



**ANALYSIS OF THE EFFECTS OF MICRORNA 130A
SUPPRESSION ON A549 CELL LINE**

BY

NABIL IZZAATIE BINTI MOHAMAD SAFIAI

**DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF
SCIENCE (MEDICAL RESEARCH)**

**ADVANCED MEDICAL AND DENTAL INSTITUTE
(AMDI)
UNIVERSITI SAINS MALAYSIA
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DECLARATION

I hereby declare that this research has been sent to Universiti Sains Malaysia for the degree of Master of Science in Medical Research. It is not to be sent to any other universities. With that, this research might be used for consultation and can be photocopied as reference.



Nabil Izzaatie binti Mohamad Safiai

P-IPM 0044/17

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TABLE OF CONTENTS

	Page
Acknowledgements	i
Table of Contents	iii
List of Figures	v
Selected List of Abbreviations	vi
Selected List of Symbols	viii
CHAPTER 1 INTRODUCTION	1
1.1 INTRODUCTION	1
1.2 PROBLEM STATEMENT	3
1.3 HYPOTHESIS	4
1.4 OBJECTIVES	4
1.5 LITERATURE REVIEW	5
1.5.1 LUNG CANCER	6
1.5.2 CURRENT MODALITIES OF LUNG CANCER TREATMENT	6
1.5.3 PERSONALISED MEDICINE FOR CANCER TREATMENT	7

1.5.4	MICRORNAS AS POTENTIAL TARGET FOR CANCER THERAPY	8
1.5.4 (a)	Biogenesis of miRNA	9
1.5.4 (b)	miR-130a in Cancer	12
CHAPTER 2	METHODOLOGY	14
2.1	CELL LINES AND CULTURE	14
2.2	SELECTION OF POST-TRANSFECTED CELLS	15
2.3	A549 GROWTH PROLIFERATION ASSAY	15
2.4	ATTACHMENT AND DETACHMENT ASSAY	15
2.5	SCRATCH WOUND HEALING ASSAY	16
2.6	STATISTICAL ANALYSES	17
CHAPTER 3	RESULTS	18
3.1	MORPHOLOGY OF A549 CELLS	18
3.2	A549 GROWTH PROLIFERATION ASSAY	26
3.3	ATTACHMENT AND DETACHMENT ASSAY	28
3.4	SCRATCH WOUND HEALING ASSAY	32
CHAPTER 4	DISCUSSION	36

CHAPTER 5 CONCLUSION AND FUTURE RECOMMENDATIONS	40
5.1 CONCLUSION	40
5.2 RECOMMENDATIONS FOR FUTURE RESEARCH	40
REFERENCES	42

LIST OF FIGURES

Page	
Figure 1.0: Structure of miR-130a shows three different regions of 5p, 3p and stem loop of where miR-130a was targeted.....	3
Figure 1.1: Schematic diagram of miRNA biogenesis.....	11
Figure 3.0: Morphology of A549-CT cells.	19
Figure 3.1: Morphology of A549-3p cells	21
Figure 3.2: Morphology of A549-5p cells	23
Figure 3.3: Morphology of A549-SL cells.....	25
Figure 3.4: Down-expression of miR-130a in A549 cells resulted in significant growth of A549-3p cells, A549-5p cells and A549-SL cells.	27
Figure 3.5: Cell attachment assay showed that only A549-3p cells have significant result.	29
Figure 3.6: All A549 cells showed significant result for cell detachment assay.....	31
Figure 3.7: A549 cells migration assessed by scratch wound healing assay.	33
Figure 3.8: Percentage of cell closure indicate the rate of cell migration.	35

SELECTED LIST OF ABBREVIATIONS

<i>ALK</i> gene	-	Anaplastic Lymphoma Kinase gene
CAR-T	-	Chimeric Antigen Receptor T cell
CCAT1	-	Colon cancer-associated transcript-1
CRISPR-Cas9	-	Clustered Regularly Interspaced Short Palindromic Repeats-Cas9
DGCR8	-	Digeorge Syndrome Critical Region Gene 8
DMSO	-	Dimethyl sulfoxide
<i>EGFR</i> gene	-	Epidermal Growth Factor Receptor gene
EMT	-	Epithelial-Mesenchymal Transition
FBS	-	Fetal bovine serum
FOSL1	-	FOS-like Antigen 1
HCC	-	Hepatocellular carcinoma
HER2	-	Human Epidermal Growth Factor Receptor 2
<i>MET</i> gene	-	Mesenchymal-Epithelial Transition gene
MiRNA	-	MicroRNA
MiR-130a	-	MicroRNA-130a

NPC	-	Nasopharyngeal cancer
NSCLC	-	Non-small Cell Lung Cancer
PBS	-	Potassium buffered solution
RAN-GTP	-	RAN-guanosine triphosphate
RISC	-	RNA-induced silencing complex
TRAIL	-	TNF-related apoptosis inducing ligand
TRBP	-	TAR RNA Binding Protein
UTR	-	Untranslated region
ZO-1	-	Zonula Occludens-1

SELECTED LIST OF SYMBOLS

°C	-	Degree Celsius
H	-	Hour
%	-	Percentage
μm	-	Micrometre
μl	-	Microlitre
μg/ml	-	Microgram per millilitre

