THE ROLE OF MYOFERLIN AND DYSFERLIN IN LUNG EPITHELIAL CELL MEMBRANE FUNCTIONS AND LUNG CANCER

by

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Abstract

Lung cancer is the deadliest cancer in the world. Almost all lung cancers originate from epithelial tissues within the lung. Lung epithelium provides a barrier to inhaled particulate matter and pathogens; hence, maintaining the normal epithelial integrity is important in defending against these foreign substances. Maintenance of lung epithelial integrity involves the collaboration of transmembrane and cytoplasmic proteins to form cellular junctions. Delivery of these junctional proteins to the cell membrane requires vesicle trafficking and membrane fusion events. Additionally, in the setting of lung tumors, vesicle trafficking and membrane fusion are also important in membrane remodeling and cell survival to facilitate tumor growth. Myoferlin and dysferlin are two transmembrane proteins that contribute to the repair of damaged cell membranes and expression of membrane proteins in multiple cell types. Based on the known functions of myoferlin and dysferlin in non-lung cells, I hypothesized that myoferlin and dysferlin have distinctive roles in normal and neoplastic lung epithelial cells. I provide evidence that attenuated myoferlin expression in human airway epithelial cells causes cell appearance changes, loss of adhesion and ultimately apoptosis. Loss of cell adhesion was associated with decreased expression of junctional protein zonula occludens 1 (ZO-1). Dysferlin gene silencing, on the other hand, did not have any effect on cell appearance, adhesion or ZO-1 expression. The pathophysiological role of myoferlin was further investigated in lung tumor. Myoferlin knockdown decreased both mouse lung tumor cell proliferation *in vitro* and solid lung tumor growth in a mouse tumor xenograft model. Subsequent investigation revealed that myoferlin knockdown impaired the resealing of ruptured membrane. These data have implications for the role of myoferlin in membrane remodeling that is essential for rapidly dividing tumor cells. In human lung tumor

cells, myoferlin knockdown decreased both cell proliferation and invasion and these were attributed to decreased Akt activity. Since Akt activity is frequently hyperactivated during tumor development, these findings further support the possibility for new therapeutic strategies targeted at myoferlin for lung cancer. In summary, my work suggests that myoferlin has important functional roles in both normal and neoplastic lung epithelia.

Preface

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Chapter 5. All figures and text have been published as a research article entitled "Expression of myoferlin in human and murine carcinoma tumors: role in membrane repair, cell proliferation, and tumorigenesis". Leung C, Yu C, Lin MI, Tognon C, and Bernatchez P. *American Journal of Pathology*. 2013 May; 182(5): 1900-1909. Pascal Bernatchez and I designed and performed all the experiments as well as writing and reviewing the manuscript.

All human studies were approved by the Providence Health Care Research Ethics Committee of the University of British Columbia (certificate number H0-50110).

All animal studies were performed in accordance with the guidelines of the University of British Columbia Animal Care Committee (certificate number A07-0782).

Table of Contents

List of Tables

List of Figures

List of Acronyms and Abbreviations

AEI: Alveolar epithelial type I

AEII: Alveolar epithelial type II

AJ: Adherens junction

Akt: Protein kinase B

APAF-1: Apoptotic protease activating factor 1

ATCC: American Type Culture Collection

ATP: Adenosine triphosphate

BAEC: Bovine aortic endothelial cells

BaP: Benzo[a]pyrene

BASC: Bronchioalveolar stem cells

Bcl2: B cell leukemia/lymphoma 2

CaM: Calcium/calmodulin

CDKs: Cyclin-dependent kinases

cIAP-1: Cellular IAP-1

cIAP-2: Cellular IAP-2

cm: Centimeters

COPD: Chronic obstructive pulmonary disease

CYP450: Cytochrome P450

DAPI: 4,6-diamidion-2-phenylindole

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic acid

Dyn-2: Dynamin-2

Dysf: Dysferlin

ECM: Extracellular matrix

EGF: epidermal growth factor

EGFR: Epidermal growth factor receptor

EHD1: Eps15 homology domain containing 1

EHD2: Eps15 homology domain containing 2

EMT: Epithelial-mesenchymal transition

FBS: Fetal bovine serum

G1: Gap 1

G2: Gap 2

h: Hours

HER: human epidermal growth factor receptor

HFL: Human fetal lung fibroblasts

HSP90: Heat shock protein 90

IAPs: Inhibitor of apoptosis proteins

IGF: Insulin growth factor

IGF1R: Insulin-like growth factor 1 receptor

IL-6: Interleukin 6

IL-8: Interleukin 8

JAMs: Junction adhesion molecules

LGMD2B: Limb-girdle muscular dystrophy type 2B

LLC: Lewis lung carcinoma

M: Extent of metastasis

mL: Millilitres

MM: Miyoshi myopathy

MMPs: Matrix metalloproteinases

mRNA: Messenger RNA

Myof: Myoferlin

N: Degree of localized lymph node involved

nM: Nanomolars

NEBs: Neuroendocrine bodies

NF-κB: Nuclear factor-kappa B

NNK: Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

NSCLC: Non-small cell lung cancer

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffer saline

PCNA: Proliferating cell nuclear antigen

PECAM-1: Platelet endothelial cell adhesion molecule 1

PI: Propidium iodide

PI3K: Phosphatidylinositol-3-kinase

pNA: p-nitroaniline

RT: Room temperature

RTK: Receptor tyrosine kinase

S: Scrambled siRNA

SCLC: Small cell lung cancer

SDS: Sodium dodecyl sulfate

siRNA: Small interfering ribonucleic acid

SNAPs: Synaptosome-associated proteins

SNARE: N-ethylmaleinide-sensitive factor attachment protein receptor

STBs: Syncytiotrophoblasts

T: Size of tumors

TGFα: Transforming growth factor alpha

TJ: Tight junction

TKIs: Tyrosine kinase inhibitors

VEGFR-2: Vascular endothelial growth factor receptor 2

XIAP: X-linked IAP

ZO: Zonula occludens

μg: Micrograms

μL: Microlitres

μm: Micrometers

μM: Micromolars

2D: Two dimension

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Dedication

This dissertation is dedicated to:

