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Master's Thesis:

MiR-9, an IKKα-regulated miRNA in lung cancer

Inter-institutional Interdepartmental Program of Postgraduate Studies
Molecular and Cellular Biology and Biotechnology

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Summary

Lung cancer is the leading cause of cancer-related death worldwide and the second most common cancer in both men and women. Despite the development of new therapeutic approaches, the 5-year survival rates are still low and resistance to therapy often arises.

The NF-κB transcription factors (TFs) which can either activate or suppress gene expression, are also aberrantly activated in pulmonary diseases and non-small cell lung cancer (NSCLC), where they promote chronic inflammation, cell proliferation and survival. NF-κB transcription factors regulate the expression of protein coding and non-coding genes, such as microRNAs, however the intricate complexity of the NF-κB and miRNA regulatory network is yet to be fully understood.

The NF-κB TFs can be activated mainly by two signalling pathways: A canonical IKKβ-mediated NF-κB signalling pathway leading to the activation and nuclear translocation of the RelA/p50 heterodimeric TF where it binds to gene target promoters influencing gene expression, and a non-canonical IKKα-mediated NF-κB signalling pathway leading to the activation and nuclear translocation of the RelB/p52 heterodimeric TF where it binds to a distinct set of gene target promoters influencing gene expression. In addition, the upstream IKKβ and IKKα Ser/Thr activating kinases have also NF-κB independent effects.

The IKKα Ser/Thr kinase has been shown to act as a tumour suppressor in NSCLC, in an NF-κB independent manner. Our laboratory has previously demonstrated that IKKα silencing causes upregulation of the hypoxia inducible factor HIF-1α, promoting tumour growth under hypoxic conditions in two *in vivo* mouse and human lung cancer models. To further explore the mechanisms by which IKKα acts as a tumour suppressor in NSCLC, this thesis aimed to explore the miRNA expression profile of IKKα depleted cells and study their mechanism of action.

To identify miRNAs that are regulated by IKKα, the lung adenocarcinoma cell line A549 was chosen, and an IKKα knockdown (KD) stable cell line was generated. Total RNA from control and $IKK\alpha^{KD}$ cells was isolated and Nanostring miRNA analysis was employed to study the expression levels of 800 miRNAs in the samples. MiR-9-5p was identified as the most significant hit, where loss of IKKα resulted in the upregulation of miR-9-5p expression. MiR-9-5p is a known tumour promoter miRNA and has been implicated in lung cancer progression and metastasis, although there is still lack of knowledge regarding its exact mechanism of action. Therefore, we chose to further study the role of miR-9-5p in NSCLC and miR-9-5p overexpressing stable cell lines were generated in A549 and H1299 cell lines.

Phenotypic assays were performed to study the effects of miR-9-5p on cell behaviour. While cell proliferation and cell cycle progression *in vitro* were not affected by the overexpression of

miR-9-5p, cell invasiveness was significantly increased, implicating miR-9-5p in epithelial to mesenchymal cell transition (EMT). In contrast, *in vivo* growth of A549 and H1299 miR-9-5p as xenografts in immunocompromised NSG mice resulted in the formation of larger tumours, with A549 miR-9-5p tumours grown as xenografts weighing 2 times more and H1299 tumours weighing 2.2 times more than their control counterparts. In order to identify miR-9-5p potential targets, the mirDIP database was used to access predicted miR-9-5p targets, and the resulting set of genes was further reduced to Wnt-associated genes due to their well-described role in EMT. We found that miR-9-5p targets *CDH1*, the gene encoding for the critical adherens junction transmembrane protein, E-Cadherin. We showed that miR-9-5p-mediated loss of E-Cadherin promoted the upregulation of active β-catenin, the main downstream regulator of the Wnt pathway. In addition, the main inhibitor of β-catenin, GSK-3β, was found inactivated, while the positive regulator of β-catenin, Akt-1, was found activated in miR-9-5p overexpressing cells. The EMT markers N-Cadherin and Vimentin were also upregulated in miR-9-5p overexpressing cells, further confirming the induction of an EMT-like phenotype. In conclusion, IKKα acts as tumour suppressor by suppressing the expression of the oncogenic miR-9-5p. We have demonstrated that miR-9-5p acts as a tumour promoter in NSCLC by targeting E-Cadherin, activating the Akt-1/GSK-3β/β-catenin pathway and promoting the expression of EMT related factors N-Cadherin and Vimentin, leading to increased cell invasion and tumour growth.

Περίληψη

Ο καρκίνος του πνεύμονα αποτελεί το δεύτερο συχνότερο τύπο καρκίνου και την κύρια αιτία θανάτου από καρκίνο παγκοσμίως. Παρά την ανάπτυξη νέων θεραπευτικών προσεγγίσεων, το ποσοστό πενταετούς επιβίωσης των ασθενών παραμένει χαμηλό λόγω της διάγνωσης σε προχωρημένα στάδια της νόσου αλλά και της εμφάνισης ανθεκτικότητας στη θεραπεία.

Οι μεταγραφικοί παράγοντες (TFs) NF-κB δρουν επάγοντας ή καταστέλλοντας την έκφραση μιας μεγάλης ποικιλίας γονιδίων στόχων. Σε χρόνιες φλεγμονώδεις νόσους του πνεύμονα, καθώς και στον καρκίνο του πνεύμονα, η σηματοδοτική πορεία του NF-κB συχνά απορρυθμίζεται με αποτέλεσμα την ιδιοστατική ενεργοποίησή της. Η απορρύθμιση αυτή οδηγεί σε χρόνια φλεγμονή, προαγωγή του κυτταρικού πολλαπλασιασμού και της κυτταρικής επιβίωσης. Οι μεταγραφικοί παράγοντες NF-κB ρυθμίζουν την έκφραση γονιδίων που κωδικοποιούν πρωτεΐνες αλλά και μη κωδικά μόρια RNA, όπως τα microRNA (ή μικρά μόρια RNA), ωστόσο η σχέση μεταξύ NF-κB και miRNA δεν έχει ακόμη πλήρως αποσαφηνιστεί.

Η ενεργοποίηση των μεταγραφικών παραγόντων NF-κB επιτυγχάνεται με δύο κύριες πορείες σηματοδότησης: την IKKβ-εξαρτώμενη κανονική σηματοδοτική πορεία, κατά την οποία τα ετεροδιμερή RelA/p65-p50 και cRel-p50 μετατοπίζονται στον πυρήνα όπου ρυθμίζουν την έκφραση γονιδίων-στόχων τους, και την IKKα-εξαρτώμενη μη κανονική πορεία του NF-κB κατά την οποία η πυρηνική μετατόπιση των ετεροδιμερών RelB/p52 προάγει την ενεργοποίηση ενός διαφορετικού συνόλου γονιδίων στόχων. Οι κινάσες σερίνης/θρεονίνης IKKβ και IKKα είναι οι κύριοι ανοδικοί ενεργοποιητές της κανονικής και μη κανονικής πορείας του NF-κB, αντίστοιχα, ωστόσο συχνά εμφανίζουν και δράσεις ανεξάρτητες από την ενεργοποίηση των μεταγραφικών παραγόντων NF-κB.

Η IKKα εμφανίζει ογκοκατασταλτική δράση στον NSCLC, ανεξάρτητα από τη μη κανονική πορεία του NF-κB. Το εργαστήριο μας ανέπτυξε δυο μοντέλα NSCLC ποντικού και ανθρώπου *in vivo*, όπου η αποσιώπηση της IKKα οδήγησε στην ενεργοποίηση του μεταγραφικού παράγοντα HIF-1α, προάγοντας την ανάπτυξη όγκων σε συνθήκες υποξίας *in vivo*. Για την περαιτέρω διερεύνηση των μηχανισμών της ογκοκατασταλτικής δράσης της IKKα στον NSCLC, σκοπός της παρούσας διατριβής ήταν η μελέτη του προφίλ έκφρασης των miRNA σε κυτταρικές σειρές NSCLC μετά από την αποσιώπηση της IKKα και η μελέτη του πιθανού μηχανισμού δράσης τους.

Για τη μελέτη των miRNA που ρυθμίζονται από την κινάση IKKα, χρησιμοποιήθηκε η κυτταρική σειρά NSCLC, A549, και αρχικά κατασκευάστηκε μια σταθερή κυτταρική σειρά με μειωμένα επίπεδα έκφρασης της IKKα (IKKα knockdown, IKKα^{KD}). Στη συνέχεια απομονώθηκε ολικό RNA από κύτταρα ελέγχου και κύτταρα IKKα^{KD} και αναλύθηκε η έκφραση των miRNA μέσω της τεχνολογίας Nanostring. Από τα διαφορικά εκφραζόμενα miRNA, το miR-9-5p παρουσίασε τη

μεγαλύτερη αύξηση, κατά 2.13 φορές, στα κύτταρα IKKα^{KD} σε σύγκριση με τα κύτταρα ελέγχου. Το miR-9-5p εμφανίζει ογκογόνο δράση σε διάφορους τύπους καρκίνου και η αυξημένη έκφρασή του έχει συσχετιστεί με προχωρημένα στάδια της νόσου καθώς και την ύπαρξη μεταστάσεων στον καρκίνο του πνεύμονα, ωστόσο ο μηχανισμός δράσης του δεν έχει μελετηθεί πλήρως. Επομένως, επιλέξαμε να μελετήσουμε τον ρόλο του miR-9-5p στο NSCLC και για το σκοπό αυτό κατασκευάστηκαν σταθερές κυτταρικές σειρές A549 και H1299 οι οποίες υπερ-εκφράζουν το miR-9-5p.

Για τη διερεύνηση του ρόλου του miR-9-5p στην ανάπτυξη του NSCLC μελετήθηκε αρχικά η επίδραση του miR-9-5p στον πολλαπλασιασμό και την πρόοδο του κυτταρικού κύκλου των καρκινικών κυττάρων *in vitro*. Η υπερ-έκφραση του miR-9-5p δεν είχε καμία στατιστικά σημαντική επίδραση στον πολλαπλασιασμό των καρκινικών κυττάρων, όπως καταδείχθηκε από τις καμπύλες ανάπτυξης και την ανάλυση του κυτταρικού κύκλου με κυτταρομετρία ροής. Αντίθετα, η υπερ-έκφραση του miR-9-5p οδήγησε σε σημαντική αύξηση της κυτταρικής διήθησης (cell invasion), υποδεικνύοντας ένα πιθανά σημαντικό ρόλο του miR-9-5p στη διαδικασία της επιθηλιακής προς μεσεγχυματικής κυτταρικής μετατροπής (epithelial to mesenchymal cell transition, EMT).

Για την εύρεση πιθανών στόχων του miR-9-5p, αξιοποιήθηκε η βάση δεδομένων mirDIP, και από το σύνολο των πιθανών γονιδίων στόχων που ταυτοποιήθηκαν επιλέχθηκαν τα γονίδια που σχετίζονται με τη σηματοδοτική πορεία Wnt, μια από τις κύριες πορείες που ρυθμίζουν τη διαδικασία της EMT. Ολικά πρωτεϊνικά εκχυλίσματα αναλύθηκαν με ανοσοαποτύπωμα κατά Western για την έκφραση της E-καντερίνης, η οποία διαδραματίζει βασικό ρόλο στον σχηματισμό των συνδέσμων πρόσφυσης (adherens junctions) μεταξύ γειτονικών επιθηλιακών κυττάρων. Τα αποτελέσματα κατέδειξαν την κατακόρυφη μείωση των επιπέδων έκφρασης της E-καντερίνης μετά από την υπερ-έκφραση του miR-9-5p γεγονός που καταδεικνύει το γονίδιο *CDH1* ως στόχο του miR-9-5p. Η απώλεια της E-καντερίνης έχει ως αποτέλεσμα την ενεργοποίηση της β-κατενίνης, που αποτελεί τον κύριο καθοδικό παράγοντα της σηματοδοτικής πορείας Wnt, γεγονός που επιβεβαιώθηκε με ανοσοαποτύπωμα για την έκφραση της β-κατενίνης. Επιπλέον, βρέθηκε ότι ο αναστολέας της β-κατενίνης, GSK-3β, απενεργοποιείται, ενώ ο θετικός ρυθμιστής της β-κατενίνης, Akt-1, ενεργοποιείται στα κύτταρα που υπερ-εκφράζουν το miR-9-5p. Ακόμη, τα επίπεδα των πρωτεϊνικών δεικτών της EMT, N-καντερίνης και βιμεντίνης, βρέθηκαν αυξημένα στα κύτταρα που υπερ-εκφράζουν το miR-9-5p, επιβεβαιώνοντας την ενεργοποίηση της διαδικασίας της EMT.

Τέλος, για τη μελέτη του ρόλου του miR-9-5p στο NSCLC *in vivo*, κύτταρα A549 και H1299 που υπερ-εκφράζουν το miR-9-5p αλλά και τα αντίστοιχα κύτταρα ελέγχου εμβολιάσθηκαν υποδόρια

σε ανοσοκατεσταλμένους μύες NSG για την ανάπτυξη ξενομοσχευμάτων. Η έκφραση του miR-9-5p οδήγησε στο σχηματισμό όγκων μεγαλύτερων κατά 2 φορές στα κύτταρα A549 και κατά 2,2 φορές στα κύτταρα H1299 σε σύγκριση με τα αντίστοιχα κύτταρα ελέγχου, καταδεικνύοντας τον ογκογόνο ρόλο του miR-9-5p στο NSCLC *in vivo*.

Συνοψίζοντας, η IKKα έχει ογκοκατασταλτική δράση στον NSCLC καταστέλλοντας την έκφραση του ογκογόνου miR-9-5p. Η παρούσα μεταπτυχιακή διατριβή καταδεικνύει το miR-9-5p ως ένα ογκογόνο miRNA στο NSCLC, όπου δρα στοχεύοντας την E-καντερίνη και επάγοντας την πορεία Akt-1/GSK-3β/β-κατενίνης, η οποία ρυθμίζει θετικά την EMT, με αποτέλεσμα την αυξημένη διεισδυτική ικανότητα των κυττάρων και τελικά την ανάπτυξη του όγκου.

1. Introduction

1.1. Lung cancer and its molecular mechanisms

Lung cancer is one of the most common cancers in men and women worldwide, with high incidence and mortality rates (1, 2), despite advances in therapeutic regimens (3). Lung cancer is the leading cause of cancer death worldwide and the second most common type of cancer after breast cancer, resulting in 1.8 million deaths and 2.2 million new cases in 2020 (4). While the main cause of lung cancer is tobacco smoking, where prevalent carcinogens known as polycyclic aromatic hydrocarbons induce mutations through covalent binding to DNA (DNA adduct formation) (5), other factors have also been linked to lung cancer development such as air pollutants, occupational exposure to carcinogens and genetic predisposition (6). Despite being one of the most common cancer types, with millions of people receiving treatment every year, survival rates remain poor, with a 15% 5-year survival rate, due to lack of symptoms and late diagnosis (7). Treatment of lung cancer usually follows a routine regimen, with conventional chemotherapy and radiotherapy being the principal approaches, sometimes with additional targeted therapies such as EGFR inhibitors. Like most types of cancer, treatment failure usually results from chemoresistance and radioresistance in cancer cells, leading to relapse, highlighting the necessity for alternative therapies (8-10).

Lung cancer is clinically divided into two distinct histological types, small cell lung cancer (SCLC) which accounts for approximately 15% of cases and mostly originates from neuroendocrinal cells (11), and non-small cell lung cancer (NSCLC) accounting for 85% of all lung cancer cases (12).

Histologically, NSCLC is divided into three major subtypes: adenocarcinoma (LUAD) which develops more distal to the airways and accounts for 70% of the squamous cell carcinoma (LUSC) (~20%), which arises in the proximal airway (13), and large cell lung carcinoma (LCLC) (~10%) (6, 14). LCLC is a diagnosis of exclusion. It is an undifferentiated malignant tumour lacking cytological features seen in other lung carcinomas. The precise cellular origin of lung cancer remains elusive, although research has shown that it could originate from stem cell-like populations found in the normal lung epithelium (15, 16).

1.1.1. Genetic changes in lung cancer

The molecular mechanism of lung adenocarcinoma initiation and development involves the accumulation of genetic and epigenetic changes (15-18). Among these, oncogene mutations play

a central role, with *K-Ras* mutations occurring in 30% of cases. *K-Ras* mutations generally appear in early stages of LUAD, playing an important role in tumour initiation and progression. The second most common oncoprotein found mutated in LUAD is *EGFR*, which is mutually exclusive with *K-Ras* mutations is found mutated in 15% of primary tumours and 30% of metastatic tumours (19). A small but significant subset of cases are characterised by oncogenic fusions of *ALK*, *ROS1*, *RET*, *NRG1* and *NTRK1* genes, as well as by mutations of *BRAF*, *PIK3CA*, *ERBB2/HER2* that act as tumour promoters (19-22). Kinase inhibitors that target specific kinases are an emerging class of drugs in cancer therapy since the FDA's approval of Imatinib in 2001 for the treatment of chronic myeloid leukaemia (23, 24). Tumour suppressor proteins also play an important role in the development of LUAD, with *TP53* mutations occurring in 50% of the cases, while other suppressors such as *STK11/LKB1, KEAP1, NF1, SMARCA4, RB1* and *CDKN2A* are also frequently found mutated in LUAD (19-22). *TP53* mutations mostly appear in advanced stages of LUAD and are associated with higher risk of mortality (25).

These alterations affect key pathways and mechanisms, the most common being the activation of the RTK/RAS/RAF and PI3K-mTOR pathways, disruptions in the p53 pathway, alterations in cell cycle regulation, oxidative stress pathways, chromatin remodelling mechanisms and the RNA splicing machinery (20). Researchers have developed several murine lung cancer models to study how genetic mutations affect LUAD initiation, development and progression (26, 27). Importantly, numerous studies have established a connection between the presence of oncogenic *EGFR* (28-32) or *K-Ras* (33-40) mutations and elevated canonical NF-κB activity in NSCLC (41).

Various therapeutic interventions have been developed over the years, the most effective being inhibitors for EGFR-mutant, ROS1- and ALK1- rearranged LUAD cases with vast improvement of patient survival after therapy, compared to platinum-based therapies that were previously the standard of care for LC (19). However, a large percentage of LUAD patients harbour *KRAS* activating mutations, which are still largely undruggable. A recent breakthrough was the first K-Ras inhibitor, Sotorasib, being FDA-approved for the treatment of $KRAS^{G12C}$ NSCLC, however this is only one of many commonly detected K-Ras mutations (42). Following the approval of Sotorasib, in December 2022 the covalent inhibitor Adagrasib targeting $KRAS^{G12C}$ was also approved by the FDA for the treatment of advanced or metastatic NSCLC (43, 44). Currently, various small molecules are being developed and tested for the inhibition of mutated K-Ras, either by selectively targeting specific K-Ras mutations or by having a pan-K-Ras approach, with the same inhibitor binding to different mutations of K-Ras (45-50). Unfortunately, a large percentage of LUAD patients do not harbour any major genetic alterations and therefore are not suitable for any known targeted treatment.