ANALYSIS OF THE EFFECTS OF MICRORNA 130A SUPPRESSION ON A549 CELL LINE

ABSTRAK

MikroRNA (miRNAs) mengawal proses biologi sel dan sangat penting dalam mengatur proses percambahan, pembezaan dan kematian sel. MiRNA juga sangat kritikal dalam pelbagai jenis kanser dan penyakit seperti leukemia limfosit kronik, B sel limfoma, kanser paru-paru dan jangkitan virus. Banyak hasil kajian telah menunjukkan bahawa miRNA-130a (miR-130a) dari keluarga miRNA pengawal bukan mengkod-protein memainkan peranan penting dalam pertumbuhan dan metastasis kanser paru-paru. Namun begitu, ilmu mengenai kesan pengekangan miRNA yang disasarkan pada kawasan-kawasan sasaran yang berbeza di dalam gen miR-130a di dalam sel kanser paru-paru masih terlalu sedikit. Dalam kajian ini, proses pengekangan miR-130a pada kawasan 3p, 5p dan pangkal yang bergelung (stem loop) telah ditentukan di dalam A549 titisan sel karsinoma paru-paru manusia. Ujian percambahan dan ujian migrasi telah dilaksanakan untuk mengkaji proses pertumbuhan dan kebolehan metastasis bagi titisan sel A549. Keputusan kami menunjukkan bahawa pengekangan pada miR-130a-3p meningkatkan proses pertumbuhan titisan sel A549 secara paling ketara. Apabila dibandingkan dengan proses pengekangan pada kawasan sasaran yang lain, hanya pengekangan pada 3p memberikan keputusan secara konsisten dalam ujian pengikatan sel dan penyisihan sel dan ujian penyembuhan luka sel. Kesimpulannya, hasil kajian menunjukkan bahawa miR-130a-3p memainkan peranan kritikal dalam proses perkembangan dan metastasis kanser paru-paru. Justeru itu, miR-130a-3p berkemungkinan besar menjadi sasaran yang lebih baik dengan potensi yang sangat besar

dalam bidang diagnostik dan ilmu terapi, dan akan menjadi rujukan dasar yang penting bagi kajian masa hadapan terutamanya dalam rawatan kanser paru-paru.

ANALYSIS OF THE EFFECTS OF MICRORNA 130A SUPPRESSION ON A549 CELL LINE

ABSTRACT

MicroRNAs (miRNAs) control the biological processes of cells and are crucial in regulation of proliferation, differentiation and apoptosis. MiRNAs are also critical in regulation of various types of cancer and diseases such as in chronic lymphocytic leukemia, B cell lymphoma, lung cancer and viral infections. In recent years, a lot of evidence arises demonstrating that miRNA-130a, from the family of regulatory non-protein coding miRNAs play important role in development and metastasis of lung cancer. However, there is still scarce knowledge about the effect of miR-130a suppression targeting at different miR-130a gene regions in lung cancer cells. In this study, suppression of miR-130a at 3p, 5p and stem loop region was established in the A549 human lung carcinoma cell line. Proliferation and migration assays were performed to study the role of miR-130a in the growth and metastatic ability of A549 cell line. Our result demonstrated that miR-130a-3p suppression significantly increased the growth of A549 cell line. Consistently, among different sites of miR-130a downregulation, only suppression at 3p showed significant increase in cell attachment and detachment assay and scratch wound healing assay. As a conclusion, our findings suggest that miR-130a-3p may plays critical role in the development and metastasis of lung cancer. Hence, miR-130a-3p might be a better target with huge potential for diagnostic and therapeutic approaches and may serve as an important fundamental reference for future research especially for the treatment of lung cancer.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

According to WHO (2017), a total of 1.69 million death was reported due to lung cancer in 2015. Steward and Wild (2014) reported that 51% of lung cancer cases come from Asian continent. Lung cancer is undeniably listed as the top leading cause of cancer deaths (IARC, 2012).

It is estimated that the adult 5-year net survival is 9.5% and it become much lower for ten-year net survival with 4.9%. Young age and early stage are good prognostic factors that gives better chance of survival (C. R. UK, 2011). Among different types of lung cancer, 80% was attributed by non-small cell lung cancer (NSCLC) with its major histological types, squamous cell lung cancer and adenocarcinoma (Ohno *et al.*, 2015).

Currently, many modalities of lung cancer treatment has emerged, from conventional therapies such as surgery, chemotherapy and radiotherapy to targeted therapies and personalised medicine. Surgical resection is commonly performed for operable tumour of NSCLC. Unfortunately, not all tumours are resectable, therefore, chemotherapy and radiotherapy become the mainstay treatment of non-resectable tumours of NSCLC. Regrettably, chemotherapy and radiotherapy give many harmful side effects and are intolerable to some patients. Furthermore, the major hindrances to effective treatment include metastatic cancer and relapse of the cancer (Wei *et al.*, 2015). Hence, it is important to find new alternative treatment for lung cancer.

Targeted therapy is available for patient with *ALK* and *EGFR* gene mutation, however its role is not properly evaluated for stage I, II and III (Postmus *et al.*, 2017). Gene therapy is another form of personalised medicine and is regarded as a targeted therapy. The first gene therapy approved by FDA in 2017 is CAR-T immunotherapy. It could be used for treatment of acute lymphoblastic leukemia and Non-Hodgkin lymphoma. CRISPR-Cas9 has emerged over the past few years as a promising gene editing tool for gene therapy (Fan, 2018). MiRNAs could be one of the targets for treatment of cancer.

MicroRNAs (miRNAs) are 17-23 nucleotides of non-protein coding RNAs that play an important role in post-transcriptional modification (Bartel, 2004). In 1993, Lee *et al.*, (1993) discovered lin-4 which is a stem-loop miRNA inside *Caenorhabditis elegans* can negatively regulate LIN-14 protein expression level. MiRNAs are found to be the important regulator in development, differentiation and apoptosis of cells (Kloosterman and Plasterk, 2006; Bushati and Cohen, 2007).

In a recent study, Chakraborty *et al.*, (2018) reported that miRNAs had been used for cancer treatment, and that include miRNAs replacement therapy, miRNAs inhibition therapy and miRNAs combination therapy. The study also reported that miRNAs replacement therapy in particular, had entered clinical trial phase I for treatment of hepatocellular carcinoma (HCC). In the clinical trial phase I, MRX34 which is a liposome-based miR-34a mimic, can be delivered to patients with HCC through intravenous route. In another study, Zhang *et al.*, (2017) showed that co-delivery of docetaxel with miR-34a for treatment of metastatic breast cancer give synergistic therapeutic effect.

The important role of miRNAs and recent discovery gives a glimpse about the potential role of miRNAs for therapeutic purposes. Hence, it is urgent to fully understand and investigate the function of miRNAs for future therapeutic application.

In this study, we aim to investigate the effects of targeting miR-130a gene at 3p, 5p and stem loop region. The three different regions of miR-130a are shown in Figure 1.0.

