-miRNA and affects target mRNA possibilities (increasing or decreasing the affinity), modulating cancer susceptibility. A CpG island was found upstream this gene which influences its expression. This SNP can have different effects depending on race (Hoffman, *et al*., 2009; Christensen, *et al*., 2010; Chu, *et al*., 2011). However, a clear link between genetic variations and functional alternations remains to be established.

Regarding miR-196a1, it is transcribed from the minus strand [4] and has a 9 nucleotide seed region which is longer than most miRNAs. The expression of this miRNA is spatio-temporally regulated during development and pathologies (Tripurani, *et al*., 2011).

Additionally, cell culture experiments supported high miR-196a1 levels could supress the activities of various cancer related genes, such as *ANXA1* (annexin A1), suppression of which was well documented in various cancers (Tian, *et al*., 2010).

Although miR-196a1 generally has a pro-oncogenic influence in human cancers in some studies on breast cancer and melanoma it seems to have an onco-supressor behavior (Schimanski, *et al*., 2009; Li, *et al*., 2010).

Scope of the thesis

II. Scope of the thesis

The aim of this project is to discover the potential link between deregulation of the PI3K/AKT pathway and deregulation of miR-196a expression in non-small cell lung cancer. Furthermore, it is intended to unravel the role of miR-196a in cellular processes related to tumour progression such as influence in replicative potential and migration *in vitro* and potential to induce tumour development *in vivo*.

Materials and Methods

III. Material and Methods

With the bioinformatic tool Target Scan and the site microRNA.org three interesting targets were predicted for miR196a - FoxO1, FoxO3 and p27. These targets were chosen because they are involved in the PI3K/AKT pathway, and one of the aims of this work is to correlate miR-196a1 with this pathway in NSCLC. The first experiments done were to confirm these genes as true targets of miR-196a1.

The predicted target genes were amplified by PCR and cloned into pGL3 control vector, represented in Figure 8. This vector contains a restriction site for XbaI endonuclease downstream of a gene that encodes luciferase (luc^+), so this property is used to insert our 3'UTR in this place. If these are really target genes for the microRNA in study, is expected to see a downregulation of luciferase expression. After the insertion of the target gene in the pGL3 control vector, the vector name will be pGL3 control-3'UTR target gene.

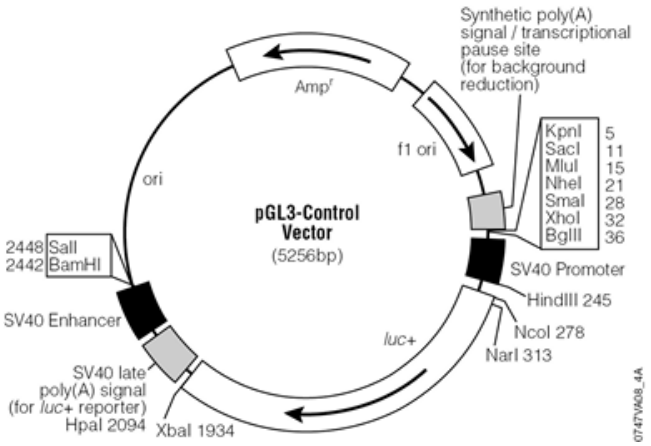


Figure 8 - pGL3-Control Vector. Source: [7]

III. 1. PCR to amplify the 3'UTR of target genes

The 3'UTR of target genes were amplified by PCR with specifically designed primers, represented on table 1. The *Taq* polymerase utilized was from Euroclone and the instrument used was DNAEngine® Peltier Thermal Cycler (BioRad).

Primers	Sequence
Primer Forward FoxO1	CCCAATGTGTGCAGGTTATG
Primer Reverse FoxO1	AGGTCCAAGGCTGTTCAATG
Primer Forward FoxO3A	GGCGGACTTTGTGTTTGTTT
Primer Reverse FoxO3A	CCATGCCAGGCTAATTTTGT
Primer Forward p27	TTCATGGAATGGACATCCTGT
Primer Reverse p27	CCTTCCCCAAAATTGCTTCT

Table 1 - Primers used to amplify the 3' UTR of chosen target genes.

The PCR products were analysed by agarose gel electrophoresis at 1% with incorporated ethidium bromide. The electrophoretic run occurred at 100 V, in an electrophoretic tank containing 1X TAE and the gel image was visualised with an UV transiluminator, using the QuantityOne 4.6.1 software.

III. 2. Purification of 3'UTRs from PCR products

After confirming that the 3'UTRs were amplified, the DNA had to be purified from the PCR products. The QIAquick PCR Purification Spin Protocol was preformed to this effect, as manufacturer's recommendations.

The DNA obtained was quantified in a NanoDrop spectrophotometer.

III. 3. Nucleic Acid quantification

Quantification of nucleic acids was made with a NanoDrop spectrophotometer ND-1000.

First the equipment was initialized and calibrated using 2 µl of H_2O milliQ. Then, the purity values (ratio between the absorbance at 260 nm and 280 nm and between the absorbance at 260 nm and 230 nm), and the concentration of nucleic acids was measured in ng/µl, depositing 2 µl of each sample in the instrument.

III. 4. Quick Ligation Protocol

First it was combined 50 ng of pGL3-Control vector with a 5-fold excess of insert, adjusting the volume to 10 µl with H_2O milliQ. Then it was added 10 µl of 2X Quick Ligation Buffer and the solution was mixed. Subsequently it was added 1 µl of Quick T4 DNA Ligase followed by a thoroughly mix. The reaction was incubated at

room temperature for 15 minutes, after what it was chilled on ice and then used to transform bacteria.

For each ligation protocol, there were made two different controls: α and β. The α control contains only the vector, the buffer and H_2O to evaluate the efficiency of the vector's digestion. On the other hand, the β control contains also the enzyme and therefore consents to evaluate the efficiency of the dephosphorilation of the vector.

III. 5. Transformation Protocol

Initially, the competent cells were thawed on ice for defrosting and it was transferred 5 µl of the ligation mixture in a 1.5 ml microcentrifuge tube. After defrosting, there were added 50 µl of competent cells to the DNA followed by a gentle mix by pipetting up and down. The mixture was incubated on ice for 30 minutes, pursued by a heat shock at 42ºC for 45 seconds. Meanwhile, it was prepared SOC medium (SOB and glucose [0,4%]). After the heat shock it was added 1 ml of room temperature SOC to each microcentrifuge tube, and the tubes were incubated at 37ºC for 60 minutes at 1000 rpm in a thermomixer.

Once the incubation was finished the microcentrifuge tubes were centrifuged at 5000 rpm for 5 minutes, to pellet the bacteria. The supernatant of each tube was discarded leaving about 150 µl on which the cellules were resuspended. Following the resuspension, the cells were plated onto LB + ampicillin selection plates and placed in an greenhouse overnight at 37ºC. After this last incubation, the plates can be stored at +4ºC or the colonies formed can be analyzed right away by PCR.

III. 6. PCR on colony

The bacteria colonies were screened by PCR to verify the presence of the pGL3-control vector containing the 3'UTR of the target genes. For that, the colonies were picked with a tip which was then mix in 20 µl of water. 3 µl of the bacteria solution were used to amplify by PCR and the remaining volume was conserved at +4ºC.

The PCR primers are represented on table 2, considering that the primer reverse is equal to every samples once it is complementary to the vector. The *Taq* polymerase utilized was from Euroclone and the instrument used was DNAEngine® Peltier Thermal Cycler (BioRad).

Primers	Sequence
Primer Forward FoxO1	CCCAATGTGTGCAGGTTATG
Primer Forward FoxO3	GGCGGACTTTGTGTTTGTTT
Primer Forward p27	TTCATGGAATGGACATCCTGT
Primer Reverse universal	CGCGGAGGAGTTGTGTTTG

Table 2 – Sequences of the primers used to amplify the insert from the bacteria.

After the complete PCR program, the samples were run in an 1% agarose gel electrophoresis with ethidium bromide incorporated, so the band corresponding to the molecular weight of the 3'UTR of the gene of interest was seen. To discard the false positive results, the samples positive for PCR were subsequently analyzed through digestion with XbaI.

III. 7. Miniprep

To make the digestion with XbaI, plasmid DNA had to be amplified in bacteria. For the positive clones, the remaining volume of the suspension of bacteria in water, that was not used for PCR on colony, was grown in 3 ml of LB supplemented with ampicillin overnight at 37ºC at 250 rpm. Then, 2 ml of the liquid culture were centrifuged at 6000 rpm for 5 minutes in a microcentrifuge tube.

Afterwards, the QIAprep Spin Miniprep kit by QIAGEN was used to extract plasmid DNA as manufacturer's recommendations except for the elution volume used, which was 30 µl of H_2O milliQ. Plasmid DNA was quantified using NanoDrop spectrophotometer.

III. 8. Digestion with XbaI

The plasmid DNA obtained from the Miniprep protocol was digested with XbaI enzyme, in order to rule out some false positive results from the PCR on colony protocol. The mix composition is showed on table 3.

Reagents	Volumes/ sample (µl)
DNA	3
Buffer 2	2
10X BSA	2
XbaI	0.5
H_20 milliQ	12.5
Total Volume	**20**

Table 3 – Mix used to preform the digestion of the plasmid with XbaI.

After mixing all the components, the solution was incubated at 37ºC for 1 hour without agitation in a Thermomixer comfort (Eppendorf). The samples were run in an 1% agarose gel electrophoresis with ethidium bromide, using the non-digested vector pGL3-control as negative control.

III. 9. Sample sequencing

After observing which clones were also positive for digestion with the endonuclease XbaI, samples were prepared for sequencing at Primm. Samples for sequencing were concentrated 150 ng/µl in a total of 10 µl from purified plamid DNA.

III. 10. Maxiprep

After confirming which clones were positive for digestion and for sequencing a plasmid DNA extraction was made, this time using the QIAGEN® Plasmid Maxi Kit. The remaining 1ml of liquid culture that was not used to do the Miniprep was added to 500 ml of LB supplemented with ampicilin, and the bacteria were allowed to grow at 37ºC at 250 rpm overnight. QIAGEN protocol was followed as recommended. The DNA pellet was resuspended in 150 µl of H_2O milliQ and the concentration was measured with a NanoDrop spetophotometer.

III. 11. Cellular culture

NCI-H460 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) in petri plates treated for cell growth, as manufacturer's recommendation.

On the other hand, HEK-293T were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% P/S.

All cells were maintained in standard conditions in an incubator at 37ºC in 5% CO_2 and splitted every two or three days, using trypsin.

III. 12. Cellular system

In order to evaluate the effects of miR-196a1 in lung cancer and correlate it to the PI3K/AKT pathway simultaneously, a complex cellular system was chosen. The cellular system is perceptible in Figure 9, where each line represents a different infection, and each square a stable cell line.

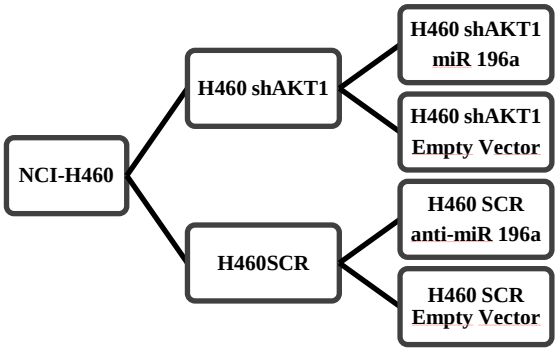


Figure 9 - Cellular system used.