Dad

Mom

Benny

Candy

Nicolas

Ealing

Haylie

Carson

1. Introduction

1.1. Thesis overview

Current treatments for late-stage lung cancer do not effectively cure or prolong the lifespan of patients for more than a year, making ongoing studies to develop therapeutic targets to control this killer a priority. In addition to the heritable risk of lung cancer, it can occur as a result of repeated exposure to inhaled airborne particulates and gases that result in repeated cycles of injury and repair of the lung epithelium. Maintaining the integrity of normal lung epithelium is, therefore, crucial as it provides the first structural barrier to the inhaled environment. Inhaled toxins not only rupture the epithelial layer of the lung macroscopically, but also the plasma membrane of individual lung epithelial cells microscopically. Damage to the plasma membrane activates repair mechanisms to re-establish homeostasis in lung epithelium. In this thesis, I describe the role of two membrane repair proteins, myoferlin and dysferlin, in maintaining the homeostasis of normal airway epithelium in Chapter 4. I demonstrate the respective role of myoferlin and dysferlin in regulating membrane repair, proliferation and growth of mouse lung adenocarcinoma in Chapter 5. Lastly, in chapter 6 I describe the importance of myoferlin in mediating a molecular pathway that promotes lung cancer metastasis.

1.2. Lung cancer epidemiology and risk factors

Lung cancer is the leading cause of cancer death in the world. Every year, more than 1.5 million new cases of lung cancer are diagnosed worldwide [1]. Although lung cancer is the second most commonly diagnosed cancer in both men and women in Canada, preceded by

prostate cancer in men and breast cancer in women, lung cancer alone accounted for 26,100 new cases and 20,500 cancer deaths in 2014 [2]. The mortality rate of lung cancer is high at 27% of all cancer deaths, and the relative 5-year survival rate for lung cancer patients is only 17% [2]. Such high mortality rates for lung cancer are due to the poor prognosis of the disease and the ineffectiveness of conventional therapy, reflecting the immediate need for better therapeutic treatments as well as preventive measures for lung cancer. Lung cancer not only carries a high cost to human life, but is also a tremendous economic burden on society. Lung cancer costs alone per year are estimated at $17.4 billion, placing a significant financial burden on any health care system [2].

Many factors have been identified to increase the risk of developing lung cancer and these factors are categorized into two main groups: extrinsic and intrinsic. Extrinsic risk factors are modifiable and external to individuals, including tobacco smoke, environmental contaminant exposure and diet. Intrinsic factors, on the other hand, are non-modifiable and inherited, and include factors such as age, race, gender, family history, and previous respiratory diseases [3].

1.2.1. Extrinsic factors

Among all the risk factors, tobacco cigarette smoking contributes to 80-90% of lung cancer cases and is considered the greatest risk factor [3]. The association between tobacco cigarette smoking and lung cancer is dose-dependent, meaning that heavy smokers have a higher chance of lung cancer than intermittent smokers [4]. At least 60 carcinogens, such as polycyclic aromatic hydrocarbons, nitrates and tobacco-specific N-nitrosamines, have been identified in tobacco smoke as the main carcinogens associated with the cause of lung cancer [5]. The second-hand smoke exhaled by tobacco smokers additionally releases the same

carcinogens to the environment and can increase the risk of lung cancer for non-smokers. Recently, the cancer agency of the World Health Organization, namely the International Agency for Research on Cancer, additionally stated that air pollution is a significant cause of lung cancer cases [6]. Air pollutants contain carcinogenic substances, including but not limited to ozone, radon, and emission from vehicles and industrial processes, all of which can cause lung cancer. In addition, people who are constantly occupationally exposed to environmental carcinogens such as asbestos, radiation, wood dust and tobacco smoke tend to have a higher incidence of lung cancer [7].

1.2.2. Intrinsic factors

In a study that investigated the racial difference among smokers, 72.7 out of 100,000 African American smokers and 63.3 out of 100,000 white Caucasian smokers developed lung cancer in the same-age cohorts between 2004 and 2008, indicating that African American are more likely to develop lung cancer than white Caucasians [8]. In comparison, American Indians/Alaska Natives, Asians/Pacific Islanders and Hispanics had significantly lower incidence rates with only 44.5, 39.0 and 32.5 per 100,000, respectively [8]. Similar trends of racial disparity also apply to the mortality rate of lung cancer [8]. With regards to gender disparities, studies have reported contradicting findings with some studies showing that male smokers are more likely to develop lung cancer than female smokers [8, 9] while others show the opposite findings [10-12] irrespective of age and race. However, there is an increasing trend of lung cancer incidence in females, while in males the incidence is decreasing [13], mostly likely due to the rising trend of female smokers. In addition to race and gender, the risk of lung cancer incidence increases with age as recent statistics demonstrated that the majority of new lung cancer cases are diagnosed at an average age of 70 years [14]. Previous

history of respiratory diseases such as tuberculosis, chronic bronchitis and emphysema (which correspond to 1.48-, 1.57-, and 2.44-fold increased risk, respectively), also poses an elevated risk in developing lung cancer [15].

1.3. Types of lung cancer

Lung cancer is classified into four main types based on the morphological features of the cancer cells by light microscopy: adenocarcinoma, squamous cell carcinoma, large cell carcinoma and small cell carcinoma (SCLC). Based on treatment and prognosis, adenocarcinoma, squamous cell carcinoma, and large cell carcinoma can be grouped together as non-small cell lung cancer (NSCLC) [16]. The majority of lung cancer cases are NSCLC, which accounts for 85-90% of all lung cancer cases, whereas SCLC only accounts for 10-15% [17, 18]. Although smokers tend to have an increased risk of developing lung cancer, non-smokers can also develop lung cancer, mostly adenocarcinoma, through exposure to environmental carcinogens. In addition to primary lung tumors, nearly 30-50% of all malignant diseases also involve the lung, as it is the most frequent organ where metastatic neoplasms are found [16, 19, 20]. Among all the lung cancer types, lung adenocarcinoma is the most frequent type in North America while squamous cell carcinoma is more common in Europe and Australia [21]. Because of the high prevalence of NSCLC, this thesis focuses specifically on NSCLC, and in particular, lung adenocarcinoma.