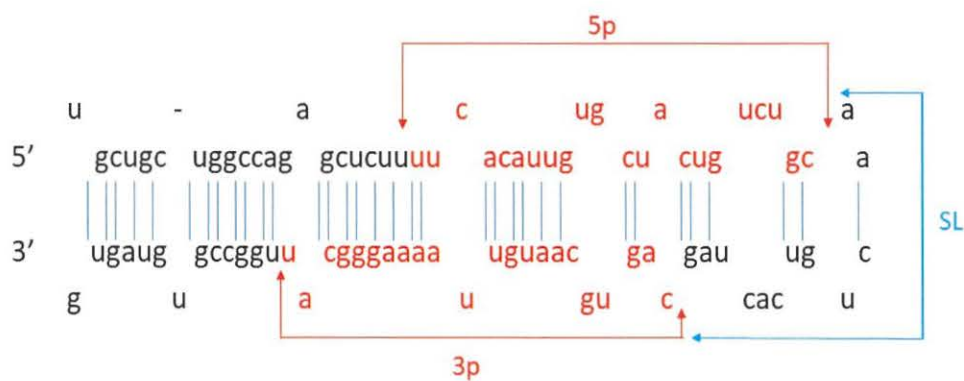


Figure 1.0: Structure of miR-130a shows three different regions of 5p, 3p and stem loop of where miR-130a was targeted.

1.2 PROBLEM STATEMENT

In lung cancer, Croce (2009) found that miR-130a is expressed at low level. In UK, lung cancer survival only change from 3% to 5% over the last 40 year (C.R. UK, 2011). Despite advancement and emergence of many therapeutic modalities, the prognosis of lung cancer remain dismal. Personalised medicine such as gene therapy might give a new hope to solve this problem.

MiR-130a is highly regulated in lung cancer and may serve as potential target for therapy. Although several studies had demonstrated the effect of miR-130a suppression in lung cancer, there is still scarce knowledge regarding modification of miR-130a in lung cancer. MiR-130a may be suppressed at different sites in its sequential structure and this condition might affect proliferation and migration of lung cancer. Therefore, further investigation is needed to study the effect of miR-130a suppression on the proliferation and migration of lung cancer cells as a foundation for better therapeutic purposes.

1.3 HYPOTHESIS

Suppression of miR-130a level targeted at 3', 5' and stem loop region will increase proliferative and migratory ability of A549 cells.

1.4 OBJECTIVES

The objectives of the present study were as follow:

- 1) To investigate the effect of miR-130a suppression on cell proliferation of A549 cell line.
- 2) To determine the effect of miR-130a suppression on migratory ability of A549 cells.

1.5 LITERATURE REVIEW

Cancer cells acquire distinctive attributes that differentiate themselves from normal cells. They achieve unlimited replicative ability and self-sufficiency in proliferative signaling, able to hide from immune surveillance, sustained their own growth signaling and angiogenesis, insensitive to growth suppressor, able to modify its metabolic activity and prompt invasion and metastasis (Hanahan and Weinberg, 2011).

Cancer cells also acquire limitless replication and able to maintain their continuous proliferative activity. These cells are capable to locally invade the area of the primary site and metastasize to another distant sites. Epithelial-mesenchymal transition (EMT) is the programme that enable the cancer cells to gain its invasiveness and migratory ability. EMT was first discovered in embryogenesis around 1980s and later was reported to be involved in cancer progression and metastasis (Hanahan and Weinberg, 2011).

Metastasis is a process in which the cancer cells disseminate from a primary site to distant part in the body. Detachment from the primary tumour is the first step to initiate the process. Detached neoplastic cells will attach and invade another adjacent area or intravasate the blood vessels to propagate to another distant organ through blood circulation (Wei and Yang, 2016; Gkretsi and Stylianopoulos, 2018).

Migration assays are available to examine the migratory ability of the cells. Among the most common migration assays are scratch wound healing assay, stopper-based assay and transwell assay. However, scratch wound healing assay might be a better choice due to its simple set-up and its cost-effectiveness. Furthermore, it allow adjustment to eliminate confounding factor from proliferation activity. The assay is also able to imitate

in vivo environment due release of cytokines from the wounded area (Goetsch and Niesler, 2011).

1.5.1 LUNG CANCER

Primary lung cancer could be divided into two main types which are small cell lung cancer and non-small cell lung cancer. In UK, only 12 % of lung cancer diagnosed would have small cell lung cancer type, and the majority of lung cancer diagnosed are non-small cell lung cancer. Based on histological classification, there are three different types of lung cancer which are adenocarcinoma, squamous cell lung cancer and large cell carcinoma. However, if the cells are undeveloped and pathologist could not tell which type it belongs to, it could be classified as undifferentiated NSCLC (C. R. UK, 2017).

Doctors usually will decide the suitable treatment for patient with lung cancer depending on the staging of the cancer. The most common staging used is TNM staging, which refer to T-Tumour, N-Nodes and M-Metastasis. Staging is categorized from stage one to stage four (C. R. UK, 2017).

1.5.2 CURRENT MODALITIES OF LUNG CANCER TREATMENT

The new 2011 NICE Clinical Guidelines suggested to advise lung cancer patient on smoking cessation as smoking will increase risk of complication after surgery. Patient should be encouraged to stop smoking once the diagnosis of lung cancer is suspected. Moreover, the guideline suggested that any medically fit patients who are suitable for treatment with curative intent should be offered surgical resection as their first choice treatment.

Patients with stage I, II and III who has good performance status are indicated for treatment with radical radiotherapy. Meanwhile, patient with inoperable tumour of stage II and III NSCLC should be considered for chemotherapy. For non-curative intent treatment, NSCLC patients with stage III and IV with good performance status should be offered chemotherapy for the aim of improving survival, controlling the disease progression and acquiring better quality of life. Besides, the chemotherapy for advanced NSCLC should be a combination of platinum drug with any single third generation drug such as docetaxel, gemcitabine, paclitaxel and vinorelbine (NICE), 2011).

1.5.3 PERSONALISED MEDICINE FOR CANCER TREATMENT

Personalised medicine is a term use for tailoring clinical characteristic of patients with suitable treatment so that they respond well to the treatment and get benefit from the treatment. Personalised medicine has emerged as an alternative approach in patient care and become particularly crucial in oncology due to critical selection of treatment with aim to minimise short-term toxicities from surgical and radiotherapies, and to maximise the efficacy of treatment and long-term function (Sarah E. Jackson and Chester, 2014).