The lenti-vectors used to infect the cells were purchased in System Biosciences and are represented in Figure 10a and b.

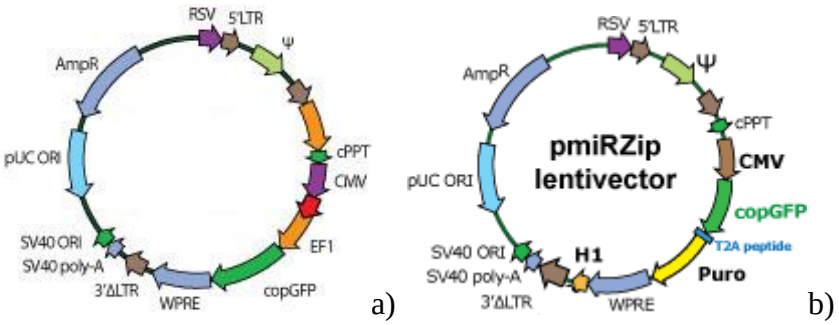


Figure 10 -- Lenti-vectors used to infect the cell lines. (a) This vector as it is represented in the figure, without any further insert is called troughout this work as "Empty Vector" and serves as a control vector. hsa-miR-196a was inserted in the place represented by the red arrow, being under the control of constitutive CMV promoter. (b) Vector used to block hsa-miR-196a.

III. 13. Dual-Luciferase® Reporter Assay (Promega)

The first step was to transfect HEK-293T cells with the siPORT™ *Neo*FX™ transfection agent, following the protocol as manufacturer's recomendations. Depending on the condition to be tested, the samples were prepared as represented in Table 4.

Condition number	Vector pGL3 control	pGL3 control-3'UTR target gene	Renilla	Small RNA
1	-	FoxO1	√	Negative control
2	-	FoxO1	√	miR-196a1
3	-	FoxO3	√	Negative control
4	-	FoxO3	√	miR-196a1
5	-	p27	√	Negative control
6	-	p27	√	miR-196a1
7	√	-	√	Negative control
8	√	-	√	miR-196a1
9	-	-	√	Negative control
10	-	-	√	miR-196a1

Table 4 – In this table there are represented the ten conditions (including controls) used to perform the Dual-Luciferase® Reporter Assay.

The luciferase assay was also done as suggested by the manufacturer.

III. 14. Production of stable clones

III. 14.1. Transfection of HEK-293T

First the HEK-293T cells were grown to 60 to 80% confluence on the day prior to the infection to produce the virus. The following quantities are indicated for one construct of interest and for one petri dish of HEK-293T cells at the right confluence and this procedure is to be done under a class II laminar flux chamber.

Afterwards, a mix is prepared containing 13 µg of the vector of interest, 12 µg of VSVG (envelope protein) and 18 µg of Δ8.9 (allows packaging). This mix is then added to $CaCl_2$ (0.25 M) in a microcentrifuge tube. BES (®Sigma) was added to a conical tube of 50 ml by BD Falcon™, and the previous solution of $CaCl_2$ and DNA is added to BES, drop by drop, while air is inflated to allow the reaction between them to occur. The reaction is incubated at room temperature for 30 minutes, allowing the crystals between the calcium of $CaCl_2$ and the fosfate of BES to form and then, the solution is added drop by drop to the cellular culture, homogenizing at the end.

The culture medium is changed after 16 hours, adding fresh medium, and after 8 hours (24h from the transfection) the first lentiviruses are recovered and filtered. Fresh medium was added again to the HEK-293T plates and at 48h from the transfection, lentiviruses are recovered again. The lentiviruses that are not used to infect cells at the moment of the recover are stored at -80ºC.

The bivalent ions promote the entering of DNA trough the membrane of the cells. This mechanism is not clear but it is thought to be by endocitosis.

III. 14. 2. Infection with lentiviruses

On the day previous to the infection the cells to infect were plated on petri plates ($1x10^5$ for NCI-H460) of 60mm with complete medium. The lentiviruses were produced by the protocol described formerly.

When the lentiviruses were recovered at 24h from the transfection, the suspension was used to substitute the medium of the cells in which was mixed 1 µl of polybrene (8 mg/ml) for each ml of suspension added to the cell culture. This polymer is added because it helps the process of transduction by camouflaging the negative charges present in the cellular membrane, and therefore permeabilising it. The first cycle was done overnight.

The morning after, the viral suspension was changed, starting a 10 hours incubation followed by another overnight incubation after changing the viral suspension again. The last viral incubation was a 10 hour one after which the viral suspension was recovered and fresh medium was added to the cells.

After 36 hours the medium was changed to medium containing 1.5 µg/ml of puromincin, so our cells of interest were selected.

III. 14. 3. Fluorescent Clone picking

After one week in selection medium, the cells were splitted and plated at a very low confluence so that the cells were completely separated from each other. The cells were then allowed to grow and form clones. The clones were observed in an inverted microscope, with a fluorescent lamp, and the fluorescent ones were signed. Under a laminar flux chamber the clones were picked with a micropipette of 200 µl with 50 µl of trypsin. The tip was positioned vertically over the clone and it was permitted the contact by capillarity between the clone and the trypsin for about one minute. Afterwards, it was dispensed just a little of trypsin over the clone and it was aspired. The cells in the clones were separated by pipeting up and down in a well of 24-well plate, and were kept in medium with selection, during amplification.

III. 15. Total protein extraction

To extract proteins from cells grown in adherent culture, the culture medium was removed and the cells were washed with 1X PBS, being detached mechanically from the plate with a scraper. The cells were recovered to a microcentrifuge tube of 2 ml and the plate was washed 1X PBS to remove any remaining cells. The cells were then pelleted in a refrigerated microcentrifuge at 4ºC for 5 minutes at 6000 rpm.

All the steps of this protocol were done using ice and the solutions were also kept in ice.

The ELB (Lysis Buffer) consists on an aqueous solution containing 50mM of Hepes (pH 7), 250mM of NaCl and 5% Nonidet-p40 (NP-40).

In order to avoid protein degradation by proteases, protease inhibitors (10X Protease inhibitor SIGMA*FAST*™ Protease Inhibitor Tablets), phosphatases inhibitors (1 mM of Na_3Vo_4, 1 mM of NaF, 1 mM of PMSF, 10 nM of Okadaic Acid) and a reducing agent (1 mM of DTT) must be added to ELB before lysing the cells. The final solution was prepared, just before using it, otherwise the inhibitors may become inactive.

The pellet was resuspended in complete lysis buffer in a variable volume based on the size of the pellet. The cell suspension was incubated on ice for 30 minutes and then centrifuged in a refrigerated centrifuge at 4ºC for 30 minutes at 13000 rpm. The supernatant was recovered to a microcentrifuge tube of 1.5 ml and protein concentration was determined by a standard Bradford Assay (BioRad) in a Beckman DU 530 spectrophotometer.

III. 16. Western Blot

This technique was used to analyse the protein expression in the different cell lines.

Poliacrylamide gels were prepared at two different acrylamide concentrations, 10 or 12.5%, according to the molecular weight of the proteins in study.

After complete polymerization of the gel, it is transferred to a electrophoresis tank that was filled with a running buffer containing 25 mM of TRIS, 250 mM of

Glicine (pH 8.3) and 0.1% of SDS. 50 µg of protein for each cell line were dispensed in the wells, and the gels were run at 100 V.

When the proteins were separated between them, the run was stopped and fractionated proteins were transferred from the gel to a nitrocellulose membrane (Protan Nitrocellulose Transfer Membrane, Schlicher & Schuell, Germany) in a 1X transfer buffer containing 25 mM of TRIS, 250 mM of Glicina and 20% of methanol, for 1.5 hours at 100 V.

After transferring, the membranes were colored with 1X Red Ponceau (ATX Ponceau S Red staining solution, FLUKA) for 1 minute at room temperature in order to validate the transferring homogeneity and quality. Next, the membranes were washed, first in H_2O bidistillated, then in TTBS (1X TBS and 0,1% Tween) and were pre-hybridized for 1 hour in a solution of TTBS containing also 5% Nonfat dried milk powder (AppliChem) and 3% BSA in order to block the non-specific hybridization sites of the primary antibody. Subsequently 3 washes of 5 minutes each were made before incubating with the primary antibodies diluted in 1X TBS, 3% BSA, 0.02% NaAzide and 100 mg of Phenol Red, overnight at 4ºC, with agitation.

The next day, the primary antibody was recovered and the membranes were washed for 5 minutes, 3 times with TTBS. After that, the membranes were incubated with the respective secondary antibody (rabbit or mouse), diluted at 1:2500 in a solution of TTBS with 5% of nonfat dried milk powder, for 1 hour, followed by 3 washes of 5 minutes each with TTBS.

Next the membranes were incubated for 1 minute with an amplified chemiluminescence kit, ECL (Amersham Inc.), which allows the operator to see the chemiluminescence on High performance chemiluminescence film (Amersham Inc.) due to the reaction between the substrate present on ECL and the peroxidase covalently bound to the secondary antibody. The primary antibodies used in this thesis were the following: Anti-FoxO3 (rabbit), diluted 1:400 (Cell Signaling TECHNOLOGY®, #2497); Anti-FoxO1 antibody (rabbit), diluted 1:500 (Cell Signaling TECHNOLOGY®, #2880); Anti-p27 antibody (mouse), diluted 1:500 (Santa Cruz Biotechnology, sc-1641); Anti-α-Tubulin (mouse) concentrated 0.25 µg/ml (NeoMarkers, #MS-581).

III. 17. RNA extraction

To extract RNA from cells grown in adherent culture, a similar procedure to that for proteins was performed to remove cells from the petri plates. For total RNA isolation, TRIzol Reagent (Invitrogen) protocol was followed as company's suggestions. The RNA concentration was determined using the NanoDrop spectrophotometer, as described before.

III. 18. cDNA production

cDNA from miR-196a1 and genes chosen was obtained following QIAGEN protocol, using miScript Reverse transcription procedure. 1 µg of RNA was used, and the concentration of cDNA obtained was measured in a NanoDrop spectrophotometer.

III. 19. Real-Time PCR quantification

miR-196a1 expression levels were evaluated by Real-Time quantitative PCR. The miScript Real-Time PCR for detection of miRNA or Noncoding RNA procedure by QIAGEN was performed using 20 ng of cDNA. RNU6B was used to normalize the Ct values.

To confirm the target genes of miR196a, the quantitative expression of those genes was also analyzed by Real-Time PCR using 1X Sybr Green PCR Master mix (Applied Biosystems) and a total of 20 ng of cDNA. HPRT was the housekeeping gene chosen to compare Ct values and the primers, by Primm srl, used are represented in table 5.