1.1.2. Epigenetic changes in lung cancer

The study of epigenetics and its impact on cancer development has reshaped our understanding of what was previously considered solely a genetic disease. DNA and histone modifications result in global chromatin structure changes, a phenomenon known as chromatin remodelling. This remodelling facilitates the aberrant expression of oncogenes while simultaneously silencing tumour suppressor genes and supporting genomic instability. Consequently, the study of epigenetic regulators could offer new approaches to lung cancer therapy (51, 52). One of the main epigenetic mechanisms affecting lung cancer progression is DNA methylation, which involves the addition of methyl groups to cytosine residues in DNA by enzymes known as methyltransferases (DNMTs). DNA methylation occurs in CpG islands which correspond to 1% of the genome and are mostly found in gene promoters. In lung cancer, DNA methylation is frequently affected by smoking, due to tobacco carcinogens such as nitrosamines, and the presence of chronic inflammation (52-54).

Alterations in DNA methylation patterns appear in the early stages of lung cancer and could provide a useful diagnostic tool for clinical practice (55-57). Global DNA de-methylation occurring in cancer cells results in oncogene activation and genomic instability due to increased mitotic recombination. More specifically, DNA methylation of tumour suppressor gene promoters is mainly mediated by DNMT1 methyltransferase and results in gene silencing and thus reduced levels of tumour suppressor proteins (52, 55, 58, 59). One of the hypermethylated genes found in lung cancer is *CDKN2A*, which encodes for the cell cycle regulator $p16^{INK4A}$, a factor that inhibits transition from G_1 to S phase in the presence of oncogenic signalling, reactive oxygen species and DNA damage through inhibition of CDK4/6 (52). This hypermethylation reduces the levels of $p16^{INK4A}$ protein, leading to cell cycle dysregulation. Another common hypermethylated gene associated with tumour suppressor activity is *CDH1*, encoding E-Cadherin, a transmembrane protein that forms cell-cell adhesions in normal tissue epithelium. Loss of E-Cadherin in cancer cells promotes a more motile and invasive phenotype, leading to epithelial to mesenchymal transition (EMT) (60). The DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) is also found methylated, resulting in a defective DNA mismatch repair mechanism, leading to increased mutagenesis arising from unrepaired O6-methylguanine causing mismatching during DNA replication (61).

Another epigenetic alteration in lung cancer involves histone modifications, such as methylation and acetylation. Post-translational modification of histones, particularly in lysine residues through methylation, acetylation, and phosphorylation, result in changes to chromatin structure. These changes, in turn, influence gene expression and genomic stability. Researchers

have been exploring modification patterns that could potentially serve as biomarkers and therapeutic targets (62). Both DNA methylation and histone modification seem to be linked and affect each other, that is there is a crosstalk between DNA and histone modifications (63). A promising therapeutic approach would be to use inhibitors targeting enzymes responsible for these modifications, such as histone acetylases and deacetylases, methyltransferases and demethylases. While many inhibitors are under investigation in a pre-clinical setting as well as clinical trials, this field is still underexplored and holds potential for future investigation (64, 65).

Another field of epigenetics focusses on the post-transcriptional regulation of gene expression through microRNAs (miRNAs; miRs). These small, non-coding RNA molecules, typically 22 nucleotides long, exhibit partial complementarity to the 3' UTR of their target genes, inhibiting their translation. miRNAs can act as tumour promoters or tumour suppressors, while in some cases their role seems to be cell type dependent. They have been implicated in numerous types of cancer, where their dysregulation leads to significant alterations in the expression of critical proteins, rendering them key epigenetic regulators (66).

1.2. The role miRNAs in cancer regulation

Gene expression is regulated at many different levels, allowing the process to be tightly controlled by different intracellular and extracellular factors. While most of the regulatory activity takes place pre-transcriptionally, with many specific and non-specific transcription factors contributing to gene expression, post-transcriptional regulation has an important role as well, where mRNA stability, nuclear export and successful translation can all be regulated by different mechanisms. miRNAs are small, non-coding RNAs that act by inhibiting translation and promoting mRNA degradation of their target genes (67). Although miRNAs play an important role in normal development, they are also strongly associated with numerous human diseases (68, 69), including cancer (66). MiRNA research began 30 years ago, with the discovery of lin-4 and let-7 in *C. elegans* through developmental studies. Lin-4 was the first miRNA to be discovered in 1993, where Lee *et al.* (70) were studying the regulation of developmental stages of *C. elegans* in larval stages. They found that the lin-4 gene produces two transcripts, 22 and 61 nt long, which have partial complementarity the 3' UTR of lin-14 mRNA. They suggested that lin-4 inhibits the translation of lin-14 by interacting with it in an antisense RNA-RNA interaction. Similarly, a few years later in 2000, let-7 was discovered by two independent research groups (71, 72), while they were studying the late developmental stages of *C. elegans*. Their findings showed that let-7 targets and regulates the expression of a series of key developmental genes in a time-regulated manner. After the discovery of those two miRNAs, many more miRNAs were discovered in *C.*

elegans and across the animal kingdom, some of them being highly conserved across many species (73-75). More recently, research interest was headed towards their functional role in development and disease (76).

1.2.1. MicroRNA biogenesis

MiRNA genes are typically found in introns of protein coding genes where they are transcribed simultaneously with the gene (intragenic), however some others are found to be intergenic, having their own promoter and being transcribed independently, similar to normal protein-coding genes, by RNA polymerase II (77). After transcription, primary miRNAs (pri-miRNAs), which are typically more than 1kb long, form a stem loop structure. Two different proteins form a complex called microprocessor that binds to the pri-miRNAs and consists of one molecule of Drosha and two molecules of DGCR8 (DiGeorge syndrome critical region 8) (78). Drosha is a class III ribonuclease (RNase III) that catalyses the cleavage of the 3' and 5' tails of the pri-miRNA, releasing a hairpin-like RNA molecule called precursor miRNA (pre-miRNA) which is 65-70 nucleotides long (79). The essential cofactor of Drosha, DGCR8, acts as a scaffold between pri-miRNAs and Drosha and assists catalysis. It is important that pri-miRNA cleavage occurs precisely as it affects the mature miRNAs specificity. After the release from the microprocessor, pre-miRNAs form a complex with the protein exportin 5 and exit the nucleus through the nuclear core complex via binding to Ran-GTP (80). In the cytoplasm pre-miRNA is cleaved by Dicer, another RNase III, on both sides of the loop terminal to form a double stranded RNA molecule, called a miRNA duplex (81, 82). The miRNA duplex then associates with the protein Argonaute (AGO), and forms a complex called RNA-induced silencing complex (RISC). One of the two RNA strands is then cleaved, either the 5' or the 3', leaving the complex with a single RNA strand attached to AGO, which is now the mature miRNA (83). The strand of miRNA that remains attached to the RISC complex is cell and tissue type-dependent (84). The 5' arm of the miRNA will result in miRNA-5p, while the 3' in miRNA-3p, and since the two mature miRNAs differ in sequence, they can have different targets, or they may target the same gene but in different regions (85, 86). The chosen miRNA will act as a guide and will bind to complementary RNA sequences found in 3' UTR of gene targets, downregulating their expression (87). The process of miRNA biogenesis is summarised in Figure 1.1.

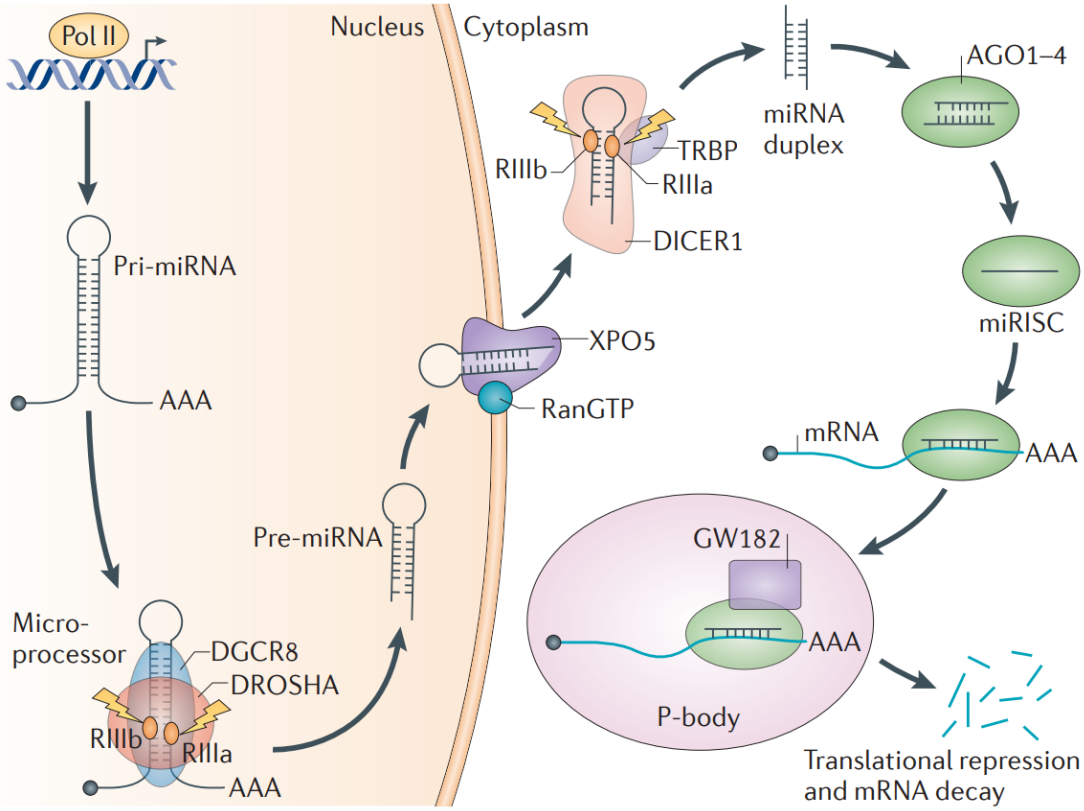

Figure 1.1. *Schematic representation of miRNA biogenesis*. RNA pol II transcribes miRNA genes into pri-miRNAs, which are subsequently cleaved by the microprocessor complex, consisting of the RNase III Drosha and the protein DGCR8. The cleaved product, called pre-miRNA, exits the nucleus by exportin 5 (XPO5) where it is further cleaved by the RNase III Dicer, resulting in a miRNA duplex. Argonaute (AGO) protein binds to the miRNA duplex, where one of the two strands remains attached to the protein, forming the miRNA-induced silencing complex (miRISC) complex. The mature miRNA recognises complementary target mRNAs, resulting in inhibition of mRNA translation as well as mRNA degradation, often occurring within cellular structures known as processing bodies (P-bodies) (88).

1.2.2. MiRNA mechanism of action

MiRNAs recognise and bind to complementary RNA sequences found in the 3' UTR of their target genes. The nucleotides 2-8 are called the seed region of the miRNA, which is the complementary sequence to the target. Binding to the target mRNA causes inhibition of translation and mRNA degradation. AGO recruits the glycine-tryptophan protein of 182 kDa (GW182), which, in turn, interacts with polyadenylate-binding protein (PABPC), a poly-A coating protein. This causes the recruitment of deadenylation complexes poly(A) nuclease 2 (PAN2)-PAN3 and carbon catabolite repressor protein 4 (CCR4)-NOT. Deadenylation of mRNAs promotes decapping by decapping enzyme subunit 1 (DCP1)-DCP2, which further destabilises them and leads to

degradation by 5′–3′ exoribonuclease 1 (XRN1) (89, 90). In addition, translation is inhibited by disrupting the association of factors eIF4A-I and -II from the 5’ UTR of the target mRNA, proteins essential for translation initiation (91-93). The mechanism of action of miRNAs is summarised in Figure 1.2 (94).

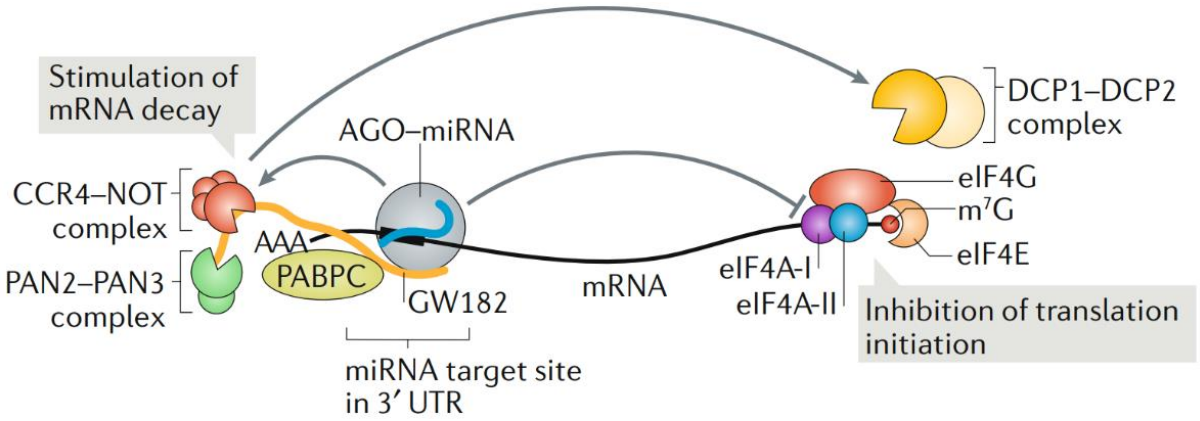

Figure 1.2. *MiRNA mechanism of ac*tion. miRNAs inhibit the mRNA translation initiation by releasing the eIF4A1 and eIF4A2 from the 5’ UTR of the mRNA target. They also facilitate mRNA degradation by interacting with the protein GW182 which recruits PABPC, a protein that associates with the poly-A tail, and the deadenylation complexes PAN2–PAN3 and CCR4–NOT. Following deadenylation, the process continues with decapping by the complex DCP1–DCP2, ultimately leading to 5’−3’ mRNA degradation (94).

1.2.3. MiRNAs in cancer

MiRNAs have various physiological roles in animal biology, such as embryonic development and cell differentiation, cell cycle regulation, apoptosis and signal transduction (95-98). In cancer, miRNAs are often dysregulated, by deletion or amplification of miRNA genes, as they are often located in fragile chromosome regions, or by transcriptional regulation (66, 99). MiRNA expression is also regulated epigenetically, by altering DNA methylation patterns of their promoter regions and histone modifications (100). They can also acquire mutations in their seed region, which alters their targets and therefore their action (101). Dysregulation of the miRNA biogenesis machinery, including mutation or abnormal expression of the catalytic and regulatory proteins of the machinery, also leads to altered miRNA expression in cancer (102). MiRNAs can act as tumour suppressors or tumour promoters, depending on their target genes (66).

The first study that linked miRNAs to cancer was in 2002 (103), where miR-15 and miR-16 were identified to be frequently deleted from the chromosomal region 13q14 in chronic lymphocytic leukaemia. This study raised interest in the link between miRNA expression and cancer. Subsequently, researchers began investigating miRNA expression profiles in various

other cancer types (104). *In vivo* mouse models further demonstrated the impact of miRNAs, where they showed that miRNAs act as oncogenes affecting tumour formation (105). In this study, researchers ectopically expressed the miRNA polycistron miR-17-92 in the population of hematopoietic stem cells of a B-cell lymphoma mouse model and found that miR-17-92 and c-Myc drastically accelerated tumourigenesis compared to c-Myc induced carcinogenesis. The polycistron miR-17-92 is a group of miRNAs found in the chromosomal locus 13q31, which is often amplified in B-cell lymphoma. The polycistron encodes seven different miRNAs, miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20, miR-19b-1 and miR-92-1, which are all simultaneously expressed. This was the first study showing that miRNAs can act as oncogenes *in vivo*, linking their altered level of expression in tumours with a tumourigenic phenotype. In addition, miRNA expression was shown to be regulated by known tumour promoter transcription factors, revealing a tight connection between these factors and miRNAs in cancer progression. c-Myc was found to bind to promoter regions of miRNAs in chromosome 13, promoting their expression (106). It is now known that c-Myc regulates the expression of various miRNAs by directly binding to their promoter, but also by regulating the expression of miRNA biogenesis machinery proteins such as Drosha (107). Many different miRNAs target c-Myc mRNA affecting its translation, adding to c-Myc/miRNA pathway complexity, reflecting the high intricacy of miRNA-transcription factor networks (108).

Clinical trials are currently exploring the potential of miRNA profiling as a means of cancer diagnosis, prognosis and prediction of response to therapies, where miRNAs could serve as biomarkers for specific types or subtypes of cancers and predicting prognosis or monitoring for treatment resistance (104, 109-111). These screening techniques could elucidate the stage of cancer, the malignancy grade and metastatic status, therefore ultimately assisting in personalised treatment strategies (112).

1.2.4. MiRNAs in lung cancer

A vast number of miRNAs are implicated in lung cancer development. Among these, the well-studied miRNA, let-7, is often found downregulated in lung cancer tissue due to chromosomal deletion (99). Low expression levels of let-7 are also correlated with unfavourable prognosis (109, 113). The physiological role of let-7 in cell fate determination and cell differentiation reflects its tumour suppressor role in most cancers, as well as lung cancer. Overexpressing let-7 in A549 cells (a LUAD cell line) decreases cell proliferation rates by cell cycle arrest (113) and ectopic expression in mouse xenograft models hinders tumour growth (114). Its tumour suppressor role is a result of targeting oncogenic proteins, including MYC, RAS and HMGA2, therefore low levels

of let-7 lead to aberrant overexpression of these oncoproteins and upregulation of oncogenic processes in cells. Additionally, let-7 targets key cell cycle regulators cyclin D2, CDK6 and CDC25A, promoting cell cycle arrest, therefore let-7 downregulation leads to aberrant cell cycle progression (113, 115, 116). Another example of miRNAs acting as tumour suppressors in lung cancer is miR-15a and miR-16, where they are often found deleted or downregulated in LUSC and LUAD. Bandi *et al* found that miR-15 and miR-16 target the cell cycle proteins Cyclin D1, D2 and E1, which are responsible for G_1 to S transition, while when they are overexpressed, they result in cell cycle arrest (117). The downregulation of miR-15a and miR-16 in lung cancer therefore leads to overexpression of Cyclins D1, D2 and E1, promoting G1/S transition and contributing to cell cycle dysregulation.