The term personalised medicine is also use interchangeably with 'precision medicine', 'genomic medicine' and 'precision oncology' (Ciardiello *et al.*, 2014). An excellent example of the iconic usage of targeted therapy is trastuzumab in breast cancer. The recombinant monoclonal antibody targeted HER2 in patient with metastatic breast cancer who overexpress the growth factor receptor, HER2. The amplified gene encoded for the overexpression of HER2 cause the poor prognosis of the metastatic breast cancer,

however, it also predicts the better response with treatment using trastuzumab in combination with chemotherapy (Slamon *et al.*, 2001).

Another example of targeted therapy is cetuximab in colorectal cancer. Cetuximab is a human monoclonal antibody targeting Epidermal Growth Factor Receptor (EGFR). Cetuximab has shown beneficial effects in treating colorectal cancer patients who express wild-type KRAS status (Sarah E. Jackson and Chester, 2014). Meanwhile in NSCLC, Tyrosine Kinase Inhibitor such as erlotinib, afatinib and gefitinib will target mutant Epidermal Growth Factor Receptor. Patients who received Tyrosine Kinase Inhibitor as their first line treatment in NSCLC were found to have higher overall response rate (ORR), progression free survival (PFS) and better quality of life compared to patients who received chemotherapy (Chan and Hughes, 2014).

1.5.4 MICRORNAS AS POTENTIAL TARGET FOR CANCER THERAPY

MiRNAs are short stranded RNAs that participate in various biological activities such as regulation of apoptosis, cell differentiation, cell proliferation, cell migration and others (Bartel, 2004). Several studies have shown that miRNAs play crucial role in the development of carcinogenesis in cancer patients.

Huang *et al.*, (2017) has discovered six miRNAs were upregulated in gastric cancer and played important role in the progression of the cancer. These microRNAs are miR-10b-5p, miR-132-3p, miR-185-5p, miR-195-5p, miR-20a-3p and miR-296-5p could be potential biomarkers for gastric cancer. In colorectal cancer, miR-19a-3p, miR-21-5p and miR-425-5p were found expressed at high levels in the serum of the patients. Those

miRNAs were important in tumorigenesis and proliferation of colorectal cancer (Zhu *et al.*, 2017).

MiRNAs was also found to be associated with EMT. Histologically, cancer cells usually lost its epithelial shape and acquired more spindle shape looking like a mesenchymal cell. Fortunately, epithelial cells that underwent mesenchymal transition can be reverted to their former state through reversal programme, the mesenchymal-epithelial transition (MET) programme (Song and Li, 2016; Yang *et al.*, 2015; Ma *et al.*, 2015).

Interestingly, in Entrez gene summary and CIViC summary, it is stated that mesenchymal-epithelial transition (*MET*) proto-oncogene encoded for the receptor of tyrosine kinase receptor family. This product of *MET* gene is also known as MET protein receptor for hepatocyte growth factor ligand.

1.5.4 (a) BIOGENESIS OF MICRORNA

As shown in Figure 1.1, RNA polymerases II and III transcribe miRNAs from intergenic region of introns and exons into primary miRNA (pri-miRNA) inside the nucleus (Ambros, 2001; Lagos-Quintana *et al.*, 2001; Lagos-Quintana *et al.*, 2003; Lim *et al.*, 2003). Pri-miRNA consist of nucleotides ranging from two hundreds to thousands in length and comprises of a stem loop structure. Pri-miRNA has 3' polyadenylated tail and its 5' region capped by the promoter element (Cai *et al.*, 2004). In human, Drosha which is a member of RNase III with the help of its co-factor Human Digeorge Syndrome Critical Region Gene 8 (DGCR8), will cleave the pri-miRNA and transform it into precursor miRNA (pre-miRNA) (Lee *et al.*, 2003; Denli *et al.*, 2004; Landthaler *et al.*, 2004).

The newly formed pre-miRNA compose of approximately 70 nucleotides are remain in its stem loop structure and has acquired new characteristic of having two 3'-nucleotides overhang (Lee *et al.*, 2003). Next, exportin-5 (Exp5) with the presence of its co-factor Ran-GTP which is a Ras-related nuclear protein (RAN) bounded to guanosine triphosphate (GTP) bind to 3'-nucleotides overhang of the pre-miRNA and exported it to cytoplasm (Lund *et al.*, 2004; Yi *et al.*, 2003).

Once in cytoplasm, Dicer-TRBP complex cleave pre-miRNA to produce miRNA duplex of around 17-23 nucleotides. Following the cleavage, Argonaute 2 (Ago2), Dicer and TRBP form a trimeric complex called RNA-induced silencing complex (RISC) of where the RISC complex bind to one of the miRNA duplex to form mature miRNA (Chendrimada *et al.*, 2005; Hammond *et al.*, 2000; Jiang *et al.*, 2005; Gregory *et al.*, 2005; Maniataki and Mourelatos, 2005).

The mature miRNA will regulate gene expression through its binding to 3'UTR of target mRNA and control either translational repression, deadenylation or mRNA target cleavage. Meanwhile, the other miRNA duplex which is not incorporated into RISC is degraded. (Chendrimada *et al.*, 2005; Hammond *et al.*, 2000; Jiang *et al.*, 2005; Gregory *et al.*, 2005; Maniataki and Mourelatos, 2005; Winter *et al.*, 2009).