Primers	Sequence
Primer Forward FoxO1	AAGGGTGACAGCAACAGCTC
Primer Reverse FoxO1	TTCTGCACACGAATGAACTTG
Primer Forward FoxO3A	CTTCAAGGATAAGGGCGACA
Primer Reverse FoxO3A	CGACTATGCAGTGACAGGTTG
Primer Forward p27	TTTGACTTGCATGAAGAGAAGC
Primer Reverse p27	AGCTGTCTCTGAAAGGGACATT
Primer Forward HPRT	GGCCAGACTTTGTTGGATTTG
Primer Reverse HPRT	TGCGCTCATCTTAGGCTTTGT

Table 5 – Primers used for Real-Time PCR

The experiment was conducted in Applied Biosystem 7900HT Fast Real-Time PCR System, using the program defined for Fast SYBR Green Master Mix (Applied Biosystems) and analyzed with SDS Enterprise Database software.

III. 20. Biological Assays

III. 20. 1. MTT Proliferation Assay

2000 cells were plated for each well on a 96-well plate in RPMI-1640 complemented with 1% FBS. The experiment lasted for 4 consecutive days, so 4 different plates were made and 1 was used on each day. On the measuring day, 10 µl of MTT (5 mg/ml) were added to each well. The MTT was allowed to incubate with the cells for 2 hours inside the cell incubator and after that it was removed and 100 µl of isopropanol were placed on each well. The plate was then incubated at room temperature with agitation for 10 minutes after what a violet precipitated was observed. The absorbance at 570 nm was read in EnVision™ 2103 Multilabel Reader (Perkin Elmer).

III. 20. 2. Migration Assays

III. 20. 2. 1. Wound Healing Assay

Cells were grown in a 6-wells plates and when they reached 100% confluence there were made 3 scratches with a tip of 10 µl in each well, for each scratch there were chosen and signed 3 fields to photograph and follow the migration of the cells for 48h. Photos were taken at 0h, 12h, 24h, 36h and 48h. Once the scratches were made, the cells were maintained in RPMI 1640 complemented with 0.5% of BSA, so that the effect observed was mainly due to cellular migration and not to proliferation. Before each photo was taken, it was made a wash with PBS and a culture medium substitution.

III. 20. 2. 2. Transwell Migration Assay

In a Multiwell of 24 wells was added to half of the wells 700 µl of complete culture medium, then the modified Boyden chambers were placed in those wells.

The cells of each line were counted with the Trypan Blue method, using a Bürker chamber. $1x10^5$ cells were resuspended in 100 µl of RPMI-1640 without any supplements and added to each modified Boyden chamber. For each cell line a duplicate was made.

After 24h the cells were fixed and colored. First, the modified Boyden chambers were translocated to another well, and the medium was removed with a micropipette. A wash with PBS was carefully made and the excess of cells that have not migrated was

removed with the help of a cotton swab from the upper part of the chamber. The PBS was then discarded.

It was added to each modified Boyden chamber (in the upper and in the lower part) 1 ml of cold methanol. The multiwell was put at -20ºC for 20 minutes after what the methanol was removed following the same method as for the removal of the medium and PBS.

To color the cells, 1 ml of 0,01% Crystal Violet in 20% ethanol was added as it was done with the methanol, incubating at room temperature for 15 minutes. The excess of Crystal Violet that remained in the upper part of the modified Boyden chamber was removed with the help of a cotton swab, so a clearer view of the cells was possible.

Several fields were photographed for each Boyden chamber and the cells for each field were counted.

III. 20. 3. Xenograft Formation Assay

Cells ($2.5x10^6$) were resuspended in 50 µl of PBS and 50 µl of Matrigel growth factor reduced (BD Biosciences), so that the volume injected in each mouse was 100 µl on each side. Cells were then subcutaneously inoculated in nude mice of 6 weeks old.

A biologic triplicate was made for each condition, and all injections followed the scheme represented in Figure 11.

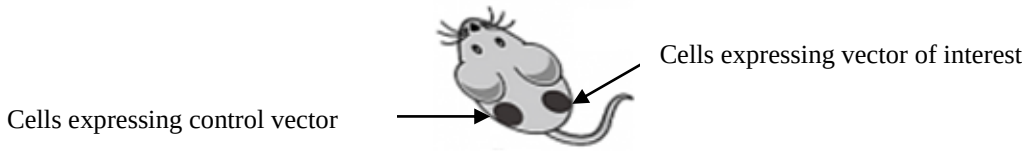


Figure 11 - Schematic illustration showing how cells were inoculated in mice. Source: [8]

III. 21. Statistical analysis

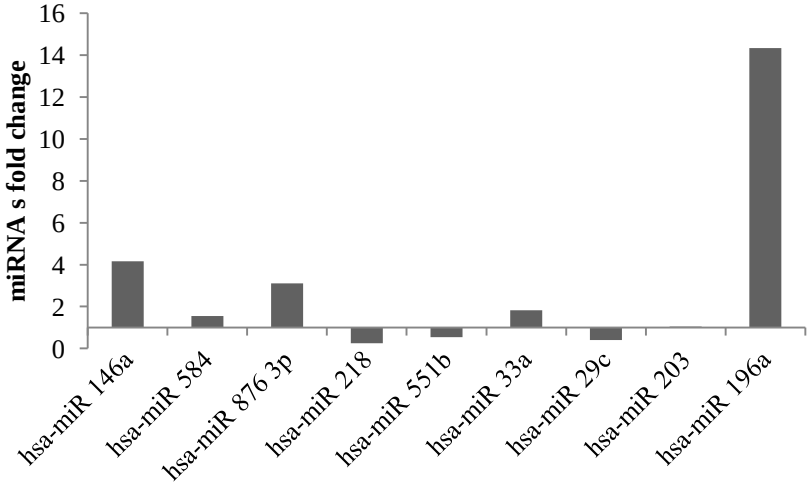
All data were statistically analyzed with the program GraphPad Prism 5, with the most correct approach – one-way or two-way ANOVA - being chosen for each experiment. The post-test used was the Bonferroni post-test.

Results

IV.1. Previous Results

Previously to the start of this thesis project there were made some experiments that allowed further developments. NCI-H460, a non-small cell lung cancer cell line was genetically modified for the knockdown of the PI3K/AKT pathway through the silencing of AKT1, AKT2 and PI3K. By microarray hybridization several differentially expressed miRNAs were identified in lentiviruses-infected cells compared to the control cell lines (from this point on called Scramble or SCR). Among them 9 miRNAs (hsa-miR-146a, hsa-miR-584, hsa-miR-876-3p, hsa-miR-218, hsa-miR-551b, hsa-miR-33a, hsa-miR-29c, hsa-miR-203, hsa-miR-196a) were chosen based on the highest fold change values. These results were validated by Real-Time PCR.

The expression of these 9 miRNAs was also analyzed in 29 human primary lung cancer samples (mostly adenocarcinomas) by Real-Time PCR. The results obtained are represented in Graphic 1.

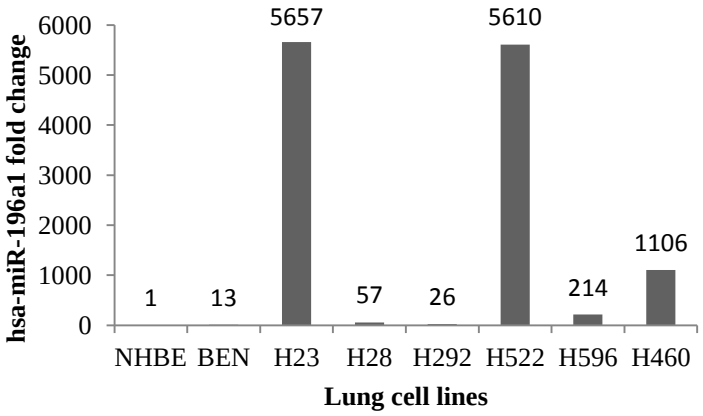


Graphic 1 - miRNA expression fold change in tumor samples when compared to normal tissue samples.

In Graphic 1 is evident the over-expression of hsa-miR196a in tumor samples when compared to normal tissue samples, being the miRNA with the major fold change in all samples. Fold changes in miRNA expression throughout this work will be considered significant when the values are higher than 2 or lower than 0.6.

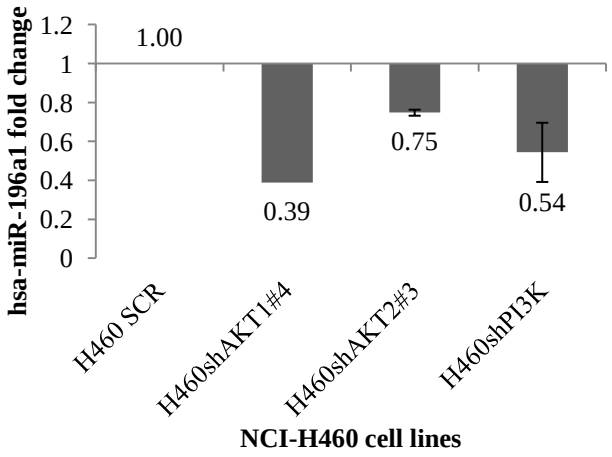
Furthermore, a Real time PCR was performed in different cancer cell lines and compared to a normal immortalized Human bronchial epithelial cells – NHBE – to see if the expression of hsa-miR-196a was modulated. The cancer cell lines are derived from different tumor types. BEN derived from squamous lung carcinoma, NCI-H23,

NCI-H522 from adenocarcinoma, NCI-H460 from large cell carcinoma, NCI- H292 from mucoepidermoid carcinoma, H28 from mesothelioma and H596 from adenosquamous carcinoma. In Graphic 2 is evident that in all cancer cell lines, miR-196a1 expression is up-regulated. Moreover, it can be observed that in adenocarcinoma cell lines this up-regulation is very emphasized followed by the cell line representing large cell carcinoma and the cell adenosquamous carcinoma. Thereby it seems that although miR-196a1 is over-expressed in all lung cancer cell lines, this is more evident in all cell lines representing NSCLC.



Graphic 2 – hsa-miR-196a1 fold change in lung cancer cell lines compared to a immortalized cell line (NHBE).

In Graphic 3 are present the results for the differential expression of hsa-miR-196a1 for NCI-H460 cell lines compared to the control cell line. This cellular system revealed a down-regulation of hsa-miR-196a1 in interfered cells respect to their control. As cells interfered for AKT1 were the ones showing a major down-regulation of hsa-miR-196a1, these cells were chosen to study the impact of miR-196a1 over-expression and to observe if this over-expression could restore the effect of AKT1 presence in cells.



Graphic 3 - hsa-miR-196a1 expression in NCI-H460 derived cell lines.

IV.2. Results

IV.2.1. Choice and Confirmation of miR-196a1 targets correlated to PI3K/AKT pathway.

IV.2.1.1. Choice of hsa-miR-196a1 targets

In order to choose hsa-miR-196a1 targets that are correlated to the PI3K/AKT pathway, two data bases were consulted TargetScan.org and microRNA.org. The predicted targets were matched to the mRNA expression profiling of NCI-H460 modified system obtained by a Microarray analysis. FoxO1, FoxO3 and CDKN1B (p27) were chosen for further studies.

IV.2.1.2. 3'UTR amplification

3'UTR of the genes of interest were amplified as described before. The amplification of 3'UTR of p27's gene is represented on Figure 13. In Figure 12 the amplification of 3'UTRs of FoxO1 in the first lane and FoxO3 in the second lane are presented.

Figure 13 - Agarose gel showing the amplification of 3'UTR of p27.

Figure 12 - Agarose gel showing the amplification of 3'UTRs of FoxO1 in the first lane and FoxO3 in the second lane.