MiR-21 is a well-established oncogenic miRNA found upregulated in many types of cancer (118). In lung cancer, miR-21 is overexpressed, and its expression appears to be controlled by the EGFR pathway (119-121). miR-21 acts by targeting PTEN in NSCLC, which is a negative regulator of the PI3K/mTOR/Akt pathway, promoting cell growth, migration and invasion (122, 123). Increased levels of miR-21 have been associated with gefitinib resistance (EGFR inhibitor), by downregulating PTEN, resulting in Akt activation (124, 125). Another tumour promoting miRNA is miR-411-5p/-3p, which is upregulated in NSCLC tissues and cell lines. Overexpressing miR-411 in NSCLC results in increased cell proliferation, migration and decreased apoptosis. This was suggested to be through the targeting of the tumour suppressor gene *SPRY4*, an inhibitor of EGFR signalling (126).

1.2.5. MiR-9-5p in cancer

MiR-9-5p is a highly conserved miRNA across species, with the mature miRNA sequence being identical across vertebrates. In humans, miR-9 has three different genes *MIR9-1*, *MIR9-2* and *MIR9-3* located in chromosomes 1 (1q22), 5 (5q14.3) and 15 (15q26.1), all of them producing an identical mature sequence (127). miR-9-5p was initially associated with neuronal differentiation and it is highly expressed in the developing and adult brain (102, 128, 129). It was later found overexpressed in oligoneural glioblastoma (GBM) (130). Furthermore, elevated expression of miR-9-5p has been identified in GBM stem cell populations (CD133+), where it inhibits differentiation by targeting the anti-proliferative transcription factor CAMTA1 (129). Since then, miR-9-5p has been associated with multiple types of cancer, where it has been shown to regulate proliferation, migration, EMT and inflammation (66, 131-136).

A key study found that miR-9-5p is upregulated in breast cancer cell lines, where it targets E-Cadherin (CDH1) and promotes EMT and metastasis (133). The researchers demonstrated that

the downregulation of E-cadherin promoted β-catenin nuclear localisation, which resulted in the increased expression of *VEGFA*, promoting angiogenesis *in vivo*. Mouse models showed that overexpression of miR-9-5p in non-metastatic cells significantly increased plasma VEGF levels, intratumoural microvessels and pulmonary micrometastasis rates. Moreover, it was established that MYC/MYCN promotes miR-9-5p expression, while a strong correlation exists between levels of MYC and miR-9 in various human cancers (133). miRNAs have many different targets even in the same tissue. Researchers also identified miR-9-5p as an EMT-promoting miRNA in colon cancer, where they showed that miR-9-5p expression is upregulated by the transcription factor PROX1 and this upregulation results in E-Cadherin downregulation, therefore promoting EMT (66, 135).

When two different mRNAs are targeted by the same miRNA, they “compete” for miRNA binding and are called competing endogenous RNAs (ceRNAs). This results in an indirect regulation of each other’s expression. In breast cancer, the FOXO1 3’ UTR competes with CDH1 for miR-9-5p binding. Consequently, when FOXO1 is overexpressed, levels of E-Cadherin increase, as FOXO1 acts as a miRNA sponge for miR-9-5p, meaning that unbound miR-9-5p in the cell can bind to the over-expressed FOXO1 3’-UTR. This results in increased levels of E-Cadherin, ultimately decreasing migration and invasion capabilities of the cells (137).

In Hodgkin’s Lymphoma, miR-9-5p positively regulates cytokine production by targeting HuR, a protein known for regulating cytokine expression by controlling their mRNA stability. It also targets DICER1, the miRNA biogenesis RNase which is found to act as a tumour suppressor in many types of cancer. When using miR-9 inhibitors *in vivo* in a Hodgkin lymphoma mouse model, researchers found that tumour growth decreases and levels of DICER1 and HuR significantly increase (136). In addition, a screening for miRNAs implicated in acute myeloid leukaemia (AML) in the murine myeloid cell line, 32D revealed that miR-9-5p inhibits neutrophil differentiation and maintains myeloblastic characteristics. This was shown to be through the targeting of ETS related gene (ERG), a transcription factor associated with the regulation of haematopoiesis. It was also found overexpressed in AML cases, suggesting an important role for miR-9 in both steady-state haematopoiesis and a role in the development of malignancy (138, 139).

MiR-9-5p was also found to promote tumour angiogenesis, by targeting the sphigosine-1-phosphate 3 ($S1P_3$) receptors in medulloblastoma and glioblastoma (140). In another study, data from the Cancer Genome Atlas (TCGA) showed that miR-9-5p is overexpressed in hepatocellular carcinoma (HCC) and is correlated with low survival rates. Researchers then studied the effects of miR-9-5p overexpression in HCC cell lines, where they found that miR-9-5p promotes cell proliferation, migration and invasion and they confirmed *ESR1* as a gene target in HCC (141). In

osteosarcoma, researchers found that overexpression of miR-9-5p promotes cell proliferation, migration and invasion and inhibits apoptosis via decreasing the protein levels of E-Cadherin, GSK-3β, FOXO3a, MMP-13 and Bcl2-L-11 (142). In gastric cancer, miR-9-5p promotes cell proliferation by targeting *CDX2*, a transcription factor that plays a tumour suppressor role by promoting cell cycle arrest by negatively regulating Cyclin D1 transcription and positively regulating $p21^{CIP1/WAF1}$ transcription. In doing this, miR-9-5p reduces the ability of cells to initiate cell cycle arrest and promotes cell cycle progression, leading to increased growth (131).

In some cases, miR-9-5p plays a dual role in cancer, acting both as a promoter and as a suppressor of carcinogenesis. In cervical cancer, miR-9-5p is overexpressed due to chromosomal gain of 1q and is associated with increased migration, anchorage-independent cell growth and increased cell viability (143). However, this appears only in cervical squamous cell carcinoma (SCC), while in cervical adenocarcinoma (AC), *MIR-9-1* is methylated and silenced. When ectopically overexpressed in cervical AC, miR-9-5p targets *TWIST1*, a key transcription factor promoting EMT, therefore it acts as a tumour suppressor inhibiting EMT and reducing cancer migration and dissemination. miR-9-5p silencing leads to increased levels of Twist, which promotes *CDH2* (N-Cadherin) expression while inhibiting *CDH1* expression, resulting in EMT. While miR-9-5p also downregulates the expression of *TWIST1* in cervical SCC, it acts as a tumour promoter by targeting a multitude of different factors contributing to the carcinogenic phenotype. Researchers suggest that miR-9-5p may still promote EMT in cervical SCC by targeting E-Cadherin (144).

In some types of cancer, miR-9-5p can act as a tumour suppressor. For example, in choroidal melanoma (CM), miR-9-5p targets the serine/threonine kinase BRAF, which is a known tumour promoter protein in melanoma involved in the MAPK signalling pathway. Overexpression of miR-9 therefore results in suppression of proliferation, migration and invasion in CM cell lines (145). In ovarian cancer, miR-9-5p is downregulated in ovarian cancer tissues compared to normal adjacent tissue. Ectopic overexpression of miR-9-5p in the ovarian cancer cell line ES-2 decreased cell proliferation and colony formation rates *in vitro*. The researchers identified *NFKB1* (p105/p50) as a direct target of miR-9-5p. *NFKB1* expression was found increased in OC tissue, leading to increased NF-κB activation which is associated with cancer cell survival and tumourigenesis (146). Knockdown of *NFKB1* decreased cell proliferation rates, while a similar effect was achieved by miR-9-5p overexpression (147, 148).

1.2.6. MiR-9-5p in lung cancer

Early studies of miRNAs expression in cancers showed that miR-9-5p is overexpressed in lung cancer compared to normal adjacent tissue (118). This has also been confirmed by later studies (149-151). Overexpression of miR-9-5p in NSCLC is associated with decreased survival and increased metastasis (151), making it an important tumour promoter in LC. In addition, miR-9 gene methylation and therefore low levels of expression are seen in early stage of LC, whereas hypomethylation and overexpression are linked with late stage and metastatic LC (152). Additionally, miR-9-5p levels are elevated in A549 cells when compared to normal lung epithelial cells NL20 (153). In another study, bioinformatics analysis using TCGA data found six miRNAs that had a significant effect on patient survival on all stages of LUAD, one of them being miR-9 (154).

A few studies have been conducted on the mechanism of action of miR-9-5p in LC. The level of miR-9-5p expression was studied in NSCLC tissues compared to adjacent normal lung tissue and found that miR-9-5p was upregulated in LC, with a three-fold difference compared to normal tissue. In addition, patients with lymph node metastasis had a two-fold higher expression of miR-9-5p compared to those without metastasis. When using miRNA mimics in A549 (LUAD) and SK-MES-1 (LUSC cell line) cells, they observed increased cell proliferation, migration and invasion and suggested that miR-9-5p produced this effect by targeting TGFBR2. They showed that TGFBR2 expression is reduced in NSCLC samples and knockdown of TGFBR2 had the same phenotypic effects as miR-9 mimics on A549 and SK-MES-1 cell lines (155).

Another study found that miR-9-5p is induced by TGF-β1 and promotes cell invasion and adhesion in A549 and HCC827 cell lines, while miR-9-5p knockdown resulted in reduced invasion and adhesion. They confirmed *SOX7* was a miR-9-5p target by overexpressing SOX7 in either TGF-b1-treated or miR-9 mimic-treated cell lines, where they found invasion and adhesion levels were significantly decreased (156). Zhu *et al* found that miR-9-5p confers increased invasion and proliferation activity in Calu-3 cells (LUAD cell line) as it targets *ID4*, coding for a protein that acts as a dominant negative inhibitor of helix-loop-helix transcription factors and acts as tumour promoter or suppressor in a tissue specific way in cancer (157).

miRNAs can have multiple targets within a cell, and their action depends on various factors. There is a clear gap in literature, especially for the tumourigenic role of miR-9-5p in lung cancer, and further research should be conducted using high standard approaches in order to decipher how this miRNA affects carcinogenesis and metastasis.

1.2.7. MiRNAs as therapy targets

As miRNAs have an important role in carcinogenesis, they could serve as new targets in cancer therapy. MiRNAs that act as tumour promoters can be targeted by inhibitors, molecules acting as an anti-sense RNA, sponging the miRNA, reducing its availability to bind mRNA. For miRNAs that act as tumour suppressors, miRNA mimics could be introduced to cells to imitate their action. There have been six clinical trials conducted using miRNA drugs for cancer therapy to date (111).

The first miRNA molecule to be pharmacologically targeted was miR-34a, a well-known p53-regulated tumour suppressor miRNA, which acts by stabilising p53, thus affecting regulation of cell cycle, apoptosis and DNA damage response. miR-34a is often found downregulated in cancers due to its potent tumour suppressor role (158, 159). The first clinical trial to use microRNA-based therapy (NCT01829971) (160), employed a synthetic double-stranded miR-34a mimic drug, MRX34. This was used to treat patients with various types of solid tumours. The clinical trial was terminated because of severe immune reactions that resulted in four patient deaths. The delivery vehicle of the drug was a liposomal nanoparticle that was not tumour-targeted, which would likely cause increased toxicity, as the miRNA mimics would also be delivered to healthy tissues as well. However, levels of miR-34a targets were found to be downregulated, thus suggesting that miRNA mimics could be of use in cancer therapy. Another miRNA mimic was developed for the tumour suppressor miR-16, TargomiR (NCT02369198), and was administered by a specialised delivery system that targets EGFR-overexpressing tumour cells, called EnGeneIC Dream Vectors (EDVs) (161). The clinical trial for the treatment of recurrent patients of NSCLC and malignant pleural mesothelioma was successful and presented early signs of tumour suppression. The moderate results could be attributed to low dose levels of miR-16 mimic, however there was low toxicity, indicating that more targeted delivery systems of the drug played a critical role in reducing side effects.

Targeting oncogenic miRNAs can be achieved by using miRNA inhibitors. The first miRNA inhibitor to be used in clinical trials was MRG-106 (Cobomarsen), a miR-155 inhibitor, that was used for the treatment of various types of lymphoma (NCT02580552) (162, 163). miR-155 is often overexpressed in lymphomas as well as solid tumours and is induced in the presence of inflammation. Phase I of the clinical trial was successful, thus a phase II was initiated but unfortunately was terminated for business reasons (NCT02580552). Nevertheless, this positive result suggests that miRNA inhibitors could be used in cancer therapy. Since the development and use of miRNA targeted therapy technology is an emerging field, there is still room for research in deploying miRNA inhibitors or mimics as therapy. In addition, most miRNAs are still largely

understudied and the mechanisms of action of each miRNA can differ drastically in a tissue-specific way. Thus, better understanding of their function can aid to developing new and improved therapies in cancer (111).

1.3. The NF-κB pathway in cancer

NF-κB transcription factors (TFs) which can either activate or suppress gene expression, are also aberrantly activated in pulmonary diseases and NSCLC. IKK/NF-κB TFs are the main regulators of immunity and of genes implicated in cell cycle progression, survival, inflammation and metabolism, but also miRNA genes. MiRNAs are involved in the negative or positive regulation of biological processes including cell cycle progression, DNA damage responses (DDR), EMT, cell motility and stemness through complex interactive TF-miRNA regulatory networks. The interplay between NF-κB and miRNAs creates feedback loops, which control cell fate impacting on inflammation in cancer (66, 148, 164).

The NF-κB pathway is a crucial signalling pathway involved in regulating the expression of genes responsible for inflammatory and stress-like responses, innate and adaptive immunity, cell survival and proliferation. In normal cells it can act as an equilibrator to stress response and cell survival, but when dysregulated it can lead to chronic inflammation and diseases such as cancer and autoimmune diseases. Most target genes involve cytokines and chemokines, anti-apoptotic factors, cell proliferation and cell adhesion proteins and immune response genes (165).

The main components of the NF-κB signalling pathways are five the transcription factors (TFs) RelA/p65, RelB, c-Rel, p105/p50 and p100/p52. The NF-κB TFs act as hetero- or homo-dimers and when dimerised, they can bind to DNA and regulate gene expression. All five NF-κB factors have a DNA binding and dimerisation domain, called Rel-homology domain (RHD) found in the N-terminal. TFs RelA, RelB and c-Rel also contain individual C-terminal transcriptional activation domains, which result in distinct functions (165, 166).

In unstressed, unstimulated cells, the inhibitors of NF-κB (IκBs) (IκBα and IκBβ being the main ones) interact with the TF subunits and inhibit their nuclear translocation by masking their nuclear localisation signal (NLS), found in the RHD of the TFs. NF-κB signalling pathway activation depends on the upstream signalling IKK kinases. The IKK complex, consisting of the serine/threonine kinases IKKα and IKKβ and the regulatory protein NEMO (IKKγ), phosphorylate IκBs triggering their ubiquitination and proteasomal degradation, allowing the TFs to move freely to the nucleus. In the nucleus, the NF-κB dimers recognise and bind to κB elements in promoter regions of their target genes, regulating their expression.

There are two main pathways by which NF-κB is activated, the IKKβ-dependent canonical pathway, where heterodimers p65/p50 and c-Rel/p50 act as the main TFs, and the IKKα-dependent non-canonical pathway, where the heterodimer RelB/p52 acts as the main TF regulating a different set of target genes (166-168). The canonical pathway is activated by various stimuli, including the inflammatory cytokines, TNFα and IL1, as well as bacterial and viral products. It plays a role in initiating a rapid response to inflammatory stimuli and infection, and it is short lived, whereas the non-canonical pathway is activated in later stages of inflammation and has a more sustained effect. Upon activation, IKKβ gets phosphorylated in Ser177/181 in the T-activation loop and in turn phosphorylates IκBα on two N-terminal serine residues (Ser32/36), signalling its ubiquitination and subsequent degradation. The heterodimer p50/p65 moves freely to the nucleus where it acts by regulating the expression of target genes (166, 168).

The non-canonical pathway is activated by stimuli that bind to a subset of TNFR receptors, CD40, BAFFR and lymphotoxin β receptor (LTβR), and it is involved in B cell maturation, adaptive immune response, and lymphoid organ development (169, 170). Upon activation, NF-κB-inducing kinase (NIK) phosphorylates and activates IKKα at Ser176/180 (171). This leads to p100 processing to p52, where it dimerises with RelB and translocates to the nucleus (166, 168). An atypical IKK-independent pathway activating p50/p65 heterodimers also exists (172, 173). A simplified version of the canonical and non-canonical NF-κB pathways is shown in Figure 1.3.

In addition to their role in NF-κB signalling, IKKα and IKKβ have distinct, NF-κB-independent actions. Activation of IKKβ promotes anti-apoptotic, anti-inflammatory and proliferative pathways. IKKα phosphorylates various targets such as kinase Aurora A, the transcription factor FOXO3A inhibiting its transcriptional activity and tumour suppressor function, and autophagy-involved protein TSC1. IKKα also phosphorylates the co-activator CBP enhancing its activity, and the SMRT co-repressor while it is bound to NF-κB complexes on the promoter, resulting in the release of chromatin-bound HDAC3, leading to de-repression of NF-κB-dependent transcription. IKKα regulates chromatin remodelling and facilitates gene expression by functioning as a histone H3 Ser10 kinase. In addition, IKKα regulates cyclin D1 and suppresses maspin expression, a suppressor of metastasis (165-168).

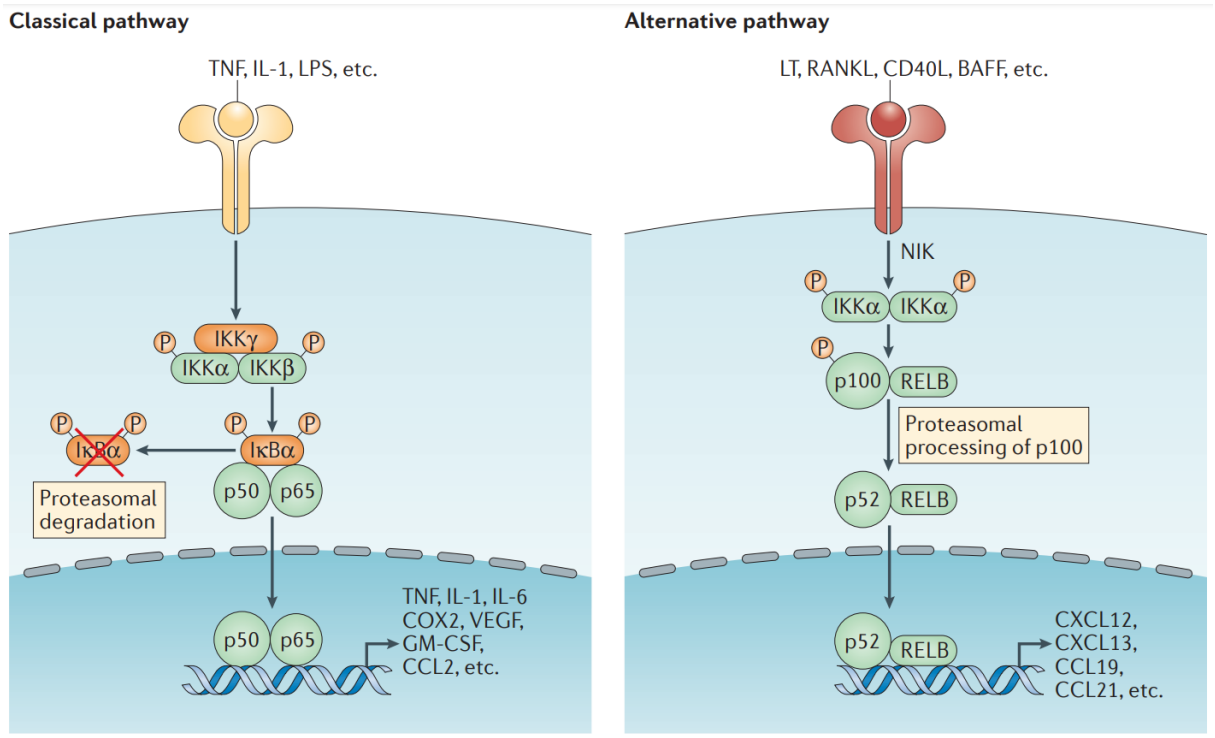

Figure 1.3. *Canonical and non-canonical NF-κB signalling pathways.* Classical/Canonical NF-κB pathway: Various stimuli such as TNFα, IL-1 and LPS bind to receptors and signal the activation of the IKK complex consisting of IKKβ, IKKα and NEMO (IKKγ). The IKK complex, with IKKβ as the main kinase, phosphorylates the inhibitor of NF-κB, IκBα, and signals its degradation. This frees the hetero-dimers of NF-κB transcriptions factors, p65/p50, which translocates to the nucleus where it binds to κB elements in target genes promoters and enhancers, regulating their expression. Alternative/Non-canonical NF-κB pathway: Stimuli such as RANKL, CD40L etc. bind to ligands and signal the activation of IKKα, which in turn phosphorylates the p100 factor of the hetero-dimer p100/RelB to signal its proteasomal processing to p52. The hetero-dimer p52/RelB then translocates to the nucleus to regulate the expression of target genes (167).