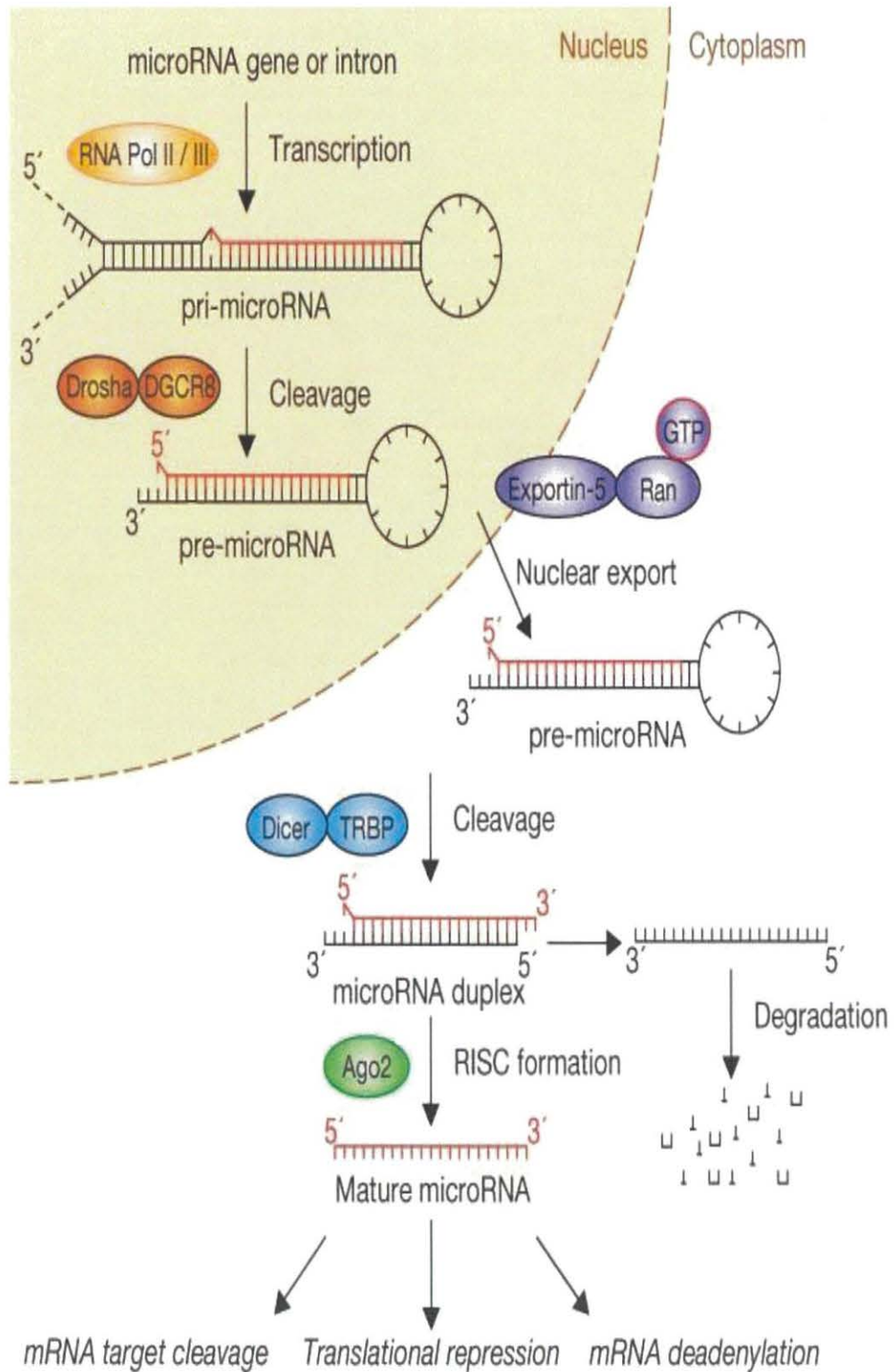


Figure 1.1: Schematic diagram of miRNA biogenesis. Two-step processing pathway produce a mature miRNA. Taken from (Winter *et al.*, 2009).

1.5.4 (b) MIR-130A IN CANCER

MiR-130a in particular, had been studied numerous times and could either promote or prevent cancer growth in different types of tumours depending on its level of expression. In a study, Ramalho-Carvalho *et al.*, (2017) showed that miR-130a was expressed at low level in prostate cancer and restoration of miR-130a level reduced the malignant phenotype of the cancer.

In addition, Chen *et al.*, (2018) has discovered that miR-130a has low level of expression in breast cancer patients. Further experiment by rescuing the downregulated miR-130a inhibited the invasion and migration of invasive breast cancer. The study found that the mechanism is through miR-130a targeting the FOSL1 mRNA at its 3'-UTR and eventually reduce the inhibition of tight junction protein ZO-1 by FOSL1.

In lung cancer, Wenqiang *et al.*, (2017) found that miR-130a expression was reduced and it acts as a tumour suppressor by downregulating TGF- α . It has been reported that, oncogene CCAT1 knockdown the miR-130a leading to SOX4 upregulation. This interaction contributed to cisplatin resistant of non-small cell lung cancer (Hu *et al.*, 2017).

On top of that, miR-130a was also associated with MET programme. In NSCLC, *MET* proto-oncogene was found to be regulated by miR-130a through modulation of miR-221 and miR-222. In a study done by Acunzo *et al.*, (2012), miR-130a downregulate *MET* gene expression and eventually increase TNF-related apoptosis inducing ligand (TRAIL) sensitivity in NSCLC. In another study, Xin Chen *et al.*, (2017) discovered that miR-130a-3p inhibited the invasive and migratory ability of nasopharyngeal carcinoma (NPC) cells and might involve in EMT process. Meanwhile, Shanshan Wang *et al.*,

(2017) found that upregulation of miR-130a-3p suppressed invasion, migration and MET process in gastric cancer cells. Further experiment *in vivo* using modified chick embryo chorioallantoic membrane (CAM) showed that miR-130a-3p also inhibited tumour growth and reduced metastatic lung lesions.

CHAPTER 2 METHODOLOGY

2.1 CELL LINES AND CELL CULTURE

The A549 cells with miR-130a suppression were established using CRISPR-Cas9 system by Ainina et al. (2018) (manuscript in preparation). The parent untransfected A549 cells were transfected with CRISPR-Cas9 plasmid with gRNA designed to target at either 3p, 5p or stem loop region of miR-130a to produce A549-3p, A549-5p and A549-SL respectively. As a control, the A549 cells were transfected with empty plasmid to produce A549-CT. The plasmids transfected to the cells using Lipofectamine and successfully transfected cells were selected by puromycin. The stably transfected cells were cultured in RPMI 1640 media enriched with L -glutamine (Gibco, Life Technologies), 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Nacalai Tesque)(Ainina *et al.*, 2018) . Cells were placed in an incubator at 37°C in 5% carbon dioxide . After the cells reached confluency, the cells were collected and stored in 10% dimethyl sulfoxide (DMSO) at -80°C until further use. The suppression level of miR-130a-3p and miR-130a-5p were measured previously using qRT-PCR. Result showed that the A549-3p cells exhibited low level of miR-130a-3p, while A549-SL showed low level of miR-130a-5p. However, no significant difference in the level of miR-130a was observed in the A549-5p cells (Ainina *et al.*, 2018).