IV.2.1.3. Colonies screening by PCR

After the amplification of the 3'UTR of the genes of interest, these inserts were cloned into pGL3-control vector. Bacteria were transformed to amplify this construct. The colonies which were successfully transformed with the pGL3-control vector containing the 3'UTRs are named positive clones. In order to identify the positive clones, the protocol of PCR on colony was performed and the PCR products were run in an agarose gel.

In Figures 14, 15 and 16 are presented the agarose gels which allowed the identification of the true positive clones used in further procedures. The lanes which do not show any visible band represent negative clones, thus, colonies that have not integrated the pGL3-control vector with the insert.

Figure 14 - Screening of colonies positive for p27 3'UTR.

Figure 15 - Screening of colonies positive for FoxO1 3'UTR.

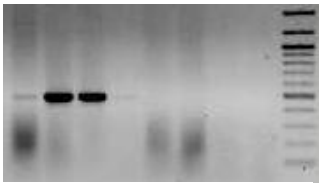


Figure 16 - Screening of colonies positive for FoxO3 3'UTR.

IV.2.1.4. Digestion with XbaI

The samples positive for PCR were subsequently analyzed through digestion with endonuclease XbaI, which restriction site is present in pGL3-control vector, to rule out the false positives.

To make the digestion with XbaI, plasmid DNA was extracted from the bacteria following the Miniprep protocol. Figures 17, 18 and 19 show the digestion of plasmid DNA of positive clones for PCR.

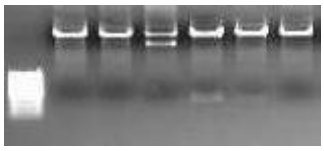


Figure 17 - Digestion of positive clones for (for PCR) p27 with XbaI.

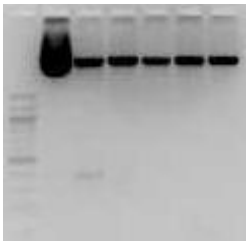


Figure 19 - Digestion of positive clones (for PCR) for FoxO1 with XbaI.

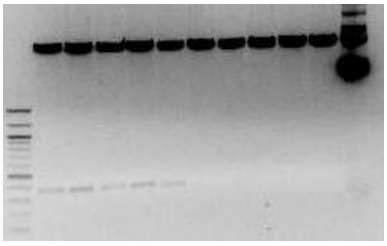


Figure 18 - Digestion of positive clones (for PCR) for FoxO3 with XbaI.

IV.2.1.5. Sequencing results

In order to be sure that the clones were truly positive, the samples positive for digestion with XbaI were sequenced. The results of sequencing were analyzed with the FinchTV software, so as to confirm that the amplified sequence is the sequence of the 3'UTR expected and to confirm the presence of the miRNA Responsive Element (MRE) for hsa-miR-196a1. In Figures 20, 21 and 22 are presented the images extracted from FinchTV software, highlighting the MRE for hsa-miR-196a1. These constructs will be used further on to perform the Dual Luciferase Report Assay to validate the binding of hsa-miR-196a to the 3'UTRs of the target genes.

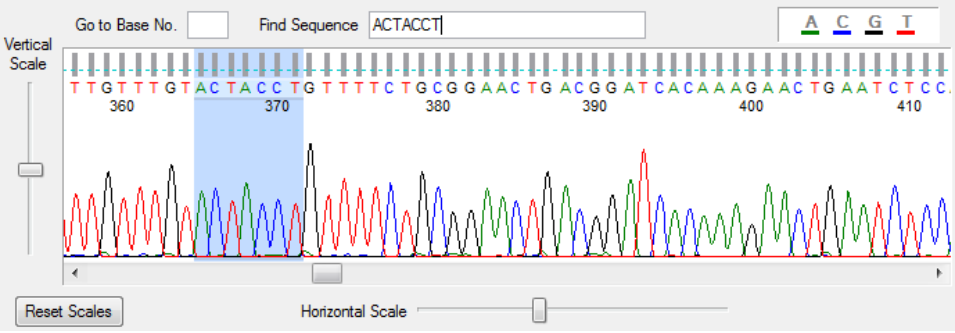


Figure 20 - Sequencing results for the positive clone for FoxO1 used in further experiments. The highlighted region represents the sequence recognized by hsa-miR-196a1.

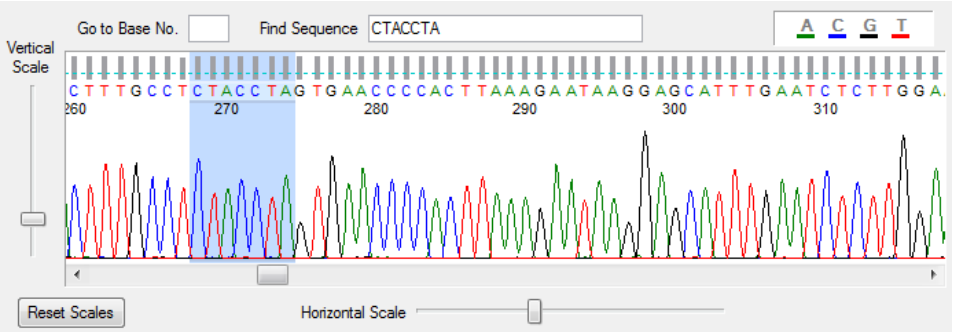


Figure 21 - Sequencing results for the positive clone for FoxO3 used in further experiments. The highlighted region represents the sequence recognized by hsa-miR-196a1.

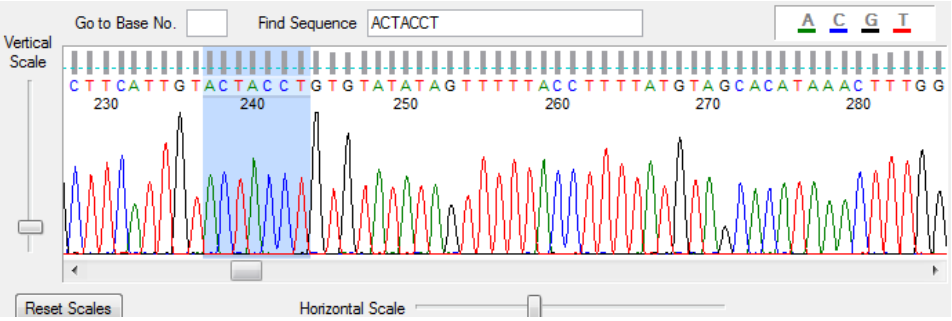


Figure 22 - Sequencing results for the positive clone for p27 used in further experiments. The highlighted region represents the sequence recognized by hsa-miR-196a1.

IV.2. Production of stable clones

Previously to this microRNA approach, the PI3K-AKT pathway in lung cancer was already being molecularly dissected by co-workers, so the cell lines to see the specific effect of some alterations to this pathway had already been obtained. Previous data led to the choosing of cell lines to be further infected. The main goal was now to produce stable clones where an inversion of the expression pattern of hsa-miR-196a was observed, by infecting the cell line where it was over-expressed with an anti-miR-196a vector, the line that had a knockdown expression with a hsa-miR-196a vector, and both lines with the equivalent Empty Vector. As the vectors used to infect the cells contain the gene which encodes GFP, the expression of this protein allowed the picking of fluorescent clones and further amplification. Figures 23 to 26 show the cell lines used to make further experiments. It is clear that the nearly 90% of these cells express the vector of interest, which gives reliability to the results presented using these cell lines as a start point.

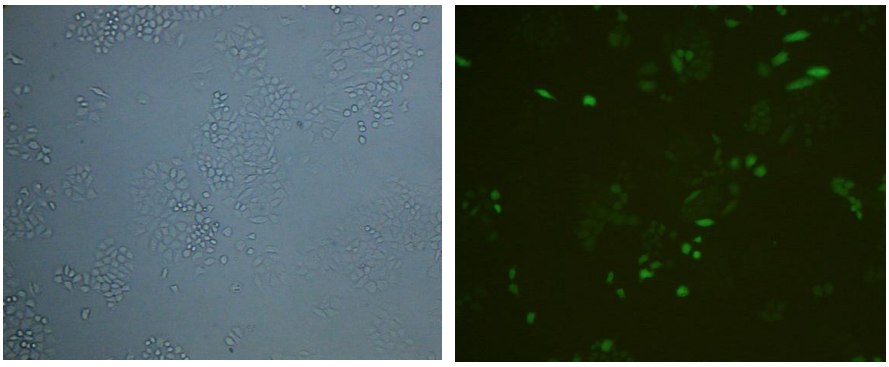


Figure 23 - NCI-H460 shAKT1 infected with the Empty Vector (H460 shAKT1 EV). At the left a photo of a field without fluorescence light and at the right a photo of the same field taken with fluorescence light.

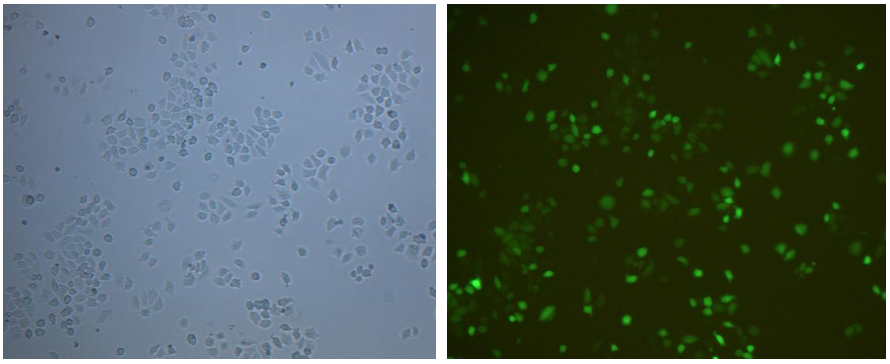


Figure 24 - NCI-H460 shAKT1 infected with the hsa-miR-196a1 Vector (NCI-H460 shAKT1 miR-196a1). At the left a photo of a field without fluorescence light and at the right a photo of the same field taken with fluorescence light.

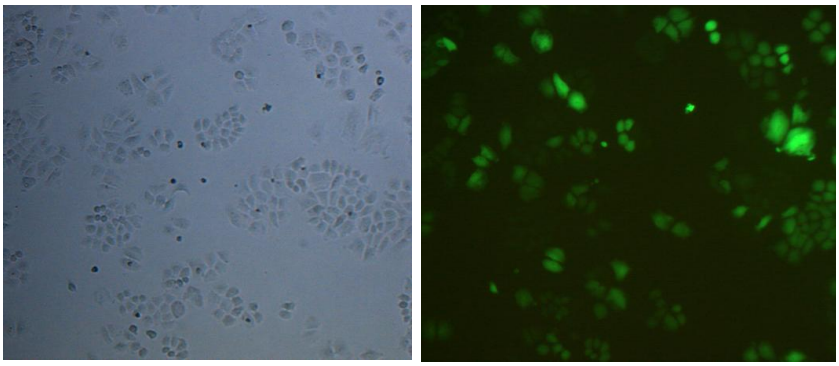


Figure 25 - NCI-H460 SCR infected with the Empty Vector (H460 SCR EV). At the left a photo of a field without fluorescence light and at the right a photo of the same field taken with fluorescence light.