1.3.1 NF-κB signalling in lung cancer

The canonical NF-κB pathway has been widely implicated with NSCLC formation (174, 175). Main players of the pathway have been found overexpressed in NSCLC, such as TFs p65 (176-178) and p50 (179) as well as other NF-κB components (176). In addition, p65 overexpression was correlated with the presence of *K-Ras* and/or *EGFR* mutations (178). In a malignant pleural effusion murine model, researchers showed that elevated tumour NF-κB activity promoted pleural tumour formation, while this effect was abrogated after NF-κB inhibition (180). Transgenic mouse lung cancer models have shown that IKKβ-mediated canonical NF-κB signalling promotes both chemically induced (181) and K-Ras oncogene induced NSCLC (34, 36, 37).

Genetic or pharmacological inhibition of NF-κB has been shown to inhibit NSCLC development (40, 181, 182). In two different mouse models with chemically (tobacco nitrosamine NNK) and genetically (*KRAS* activation) induced lung cancer, researchers demonstrated that exposure to tobacco smoke promotes carcinogenesis by promoting inflammation. It was found that IKKβ was the main factor responsible for the inflammatory response in myeloid cells, and when depleted, the tumour-promoting effect of tobacco smoke was almost eliminated (40). Additionally, NF-κB is activated in urethane-induced lung cancer in mice, where pharmacological inhibition of the pathway increases apoptosis and inhibits tumour formation (181). Our lab has shown that RelA/p65, the main canonical NF-κB pathway TF, plays a tumour promoter role in LUAD by promoting EMT. p65 was shown to be essential for tumour growth in xenografts in immunocompromised mice. Transcriptomics analysis identified CD82 as a key target of NF-κB pathway, a known metastasis suppressor, the loss of which was linked with increased malignancy. The proposed mechanism of action suggests that p65 promotes EMT by downregulating CD82, which regulates the expression of Akt1, Rac1 and ERK (182).

Similarly, the main non-canonical NF-κB pathway transcription factor, p52 (*NFKB2*), has been shown by our lab to promote NSCLC in mice xenograft models (183). *NFKB2* is correlated with poor prognosis in stage I lung cancer patients (184), while overexpression of p52 promotes gene expression of genes highly associated with unfavourable prognosis (185). However, the role of IKKα in lung cancer is still largely unknown, as it has been shown to act both as tumour suppressor and tumour promoter in a tissue-specific manner (186). The first study to identify IKKα as a tumour suppressor in lung cancer found that transgenic, kinase-dead mutant IKKα ($IKK\alpha^{KA/KA}$) mice developed spontaneous lung squamous cell carcinoma with elevated levels of T cell, macrophage and neutrophil infiltration (187). In a more recent study, IKKα ablation in mice promoted spontaneous LUAD initiation while it also facilitated the development of $Kras^{G12D}$ LUAD, one of the more common K-Ras mutations found in lung cancer. IKKα ablation also led to increased cell proliferation, accumulation of reactive oxygen species and hindered cell senescence (188). The same research team later published another study where they discovered that IKKα impedes tumour infiltration by monocyte-derived macrophages and regulatory T cells, both cell types promoting carcinogenesis in $K\text{-}Ras^{G12D}$ driven LUAD mouse models (189).

Our lab has shown that IKKα acts as a tumour suppressor in LUAD, using two different *in vivo* mouse models. Using a transgenic mouse strain, IKKα was ablated in alveolar type II lung epithelial cells with the administration of tamoxifen. $IKK\alpha^{f/f}$ mice developed significantly more and larger adenomas as well as carcinomas in comparison to $IKK\alpha^{WT}$ mice, after exposure to the chemical carcinogen urethane. In parallel, $IKK\alpha^{KD}$ NSCLC cell lines were generated and were

used to create tumour xenografts in immunocompromised mice. IKKα^{KD} xenografts were significantly larger than control tumours. Transcriptomics analysis showed that loss of IKKα leads to the upregulation of the hypoxia factor HIF-1α, which enhances tumour growth in hypoxic environments (183). As we would like to further study the tumour suppressor effects of IKKα in LUAD, we conducted a Nanostring analysis using IKKα^{KD} cell lines, in order to investigate the miRNAs being influenced by IKKα in LUAD.

Aims

Lung cancer is the leading cause of cancer death worldwide and the second most common cancer type. It is often diagnosed at a late stage due to lack of symptoms in early stage lung cancer and effective treatments are limited. Lung adenocarcinoma is the most common lung cancer subtype and results from the accumulation of genetic and epigenetic changes, such as *K-Ras* and *EGFR* activating mutations and *TP53* inactivating mutations. The NF-κB inflammatory signalling pathway is often found activated in lung adenocarcinoma (LUAD) and promotes carcinogenesis by sustaining chronic inflammation. However, the non-canonical NF-κB kinase, IKKα, appears to act as a tumour suppressor in lung adenocarcinoma. The NF-κB pathway regulates the expression of protein coding and non-coding genes, such as miRNAs, although the link between IKK/NF-κB and miRNAs is poorly understood. MiRNAs control the expression of their target mRNAs, affecting crucial cell functions and are widely dysregulated in different cancers.

The purpose of this thesis was to identify an alternative mechanism accounting for the tumour suppressive function of IKKα in NSCLC, in addition to its role in regulating HIF and HIF-mediated process under hypoxia *in vivo* (183).

The aims of this thesis were to:

- Identify miRNAs regulated by IKKα in LUAD that account for its tumour suppressive function
- Explore phenotypic effects of the overexpression of selected miRNA(s) in LUAD cell lines and *in vivo*
- Identify new mRNA targets of the selected miRNA and study their effects and mechanism of action in LUAD

2. Materials and Methods

2.1. Bacterial cell methods

2.1.1. Preparation of L-broth (Luria-Bertani; LB) and L-agar plates

The preparation of the L-agar plates was performed concurrently with the preparation of the L-Broth solution. 10 g of tryptone (LAB M), 5 g of yeast extract powder (LAB M), and 10 g of sodium chloride (LAB M) were dissolved by stirring in 1 l distilled water, and the solution was transferred to two 500 ml bottles. In one bottle, 7.5 g of agar (LAB M) was also added, and after autoclaving, the bottle was left at room temperature to reach 55°C. The antibiotic ampicillin (100 μg/ml) was added and the solution was poured into petri dishes to solidify at room temperature. The plates were stored at 4°C for future use.

2.1.2. Preparation of competent bacteria

The preparation of bacteria susceptible to transformation requires their treatment with agents that create pores in their cell walls, allowing the passive diffusion of exogenously added DNA. The bacterial strains used for the preparation of competent cells capable of transformation with plasmids were the *E. Coli* Stable 3 strain.

Non-competent bacteria were used to inoculate 4 ml of L-Broth and incubated in a heated orbital shaker at 37°C overnight to grow. The next day, 0.2 ml of the overnight bacterial culture was used to inoculate 20 ml of L-broth in a sterilised conical flask and incubated for 2-3 hours at 37°C in an orbital shaker at 250 RPM until they reached OD_{600}=0.5. Subsequently, the culture was centrifuged for 10 minutes at 3000 x g at 4°C. The bacterial pellet was resuspended in 4 ml of calcium chloride ($CaCl_2$) solution and centrifuged for 10 minutes at 2000 x g at 4°C. The pellet was resuspended again in another 4 ml of $CaCl_2$ and incubated for 30 minutes on ice. Then, centrifugation was performed for 7 minutes at 2500 x g at 4°C. The pellet was resuspended in 1.5 ml of $CaCl_2$, and samples of 100 μl of bacteria were placed in sterilised Eppendorf tubes and stored at -80°C for future use.

Calcium Chloride Solution ($CaCl_2$): 60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES pH 7.0
PIPES 0.1 M: 3.46 g PIPES, ddH2O for total volume of 100 ml. Adjust pH to 7.0

2.1.3. Bacterial Transformation with plasmid DNA

100 µl of competent *E. coli* Stable 3 bacteria were thawed on ice and then mixed with 50 ng of plasmid DNA and incubated on ice for 10 minutes to allow the plasmid to enter the bacteria cells. Subsequently, the bacteria were plated on L-agar plates containing 100 µg/ml ampicillin. The plates were incubated at 37°C overnight to allow the formation of antibiotic-resistant bacterial colonies.

The next day, colonies were selected and added to 4 ml of LB Broth and incubated overnight at 37ºC, in an orbital shaker at 250 RPM to allow growth. The next day, bacterial glycerol stocks were prepared by mixing 9 parts of the cultured liquid bacterial culture with 1 part of 100% glycerol (10% final glycerol concentration). The stocks were stored at -80°C for future use.

2.1.4. Isolation of Plasmid DNA on a Small Scale (Mini Prep)

1.5 ml of liquid bacterial culture derived from colonies were placed in a sterilised Eppendorf tube and centrifuged at 14500 x g for 2 minutes at 4°C. The supernatant was discarded and the bacterial pellet was resuspended by vortexing in 70 µl of STET buffer and 25 µl of lysozyme (10 mg/ml). After 5 minutes of incubation at room temperature, the samples were heated to 100°C for 1 minute then centrifuged for 15 minutes at 14500 x g. The supernatant was carefully collected and transferred to a clean Eppendorf tube, where 100 µl of isopropanol was added. The plasmid DNA was allowed to precipitate for 5 minutes at -80°C and then collected by centrifugation for 7 minutes at 14500 x g. The pellet was resuspended in 25 µl of 1X TE buffer and stored at -20°C for further use.

STET Buffer: 8% sucrose, 5% Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0
1X TE pH 8.0: 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0

2.1.5. Isolation of Plasmid DNA on a Large Scale (Midi Prep)

The isolation of plasmid DNA was performed using the Nucleobond kit Xtra Midi EF/Maxi EF (Macherey-Nagel) using 100 ml of bacterial culture, following the manufacturer's protocol. After isolation, the DNA was stored at -20°C for future use.

2.2. Tissue culture methods

2.2.1. Cell culture

A549, NCI-H1299 and NCI-H1437 non-small cell lung cancer cell lines and the HEK293T cell line, were obtained from ATCC. A549 and H1299 were cultured in low glucose DMEM (Sigma, cat. no. D6046-500ML), supplemented with 10% Fetal Bovine Serum (FBS) (Biowest, cat. no. S181B-500), 1% Penicillin-Streptomycin (Biowest, cat. no. L0022-100) and 2mM L-Glutamine (Biowest, cat. no. X0550-100). H1437 cells were cultured in RPMI (Sigma, cat. no. R8758-500ML) supplemented with 10% FBS, 1% Pen/Strep and 2mM L-Glutamine. HEK293T and Phoenix cell lines were cultured in high glucose DMEM (Sigma, cat. no. D6429-500ML) supplemented with 10% FBS, 1% Pen/Strep and 2mM L-Glutamine.

For sub-culturing, cells reaching 80-90% confluency were gently washed once with phosphate buffered saline (PBS) and treated with Trypsin-EDTA (Biosera, XC-T1717/100) until they detached from the plate surface. After detachment, cells were resuspended in complete media and transferred to new plates with the appropriate dilution, supplemented with additional media. Cells were kept in a humidified incubator, with 5% CO_2 at 37^{o}C.

2.2.2. Cryopreservation of cell lines

For the preservation and deep freezing of cells, dishes with cells in a semi-confluent state were selected and detached using trypsin-EDTA. Subsequently, media was added, and the cell suspensions were transferred to sterilised 15 ml Falcon tubes and centrifuged for 10 minutes at 1500 rpm. The cell pellets were resuspended in FBS solution containing 10% dimethyl sulfoxide (DMSO) and then transferred to 2 ml cryovials. The cryovials were placed in an isothermal container containing isopropanol and moved to -80°C for gradual freezing (1°C/minute). The next day, they were transferred to liquid nitrogen for long-term preservation.

For their revival, the cryovials from liquid nitrogen were placed in a water bath at 37°C for approximately 1-2 minutes to thaw and the contents were transferred to 100 mm cell culture dishes with the appropriate media. The cells were maintained in a humidified cell culture incubator at 37°C, 5% CO_2.

2.2.3. Stable cell line generation

For the establishment of A549 IKKα^{KD} cell line, the retro-vector plasmids pSUPER-Retro and pSUPER-shIKKα-A3 were used (190, 191). The day before transfection, Phoenix cells were seeded in 100mm plates at a concentration of 2.5x10^{6} cells/plate. The next day, media was

changed and cells were transfected with a mixture of 10 µg of the desired plasmid and 30 µg of the cationic polymer polyethylenimine (PEI) diluted in 100 µl of serum free media. PEI forms a positively charged PEI:DNA complex which interacts with the negatively charged cell membrane and enters the cells by endocytosis (192). After 24 hours, the media was replaced, and the transfected cells were incubated for an additional 24 hours to generate retroviral particles. Retrovirus-containing media was collected at 48 and 72 hours post transfection, filtered through a 0.45 µm filter syringe and stored overnight at 4°C with Polyethylene Glycol 8'000 (Sigma, cat. no. 81268) to precipitate viral particles. The retro-media was then centrifuged at 1500 x g for 30 minutes, at 4°C. The pellet was resuspended in 200µl PBS, aliquoted and stored at -80°C for later use.

A549 cells were plated in 24 well plates with a concentration of $1x10^4$ cells/well. After 24 hours, fresh media was added with 8 µg/ml Polybrene (Merck, cat. no. TR-1003-G), a cationic polymer which facilitates lentiviral infection. 50 µl of retrovirus was added to each well and the cells were incubated for 72 hours. After 72 hours, media was changed and selection antibiotic puromycin was added at a concentration of 1 µg/ml. The cells were selected for a total of 7 days, with media being renewed every 2 to 3 days. After selection, the cell lines were expanded and used for downstream applications.

For the establishment of miR-9-5p overexpressing NSCLC cell lines, the lenti-vector plasmids Twl-PGKgfp-H1 for control and Twl-PGKgfp-H1-miR-9-5p (Figure 2.1) for miR-9-5p overexpression were used, which were kindly provided by Prof. Alessandro Barbon, University of Brescia (34458512).

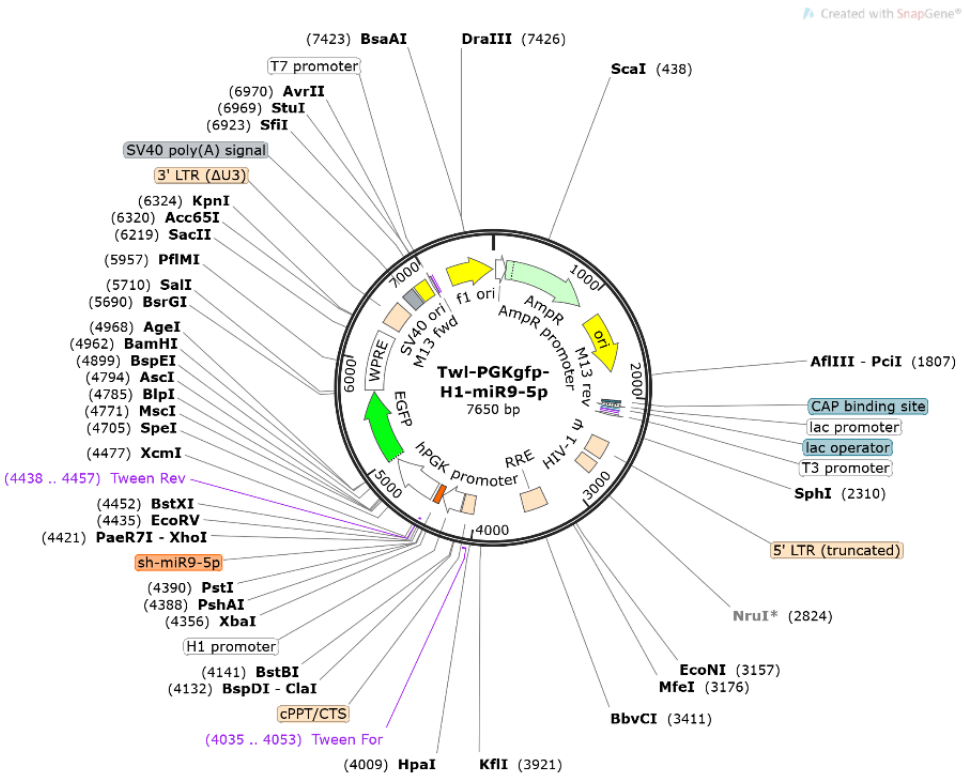

Figure 2.1. *Plasmid map of lentiviral vector, Twl-PGKgfp-H1-miR9-5p carrying miR-9-5p insert, kindly gifted by Dr Barbon* (193).

The day before transfection, HEK293T cells were seeded in 100mm plates at a concentration of $7x10^6$ cells/plate. The next day, at 50-60% confluency, media was changed and cells were transfected with a mixture of the below plasmids and PEI:

Twl-PGKgfp-H1/-miR-9-5p	4.5 µg
pΔ8.91 (packaging)	3.3 µg
pCMV-VSVG (envelope)	2.2 µg
Serum free DMEM	100µl
PEI	30 µg

After 24 hours, the media was replaced, and the transfected cells were incubated for an additional 24 hours to generate lentiviral particles. Lentivirus-containing media was collected at 48 and 72 hours post transfection, filtered through a 0.45 µm filter syringe and stored overnight at 4ºC with Polyethylene Glycol 8'000 (Sigma, cat. no. 81268) to precipitate viral particles. The

lenti-media was then centrifuged at 1500 x g for 30 minutes, at 4°C. The pellet was resuspended in 200μl PBS, aliquoted and stored at -80°C for later use.