2.2 SELECTION OF POST-TRANSFECTED CELLS

A549-CT, A549-3p, A549-5p and A549-SL were thawed from the frozen stock and cultured in initial passages of between 11 to 15. The cells were cultured in complete RPMI 1640 media for 3 to 7 days. Following that, the media was removed and cells were washed with potassium buffered solution (PBS). Cells were then continued to grow in fresh complete RPMI 1640 media with an addition of 0.75µg/ml puromycin for three days to allow the selection of stably transfected cells. After 72 hours, the media was removed and cells were washed with PBS. The cells were cultured into confluency in a complete media again. The puromycin selection procedure was done at different interval following few passages to eliminate the growth of untransfected cells.

2.3 A549 GROWTH PROLIFERATION ASSAY

A549-CT, A549-3p, A549-5p and A549-SL were seeded into 24 well plates at 2.5×10^4 cells per well and were maintained in complete RPMI 1640 media. Without changing the media, the cells were allowed to grow and harvested at 24-hour interval until day 5. Harvested cells were trypsinized and counted manually using trypan blue and haemocytometer. Live cells were counted under 10x objective (Axiovert 40 CFL). Data for the cell growth was presented in percentage increment in bar graph with 100% at initial 0 hour. The experiments were performed in three independent biological assays.

2.4 ATTACHMENT AND DETACHMENT ASSAY

The attachment and detachment assays were performed as described previously by Liu et al. (2016). In attachment assay, 5×10^4 cells were plated into 24 well-plates and

incubated for 1 hour. After an hour, the unattached cells were collected and discarded. The attached cells were incubated with 0.05% trypsin for approximately 10 minutes until cells were detached. Once detached, cells were counted under the inverted light microscope. Results were displayed as a percentage of the attached cells over total cells. Equation used for attachment assay was as follow:

$$\frac{\text{No. of cells counted after trypsinization}}{\text{No. of cells seeded } (5 \times 10^4)} \times 100 = \% \text{ attached cells}$$

For detachment assay, cells were seeded and incubated for 24 hours. Then, the media was discarded and the cells were incubated with 0.05% trypsin for 3 minutes. The culture media was added after 3 minutes to inactivate the action of trypsin. The detached cells were counted manually. Next, the remaining cells were incubated with 0.25% trypsin to detach the remaining cells. Once detached, the cells were counted. The results were displayed as a percentage of detached cells over total cells. Equation used for detachment assay was as follow:

$$\frac{\text{No. of cells detached by 0.05\% trypsin}}{\text{No. of cells detached by (0.05\% + 0.25\%) trypsin}} \times 100 = \% \text{ detached cells}$$

All experiments were performed using three independent biological assays in triplicate.

2.5 SCRATCH WOUND HEALING ASSAY

The A549 cells were plated into 24-well plates at a concentration of between 2×10^5 to 2.5×10^5 and were allowed to proliferate overnight to reach 97-100% confluency. Then the cells were incubated with RPMI media supplemented with only 1% FBS. A 200 μ l

pipette tip was used to scratch the confluence monolayer. The well was washed for three times to remove floating cells. Then, the cells were incubated to allow migration into the acellular area. Cells were photographed at 0, 24, 48 and 72-hour using light microscope (Olympus CKX41). Images taken were analysed by tracing the border of the wounded area using Image J software (Image J 1.52b version). The results were displayed as a percentage of wound closure. The equation used was

$$[(A_{t=0h} - A_{t=\Delta h}) / A_{t=0h}] \times 100 = \% \text{ of wound closure}]$$

where, $A_{t=0h}$ is the initial area of the gap measured immediately after scratching, and $A_{t=\Delta h}$ is the area of wound measured at either 24, 48 or 72 hours after scratching (Yue *et al.*, 2010; Goetsch and Niesler, 2011).

2.6 STATISTICAL ANALYSES

Data were analysed using student's t-test. P-value ≤ 0.05 was considered as significant. Data were shown as mean $\pm$ standard deviation (SD). All analyses were done using Microsoft Excel Sheet (Version 1805).

CHAPTER 3

RESULTS

3.1 MORPHOLOGY OF A549 CELLS

During culturing A549 cells, no morphological changes were observed between A549-CT cells, A549-5p cells and A549-SL cells. This morphological trait was maintained upon proliferation of these cells in different passages. A549-3p cells were observed to have more fusiform shape with lack of cytoplasm compared to other A549 cells. The cells were also noted to have very refined edges.

A549-CT was transfected with empty plasmid and acts as a control cell (Figure 3.0). A549-CT cells were maintained at passage 21. Figure 3.0 A, B and C showed the morphology of A549-CT cells when viewed under 10x objective at 24h, 48h and 96h respectively. Meanwhile, Figure 3.0 D and E showed morphology of A549-CT taken under 20x objective at 48h and 96 h respectively. The A549-CT cells were observed to have elongated fibroblastic-like morphology.