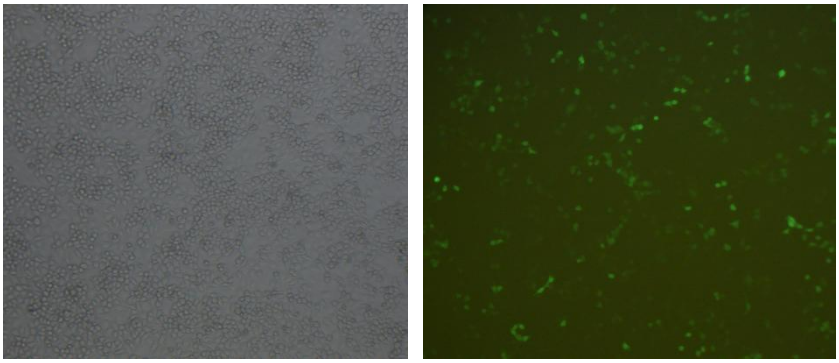
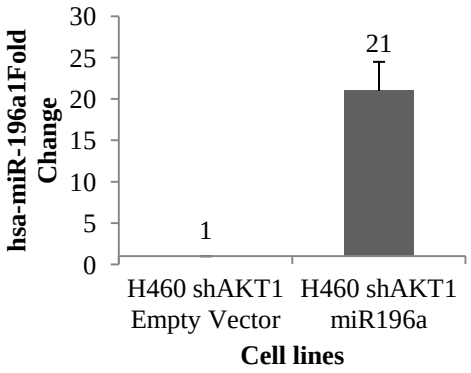


Figure 26 - NCI-H460 SCR infected with the anti-miR-196a Vector (H460 SCR anti-miR-196a). At the left a photo of a field without fluorescence light and at the right a photo of the same field taken with fluorescence light.

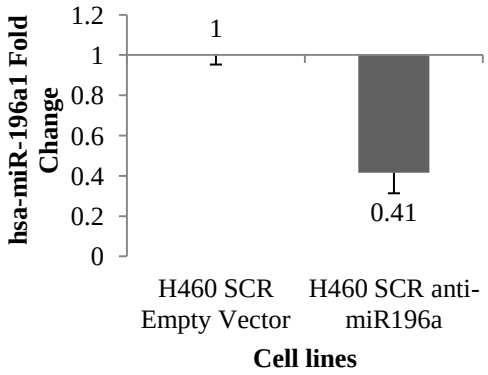
IV.2.1. Confirmation of the alteration of hsa-miR-196a1 expression in the cellular system.

In order to confirm the over-expression or down-regulation of hsa-miR-196a1, a Real-time PCR was performed. In Graphic 4, is visible the over-expression of hsa-miR-196a1 in NCI-H460 shAKT1 cells infected with the lenti-vector containing the miRNA when compared with the same cells infected with the empty vector.



Graphic 4 - Over-expression of hsa-miR-196a1 in NCI-H460 shAKT1 infected with the vector containing the miRNA when compared to NCI-H460 shAKT1 infected with the Empty Vector.

hsa-miR-196a1 knockdown in NCI-H460 SCR cell line infected with the anti-miR-196a lenti-vector compared to the same cell line infected with the empty vector is visible in Graphic 5.



Graphic 5 - hsa-miR-196a1 knockdown in NCI-H460 SCR infected with the vector containing the anti-miRNA when compared to NCI-H460 SCR infected with the Empty Vector.

Observing the last two graphics, one can conclude that there is a different modulation of hsa-miR-196a1 in the cell lines infected. Therefore, these cell lines constitute a good model to study the biological effect of this miRNA and to correlate it to the PI3K/AKT pathway.

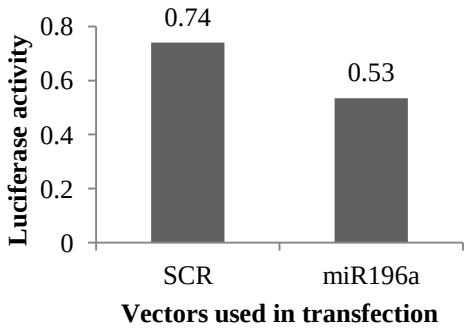
IV. 3. Confirmation of target modulation

IV.3.1. Luciferase Assay

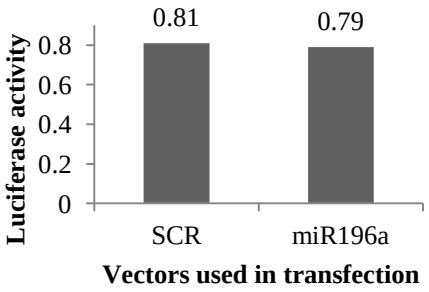
The plasmid DNA from positive clones for sequencing was amplified and used to transfect HEK-293T cells with the siPORT™ *Neo*FX™ transfection agent. The values for luciferase activity were obtained following the Dual-Luciferase® Reporter Assay (Promega).

In Graphic 6 we can see that cells that were both transfected with pGL3-control containing the insert of FoxO1 3'UTR and hsa-miR-196a show a knockdown of luciferase activity respect to the same cells transfected with negative control (SCR) and the vector of interest. This happened because when the miRNA binds to the 3'UTR of its target gene the mRNA becomes instable and as the luciferase gene is no longer expressed, is visible a down-regulation of the luciferase activity, whose gene is directly linked to the insert. Thereby, when a down-regulation of luciferase activity is evident one can infer that the insert is a target of the miRNA in study.

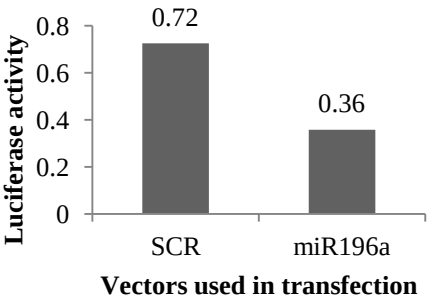
However, the results for FoxO3, represented on Graphic 7, do not show this reduction indicating that this is not a relevant target for hsa-miR-196a. On the other hand, cells transfected with the vector containing p27 3'UTR show a strong reduction of luciferase activity showing that this is a target for hsa-miR-196a1 as can be seen in Graphic 8.



Graphic 6 – Down-regulation of luciferase activity on cells transfected with pGL3-control containing the insert of FoxO1 3'UTR and hsa-miR-196a.



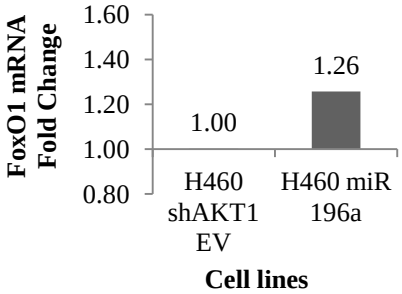
Graphic 7 - Cells transfected with pGL3-control containing the insert of FoxO3 3'UTR and hsa-miR-196a do not show a significant reduction of luciferase activity.



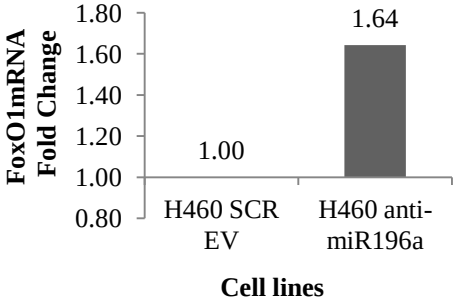
Graphic 8 – Strong down-regulation of luciferase activity on cells that were both transfected with pGL3-control containing the insert of p27 3'UTR and hsa-miR-196a.

IV.3.2. Real-time PCR Analysis

Real-time PCR was carried out in order to evaluate if there is a differential regulation of mRNAs of the genes of interest in the cellular system created. On Graphic 9a, b, is visible that the increase or knockdown of miR-196a1 does not have a significant effect on FoxO1 mRNA levels, considering the threshold values 0.5 and 2, indicating that the miR-196a1 does not promote the degradation of this mRNAs.



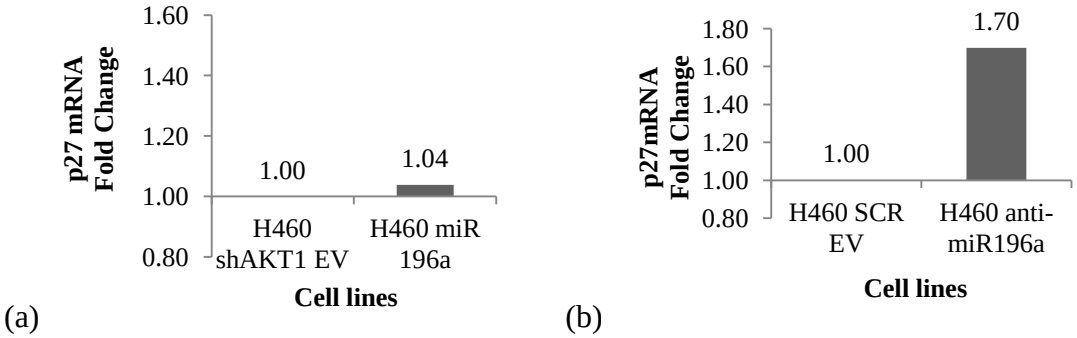
(a)



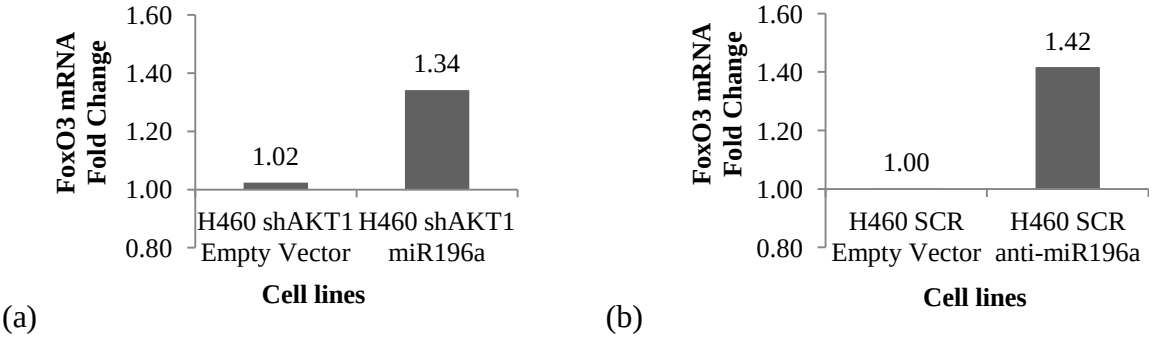
(b)

Graphic 9 – The graphics represent the modulation of FoxO1 mRNA in the cell lines created. (a) Modulation of FoxO1 mRNA in cells expressing the miR-196a1 vector is not significant compared to its control. (b) Modulation of FoxO1 mRNA in cells expressing the anti-miR-196a1 vector is not considered significant compared to its control.

Fold changes in p27 mRNA and FoxO3 mRNA showed identical results to those obtained for FoxO1 (Graphics 10a, b and 11a, b). In conclusion, miR-196a1 does not seem to modulate the levels of mRNA of any of the genes in study, indicating that its seed sequence is not fully complementary to any of the 3'UTR of the genes in study.