A549 and H1299 cells were plated in 24 well plates (A549: $2x10^4$ cells/well, H1299: $1.5x10^4$ cells/well). After 24 hours, fresh media was added with 8 μg/ml Polybrene. 50 μl of lentivirus was added in each well and the cells were incubated for 72 hours. After 72 hours, cells were transferred to larger plates to allow expansion of the cell lines. Since the lentivectors do not carry an antibiotic selection gene, successful infection was assessed by observing GFP expression in cells. In order to obtain single clone populations, the cell lines were plated in 96 well plates, with a concentration of 1 cell/well. GFP positive colonies were selected and expanded. The successful overexpression of miR-9-5p was validated by qPCR of selected clones.

2.2.4. Cell Proliferation Assay

For the study of cell proliferation rates in each cell line, cells were plated in 96well plates in a concentration of 4500 cells/well for A549 cell lines and 1500 cells/well for H1299. The plates were incubated for 96 hours to grow to full confluency. Images of the wells were taken every 6 hours using the IncuCyte® ZOOM Live Imager (Essen BioScience) and the percentage of cell density was analysed using the IncuCyte® ZOOM Software. The experiment was repeated 3 times, with 6 technical replicates.

2.2.5. Flow cytometry (FACs) for cell cycle analysis

Cell cycle analysis was performed by fixing and staining the cells with Propidium Iodide (PI), a fluorescent dye that interacts with the DNA. The fluorescent intensity of each is representative of the amount of DNA, which corresponds to the different phases of cell cycle as cells progress through G0/G1 to S phase where DNA replication occurs and G2 phase where the amount of DNA is double compared to G1 (194).

For each sample, $1x10^6$ cells were counted from a 70-80% confluent plate and resuspended in 300 μl PBS. For cell fixation, 70% ice-cold ethanol was added to the samples dropwise while vortexing, reaching total volume of 3 ml. The samples were kept on ice for 30 minutes. For PI staining, samples were centrifuged at 3200 rpm, for 5 minutes then washed once with 1 ml of PBS. After centrifugation, the cells were resuspended in 300 μl of PI staining solution (PI Staining Solution: 38 mM Sodium Citrate pH 7.4, 50 μg/ml PI, 100 μg/ml RNase) and incubated at 37°C for 30 minutes. After staining, the cells were centrifuged at 3200 rpm for 5 minutes and

resuspended in 200 µl of PBS. The cells were analysed by flow cytometry, using the flow cytometer BD FACSAria III, BD Biosciences, US.

2.2.6. Matrigel Invasion Assay

80 µl of Matrigel (Corning, cat. no. 356230) (1:5 in serum-free media) was added to transwell inserts (24 well, 8µm pore size, Sarstedt, cat. no. 83.3932.800) and allowed to solidify at 37°C for 2 hours. Cells were detached and counted before 50,000 cells were added to the transwell insert in 300 µl serum-free media. 1 ml of media containing 20% FBS was added to each well of the 24 well plate below the transwell insert. After 48 hours, the cells were fixed in 4% PFA in PBS for 15 min at room temperature and the cells were stained with 1% crystal violet in 20% Ethanol in ddH_20 for 20 minutes at room temperature. The inserts were washed in PBS and a cotton bud was used to clean the inside of the insert. 10 representative images were taken of each insert using an EVOS microscope with a 10 x objective lens and infiltrating cells were counted with ImageJ, using the mean intensity of each image.

2.2.7. Tumour xenografts

The development of *in vivo* xenografts, using A549 and H1299 cell lines, was carried out at the Biomedical Research Foundation (BRFAA) of the Academy of Athens, in collaboration with Dr. Apostolos Klinakis, Principal Investigator A' (Professor level), Dr Zoe Kanaki, Staff Research Scientist (Assistant Professor level) at the Biomedical Research Foundation of the Academy of Athens (BRFAA), following European regulations for experimental animal handling (Federation of Laboratory Animal Science Associations), current Greek legislation, and the regulations of the Animal Facilities Unit of BRFAA. The care of animals and all experimental procedures were approved by the Department for the Protection of Production Animals and Laboratory Animals, Directorate for Animal Welfare, Drugs, and Veterinary Applications, Ministry of Rural Development and Food (MRDF).

For the development of human cancer cells as *in vivo* xenografts, 4 x 10^6 cells in 200 µl of PBS were subcutaneously inoculated into immunocompromised NSG (NOD-SCID-IL2Rgamma) mice aged 5 weeks. Each animal was inoculated bilaterally, with modified cells (A549 TwI-miR-9-5p and H1299 TwI-miR-9-5p) inoculated on the right side and the respective control cells on the left side.

The animals were sacrificed using the cervical dislocation method 4 and 6 weeks for H1299 and A549 respectively after cell inoculation to isolate the tumours. The isolated tumours were

weighed and were immediately snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein isolation.

2.3. Molecular and Biochemical methods

2.3.1. RNA Isolation

For each sample, cells were collected from an 80-90% confluent 100mm plate. Cells were lysed using the QIAzol Lysis Reagent (Qiagen, cat. no. 1023537) following the company's instructions. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, cat. no. 217004) and was eluted in RNase free water. The concentration and integrity of total RNA was quantified using the Nanodrop 2000 spectrometer.

2.3.2. Total RNA extraction from mouse xenografts

Each tumour was disrupted using a mortar and pestle with the use of liquid nitrogen, until tissue was grinded into fine powder. For each sample aliquots of 50mg were made for efficient RNA extraction. To each aliquot, 700µl of Qiazol was added and the samples were homogenised by vortexing. For the RNA extraction, the miRNeasy Mini Kit (Qiagen, cat. no. 217004) was used following the manufacturer's instructions.

2.3.2. Nanostring Analysis – miRNome profiling

The study of differential expression of miRNAs was conducted using the NanoString nCounter technology (195) in collaboration with Dr. Christos Polytarchou and Maria Hatziapostolou at Nottingham Trent University, UK. This is a microarray-based analysis where it utilises probes bearing molecular barcodes to detect and quantify the total copies of miRNAs found in each sample. This technique does not require amplification of target molecules, is characterised by high sensitivity, and no additional validation with other methods such as real-time polymerase chain reaction is needed. Sample preparation was carried out at the nCounter Prep station, while detection of probes was performed using the nCounter Digital analyser.

2.3.3. cDNA synthesis

For miRNA cDNA synthesis, the reverse transcription reaction was carried out using the miRCURY LNA RT Kit (Qiagen, cat. no. 339340) according to manufacturer's instructions, with 200ng of total RNA used for each sample.

2.3.4. Quantitative Polymerase Chain Reaction (qPCR)

To quantify the levels of miR-9-5p present in each sample, qPCR reactions were performed using the miRCURY LNA SYBR Green PCR Kit (Qiagen, cat. no. 339346). miR-222-3p and miR-16-5p were used as internal controls for normalisation. The fold change of miR-9-5p levels was calculated using the $2^{-\Delta\Delta Ct}$ method. All the primers were designed and made by the company (Qiagen), with the product names hsa-miR-9-5p (Cat. no. YP00204513), hsa-miR-16-5p (YP00295702) and hsa-miR-222-3p (YP00204551).

2.3.5. Target Prediction Algorithms

For the prediction of miR-9-5p mRNA targets, the database mirDIP was used (196-198). mirDIP is an online tool that integrates various miRNA target prediction algorithms, reducing bias of each individual algorithm and resulting in higher quality target list. miRNA target algorithms are mainly based on the complementarity of seed match regions between the given miRNA and 3' UTR sequences (199, 200), the conservation of the miRNA:mRNA target interaction across species (199), the stability of the miRNA:mRNA target interaction measured as free energy (201) and the site accessibility of the mRNA complementary region (202, 203).

Wnt associated genes were accessed using the Wnt Signalling Pathway gene list deposited to the Harmonizome 3.0 platform, with Gene Ontology code (GO_0016055) (204).

2.3.6. Total Protein Extraction

For each sample, cells from a 100mm dish were washed twice with 5ml of cold PBS-1mM EDTA pH 8.0. Subsequently, 1 ml of PBS-1mM EDTA pH 8.0 at 4°C was added, and cells were scraped off and collected in an Eppendorf tube. The samples were centrifuged at 6500 rpm for 2 minutes at 4°C. Next, the cell pellets were resuspended in 100-200 µl of RIPA buffer (proportionate to the pellet size) and they were kept on ice for 30 minutes to allow cell lysis, with occasional vortexing in between. The lysate was then centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was transferred to a clean, sterile Eppendorf tube, and protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, #23225) following the manufacturer's instructions. The cell lysates were stored at -80°C for future use.

RIPA Buffer: 150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% NP-40, 0.5% Triton X-100, 1 mM EDTA pH 8.0, 0.5% Sodium Deoxycholate, 1 mM Dithiothreitol (DTT), 1 mM PMSF, 1 mM Sodium Orthovanadate (Na3VO4), and 1X protease inhibitors (Sigma, S8820).

2.3.7. Total protein Isolation from mouse xenografts

Total protein extracts from mouse xenografts were isolated using the TRIzol™ reagent (Invitrogen, #15596018) method following the manufacturer's instructions.

Briefly, after RNA isolation, 300 µl of 100% ethanol was added to the lower phase to precipitate DNA. The samples were incubated at room temperature for 2-3 minutes and then centrifuged for 5 minutes at 2000 x g at 4°C. The supernatant containing the proteins was transferred to a new Eppendorf tube, and 1 ml of isopropanol was added to precipitate the proteins. This was followed by incubation for 10 minutes and centrifugation for 10 minutes at 12,000 x g at 4°C. The supernatant was removed, and the protein pellet was washed three times with a solution of 0.3 M guanidine hydrochloride in 95% ethanol. In each wash, the protein pellet was incubated for 20 minutes with the washing solution, followed by centrifugation for 5 minutes at 7500 x g at 4°C. After the final wash, the protein pellet was resuspended in 100% ethanol. It was then incubated for 20 minutes at room temperature and centrifuged for 5 minutes at 7500 x g at 4°C. The supernatant was removed, and the protein pellet was allowed to air-dry for 5-10 minutes. Finally, the protein pellet was resuspended in 100 µl of a 1% SDS solution. This was followed by centrifugation for 10 minutes at 10,000 x g at 4°C. The supernatant containing the protein extracts was transferred to a new Eppendorf tube and stored at -20ºC until further use.

2.3.8. Western Blotting

For Western blotting, 20-40 µg of total protein was loaded for each sample. SDS-PAGE gels were loaded with 20 µl of sample and run at 80 V for 10 minutes then 140 V for 90 minutes. Proteins were separated following SDS polyacrylamide electrophoresis using 10% polyacrylamide gels and were transferred to a nitrocellulose membrane (Amersham Protran 0.45µm nc, GE Healthcare Life Science, cat. no. 10600018) following wet transfer, at 100 V for 90 minutes, at 4°C. Membranes were blocked using 5% milk in TBST (Tris buffered saline + 0.1% Tween 20) for 1 hour. Primary antibodies were diluted in 3.5% milk TBST and incubated overnight at 4°C. Antibodies used in the experiments are listed on table 1. The next day, membranes were washed 3 times in TBST for 10 minutes each. They were incubated in HRP-conjugated secondary antibodies diluted (1:4000) in 3.5% milk in TBST, for 1 hour. They were washed 3 times x 10 minutes in TSBT and the membranes were developed after incubation in the reagent Clarity Western ECL Substrate (Bio-Rad) using the Imager Molecular Imager® Chemi Doc™ XRS (Bio-Rad).

Table 1: Primary and secondary antibodies used in Western Blot experiments

Antibody	Organism	Dilution	Company	Catalogue number
E-Cadherin	mouse	1:500	Cell Signalling Technology (CST)	3195
GAPDH	mouse	1:3000	SantaCruz Biotechnology (SCB)	sc-47724
phospho-Akt (S473)	rabbit	1:500	CST	9271
phospho-GSK-3β (S9)	rabbit	1:2000	CST	9323
GSK-3β	rabbit	1:1000	CST	9315
Akt1	rabbit	1:1000	CST	2938
β-catenin	rabbit	1:20000	ProteinTech	51067-2-AP
non-phospho β-catenin (non-45)	rabbit	1:1000	CST	19807
N-Cadherin	mouse	1:500	SCB	sc-59987
Vimentin	rabbit	1:1000	CST	5741
IKKα	mouse	1:1000	Novus Biologicals	14A231
α-tubulin	mouse	1:1000	GenScript	A01410
PPP2R5D	rabbit	1:1000	ProteinTech	12068-1-AP
CSNK1A1	rabbit	1:1000	ProteinTech	55192-1-AP
RAP2A	rabbit	1:500	ProteinTech	13789-1-AP
Vinculin	mouse	1:500	SCB	sc-73614
HRP-linked anti-mouse IgG	horse	1:4000	CST	7076
HRP-linked anti-rabbit IgG	goat	1:4000	CST	7074

2.4. Statistical Analysis

GraphPad Prism 9 software was used for statistical data analysis of the experiments. For comparison of two groups, a two-tailed Student's t-test was performed and a p value < 0.05 was considered significant. Data were presented as mean ± the standard error of the mean (SEM).

3. Results

3.1. Nanostring analysis of IKKα^{KD} cell lines

Previous studies from our laboratory showed that IKKα acts as a major NSCLC tumour suppressor in NSCLC by regulating HIF-1α and hypoxia-mediated processes required for *in vivo* NSCLC growth in response to urethane, a tobacco constituent, using human and lung cancer models (183).

To further investigate the impact of IKKα on lung cancer development, we established A549 IKKα knockdown (IKKα^{KD}) cell lines to study alterations in the miRNA expression profiles. A549 cells were infected with the retroviral vectors pSuper-Retro as control and pSuper-Retro-shIKKα for silencing IKKα. Stable cell lines were generated by puromycin selection of transduced cells. Whole protein lysates of the cells were used to analyse the expression of IKKα in control and KD cell lines by Western blotting (Figure 3.1), where the silencing of IKKα was confirmed.

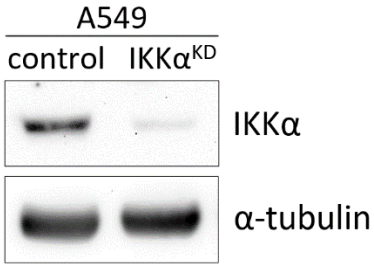

Figure 3.4. *Expression of IKKα in lentivirus-infected human lung cancer cells*. Western blotting for the expression of IKKα in A549 control (vector) and A549 IKKα^{KD} cell lines. α-tubulin was used as an internal control.

Following the establishment of A549 IKKα^{KD} cell lines, samples of total RNA were collected and subjected to MiRNome Profiling analysis using Nanostring technology in collaboration with Dr. Christos Polytarchou and Maria Hatziapostolou at Nottingham Trent University, England. Nanostring Analysis is a microarray technology which allows quantification of miRNAs using a panel of a total of 800 human miRNAs. Of these 800, 125 miRNAs were found to be expressed in the samples. Figure 3.2 represents the down- and upregulated miRNAs with the highest fold change between control and IKKα^{KD} cells. The full list of miRNAs expressed in the samples are available upon request. Interestingly, miR-9-5p exhibited the highest fold change among all miRNAs present in the samples, showing a 2-fold upregulation in the A549 IKKα^{KD} cells.

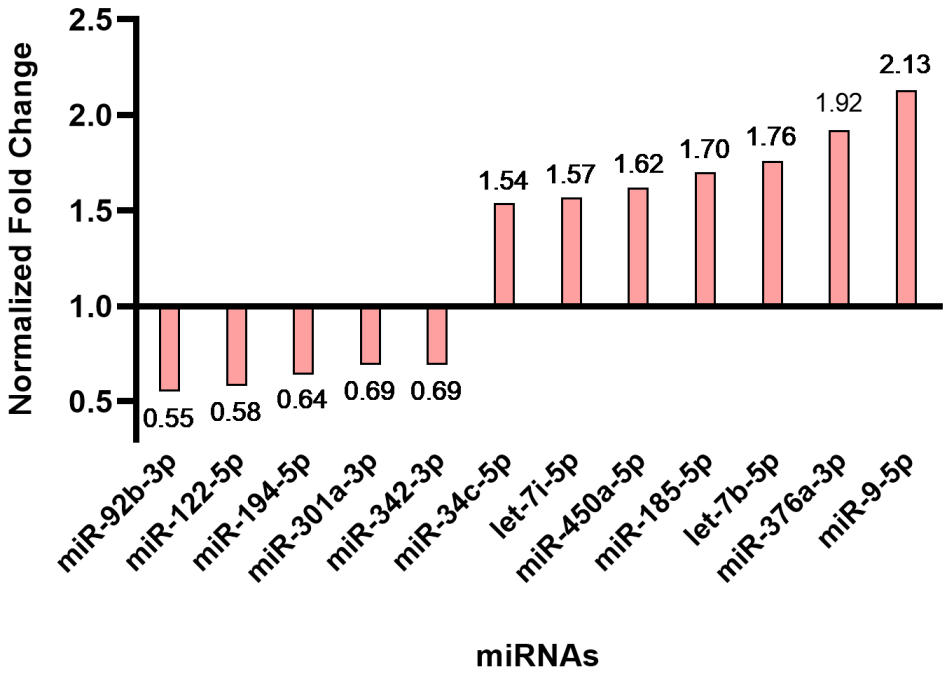

Figure 3.5. *Nanostring analysis of miRNA expression in IKKα^{KD} cells*. Fold change of miRNA levels in A549 IKKα^{KD} cells compared to control samples. Selected miRNAs are shown here, which have a fold change value above 1.5 or below 0.7. miR-9-5p has the most significant fold change (2.13) compared to other miRNAs.

This was confirmed by qPCR of A549 IKKα^{KD} cells compared to control, where the upregulation of miR-9-5p was found to be a fold change of 1.8 (Figure 3.3).

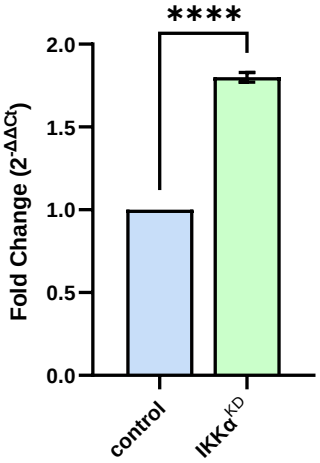

Figure 3.3. *Upregulation of miR-9-5p in IKKα^{KD} cells*. qPCR analysis of miR-9-5p levels in A549 IKKα^{KD} and control cell lines. MiR-222-3p was used as an internal control. Fold change was calculated with the ΔΔCt method (n=3, **** $p < 0.0001$). Bars represent means ± SEM.