1.4. Diagnosis of NSCLC

Due to lack of early symptoms, NSCLC is frequently considered as a silent killer. Although patients with lung cancer may experience weight loss, night sweats, fatigue and fever, these

symptoms are very common in other non-cancer related diseases and patients easily ignore these potential symptoms. Only after further spread will patients start coughing with occasional bloody sputum and experience wheezing, pain, dyspnea, stridor and sometimes post-obstructive pneumonia [22, 23]. Once the lung cancer spreads to the extrathoracic structures, patients can experience hoarseness, upper airway obstruction, Horner's syndrome (a sympathetic nerve interruption that leads to miosis, ptosis, and anhidrosis), dysphagia and elevated diaphragmatic paralysis [22, 23]. In more severe cases in which lung cancer metastasizes to distant organs, patients will experience bone pain, headache, neurological or psychiatric abnormalities, paraplegia, hepatomegaly, and pathological fractures [22, 23]. In addition, some patients may exhibit Cushing syndrome, syndrome of inappropriate secretion of antidiuretic hormone, Lambert-Eaton syndrome and Pierre-Marie-Bamberger syndrome [22, 23]. Unfortunately, the occurrence of these symptoms indicates advanced stage of lung cancer at which point treatment options are very limited.

Presence of symptoms may be suggestive of lung cancer, but additional tests are required to confirm the diagnosis. Most of the time, patients with lung cancer are first diagnosed with lesions on the lung by a chest x-ray. Then, clinical multi-detector computer tomography scans are used to determine the exact location, size, and shape of lung lesions. Bronchoscopy, positron emission tomography, endobronchial ultrasound, and mediastinoscopy may also be used for further classification of tumor type as well as searching for the metastases. Once the lung lesion is located, biopsy samples will be collected from the patients for a series of histological and pathological tests to provide further information on the lesion as well as identifying the lung cancer type. After confirming and characterizing the lung cancer type, the lung cancer stage will be determined in order to inform effective treatments for the patients.

The staging system for cancer was first introduced by Pierrre Denoix in 1940s and modified specifically for lung cancer by Dr. Clifton Mountain in 1973 [24, 25]. The advancement of technology allows a better understanding of lung cancer, and hence, the staging system is periodically revised as an ongoing International Association for the Study of Lung Cancer project by the American Joint Committee on Cancer and the International Union Against Cancer to establish applicable international protocol [24, 25]. For NSCLC, the staging system comprises three descriptors: size of tumors (T), degree of localized lymph node involved near the cancer (N), and the extent of where the tumor has metastasized in the body (M) [24, 25]. Based on the assessment of individual TNM criteria, the NSCLC is then assigned a stage according to Table 1.

Stage	TNM assessment
0	Carcinoma *in situ*
I	Tumor is found only in the lung and has not spread to lymph nodes
A	Less than 3 centimeters (cm) in size
B	Greater than 3 cm in size
II	Tumor is located in the lung and near the lymph nodes or has spread to main bronchus or pleura, but not spread to lymph nodes
A	Less than 3 cm in size
B	Greater than 3 cm in size or any size that situated in the main bronchus or pleura
III	Tumor is found in the lung and has spread to the lymph nodes
A	Any tumor size that has spread to the lymph nodes on the same side of the original tumor (locally advanced cancer)
B	Any tumor size that has spread to the lymph nodes on the opposite side of the original tumor and/or to other structures in the chest (advanced cancer)
IV	Tumor is found in the lung and spread to both sides of the lung, to the fluids around the lungs, or to other body parts (advanced cancer)

Table 1. Summary of staging system for NSCLC.

The characterization of cancer stage provides important information to properly select the best treatment option for the patients with NSCLC. However, most NSCLC patients have intrinsic resistance to conventional chemotherapy and radiation therapy that make treatment of NSCLC difficult. To effectively choose a treatment that best fits each patient's needs, it is necessary to understand the pathogenesis of NSCLC.

1.5. Pathogenesis of NSCLCs

NSCLC is a complicated and multi-centric disease that is believed to involve stepwise genetic and molecular abnormalities along with morphological changes from normal lung epithelium to preneoplastic to neoplastic lesions. Although the cascade of events that trigger the malignant transformation of lung epithelial cells is not fully understood [26], it usually begins with chronic exposure to inhaled pathogens or toxins which induces chromosomal deoxyribonucleic acid (DNA) damage or epigenetic changes in the epithelial cells. Studies from well-known carcinogens such as nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), benzo[a]pyrene (BaP), and arsenic demonstrated that the balance between metabolic activation and detoxification pathways determines the patient's susceptibility to the mutagenic effects of these carcinogens [27-36]. For example, cytochrome P450 (CYP450) enzymes in the lung metabolically activate NNK to produce electrophilic metabolites which can bind covalently to DNA nucleotides, particularly at guanine or adenine, to form DNA adducts [27-29]. With proper repair, these DNA adducts will revert to normal DNA and cause no further harm. Unfortunately, failure to repair the DNA adducts leads to miscoding of nucleotides and sometimes followed by irreversible activating mutations in oncogenes or inactivating mutations in tumor suppression genes. Cells with mutated genes are normally removed by apoptosis, but if left unchecked, they can initiate

carcinogenesis (Figure 1) [37-40]. However, inhaled carcinogens do not always induce carcinogenesis. Cells also possess detoxification enzymes such as 11-beta-hydroxysteroid dehydrogenase, carbonyl reductase 1, and aldo-keto reductase family 1 to convert the carcinogens to water soluble metabolites that are ready for excretion (Figure 1) [41].

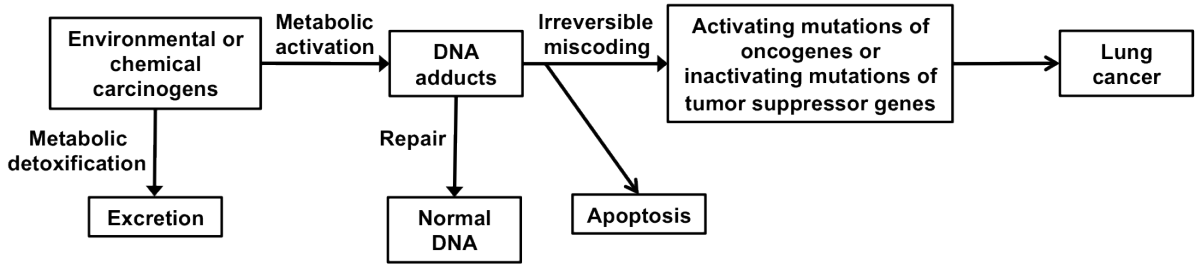


Figure 1. Fate of environmental or chemical carcinogens in the lung.