Graphic 10 - The graphics above show the modulation of p27 mRNA in the cell lines created. (a) Modulation of p27 mRNA in cells expressing the miR-196a1 vector is comparable to its control (b) Modulation of p27 mRNA in cells expressing the anti-miR-196a1 vector is not considered significant compared to its control.



Graphic 11 - The graphics represented above show the modulation of FoxO3 mRNA in the cell lines created. (a) The difference in fold change of FoxO3 mRNA in cells expressing the miR-196a1 vector is not significant when compared to its control. (b) Modulation of FoxO3 mRNA in cells expressing the anti-miR-196a1 vector is not considered significant compared to its control.

IV.3.3. Western Blot Analysis

miRNAs can also down-regulate the translation of mRNAs with which they do not have a fully complementarity. The levels of protein expression of the genes in study were thereby analyzed through Western Blot analysis and the signals were normalized with an antibody against tubulin, a protein which has a constitutive expression. In figure 28 is visible a knock-down of the expression of FoxO1 on the cell line expressing the miR-196a1 vector when compared to its control (H460 shAKT1 EV) and a up-regulation on the cell line expressing the anti-miR-196a1 vector when compared to the control cell line (H460 SCR EV). The results obtained for p27 are the similar to those obtained for FoxO1 and are shown in figure 27.

These results indicate that miR-196a1 regulates the expression of these genes at protein synthesis level, inhibiting it. The mechanism of this regulation is not known, but it falls under the scope of this project.

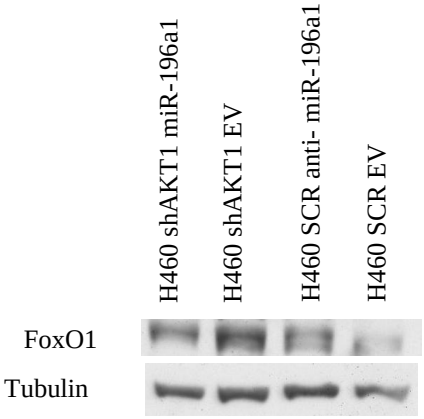


Figure 28 - Western Blot using an anti-FoxO1 antibody reveals a modulation of FoxO1 expression by miR-196a1. The signals are normalized with an anti-Tubulin antibody.

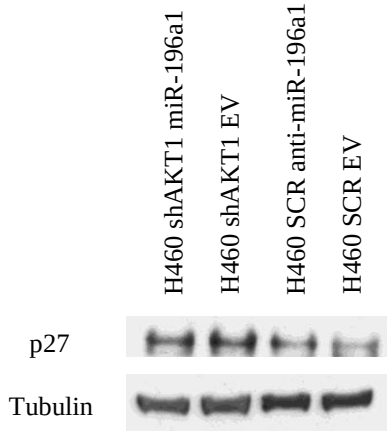


Figure 27 - Western Blot using an anti-p27 antibody shows a modulation of p27 expression by miR-196a1. The signals are normalized with an anti-Tubulin antibody.

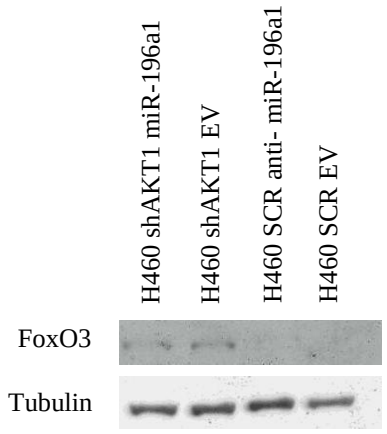


Figure 29 - Western Blot using an anti-FoxO3 antibody does not show a modulation of FoxO3 expression by miR-196a1. The signals are normalized with an anti-Tubulin antibody.

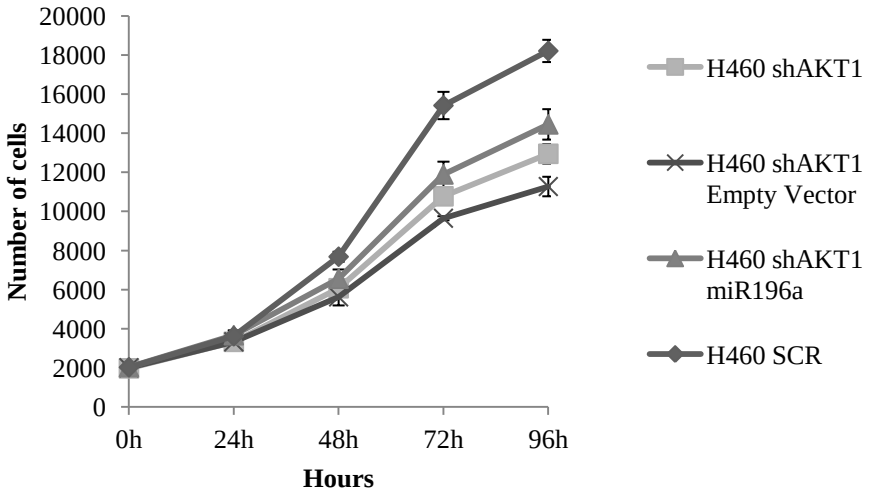
In figure 29, is visible that the protein levels of FoxO3 are low in general and a regulation of FoxO3 by miR-196a1 is not observed once the bands for H460 shAKT1 miR-196a and its control appears to have no difference between them. These observations may lead to the conclusion that FoxO3 is not a true target of miR-196a1.

IV.4. Biological Assays

IV.4.1. MTT Results

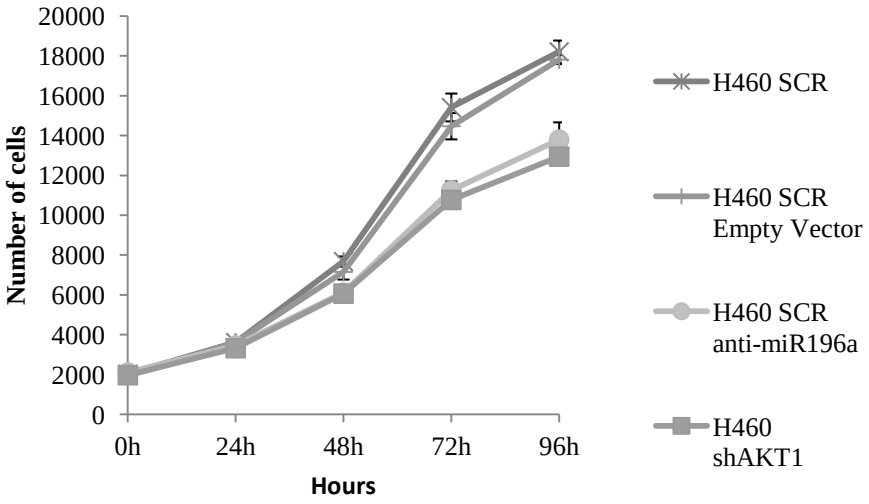
This assay was performed for all cell lines, produced in this work and parental cells, simultaneously. The number of cells was inferred from the absorbance read in EnVision™ 2103 Multilabel Reader, considering that the value 0.1 corresponded to 1000 cells.

Graphic 12 corresponds to H460 shAKT1 miR-196a1 and the respective controls. It is clear that the cell line H460 shAKT1 miR-196a1 has a proliferative advantage when compared to its direct controls, H460 shAKT1 and H460 shAKT1 EV, being the cell line that comes the closest to its indirect control H460 SCR. Statistical analysis using two-way ANOVA and the Bonferroni post-test, indicated that H460 shAKT1 miR-196a1 is statically different from H460 shAKT1 and H460 shAKT1 EV, from 72h of the beginning of the assay, presenting a *p-value* of $p<0.0001$. No statistical significance was found in the difference between H460 shAKT1 and H460 shAKT1 EV.



Graphic 12 – Growing curve of H460 shAKT1 miR-196a1 presenting direct and indirect controls for this cell line.

Graphic 13, in its turn, represents H460 SCR anti-miR-196a1 and the respective controls. The cell line H460 SCR anti-miR-196a1 shows a reduced proliferative potential when compared to its direct controls, H460 SCR and H460 SCR EV, being also very close to its indirect control H460 shAKT1. Statistical analysis using 2 way ANOVA and the Bonferroni post-test, indicated that H460 SCR anti-miR-196a1 is statistically different from H460 SCR and H460 SCR EV, from 48h of the beginning of the assay, presenting a *p-value* of $p<0.0001$. No statistical significance was found in the difference between H460 SCR and H460 SCR EV.



Graphic 13 - Growing curve of H460 SCR anti-miR-196a1 presenting direct and indirect controls for this cell line.

IV.4.2. Wound healing assay

Although photos have been made each 12 hours from the moment the scratches were made, the differences are more evident after 24 and 48 hours, so the analysis was made referring to this two time points. In figure 30 is visible the difference between the migration potential of H460 shAKT1 miR-196a1 and H460 shAKT1 EV, once after 48h the cells on the cell line H460 shAKT1 miR-196a1 have already migrated to fill almost half of the initial scratch. The same is not true for H460 shAKT1 EV that shows a reduced migratory capacity when compared to H460 shAKT1 miR-196a1.

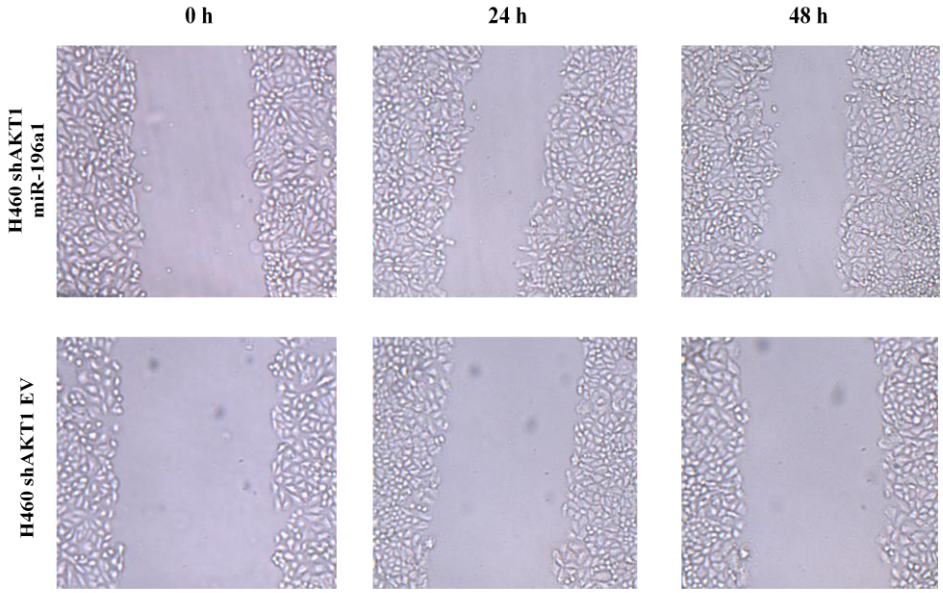


Figure 30 - Images of the same fields were taken at 24h and 48h from the moment the scratch was made. On the first row is presented the difference obtained for the cell line H460 shAKT1 miR-196a1 and the migration of the cells is clear.

In figure 31 it can be observed that the cell line H460 SCR anti-miR-196a1 has a minor migration potential than that of H460 SCR EV. After 24h is already evident that cells of the cell line H460 SCR EV have a strong migratory capacity comparing to H460 SCR anti-miR-196a1. This difference is further accentuated at 48h.