Therefore, we decided to further investigate the role of miR-9-5p in lung cancer, as it has been implicated in lung cancer malignancy (118, 151, 154-157).

3.2. Generation of miR-9-5p overexpression cell lines

To investigate the role of miR-9-5p in lung cancer, two lung cancer cell lines with different mutational backgrounds were used. These were A549 lung adenocarcinoma cells which have wild-type p53 and carry a $KRAS^{G12C}$ mutation, and the p53-null human lung cancer cell line H1299 bearing no *K-Ras* mutations.

To establish miR-9-5p overexpression cell lines, the lentivectors Twl-PGKgfp-H1 (control) and Twl-PGKgfp-H1-miR-9-5p (miR-9-5p) were used, kindly provided by Prof. Alessandro Barbon, University of Brescia (193). After lentiviral infection of the cell lines A549 and H1299, successful transduction was assessed by observing the levels of GFP expression using fluorescence microscopy. Single clone populations were obtained by seeding 1 cell/well in 96-well plates due to the absence of an antibiotic selection marker in the vectors. Following expansion of clonal populations expressing GFP (Figure 3.4a/c), total RNA was isolated followed by cDNA synthesis to determine the levels of miR-9-5p by qPCR. Both A549 and H1299 displayed significantly higher levels of miR-9-5p expression with a fold change of 40.28 and 2.97 respectively, compared to their control counterparts (Figure 3.4b/d). The difference in miR-9-5p fold change between A549 and H1299 can be attributed to low endogenous levels of miR-9-5p in A549 control cells compared to H1299.

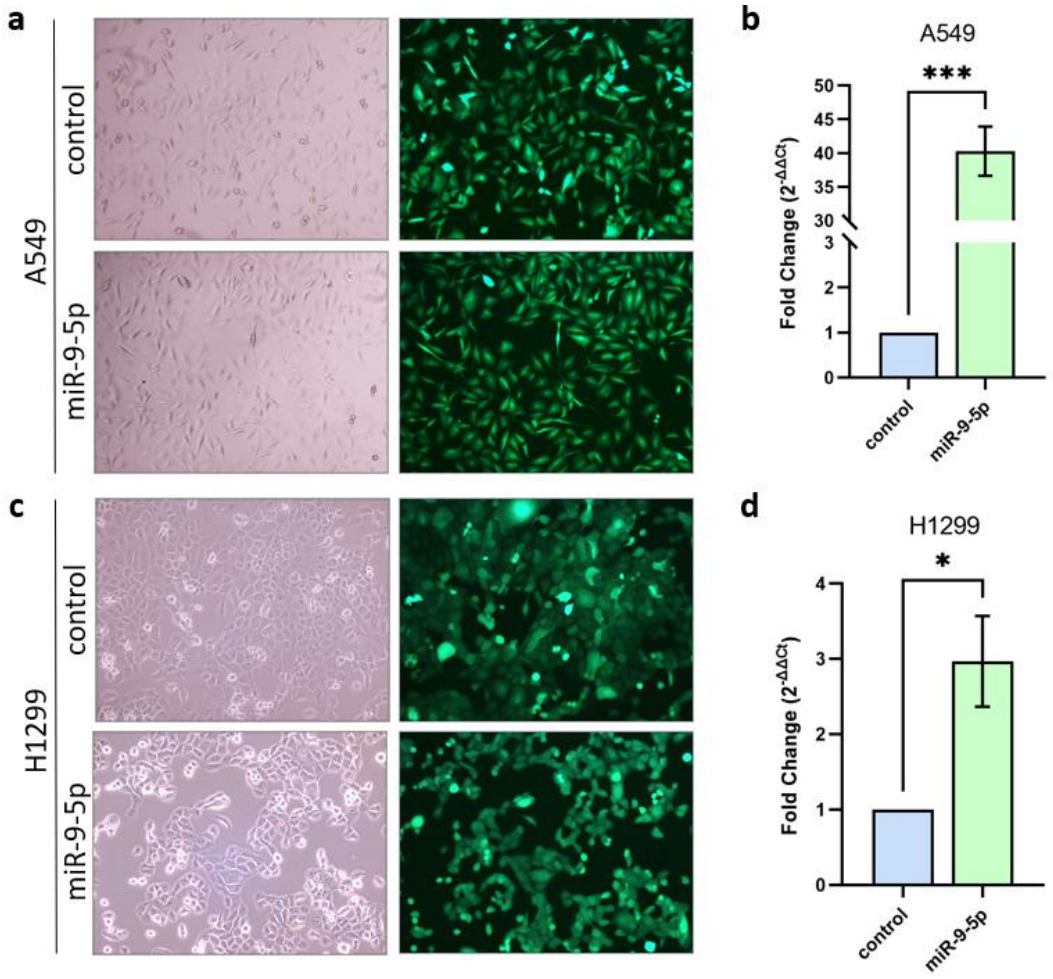

Figure 6.4. *Generation of miR-9-5p overexpressing cell lines*. (**a**, **c**) A549 and H1299 control and miR-9-5p stable cells lines expressing GFP. (**b**, **d**) qPCR analysis of miR-9-5p levels in lentivirus-infected A549 and H1299 control and miR-9-5p (overexpressing) cell lines (A549: p value = 0.0004, H1299: p value = 0.0306, t test). MiR-222-3p was used as an internal control. Fold change was calculated with the ΔΔCt method (n=3 * $p < 0.05$, *** $p < 0.001$). Bars represent means ± SEM.

3.3. miR-9-5p effect on cell proliferation, cell cycle and invasion *in vitro*

In order to investigate the phenotypic effects of miR-9-5p overexpression, the proliferation rates of the cell lines were analysed. IncuCyte® ZOOM Live Imager was used to obtain images of the cells every 6 hours and the company's software was used to analyse the percentage of surface area covered with cells in each time point. Although a slight decrease in proliferation rates in both A549 and H1299 miR-9-5p overexpressing cell lines was observed around 72 hours, the difference was not statistically significant (Figure 3.5).

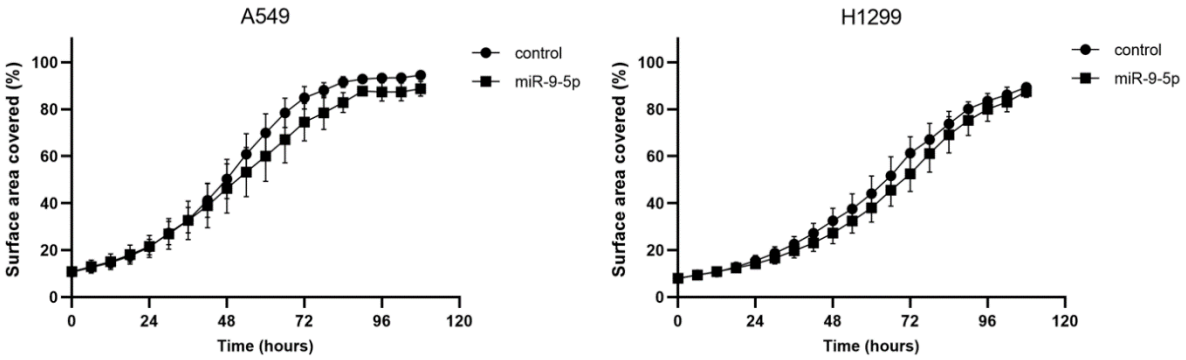

Figure 3.5. *Cell proliferation of A549 and H1299 control and miR-9-5p cell lines*. Images were taken by IncuCyte® ZOOM Live Imager every 6 hours and the cells were incubated until they reached full confluency. Surface area covered was analysed using the company's software. The graphs show means ± SEM representing 3 independent experiments.

In addition, cell cycle analysis was performed by flow cytometry, to investigate possible differences in cell cycle progression between the samples (Figure 3.6). There was no significant difference between control and miR-9-5p overexpressing cell lines, indicating that miR-9-5p overexpression does not affect cell cycle progression in these cells.

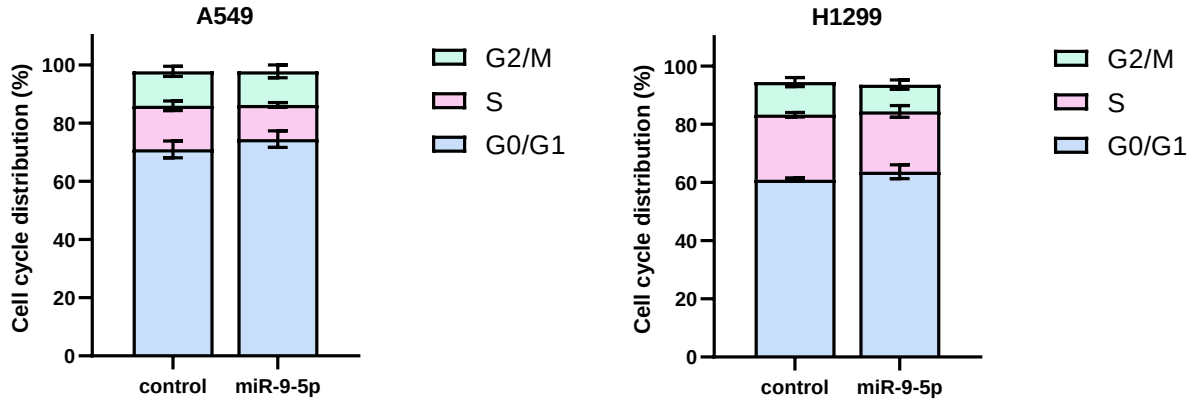

Figure 3.6. *Cell cycle analysis of A549 and H1299 control and miR-9-5p cell lines*. PI stained cells were analysed using the flow cytometer BD FACSAria III, BD Biosciences, US. The graphs show means ± SEM representing 3 independent experiments.

Since miR-9-5p has been shown to promote EMT in several types of cancer (66, 133, 135, 141, 142), we investigated whether miR-9-5p overexpression had an impact on cell invasion. A cell invasion assay was conducted using 8 μm pore transwell inserts coated with Matrigel, a protein solution derived from the mouse Engelbreth-Holm-Swarm sarcoma cells that mimics the basement membrane found in the extracellular environment of human tissues (205).

Cell invasion is assessed as the number of cells being able to migrate through the Matrigel coating and the pores of the transwell inserts. A549 control and miR-9-5p cells were plated in Matrigel coated 24 well transwell inserts and incubated for 48 hours to allow invasion. After fixation and staining of the invaded cells, the cells were counted. The results revealed that cell invasion was increased by 46% in A549 miR-9-5p cells compared to their control counterparts, showing miR-9-5p as positive regulator of cell invasion in the chosen lung cancer cell lines. Figure 3.7 displays representative images of the invaded cells (Figure 3.7a), and corresponding statistical analysis (Figure 3.7b).

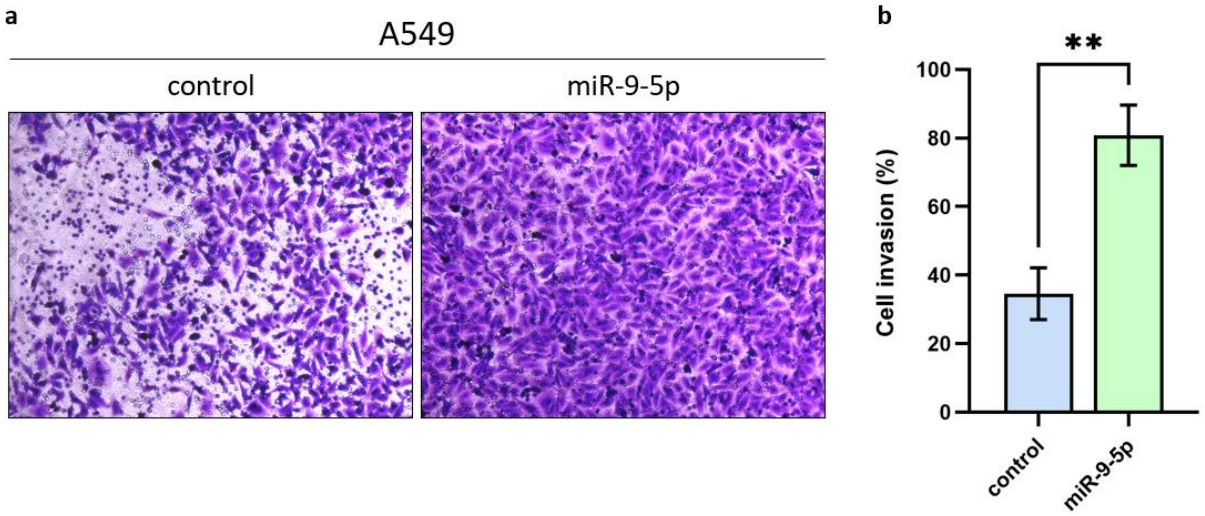

Figure 3.7. *Cell invasion Assay of A549 control and miR-9-5p cell lines*. (**a**) Representative images of A549 control and miR-9-5p invaded cells (**b**) Percentage of invaded cells in A549 control and miR-9-5p cell lines (p value = 0.0068, t test) (n=3, ** $p < 0.01$). The bars show means ± SEM representing 3 independent experiments.

3.4. Predicted targets of miR-9-5p

To investigate possible miR-9-5p targets in the established cell lines, the mirDIP database was used. mirDIP uses more than 30 miRNA target prediction algorithms to provide a list of predicted target genes for a miRNA of interest.

For miR-9-5p predicted targets, the top 5% of the hits were selected, consisting of 1036 genes. Since our *in* vitro assays demonstrated that miR-9-5p has an impact on cell invasion, the mirDIP gene list was compared to sets of genes involved in key pathways implicated in EMT, such as the Wnt pathway. Table 3.1 summarises the total selected genes that are predicted miR-9-5p targets which are involved in the Wnt signalling pathway. The Wnt signalling pathway is one of the major pathways implicated in EMT signalling (206, 207) and is also found activated in cancer (208-210), including lung cancer (206, 207, 211, 212). Moreover, an interaction between Wnt signalling and

noncoding RNAs including miRNAs was shown in human cancers (213, 214) including lung cancer (215, 216).

Table 3.1. *Selected miR-9-5p predicted target genes involved in Wnt Signalling Pathway*

Gene Symbol	Name
CDH11	Cadherin 11
CSNK1A1	Casein Kinase 1 alpha 1
CSNK1G2	Casein Kinase 1 gamma 2
CTNNA1	Catenin alpha 1
FBXW11	F-box and WD repeat domain containing 11
FZD5	Frizzled Class Receptor 5
GSK3B	Glycogen Synthase Kinase 3 beta
HLTF	Helicase-like Transcription Factor
NFATC3	Nuclear Factor of Activated T Cells 3
PCDH10	Protocadherin 10
PCDH7	Protocadherin 7
PLCB1	Phospholipase C beta 1
PPP2R5D	Protein Phosphatase 2 Regulatory Subunit B'delta
PRKCA	Protein Kinase C alpha
PYGO2	Pygopus Family PHD Finger 2
RAP2A	Rap2a, member of RAS oncogene family
SMAD4	SMAD Family Member 4
TBL1XR1	TBL1X/Y related 1
TCF7L2	Transcription Factor 7 like 2

From the list shown in Table 3.1, three targets were selected based on their action, *PPP2R5D*, a negative regulator of the Wnt pathway (217), *CSNK1A1* (CK1α), inhibitor of β-catenin (218, 219), and *RAP2A* (Rap2a), inhibitor of Akt1 (220).

Immunoblotting showed no significant difference in the protein levels of these selected targets between control and miR-9-5p overexpressing cell lines, indicating that the selected proteins are not targets of miR-9-5p in these cell lines (Figure 3.8). *PPP2R5D* could be a target in H1299 cells, as protein levels appear lower in miR-9-5p overexpressing cells, but since there was only a small

decrease of PPP2R5D and protein levels remain stable in A549 cells we chose to search for another target that would be more likely to induce the increased invasiveness phenotype observed in miR-9-5p cells.

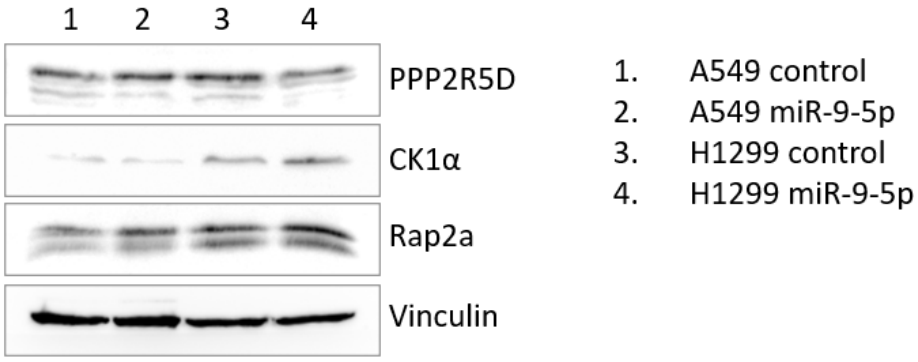

Figure 3.8. *Protein expression of selected miR-9-5p targets*. Western blot analysis of selected predicted miR-9-5p targets PPP2R5D, CK1a and Rap2a in A549 and H1299 control and miR-9-5p cell lines. Protein levels of Vinculin were used as an internal control.

3.5. miR-9-5p targets E-Cadherin in LUAD

Since the selected predicted target genes were not verified in A549 and H1299 cell lines, we sought to examine if miR-9-5p targets E-Cadherin in this system, as it is a crucial factor involved in EMT and invasiveness. Moreover, E-Cadherin interacts with β-catenin, a component of the Wnt signalling pathway (221-225).

Immunoblotting analysis was performed using A549 and H1299 control and miR-9-5p cell lysates, to determine E-Cadherin protein levels. Figure 3.9 shows that protein levels of E-Cadherin in A549 miR-9-5p cells were almost eliminated compared to the control sample. H1299 cells do not express E-Cadherin, therefore, no change was observed upon miR-9-5p overexpression.

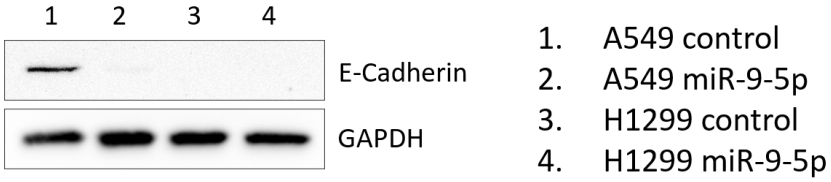

Figure 3.9. *miR-9-5p targets E-Cadherin in LUAD*. Western blot analysis of E-Cadherin expression in A549 and H1299 control and miR-9-5p overexpressing cell lines. Protein levels of GAPDH were used as a loading control.