Environmental or chemical carcinogens in the normal airway can either be metabolically activated by cytochrome P450 enzymes to form DNA adducts or detoxified by 11-beta-hydroxysteroid dehydrogenase, carbonyl reductase 1 or aldo-keto reductase family enzymes for excretion. Normal DNA can be restored by successful repair. In contrast, failure of DNA repair leads to miscoding of nucleotides that often followed with activating mutations of oncogenes or inactivating mutations of tumor suppressor genes. Ultimately, lung cancer will be formed. Modified to use with permission from *Journal of the National Cancer Institute* [42].

Many lung carcinogens are categorized as "complete" carcinogens, which means, they not only act as initiators of tumorigenesis, but also serve as tumor promoters. Genes in the mutated cells are vulnerable to additional mutations that lead to self-sufficiency of growth signals, evasion of apoptosis, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis, together yielding the "hallmarks of cancer" [43]. For example, NNK metabolites lead to mutations in the genes that include epidermal growth factor receptor (EGFR) [44], protein kinase B (Akt) [45-47], B

cell leukemia/lymphoma 2 (Bcl2) [48] and tumor suppressor protein p53 [49]. Proteins encoded by these mutated genes participate in signaling pathways that regulate bronchial, bronchiolar or alveolar epithelial cell proliferation, survival, migration and invasion [44]. Therefore, these genetic mutations cause dysplasia or preneoplastic lesions, and ultimately, malignant neoplastic lung cancer. The main histological types of lung cancer are defined on the basis of the specific tissue type from which the tumor originated. Since most lung cancers are composed of cells with an epithelial origin, I will review the normal physiology of lung epithelium and how epithelial injury leads to the development of lung cancer.

1.5.1. The lung epithelium

The respiratory system is composed of a series of conducting airways that branch up to 23 generations to allow airflow into the lung. From the trachea, conducting airways branch in an irregular and dichotomous pattern to the terminal bronchioles (generations 16), inclusively [50]. At the last generation of conducting airway (the terminal bronchiole), the respiratory zone branches regularly and dichotomously into the acinus which consists of alveolar ducts and alveoli that generate a total surface area of approximately 70 square meters to allow for gas exchange [51]. Contrary to the airways, the branding pattern of pulmonary vasculature is asymmetrical and non-dichotomous [52]. Along the airway branches, the pulmonary arteries are found. These arteries terminate in a dense, pulmonary capillary network at the alveolar ducts and surround the alveolar epithelium to form the gas exchange units. Both the airways and alveoli are lined with specialized epithelia. In the airway, the epithelium serves to prevent the entry of particulate matter, viruses and microbes through the action of mucociliary elevator, whereas in the alveoli, the epithelium is specialized to allow the exchange of oxygen and carbon dioxide to support the body's cellular metabolism. In order

to meet the complicated functions of the lung, the epithelial lining of the main airways and the terminal alveoli are composed of several cell lineages.

The pulmonary epithelium develops from the endoderm during fetal development. Through the sequential interactions between the endoderm and mesoderm along with the surrounding extracellular matrix (ECM), specialized epithelial cells are formed to achieve the final cellular composition of the lung. In the airways, the epithelial layers of trachea, bronchi (upper airways) and bronchioles (lower proximal airways) are pseudostratified, and are composed of ciliated columnar cells, mucus-secreting goblet cells, and basal cells (Figure 2A) [53]. Each polarized ciliated cell has 200 to 300 hair-like projections called cilia, and together with mucus released from goblet cells, defend against inhaled pathogens or substances in a process called mucociliary clearance [54]. This process begins with the goblet cells secreting mucus factors to trap inhaled particulate matter, viruses and bacteria [54], followed by synchronous ciliary beatings by ciliated cells to propel the trapped substances out to the pharynx [55]. Basal cells provide anchorage for the epithelium to the basement membrane through desmosome junctional proteins (Figure 2A) [56]. In damaged airway epithelium, basal cells act as progenitor cells that can differentiate into either ciliated cells or club cells (previously known as Clara cells), and goblet cells to repair the injured site and restore normal epithelial integrity [57, 58]. Unlike the upper airways, the epithelial surface of the lower distal airways is covered with dome-shaped, microvilli-containing club cells that secrete surfactant-associated proteins, leukocyte-protease inhibitors, and Clara cell proteins (Figure 2A) [59-61]. Like basal cells, club cells are also progenitor cells and can differentiate into ciliated and goblet cells to repair damaged airway epithelium [62].

In the alveoli, 95% of the epithelial surface is made up of alveolar epithelial type I (AEI) cells and the rest is type II (AEII) cells; these cells are also known as type I and type II