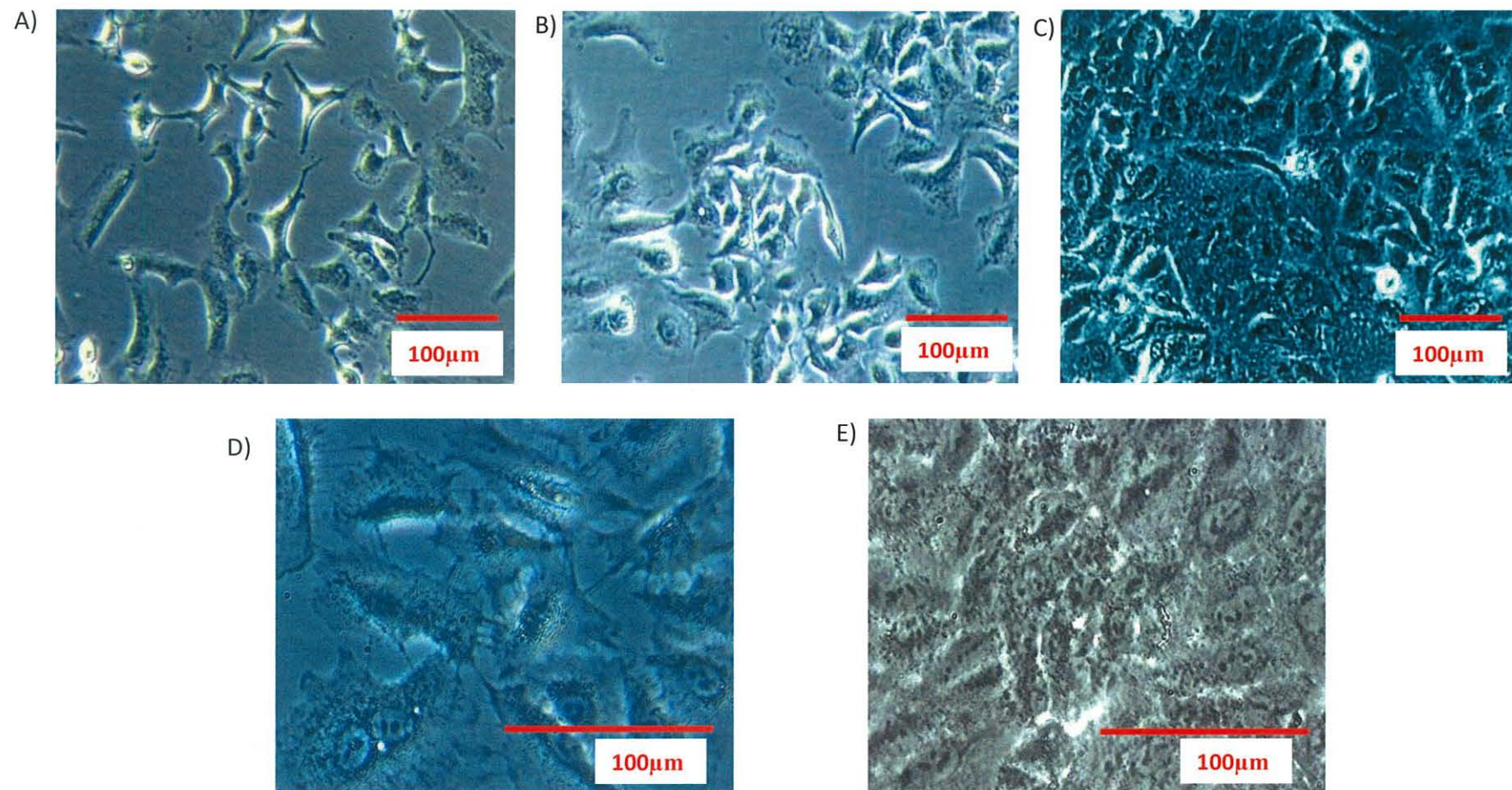


Figure 3.0: Morphology of A549-CT cells. Cells are viewed at A) 24h B) 48h C) 96h under 10x objective. Images are taken under 20x objective at D) 48h and E) 96h.

A549-3p cells which were transfected with suppressed miR-130a-3p were photographed and shown in Figure 3.1. The A549-3p cells were maintained at passage 21. Figure 3.1 A, B and C were photographed at 24h, 48h and 96h respectively under 10x objective, meanwhile Figure 3.1 D and E were a closed-up view at 20x objective photographed at 48h and 96h respectively. The A549-3p cells were noted to have more elliptical shape with less cytoplasm compared to control cells (A549-CT) and its sibling cells, A549-5p and A549-SL.