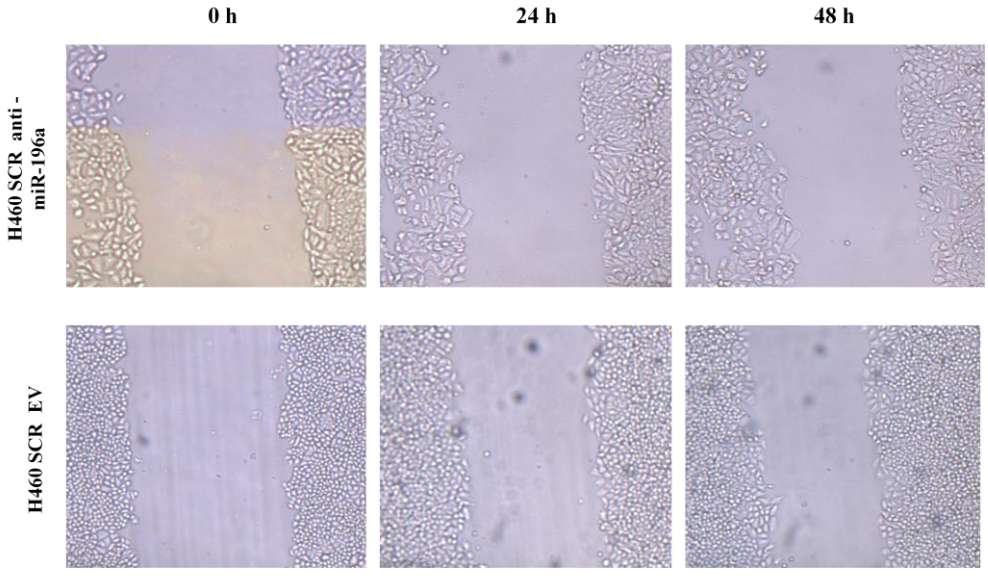
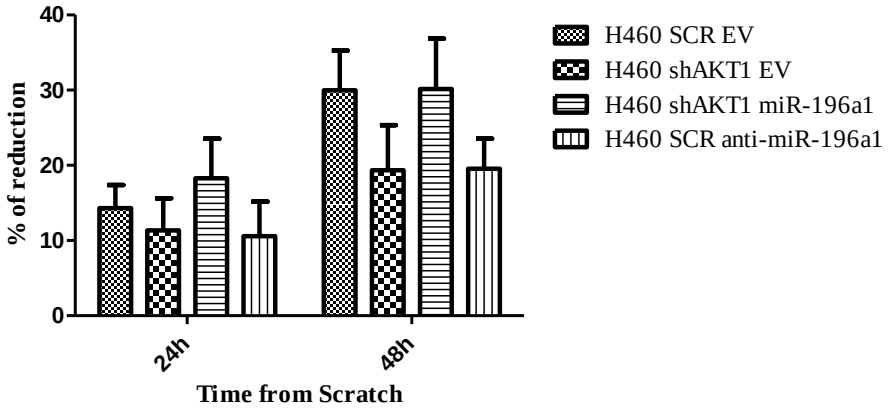


Figure 31 - Images of the same fields were taken at 24h and 48h from the moment the scratch was made. On the first row is presented the difference obtained for the cell line H460 SCR anti-miR-196a1. On the second row is visible that the cell line H460 SCR EV.

Three measures of the scratch were made for each field at different time points (approximately in the same places) and these measures were then compared to each other to infer the percentage of reduction of the scratch.

In Graphic 14 are presented the percentage of reduction obtained for each cell line. Interestingly, at 48 hours the cell line H460 SCR anti-miR-196a1 and H460 shAKT1 EV show approximately the same migratory capacity and the same happens for the cell lines H460 shAKT1 miR-196a1 and H460 SCR EV. This indicates that somehow the over-expression or knock-down of miR-196a1 is capable of inverting the phenotypes of the cells interfered for AKT1 or normally expressing AKT1 respectively, leading to the possibility that this miRNA modulates migration through the regulation of genes controlled by the PI3K/AKT pathway.



Graphic 14 – Percentage of reduction of scratches in the cell lines created. The patterns of migration obtained for the control cell lines are reversed by the over-expression or knock-down of miR-196a1.

Statistical analysis performed using two-way ANOVA and a Bonferroni post-test indicates that there is no statistical difference between H460 shAKT1 miR-196a1 and H460 SCR EV and between H460 SCR anti-miR196a1 and H460 shAKT1 EV. On the other hand, the cell lines H460 shAKT1 miR-196a1 and H460 SCR anti-miR-196a1 show a significant statistical difference from their direct controls, H460 shAKT1 EV and H460 SCR EV respectively, with a *p-value* of $p<0.01$.

IV.4.3. Tranwell Migration Assay

In order to confirm the results obtained for the wound healing assay and to understand if it the results were not influenced by the technique, another migration assay was preformed. The migration through the modified Boyden chamber forces the cells to pass through the pores of the membrane (physic barrier), as in the wound healing assay they did not have to surpass any physic obstacle to migrate. Nevertheless, the results obtained confirm a modulation of migration by miR-196a1.

Cells that have migrated are colored with a light violet shade. Figure 32 reveals that the cell line H460 shAKT1 miR-196a1 migrates more than H460 shAKT1 EV and that H460 SCR anti-miR-196a1 migrates less than its control H460 SCR EV. The pictures were taken with an inverted microscope with an amplification of 10X.

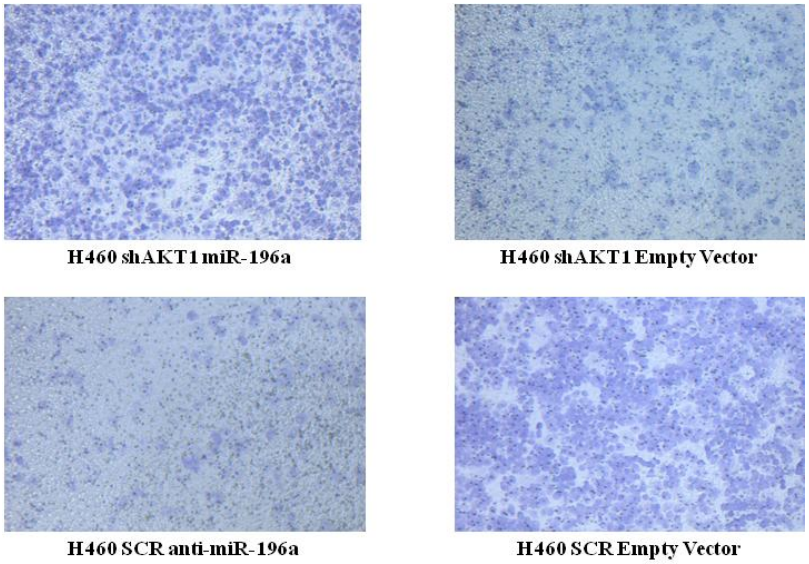
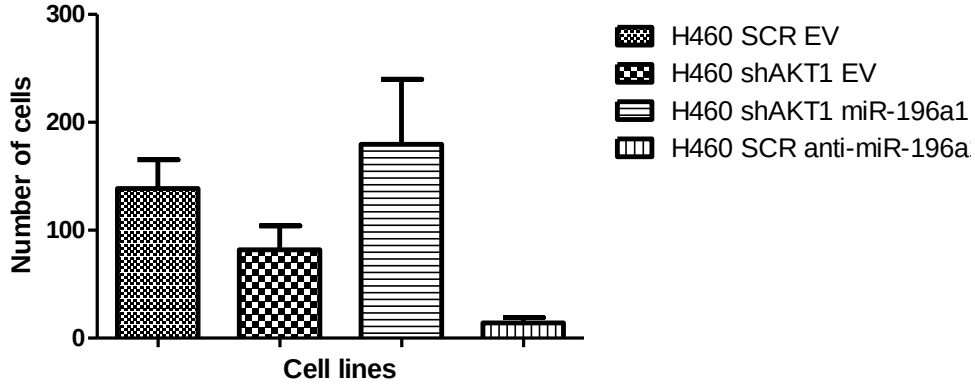


Figure 32 - In this figure are represented the images of the bottom part of the modified Boyden chambers.

Cells were counted in 8 fields randomly chosen, with an inverted microscope with an amplification of 20X. In graphic 15 are represented the average number of cells counted in each field for each cell line. Is evident that H460 shAKT1 miR-196a1 has a major number of migrating cells respect to all the other cell lines whereas H460 SCR anti-miR-196a1 shows a reduced number of migrating cells comparing to all the other cell lines.

Statistical analysis was preformed doing a one-way ANOVA with a Bonferroni post-test. The results indicate that H460 shAKT1 miR-196a1 has a significant statistical difference from its control with a *p-value* of $p<0.0001$ and H460 SCR anti-miR-196a1 also differs from H460 SCR EV, with the same *p-value*.



Graphic 15 – Average number of cells counted in each field for each cell line.

IV.4.5. Xenograft

Xenograft assay was preformed to evaluate the capacity of the cell lines created to induce tumorigenesis *in vivo*. After 3 weeks the cell line H460 SCR anti-miR-196a1 was not able to induce tumorigenesis whereas its control, H460 SCR EV, had already induced the formation of tumors bigger enough to force the ending of the experiment and the consequent sacrifice of the nude mice (Figure 33).

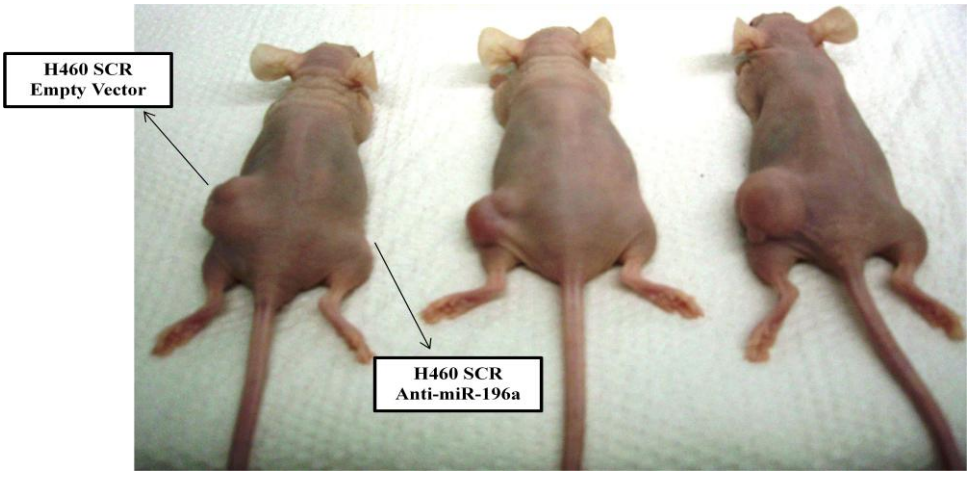
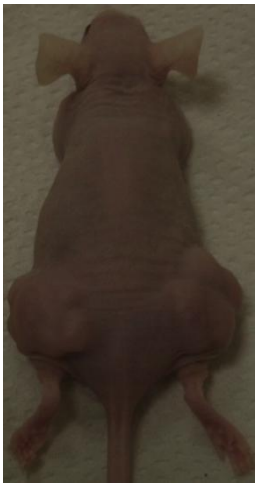


Figure 33 - Xenograft assay of the cell line H460 SCR EV (injected on the left side) and H460 SCR anti-miR-196a1 (injected on the right side).