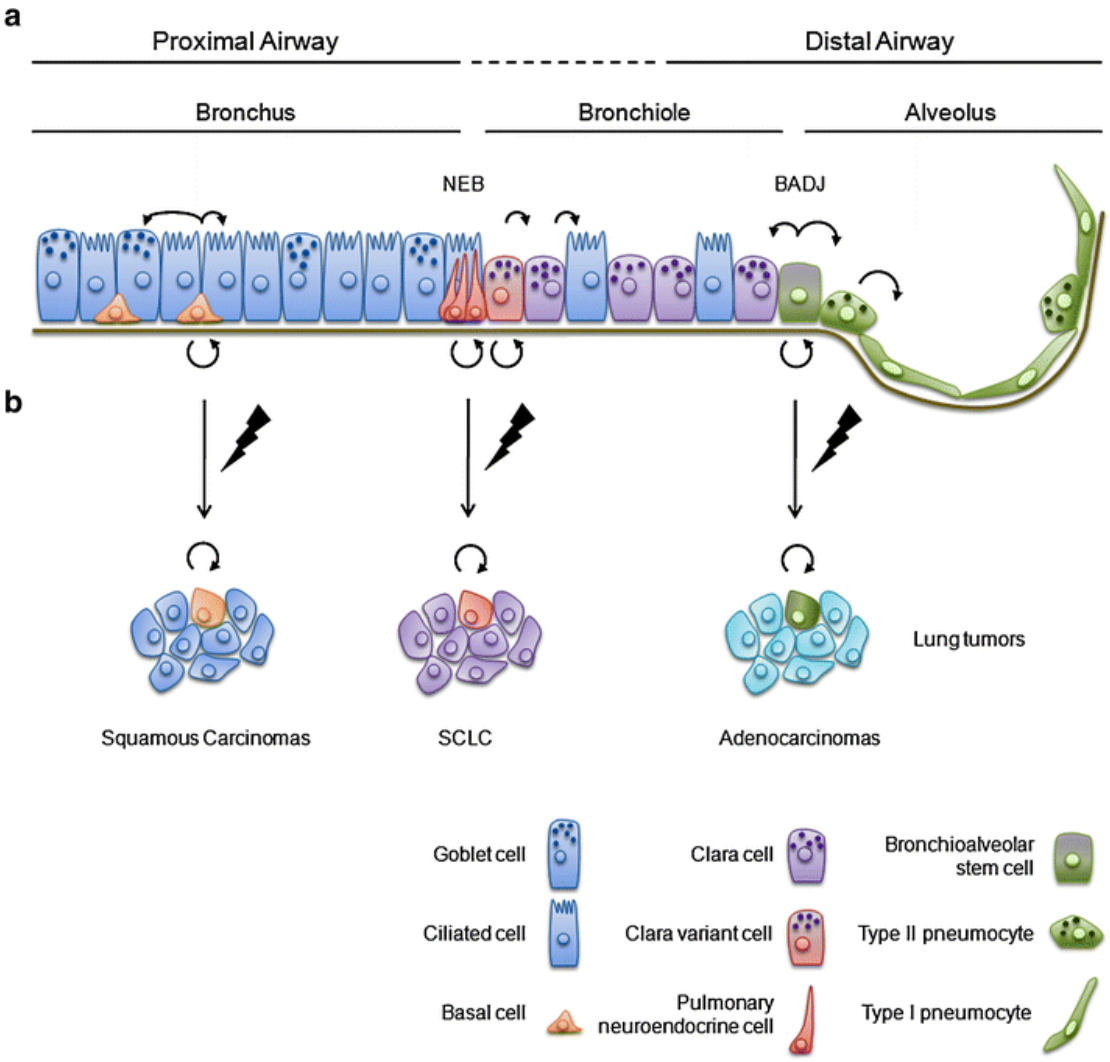


Figure 2. Origin of different types of lung cancer.

(A) Lung adenocarcinomas derive from the Club (or Clara) cells or type II pneumocytes are proposed to begin with the bronchioalveolar stem cells that are positive for Club cell and type II pneumocyte markers at the bronchioalveolar duct junction in the distal airway. Squamous cell carcinomas arise from the differentiating basal, goblet and ciliated cells in the proximal airway, particularly the bronchus. Large cell carcinomas can originate from anywhere in the lung which make this cancer type difficult to trace their origin. SCLCs initiate from the clustered pulmonary neuroendocrine cells called neuroendocrine bodies (NEBs) in the bronchiole. These neuroendocrine cells subsequently differentiate into Club cells and ciliated cells. (B) Oncogenic mutations (indicated by the lightning bolts) accumulate in various lung epithelial cell types, which ultimately lead to the development of different tumor types. Reproduced with permission from *Cancer and Metastasis Reviews* [64].

pneumocytes, respectively (Figure 2A) [63]. AEI cells are large (volume of about 2000-3000 μm^3), thin and branched in morphology, which provides a minimal diffusion barrier between the alveolar space and pulmonary capillary blood for effective gas exchange. In constrast, the small cuboidal-shaped AEII cells (volume of 450-900 μm^3) secrete high levels of surfactant proteins to reduce the surface tension of alveoli for the removal of inhaled particulates, to regulate the lung fluid balance and to provide host defense [65, 66]. AEII cells also serve as the progenitor cells during alveolar epithelial repair [67]. Following physiologic insult, AEII cells proliferate, differentiate into AEI cells and phagocytose the apoptotic AEII cells in order to repair the damaged alveolar epithelium [66]. Since AEI cells cover most of the alveolar surface area and are directly exposed to more atmospheric area, they are more sensitive to injury than AEII cells [68]. Likewise, the endothelial cells that make up the innermost cellular lining of pulmonary capillary beds around the alveoli are very susceptible to air-borne and blood-borne insults. Depending on the specific region of the lung, the lung epithelium is maintained and repaired by different types of epithelial cell populations.

1.5.2. Lung epithelial cell types and specific lung cancers

In lung cancer, the diverse spectrum of lung epithelial cell types also provides a unique nature of lung cancer phenotype. Evidence from murine models of lung cancer demonstrated that accumulated genetic changes and the type of epithelial cells dictate the lung cancer type [69, 70]. Hyperplasia of abnormal secretory epithelial cells, including Club (or Clara) cells in the bronchioles and AEII cells in the alveoli, often leads to peripherally located lung adenocarcinoma [71]. It is believed that bronchioalveolar stem cells (BASC) that stained positive for both Club and AEII cells are also able to initiate adenocarcinoma (Figure 2B) [72]. Contrary to lung adenocarcinomas, squamous cell carcinomas are more centrally

located. This type of lung cancers arises from hyperplasia, metaplasia, and dysplasia of thin and flattened differentiating epithelial cells located in tertiary bronchi (or segmental bronchi) and extends to secondary (or lobar) and primary (or trachea) bronchus [73] (Figure 2B). Unique features of squamous cell carcinoma cells, including intercellular bridging and formation of keratin pearls, allow them to be distinguished from other lung cancer types [74]. Hyperplasia of mucosal and neuroendocrine cells in the bronchial and bronchiolar epithelia is believed to be the precursor lesions of small cell (or oat cell) carcinoma (Figure 2B). If the lung tumor cannot be classified as lung adenocarcinoma, squamous cell carcinoma, or small cell carcinoma, by exclusion, it will be classified as large cell carcinoma originated from any lung region.

The distinct structure and function of airway and alveolar epithelia indicate the importance of normal epithelial integrity to protect the underlying lung tissues from the inhaled environment. Hence, control of epithelial intercellular junctions is essential for biological processes such as airway homeostasis, epithelial repair, and inflammation. Additionally, airway epithelial integrity constitutes a barrier against lung tumor invasion and metastasis [75]. Therefore, understanding the cell-cell interactions and cell-ECM interactions to maintain lung epithelial integrity will give us an insight on the lung epithelial changes during lung injury and tumor development.

1.5.3. Cellular junctions in lung epithelium

The lung epithelium is composed of pseudostratified layers of epithelial cells in the airway and a single layer of epithelial cells in the alveolus. To maintain epithelial integrity and permeability, these epithelial sheets are held together by cellular junctions, including tight junctions, adherens junctions, desmosomes, gap junctions and hemidesmosomes (Figure 3).