Next, we explored the downstream effects of E-Cadherin silencing in the miR-9-5p overexpressing cell lines. Overexpression of miR-9-5p resulted in elevated levels of active β-catenin, which is non-phosphorylated at serine 45 (non-phospho β-catenin S45) (Figure 3.10). This could be the effect of E-Cadherin downregulation, since β-catenin is typically bound to E-Cadherin near the cell membrane, where it acts as structural component of the cadherin cell junctions. Total β-catenin levels were also increased in miR-9-5p overexpressing cells, suggesting that β-catenin is either more stabilised in the presence of miR-9-5p, or that its expression is positively regulated. This indicates that the canonical Wnt signalling pathway is in the on-state.

One of the main kinases phosphorylating and inhibiting β-catenin is GSK-3β. The levels of inactive GSK-3β, phosphorylated at serine 9 (phospho-GSK-3β S9) were increased upon miR-9-5p overexpression, while total levels of the protein did not present any significant change (Figure 3.10).

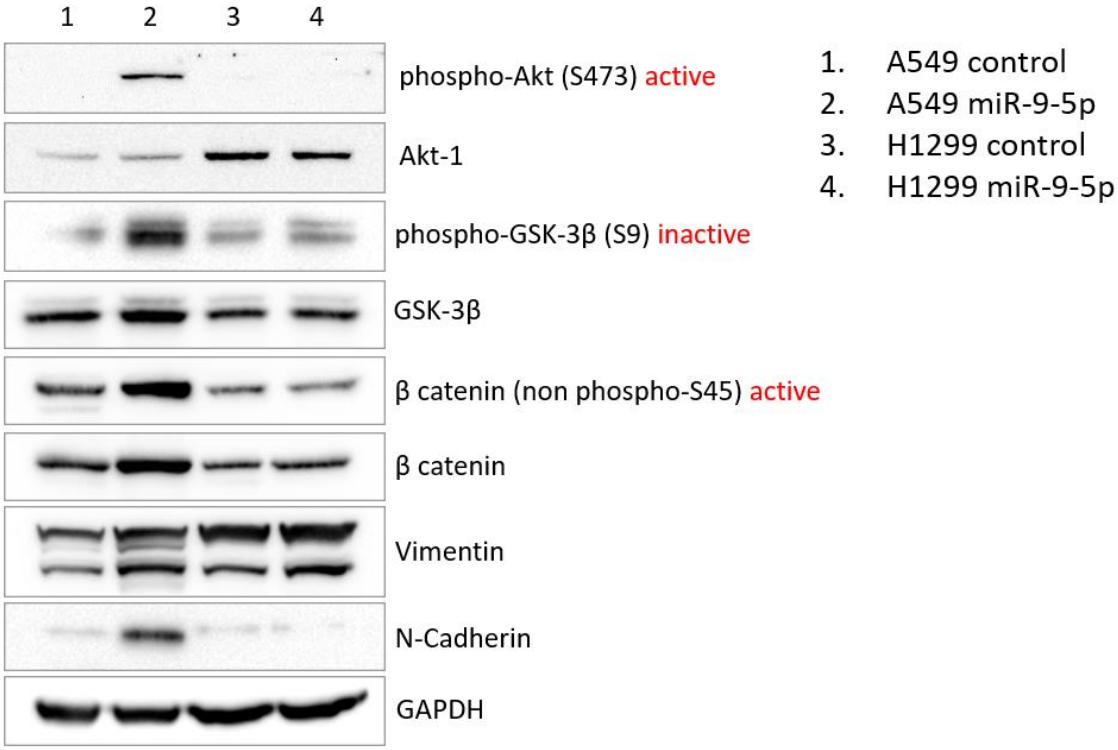

Figure 3.10. *Protein expression of proteins involved in the Wnt/β-catenin signalling pathway*. Western blotting analysis of phospho-Akt (S473), Akt-1, phospho-GSK-3β (S9), GSK-3β, non-phospho-β-catenin (S45), β-catenin, Vimentin and N-Cadherin expression in A549 and H1299 control and miR-9-5p cell lines. Protein levels of GAPDH were used as a loading control.

Since Akt1 is the main kinase phosphorylating GSK-3β, western blotting was performed for total Akt-1 as well as the active form, the phosphorylated Akt at serine 473 (phospho-Akt1 S473).

Levels of active Akt were significantly increased upon miR-9-5p overexpression. Levels of N-Cadherin were also highly increased in A549 miR-9-5p overexpression cells (Figure 3.10), a mesenchymal cell protein biomarker which is highly associated with EMT progression (226-228). Protein levels of Vimentin were also found increased in A549 miR-9-5p cells compared to control, an intermediate filament protein associated with the mesenchymal phenotype (229, 230).

3.6. miR-9-5p overexpression promotes tumour formation *in vivo*

To further examine the role of miR-9-5p in lung tumourigenesis *in vivo,* A549 and H1299 cell lines overexpressing miR-9-5p were grown as xenografts in immunocompromised NSG mice, in collaboration with Dr. Apostolos Klinakis, Principal Investigator A' (Professor level), and Dr. Zoe Kanaki, Staff Research Scientist (Assistant Professor level) at the Biomedical Research Foundation (BRFAA) of the Academy of Athens. A549 and H1299 control and miR-9-5p cells were inoculated bilaterally in the flanks of NSG mice and left to grow for 4-6 weeks.

Mice were euthanised and the tumour xenografts were resected and weighed. In both A549 and H1299, miR-9-5p overexpression significantly increased tumour size compared to the controls (Figure 3.11a, b, c, e). A549 miR-9-5p xenografts weighed 2 times more and H1299 2.2 times more than their control counterparts. In addition, tumour size was measured during their growth and tumour volume was calculated. Tumour growth curves presented in Figure 3.11 (d, f) show the size differences of control and miR-9-5p overexpression tumour xenografts. These results confirm the oncogenic role of miR-9-5p in LUAD *in vivo*.

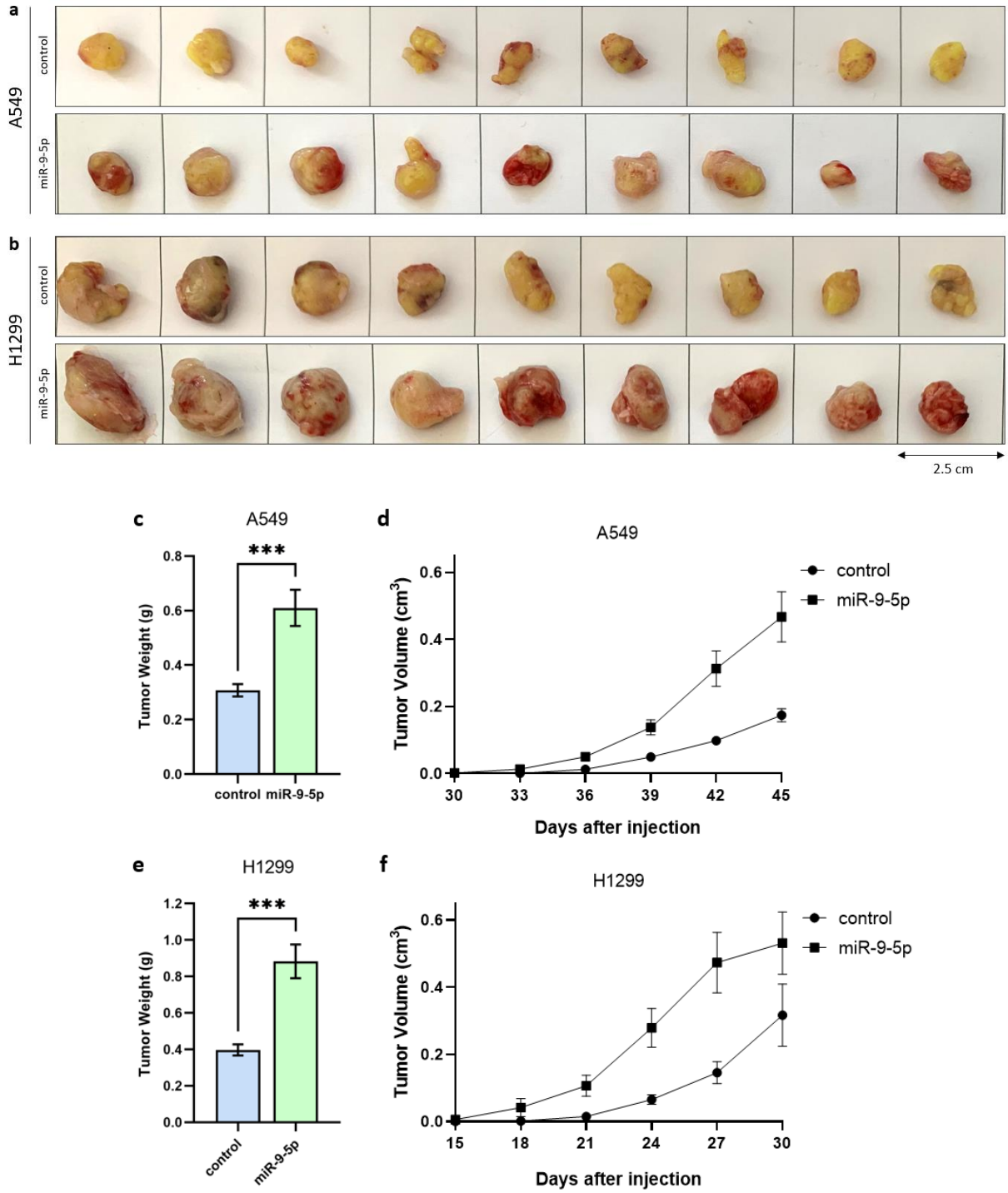

Figure 3.11. *Tumour xenografts of A549 and H1299 control and miR-9-5p cell lines.* (**a, b**) Representative images of tumour xenografts of A549 and H1299 control and miR-9-5p grown in NSG mice for 4 (H1299) and 6 (A549) weeks. Graphs show tumour weight of (**c**) A549 (p value = 0.0005, t test) and (**e**) H1299 (p value = 0.0001, t test) xenografts. Growth curves of (**d**) A549 and **(f)** H1299 xenografts were measured by tumour size in the timelines stated above (n=9, *** $p < 0.001$). Bars represent means ± SEM.

To confirm the overexpression of miR-9-5p in the tumour xenografts, total RNA was isolated from tumours and qPCR analysis for miR-9-5p was performed. Figure 3.12 shows the levels of miR-9-5p in A549 and H1299 control and miR-9-5p tumour xenografts, confirming the statistically significant overexpression of miR-9-5p accounting for the increased tumour growth in immunocompromised mice.

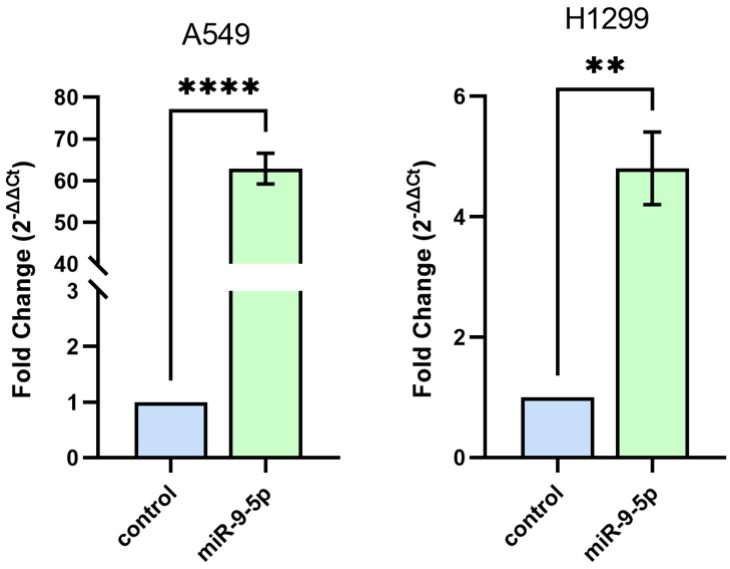

Figure 3.12. *Expression of miR-9-5p levels in A549 and H1299 control and TwI-miR-9-5p xenografts by qPCR analysis*. miR-9-5p levels are significantly higher in the miR-9-5p overexpressing cells than their respective control samples (A549: p value < 0.0001, H1299: p value = 0.0032, t test). miR-222-3p was used as an internal control. Three biological replicates were used and fold change was calculated with the ΔΔCt method (n=3, ** p < 0.01, **** p < 0.0001). Bars represent means ± SEM.

4. Discussion

4.1. Introduction

Lung cancer is the leading cause of cancer death worldwide and the second most common cancer type in men and women (1, 2). Despite its prevalence, there is still a lack of effective treatment as it is often diagnosed at a late stage and rapidly develops resistance to radio- and chemotherapy (8-10). Lung adenocarcinoma is the most prevalent non-small cell lung cancer subtype, accounting for 70% of total cases. It results from the accumulation of genetic and epigenetic changes. Key genetic mutations, such as *K-Ras* activating mutations, activate the NF-κB signalling pathways, and in particular the canonical NF-κB pathway which sustains chronic inflammation and promotes carcinogenesis (34, 37, 40, 41, 181, 182). However, the non-canonical NF-κB Ser/Thr kinase, IKKα, appears to act as both a tumour suppressor and a tumour promoter in a tissue-specific manner (186). In lung adenocarcinoma, as well as lung squamous cell carcinoma, IKKα has been shown to act as a tumour suppressor (187-189). We have previously shown that silencing of IKKα in two *in vivo* lung cancer models, a mouse and a human model, caused increased expression of the hypoxia factor, HIF-1α, to lead and sustain increased tumour growth under hypoxic environments (183). However, the tumour suppressor role of IKKα in lung cancer is still underexplored and further research is required to elucidate the molecular mechanisms of the tumour suppressive action of IKKα.

MiRNAs are small non-coding RNAs that regulate gene expression at a post-transcriptional level by binding to complimentary sequences found in 3' UTRs of mRNA targets, suppressing translation and inducing mRNA degradation (67). MiRNAs are widely dysregulated in a variety of cancers and act as both tumour promoters and tumour suppressors (66). NF-κB signalling pathways regulate the expression of various genes, including protein coding genes and non-coding RNAs, such as miRNAs, however research into the link between NF-κB signalling and miRNAs is limited (148). Therefore, there is a clear research gap between NF-κB and miRNA regulatory pathways in lung cancer, and elucidating the complexity of their relationship would result in better understanding of the disease and potential new therapeutic targets.

4.2. IKKα loss induces miR-9-5p expression

In order to further explore the tumour suppressor role of IKKα in NSCLC, we examined the miRNA expression profile of lung cancer cells upon IKKα loss. To accomplish this, we established an IKKα^{KD} cell line, using the A549 LUAD cells (Figure 3.1). Total RNA was isolated and the miRNome profile was explored by Nanostring analysis, quantifying the levels of 800 miRNAs in the samples. The most significant hit of this analysis was the upregulation of miR-9-5p, with a fold change of 2.13 compared to control cells (Figure 3.2), which was confirmed by qPCR of A549 control and IKKα^{KD} samples (Figure 3.3). miR-9-5p has been characterised as an oncogenic miRNA in different types of cancer, affecting cell proliferation and migration, EMT and inflammation (66, 131-136). In lung cancer, miR-9-5p is often found overexpressed and has been associated with metastasis (118, 151), but its mechanism of action is yet to be elucidated. Therefore, we decided to further study the effects of miR-9-5p in NSCLC development, as it appears to be an important miRNA in cancer development and could serve as a potential therapeutic target. Lentivirus based-vectors were used to establish stable overexpression of miR-9-5p in A549 and H1299 cell lines, and the overexpression was confirmed by qPCR (Figure 3.4). The stable cell lines were subjected to *in vitro* assays to examine phenotypic changes in the cells. MiR-9-5p did not have any effects on *in vitro* cell growth, as documented by cell proliferation assay (Figure 3.5) and cell cycle flow cytometry (Figure 3.6) which only showed small but non-significant changes. Interestingly, the cell invasion assay using Matrigel-coated transwell inserts, showed a statistically significant increase in invasion of miR-9-5p overexpressing cells, linking miR-9-5p to enhanced EMT properties in NSCLC cells (Figure 3.7). These results agree with previous published literature, where miR-9-5p has been shown to promote cell invasion and EMT in breast and colon cancer, hepatocellular carcinoma and osteosarcoma (66, 133, 135, 141, 142). Therefore, we decided to further investigate miR-9-5p targets that are implicated in EMT.

4.3. MiR-9-5p targets in NSCLC

Specialised algorithms can be used to predict miRNA target genes based on different factors, such as complementarity and stability between the miRNA:3'UTR sequences, the conservation of this interaction across species and the site accessibility of the mRNA complementary region (199-203). To explore predicted miR-9-5p targets, the mirDIP database was used to predict target genes by accessing data from multiple different algorithms (196-198). As the resulting gene list was wide, with 1026 results considered significant by the platform, we chose to compare the targets to genes involved in core pathways regulating EMT, such as the Wnt signalling pathway

(Table 3.1). The Wnt signalling pathway regulates cell fate determination and organogenesis during embryonic development, and is often found activated in cancer, where it promotes cell proliferation and migration. β-Catenin is the main downstream effector of the pathway, and when activated, it translocates to the nucleus where it promotes the expression of genes associated with EMT (231-233).

4.3.1. EMT and cancer

EMT is a process where cells transition from possessing epithelial to mesenchymal cell characteristics, including the loss of cell-cell junctions and cell polarity, and the gain of an invasive and motile phenotype. This process is involved in organogenesis during embryonic development (234), tissue regeneration and wound healing (235), but is also highly associated with cancer progression and metastasis (236, 237). Cancer cells often acquire partial mesenchymal cell characteristics, while still carrying epithelial qualities, having a hybrid epithelial/mesenchymal phenotype. These cells can enter the bloodstream as clusters and have high tumour-initiating properties and are highly resistant to apoptosis (238). The mesenchymal to epithelial cell transition (MET) is the reverse action of EMT and plays a role in development (239) as well as cancer progression. When circulating cancer cells extravasate to a new tissue, they can transition back to an epithelial phenotype eventually establishing a metastatic niche (240). A schematic representation EMT is shown in Figure 4.1.

Many different pathways regulate EMT, including Wnt (241), TGFβ (242) and EGF (243) pathways, hypoxia (244) and elevated levels of extracellular matrix components, such as collagen type I and hyaluronic acid (245, 246). The process of EMT is governed by extended gene expression changes, regulated by specific EMT transcription factors. The main TFs involved in EMT are the Zinc-finger transcriptional repressors SNAI1 and SNAI2 (247-249), the Zinc-finger E-box binding homeobox transcriptional repressors ZEB1 and ZEB2 (247), the basic helix-loop-helix transcription factors TWIST1 and TWIST2 (250, 251) and the transcriptional activator Brachyury, which is upregulated in lung cancer (252, 253).