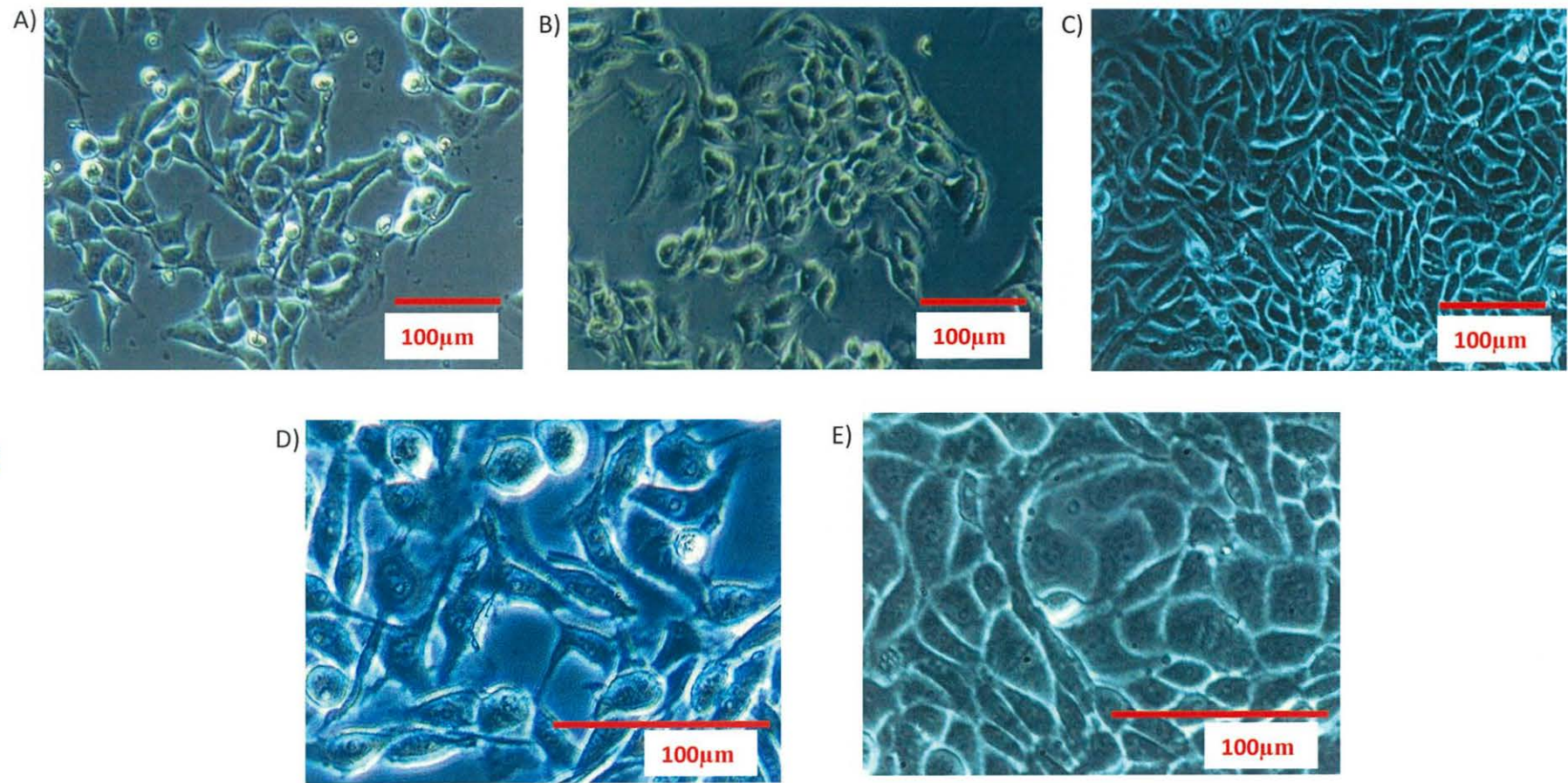


Figure 3.1: Morphology of A549-3p cells. Cells are viewed at A) 24h B) 48h C) 96h under 10x objective. Images are taken under 20x objective at D) 48h and E) 96h.

A549-5p cells were maintained at passage 22. The cells morphology was shown in Figure 3.2. Figure 3.2 A, B and C shown were taken at 24h, 48h and 96h respectively under 10x microscopic view. Figure 3.2 D and E showed A549-5p cells under 20x microscopic view at 48h and 96 h respectively. The A549-5p cells were found to have spindle fibroblastic-like shape morphology just like A549-CT cells. There is almost no different in the histological shape between A549-5p, A549-SL and A549-CT.

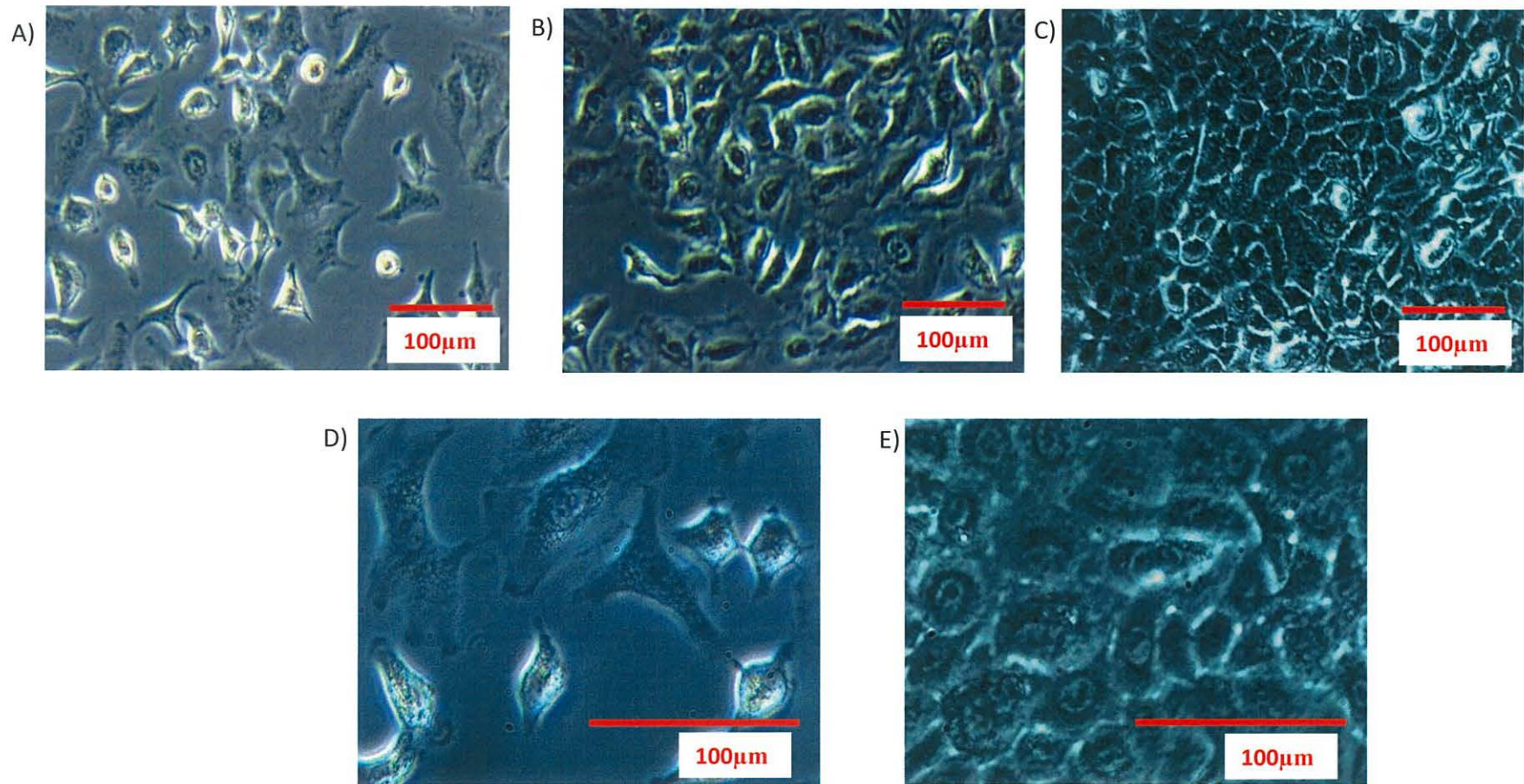


Figure 3.2: Morphology of A549-5p cells. Cells are viewed at A) 24h B) 48h C) 96h under 10x objective. Images are taken under 20x objective at D) 48h and E) 96h.

Figure 3.3 showed A549-SL cells with suppressed miR-130a inserted at stem loop region. Figure 3.3 A, B and C were viewed under 10x objective at 24h, 48h and 96h respectively. Meanwhile, Figure 3.3 D and E were taken under 20x objective at 48h and 96h respectively. The A549-SL cells were maintained at passage 17 and were noted to have fusiform shape morphology. There is almost no morphological changes between A549-SL, A549-5p and A549-CT.