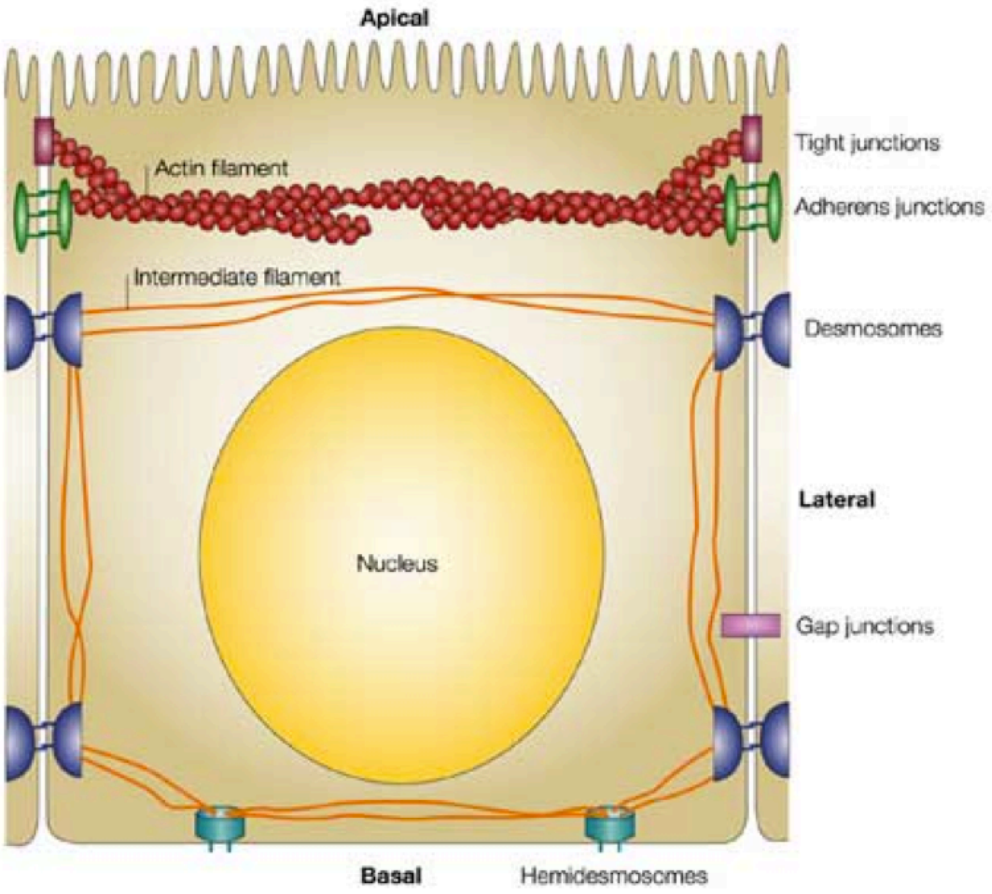


Figure 3. Intercellular junctions in airway epithelium.

Tight junctions on the apical side of cells form an intercellular barrier to regulate the diffusion of ions and molecules, and prevent penetration of pathogens. Adherens junctions hold the columnar epithelial cells together strongly. Within the epithelial cells, actin filaments attach to the adherens junctions. Desmosomal junctions provide strong adhesion among basal cells as well as adhesion between basal cells and columnar cells. Gap junctions allow the passage of ions, small molecules and cellular signals to promote intercellular communication. Hemidesmosomal junctions anchor the basal cells to the basement membrane or ECM. These junctions are responsible for forming focal adhesions by linking the intermediate filaments of cytoskeletal network to the ECM. Reproduced with permission from *Nature Reviews Molecular Cell Biology* [76].

In normal lung epithelium, tight junctions (TJs) at the apical surface of the epithelium are responsible for regulating paracellular permeability and allow movement of ions and solutes

between cells. These are often referred to as “gate” and “fence” function, respectively (Figure 3) [77-80]. Integral TJ proteins, including membrane proteins occludin, claudins 1-20, and junction adhesion molecules (JAMs) have been identified in TJs [81]. These membrane TJ proteins anchor to the cytoskeleton through peripheral membrane proteins zonula occludens (ZO)-1, -2, -3 or cingulin to regulate epithelial cell proliferation, gene expression and differentiation via intercellular signal transduction (Figure 4) [82, 83]. Changes in the expression and structure of these proteins, leading to loss of TJs, are often present in damaged lung epithelium as well as during the development of lung cancers [75, 85].

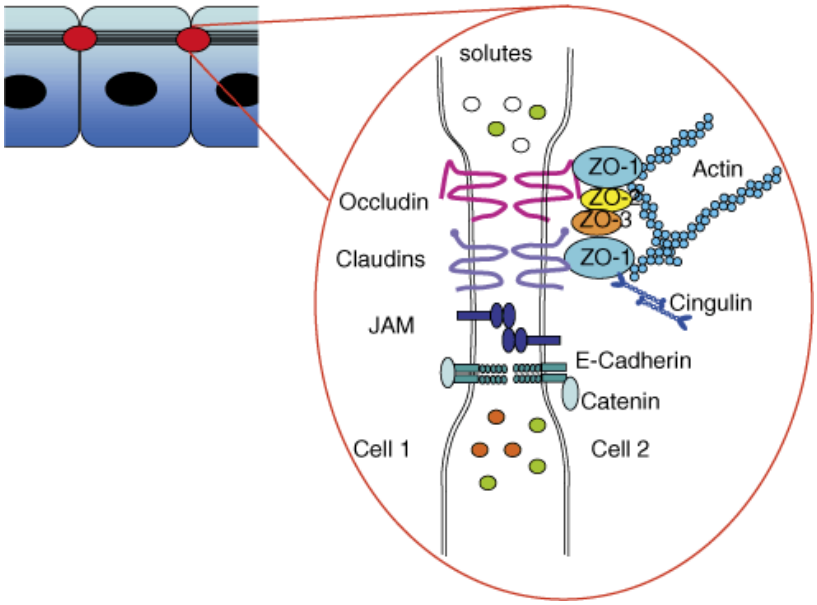


Figure 4. Proteins that form tight and adherens junctions on the apical epithelium.

Tight junctions look like continuous belts that are formed by homophilic interactions of occludin and claudins. Inside the cell cytoplasm, occludin and claudins interact with actin filaments of cytoskeleton through ZO-1 and cingulin. JAMs also contribute to the tight junction formation via homophilic interaction. Formation of adherens junctions requires the E-cadherin from both cells to interact with each other. E-cadherin also associates with cytoskeletal microtubules via catenin. Reproduced with permission from *Allergology International* [84].