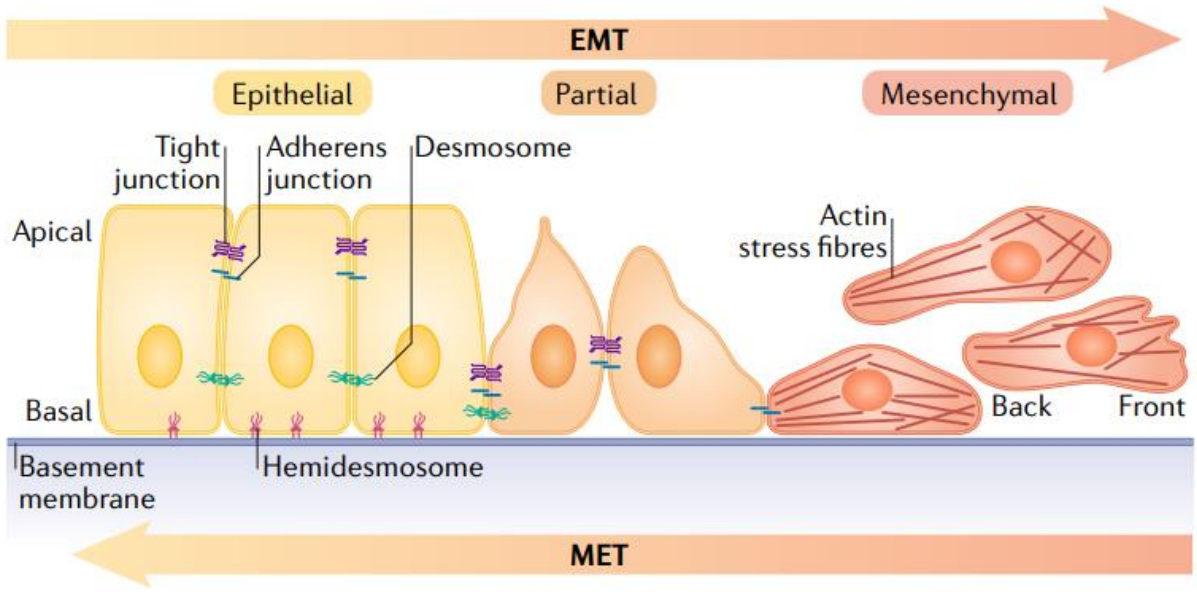

Figure 4.7. *Schematic figure of epithelial to mesenchymal transition*. A typical apical-basal polarity of epithelial cells is the result of adherens junctions, tight junctions and desmosomes between neighbouring cells, as well as hemidesmosomes between the cells and the basement membrane of the tissue. During EMT, transcription factors, such as TWIST1, SNAIL and ZEB repress the expression of epithelial factors that form the cell-cell junctions, resulting in loss of cell polarity. EMT TFs also promote the expression of mesenchymal factors, which causes cytoskeleton reorganisation and increased motility and invasiveness (254).

4.3.2. Wnt signalling pathway

β-Catenin is a key player in the Wnt signalling pathway. In the absence of stimuli, β-catenin is bound to E-Cadherin at the cell membrane, while any free cytoplasmic molecules bind to the β-catenin destruction complex, consisting of AXIN, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β), where it is phosphorylated by CK1 at Serine 45 and GSK-3β at Serine 33, 37, and 41 (219, 255, 256). The β-TrCP E3 ubiquitin–ligase complex recognises phosphorylated β-catenin and ubiquitinates it, leading to its degradation by the 26S proteasome (257, 258). In the presence of a ligand (WNT), the protein Dishevelled (DVL) binds the destruction complex to the membrane receptors Frizzled (FZD) and LDL receptor-related protein 5 or 6 (LRP5/6), allowing β-catenin to accumulate in the cytoplasm. This accumulation allows β-catenin translocation to the nucleus, where it acts as a regulator of gene expression by binding to the Wnt transcription factors TCF/LEF and attracting transcriptional activators, such as cAMP-response element binding protein (CREB) and homologs p300 and CREB-binding protein (CBP), promoting the expression of target genes (210, 259, 260). In addition to EMT markers, some other Wnt targets are the tumour promoting transcription factors

c-Myc and c-Jun, and the key cell cycle regulator Cyclin-D (261-264). Figure 4.2 shows the active and inactive states of the Wnt signalling pathway.

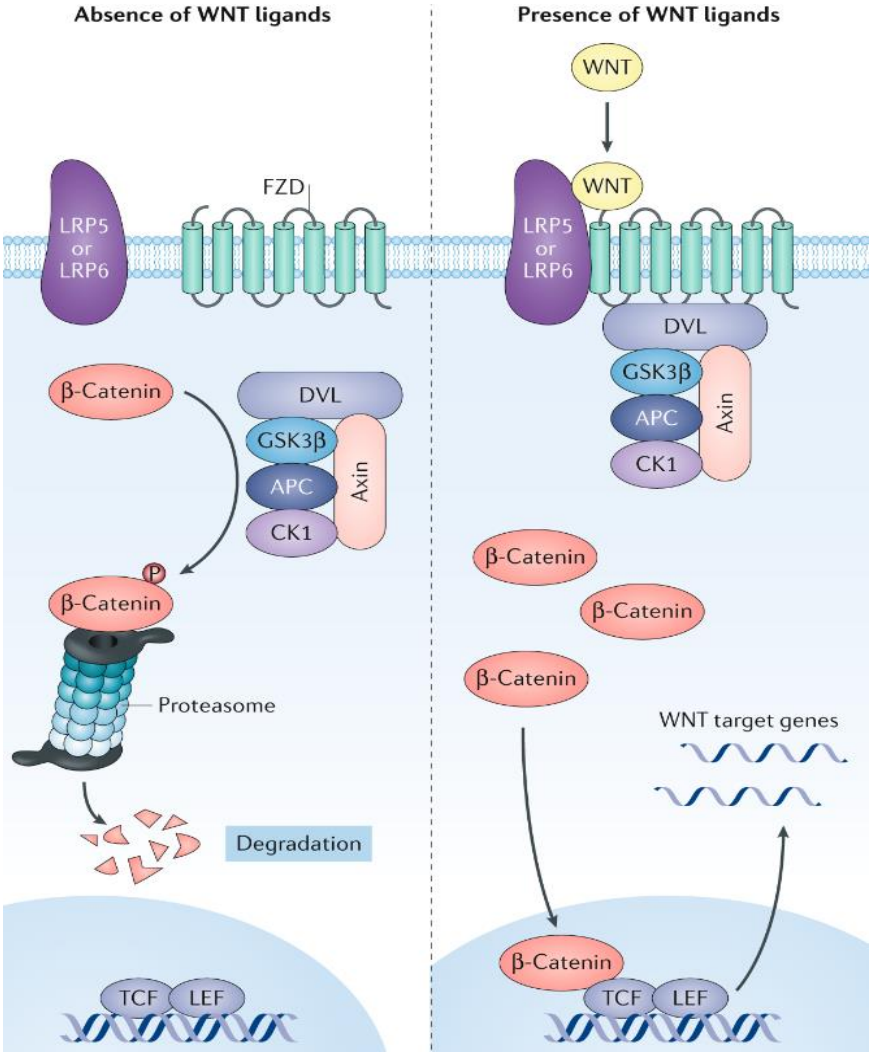

Figure 4.8. *Wnt signalling pathway*. The β-catenin destruction complex is comprised by the proteins Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β). In the absence of ligand, kinases CK1 and GSK-3β phosphorylate β-catenin, signalling its degradation by the proteasome. Upon presence of ligand (WNT), the destruction complex is bound to the membrane receptors Frizzled (FZD) and LDL receptor related protein 5 or 6 (LRP5/6) via the protein Dishevelled (DVL), allowing cytoplasmic accumulation of β-catenin. This leads to β-catenin translocation to the nucleus, where it binds to Wnt transcription factors TCF/LEF and regulates the expression of target genes (265).

The Wnt signalling pathway has a significant role in cancer biology. In a murine lung cancer model, activated Wnt signalling increased tumour initiating ability (266). Normal lung epithelial cells exposed to cigarette smoke components showed increased Wnt signalling activation (267,

268). In addition, Wnt signalling has been implicated in K-Ras induced lung cancer in mice models (269, 270) and the activation of Wnt signalling, combined with the presence of K-Ras mutations, has been shown to result in a higher rate of tumour formation as well as larger tumour size in the mice (271).

Non-coding RNAs, such as miRNAs, have been associated with EMT regulation and gene expression programming of epithelial and mesenchymal states, and can act as both inducers and inhibitors of EMT. For example, the miR-200 family has been shown to act as a repressor of EMT and a promoter of epithelial differentiation, by targeting the key EMT transcription factors, ZEB1 and ZEB2 (272, 273). ZEB1 represses the expression of miR-200 family members, showcasing the complexity of the regulatory networks involved in EMT processes (274). MiRNAs can also promote EMT, by targeting key regulators of the epithelial phenotype. MiR-92a has been shown to target E-Cadherin in oesophageal squamous cell carcinoma, promoting cell migration and invasion (275). In addition, researchers have found that TGF-β-induced expression of miR-99a and miR-99b contributes to EMT progression by downregulating the expression of E-Cadherin and ZO-1, a tight junction protein, thus promoting migration (276). Inhibition of both miRNAs was not sufficient to block EMT induced by TGF-β, indicating that many miRNAs have a complimentary role to protein factors regulating EMT, forming a complex landscape of gene expression regulation.

From the Wnt associated genes included in the mirDIP list, the following targets were selected for further investigation, based on their function: *PPP2R5D*, *CSNK1A1* and *RAP2A*. Casein kinase 1α (CK1α), coded by the gene *CSNK1A1*, forms a complex with GSK-3β and the scaffolding protein APC in the cytoplasm and acts by phosphorylating β-catenin at Ser45, priming it for subsequent phosphorylation by GSK-3β, which leads to its ubiquitination and proteasomal degradation (218, 219). PPP2R5D is a regulatory unit of the phosphatase PP2A, which is implicated in the regulation of Wnt signalling (217). Rap2a is a GTP-binding protein and a member of the Ras protein family that inhibits Akt1 activity in the brain, resulting in elevated active (unphosphorylated) GSK-3β (220). In neural stem cells, miR-9-5p acts synergistically with miR-124 by targeting Rap2a, promoting dendritic differentiation (277).

The expression of CK1α, PPP2R5D and Rap2a was analysed by immunoblot in our control and miR-9-5p overexpressing cell lines, however none of the selected genes was validated as a miR-9-5p target as they were equally abundant in both control and miR-9-5p overexpressing cells (Figure 3.8). Expression of PPP2R5D showed a slight decrease in H1299 miR-9-5p overexpression cells compared to their control counterparts, however this change was not considered significant (Figure 3.8).

4.4. miR-9-5p targets *CDH1* in lung cancer

Since the selected predicted targets were not miR-9-5p targets in our model, we decided to evaluate *CDH1*, which encodes for the protein E-Cadherin, as a possible miR-9-5p target. Loss of E-Cadherin is one of the most significant markers of EMT. In epithelial cells, E-Cadherin is a core transmembrane protein of the adherens junctions that mediate epithelial cell-cell adhesion, creating the normal structure of the epithelium. The extracellular part of E-Cadherin consists of five cadherin domains (EC1-5), which bind to EC domains of neighbouring cells and is regulated by Ca^{2+} (278-281). The intracellular domain of the protein associates with the actin cytoskeleton through binding to catenin proteins, forming the complex of E-Cadherin/β-catenin/α-catenin/P120-catenin, stabilising cell adhesion (Figure 4.3) (282-284).

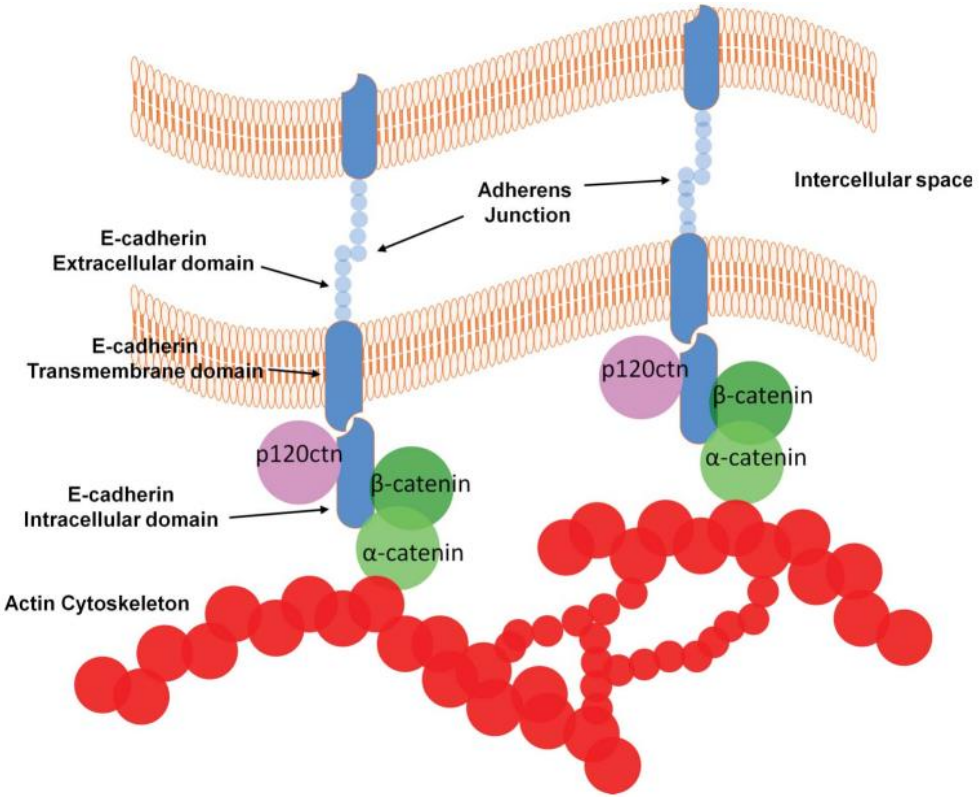

Figure 4.9. *Structure of E-Cadherin adherens junctions*. The E-Cadherin extracellular domain (EC) binds to EC domains of neighbouring cells creating adherens junctions. The intracellular domain of E-Cadherin associates with p120-catenin and β-catenin. This clustering allows α-catenin to bind to β-catenin, linking the complex to the actin cytoskeleton and securing the adherens junctions (285).

Upon loss of E-Cadherin, EMT TFs promote the expression of N-Cadherin and other EMT factors such as vimentin, fibronectin, alpha-smooth muscle actin (αSMA) and matrix

metalloproteinases (MMP-2, MMP-3, MMP-9), causing a change of the actin landscape and promoting infiltration and migration (286-288). ZEB and TWIST proteins can also promote carcinogenesis by inhibiting senescence and apoptosis induced by p53 and Rb pathways, cooperating with RAS and MYC oncoproteins (289, 290).

E-Cadherin has been previously identified as a miR-9-5p target in breast cancer (133), colon cancer (66, 135), cervical cancer (144) and osteosarcoma (142), however no studies have evaluated the ability of miR-9-5p to target E-Cadherin in lung cancer. Here, through immunoblot analysis, we showed that E-Cadherin protein levels decrease in A549 miR-9-5p overexpressing cells, correlating with the increased cell invasion observed in this cell line (Figure 3.9). While E-Cadherin levels decreased, N-Cadherin proteins levels were found to be higher in the same cell line (Figure 3.10). The switch from E-Cadherin to N-Cadherin expression in epithelial cells is highly associated with EMT and with the invasive cellular phenotype (226, 291). Vimentin, an intermediate filament protein highly implicated in EMT and cancer progression was found elevated in A549 miR-9-5p cells (Figure 3.10) (229, 230). Vimentin promotes cell migration by supporting cytoskeletal reorganisation (292-294) and is often associated with poor prognosis and cancer metastasis (295, 296), including lung cancer (296-299). In addition, miR-9-5p overexpressing cells presented elevated levels of active Akt1 and β-catenin, while GSK-3β was found inactivated, showing activation of Akt1/GSK-3β/β-catenin pathway (Figure 3.10).

4.5. miR-9-5p promotes tumour growth *in vivo*

In order to further study the role of miR-9-5p in lung cancer *in vivo*, we inoculated immunocompromised mice (MSG strain) with A549 and H1299 control and miR-9-5p overexpressing cells to grow as tumour xenografts. Tumour growth was observed by measuring the tumour size over a period of 4-6 weeks, and tumours were weighed after mice were euthanised. A549 and H1299 miR-9-5p overexpressing cells resulted in significantly larger tumours than their corresponding control counterparts, confirming miR-9-5p as a tumour promoter in NSCLC *in vivo* (Figure 3.11).

To confirm that miR-9-5p expression persisted in the tumours, total RNA was isolated from tumours and qPCR showed that miR-9-5p was overexpressed in tumours resulting from miR-9-5p overexpressing cell lines (Figure 3.12). Therefore, miR-9-5p acts as a tumour promoter in NSCLC *in vivo*.

Other studies have demonstrated the tumour promoting role of miR-9-5p in an *in vivo* setting in different cancer types. In a mouse glioma model, miR-9-5p was shown to promote growth and angiogenesis by targeting COL18A1, THBS2, PTCH1 and PHD3 (300). In breast cancer, miR-9-

5p promoted growth, angiogenesis and enhanced formation of micrometastases in the lungs by targeting E-Cadherin and increasing circulating VEGFA levels (133). In Hodgkin's Lymphoma, miR-9-5p ablation resulted in decreased tumour growth through targeting HuR and DICER1, lending evidence to miR-9-5p being a tumour promoter in different cancer types (136).

4.6. Conclusions

The aim of this project was to study the miRNA profile regulated by the non-canonical NF-κB kinase, IKKα, and the elucidation of their mechanism of action in lung cancer. Our results demonstrate that IKKα negatively regulates the expression of miR-9-5p, a tumour promoting miRNA. We showed that miR-9-5p promotes EMT by targeting E-Cadherin in A549 cells, causing the activation of the Akt1/GSK-3β/β-catenin pathway, increased cell invasion *in vitro* and tumour growth *in vivo*. These results add to the body of literature, showcasing the intricate molecular mechanisms of EMT activation in cancer, and offer new perspectives on therapy. Overexpression of miR-9-5p in H1299 cells resulted in the formation of significantly larger tumours in mouse xenografts. However, the H1299 cell line is characterised by the lack of E-Cadherin expression, therefore, miR-9-5p would not target *CDH1* in this cell line. Moreover, the H1299 miR-9-5p overexpressing cells did not show any changes in protein levels of Akt1/GSK-3β/β-catenin. This indicates that miR-9-5p acts as a tumour promoter using a different, as yet uncharacterised, mechanism in this cell line, highlighting the heterogeneity of the disease. As miRNAs have multiple targets in different tissues, further research is needed to elucidate the precise mechanism of miR-9-5p in lung cancer.

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