The results for the cell lines H460 shAKT1 miR-196a1 and H460 shAKT1 EV were not reproducible for the biological triplicate. After 5 weeks of the beginning of the experiment one nude mouse had to be sacrificed. This mouse had developed tumors induced by both cell lines, but the tumor induced by the cell line H460 shAKT1 miR-196a1 was slightly bigger than the one induced by H460 shAKT1 EV (Figure 34a).

After another two weeks the other two nude mice were sacrificed. These two mice showed the development of tumors induced only for the cell line H460 shAKT1 miR-196a1 whereas H460 shAKT1 EV was not able to successfully induce tumorigenesis (Figure 34b). The results obtained showed that the cell line H460 shAKT1 miR-196a1 seems to be more tumorigenic than H460 shAKT1 EV.

(a)



(b)

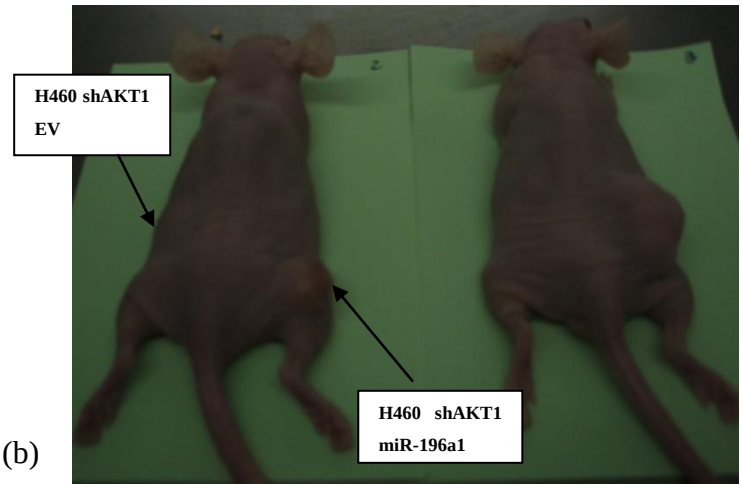


Figure 34 - Xenograft assay for cell lines H460 shAKT1 miR-196a1 and H460 shAKT1 EV. (a) Nude mouse sacrificed 5 weeks after the beginning of the experiment. (b) Nude mice sacrificed 7 weeks after the beginning of the experiment.

Discussion

V. Discussion

Since its discovery, miRNAs have been associated with the regulation of every cellular process investigated so far and the alteration of its expression is linked to several human diseases.

In cancer, they can act as oncogenes or tumour suppressor genes and may contribute to the loss of cell cycle control and increased proliferative capacity, characteristics of transformed cells. They can also contribute to the less differentiated state of tumours and can act simultaneously as apoptosis and checkpoint regulators directly or indirectly, regulating different targets.

The modification of expression of several miRNAs and miRNA processing machinery have shown to regulate autocrine/paracrine loops, anti-apoptotic genes, genes that promote tumour vasculature development, cell cycle related genes, metastasis related genes and chemoresistance genes in lung tissue, contributing greatly to the initiation and progression of lung cancer. Their deregulation in NSCLC can be used to sub-classify NSCLCs but specific miRNA profiles may also predict prognosis and disease recurrence in early-stage NSCLCs.

The role of hsa-miR-196a1 in oncogenesis seems to be cancer type-specific; nevertheless the over-expression of this miRNA is often associated with promotion of oncogenesis rather than its inhibition.

Several miRNAs have been found to normally down-regulate the PI3K/AKT pathway activation, such as miR-205, miR-331, miR-125, miR-7 and miR-126, and their knockdown is associated with the activation of the pathway and tumour progression. However, over-expression of other miRNAs, such as miR-21, miR-214 and the cluster miR-17-92 is highly associated with the activation of this pathway (Santarpia, *et al*., 2010). Schimanski *et al*., have validated an increase of phosphorylation of AKT in cells over-expressing hsa-miR-196a. Although this approach is interesting, the cellular system used in the presented work does not allow this kind of analysis once some of the parental cells are interfered for AKT1, thereby the levels of phosphorylation of AKT could not be accessed.

The target genes chosen to this study are regulated by the activation of PI3K/AKT pathway. FoxOs are one of the major targets of the PI3K/AKT signalling in the presence of growth factors and nutrients. Activation of the PI3K pathway promotes

phosphorylation, and consequent nuclear exclusion of the FoxOs resulting in the blockage of their growth inhibitory transcriptional functions. Since growth inhibitory functions of FoxOs involve activation of several proteins that include inhibitors of cell cycle such as p27, retinoblastoma protein, inducers of extrinsic as well as intrinsic apoptosis, and interactions with tumour suppressors such as p53. FoxOs are known to function in a tissue and context-dependent manner, and functional redundancy exists among them. FoxO proteins have as their transcriptional target PTEN, so their expression promotes the inhibition of PI3K/AKT pathway (Tzivion, *et al*., 2011; Zhang, *et al*., 2011).

p27 may be directly or indirectly regulated by active AKT. AKT is capable of directly phosphorylating p27, causing its cytoplasmic retention and subsequent degradation through the ubiquitin-proteasome pathway and can inhibit its transcription through FoxO phosphorylation and exclusion from the nucleus (Fujita, *et al*., 2002; Motti, *et al*., 2005; Rassidakis, *et al*., 2005).

Target genes were experimentally confirmed by Dual Luciferase reporter assay. The pGL3-Control Vector was chosen because it provides a basis for the quantitative analysis of factors that potentially regulate mammalian gene expression by *cis*- or *trans*-acting factors. The coding region for firefly (*Photinus pyralis*) luciferase has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. The assay of this genetic reporter is rapid, sensitive and quantitative and XbaI site just downstream of luc^+ gene facilitates insertions of the 3´ untranslated region of mRNAs.

The presence of SV40 large T antigen in HEK-293T promotes replication from the SV40 origin found in the promoter of pGL3-Control Vectors. The combination of the large T antigen and the SV40 origin will result in a higher copy number of these vectors, which in turn may result in increased expression of the reporter gene compared to other cell and vector combinations [9]. There are several works in which this system has proven to be useful and has given good results (Visone, *et al*., 2007; Kulkami, *et al*., 2011).

This method allowed to confirm FoxO1 and p27 as targets of miR-196a1, but not FoxO3, once there was no significant reduction of luciferase activity when this 3'UTR was inserted downstream of luc^+ gene. Nevertheless, this gene was used for further analysis to confirm if there was not a modulation.

To analyse if miR-196a1 promoted the cleavage of target genes mRNAs, Real-Time PCR was performed in order to see if there was a significant fold change in

mRNA expression levels. Analysing the obtained results, miR-196a does not seem to modulate the expression of these mRNAs.

The results for p27 are not in agreement with Sun, *et al*., 2012, which found a down-regulation of the mRNA expression of p27 in cells over-expressing hsa-miR-196a in a gastric cancer cell line. This may be explained with former observations that miRNAs may have a tissue-specific fuction.

Western blot analysis showed a modulation of FoxO1 and p27 proteins levels but not of FoxO3. On the one hand, this can lead to the possibility that FoxO3 is not a target of miR-196a1. On the other hand, this confirms FoxO1 and p27 as targets of miR-196a1. The results obtained indicate that in this cellular system FoxO1 and p27 are regulated by miR-196a1 at the protein synthesis level.

Biological assays showed an increased proliferation in cells expressing the miR-196a1 vector and an inhibition of this process in cells expressing the anti-miR-196a1 vector when compared to their respective direct and indirect controls. These results are similar to those obtained by Luthra, *et al* (2008) for endometrial, esophageal and breast cancer cell lines. Other reports in other cellular systems have also correlate FoxO1 and p27 to this phenomenon, stating that a decrease in FoxO1 levels down-regulates the transcription of p27 thus promoting proliferation (Adachi, *et al*., 2007; Saramaki, *et al*., 2009). Wu, *et al* (2012) have obtained the same results for a cancer cell line of colorectal carcinoma (HCT116) using hsa-miR-223. Nevertheless, miR-196a1 has not induced proliferation in another cellular system using the cell line SW480 (colorectal carcinoma) reinforcing the statement that miRNAs have cell line specific effects (Schimanski, *et al*., 2009).

As in this work the modulation of FoxO1 and p27 has been confirmed, this may be one of the ways in which miR-196a induces proliferation.

miR-196a1 has proven to modulate cell migration in the cellular system created. This have also been observed in the work developed by Schimanski *et al* (2009). FoxO1 has been associated with the down-regulation of Runx2, a transcription factor of genes associated with improved migration, and subsequently has also been associated with reduced migration (Potente, *et al*., 2005; Zhang, *et al*., 2011). The role of p27 in migration is not clear and has been proved to be very complex. Several reports point to modulation of this process by p27, but the results are often cell type-specific, once it can induce or inhibit migration depending on the molecules with which it associates (Besson, *et al*., 2004).

Therefore, the mechanism by which miR-196a1 induces cellular migration can be explained, in part, due to the down-regulation of the studied targets, especially FoxO1, once the influence of p27 had to be explored by other techniques.

Cells over-expressing hsa-miR-196a1 induced larger tumours *in vivo* than their control and cells expressing the anti-miR-196a1 vector were not able to induce tumour formation *in vivo*, indicating the oncogenic potential of this miRNA.

The vectors of hsa-miR-196a1 and anti-miR-196a1 were inserted in different cell lines in order to correlate the effects of over-expression or knockdown of miR-196a1 with the PI3K/AKT pathway.

Finally, this cellular system successfully allowed the correlation between miR-196a1 and the PI3K/AKT pathway, once over-expression of this miRNA in a cell line interfered for AKT1 lead to a partial restoring of the phenotype of cells expressing AKT1 normally and that have a major activation of the PI3K/AKT pathway, in functional assays.

Conclusions

VI. Conclusions

This is not a conclusive study and it opens the door to further approaches, once miR-196a1 may also be co-involved in other processes that lead to tumour progression that have not yet been studied. Additional studies can be made to access the whole miR-196a role in NSCLC. There are some data in bibliography confirming this miRNA to be involved in other cellular processes (with different cellular systems) such as colony formation, influence in apoptosis, reduced adhesion and chemoresistance. In order to fully characterize the influence of this miRNA and its correlation with PI3K/AKT pathway in NSCLC some other studies have to be performed such as anchorage-dependent and independent colony formation, invasion assays, adhesion assays, cell-cycle studies and chemotherapy sensibilization assays.

With this work, there has been proven the influence of hsa-miR-196a1 in the PI3K/AKT pathway, through the modulation of FoxO1 and p27 and partially recovering of the phenotype of active AKT in cells interfered for AKT1. The influence observed in proliferation and migration also confirms the oncogenic action of hsa-miR-196a1 in NSCLC, once it regulates processes that are associated with the first steps in tumour progression and it was able to modulate tumour formation *in vivo*.

This can be important in further therapy strategies and diagnosis approaches and reconfirms the major role of PI3K/AKT pathway in NSCLC, highlighting this pathway has an important therapy target.

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