The main component of adherens junctions (AJs), E-cadherin, also plays a key role in forming and sustaining the function of TJs (Figure 4). This is evident in the mislocalization of ZO-1, occludin and claudins when the expression of E-cadherin is reduced [86, 87]. Also, knockdown of E-cadherin expression in cultured bronchial epithelial cells with small interfering ribonucleic acid (siRNA) decreases the expression of ZO-1 that frequently results in decreased epithelial resistance [87]. Between neighbouring epithelial cells, E-cadherin normally forms homophilic interactions to maintain structural integrity of epithelium [88]. Within the epithelial cells, E-cadherin interacts with the actin cytoskeleton and microtubule network by binding to catenins (α-, β-, γ-, and p-120 catenins) and other proteins. These interactions serve to maintain cellular architecture and to initiate cell proliferation and differentiation (Figure 4) [89-92]. If E-cadherin fails to form a complex with catenin and/or connect to the cytoskeleton, the cell will lose its adhesion to adjacent cells and possibly detach from the epithelium. Thus, E-cadherin plays a key role in cell adhesion.

In addition to TJs and AJs, desomosomes and hemidesmosomes also contribute to the maintenance of structural epiehtlial integrity through interactions between the cells as well as interactions between the cells and ECM within the basement membrane, respectively (Figure 3) [93]. Adjacent epithelial cells can also communicate with each other to regulate cell growth and survival by connecting the cytoplasm and forming gap junctions at the cell membrane (Figure 3) [94, 95]. All of these features are essential for maintaining homeostasis and the structural integrity of airway epithelium. Importantly, plasticity of airway epithelium through the constant rearrangement of the epithelial junctions listed above enables cell homeostasis and repair through the active remodeling of cell junctions after injury.

1.5.4. Lung epithelial injury

Under normal homeostasis, the differentiated tracheal and bronchiolar epithelial cells are not proliferative and have a long survival time, with an average lifespans of more than 120 days [96, 97]. However, the airway epithelium has a large proliferative capacity to facilitate repair in response to injury. In the last decade, it has been recognized that the airway epithelium is a highly specialized tissue with the ability to regulate airway repair due to injury [98, 99]. In the circumstance of prolonged injury and repair this can predispose morphological and genetic abnormalities in the epithelium for tumorigenesis. When interacting with the airway epithelium, inhaled toxins can cause damage to epithelial junctional proteins. Specifically, they disrupt apical junctions (TJs and AJs) through proteolytic degradation, dissociation and dislocalization of junctional proteins, or gene mutations. Additionally, inhaled toxins can disrupt the epithelial integrity by releasing proteases that degrade occludin, claudin-1, ZO-1 and E-cadherin in epithelial cells [100-102]. Some toxins cause derangement of cytoskeletal organization and affect the cytosolic junction proteins such as ZO-1 and catenins that correspondingly link to occludin and claudin, and E-cadherin to the underlying cytoskeletal actin filaments [103]. Another mechanism of disrupting airway epithelial integrity is by pore formation. Pore-forming bacterial toxins, such as pneumolysin, α-hemolysin, streptolysin O, and anthrolysin O, have been shown to form pores at the cell membrane to disrupt the membrane integrity of lung epithelial cells [104]. These water-soluble toxins bind to the host cell surface where they oligomerize and become amphipathic, thus allowing them to insert into the target cell membrane and form a pore [105]. Furthermore, expression and stabilization of junctional proteins on the cell surface requires proper delivery of junctional proteins through vesicle trafficking [106-108]. Toxins interfering with the vesicle trafficking machinery can disrupt the translocation of junctional proteins via transcytosis [109].

Disruption of junction formation alters the integrity, permeability, polarity, intercellular transport and signal transduction of the lung epithelium. To restore the epithelial integrity, epithelial cells reassemble the junction complex by utilizing the existing junction protein components in the cytoplasm and/or by synthesizing new junction proteins. As shown in Figure 5, studies using guinea pig and rat trachea have shown that cell spreading and

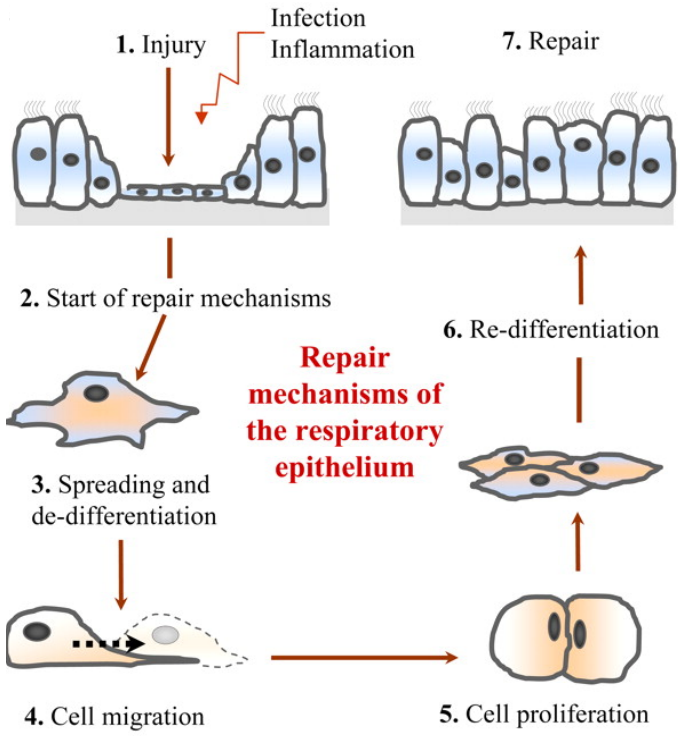


Figure 5. Mechanism of lung epithelial repair.

Injury (e.g. infection, inflammation) on the pulmonary epithelium (step 1) activates the repair mechanisms (step 2) that involve spreading and dedifferentiation of healthy epithelial cells near the wound (step 3). The dedifferentiated cells migrate to the wound (step 4) and proliferate (step 5). Epithelial cells redifferentiate (step 6) to replace the damaged ciliated and/or goblet cells. The damaged site is repaired and the integrity is restored (step 7). Reproduced with permission from *American Journal of Physiology* [110].

migration begins in the first 12-24 h after lung epithelial injury [111-115]. Such cell migration allows the damaged area to be quickly covered with an immature epithelium to provide a cell barrier [112]. Proliferation starts at 15-24 h after injury and lasts for days to weeks [111-115]. The final stage of repair involves re-differentiation of undifferentiated cells to ciliated columnar cells to completely restore the normal airway epithelial integrity and composition [112]. Therefore, the packaging and assembly of adhesion junctions is essential for the survival of a cell and the formation of a functional barrier.

1.5.5. Epithelial membrane repair and reassembly of intercellular junctions

Membrane repair and reassembly of intercellular junctions share similar mechanisms in that they both involve vesicle trafficking and fusion with the cell membrane. First, membrane damage or loss of intercellular contact initiates the influx of extracellular calcium at the site where the membrane is ruptured or the cell-cell contact is lost [116, 117]. Second, calcium activates the calcium/calmodulin (CaM)-dependent kinase to phosphorylate synapsin I, triggering the release of vesicles from actin filaments [118, 119]. These vesicles carry membrane proteins that include junctional proteins (for examples, ZO-1, occludin, claudins, and E-cadherin) and growth factor receptors (for examples, EGFR, insulin-like growth factor receptor, and vascular endothelial growth factor receptors) that are either newly synthesized or recycled from previous endocytosis. Third, vesicles are directed to the cytosol near the targeted site by kinesin on the microtubule in the presence of adenosine triphosphate (ATP) [120]. In the case of membrane repair, membrane vesicles accumulated near the damaged region fuse together to form membrane patches before docking at the damaged site [121]. In the case of intercellular junction reassembly, membrane vesicles containing junctional protein components gather near the junctional region of plasma membrane [122, 123]. After

the tethering of membrane vesicles to the targeted site, three members of the *N*-ethylmaleinide-sensitive factor attachment protein receptor (SNARE) proteins mediate the fusion of the vesicular membrane to the plasma membrane: synaptobrevin (also known as vesicle-associated membrane proteins) on the vesicular membrane, and synaptosome-associated proteins (SNAPs) and syntaxin on the plasma membrane [124]. Last, the SNARE complex undergoes conformational changes to bring the plasma membrane and vesicular membrane closer to each other to reseal the ruptured area and restore membrane integrity [124]. At the same time, the junctional proteins are delivered to the plasma membrane for re-establishing the junctional complex.

In addition to SNARE proteins, multiple C2-domain-containing proteins have been shown to be involved in membrane repair by translating the calcium influx into exocytosis of vesicles. The best-studied molecules are synaptotagmins, especially, synaptotagmin-1. Calcium binds to the cytoplasmic C2 domains of synaptotagmin-1 in the vesicles, which then bind to the SNARE complex and triggers exocytosis [125, 126]. Recently, other C2-domain-containing proteins, such as ferlins, were also found to participate in the docking and fusion of vesicles with the ruptured cell membrane [127, 128]. Ferlins belong to a family of transmembrane proteins that share a similar genetic sequence to Fer-1 and contain a transmembrane domain on the carboxyl terminal and multiple cytosolic C2 domains (Figure 6) [129]. The transmembrane domain anchors the protein to the membrane whereas the multiple C2 domains mediate lipid binding and protein-protein interactions. In *Caenorhabditis elegans*, Fer-1 mediates the calcium-dependent fusion of specialized vesicles with the plasma membrane of the spermatids during spermiogenesis [130, 131]. The process of fusing specialized vesicles with the plasma membrane also adds more membrane at the fusion site. The additional plasma membrane permits the extension of pseudopodia that are

necessary for sperm motility. The genetic and structural homologies of ferlins to Fer-1 suggest that ferlins may also mediate the docking and fusion of vesicles. Currently, six ferlin proteins have been identified in humans and they are dysferlin (Fer1L1), otoferlin (Fer1L2), myoferlin (Fer1L3), Fer1L4, Fer1L5, and Fer1L6 (Figure 6) [131-137].

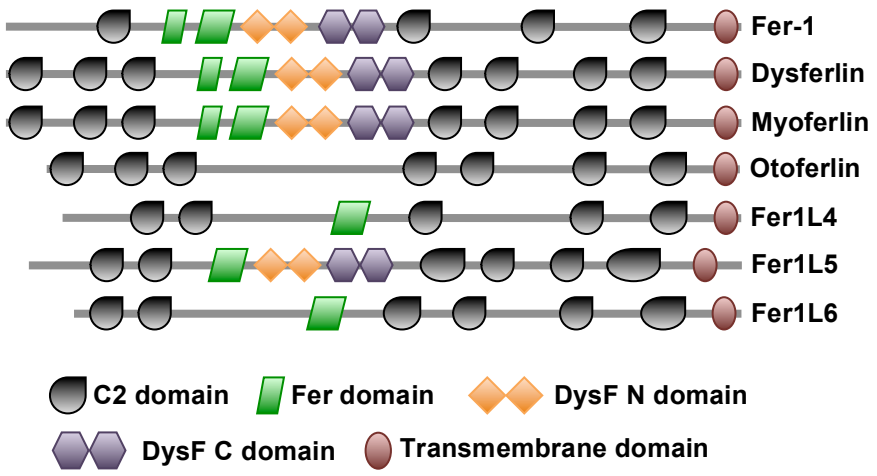


Figure 6. Schematic diagram of the Fer-1 and mammalian ferlin family proteins.

Six ferlin proteins are identified in mammalian cells: dysferlin, myoferlin, otoferlin, Fer1L4, Fer1L5, and Fer1L6. These mammalian ferlin proteins share similar domain structure as *C. elegans* Fer-1: multiple C2 domains and a single transmembrane domain on the C-terminus. The C2 domains are responsible for membrane fusion events, vesicle trafficking, and protein-protein interaction while the transmembrane domain provides the membrane-anchored feature of ferlins. Besides C2 and transmembrane domains, ferlins, except otoferlin, contain fer, dysF N and dysF C domains of unknown functions. Modified to use with permission from *Biochemical and Biophysical Research Communications* [138].

1.5.5.1. Dysferlin

Of the six ferlin proteins currently known, dysferlin was the first to be identified in mammalian cells (Figure 6) [135]. Mutations of dysferlin are linked to several types of muscular dystrophy that include limb-girdle muscular dystrophy type 2B (LGMD2B) [135,

139, 140], Miyoshi myopathy (MM) [135, 140-142] and distal myopathy [142]. Patients with reduced expression or absence of dysferlin do not show any pathological phenotype initially; however, they subsequently report various degrees of skeletal muscle weakness and wasting at a later stage of life [135, 139]. The late onset of symptoms suggests the association of dysferlin with skeletal muscle repair and regeneration rather than development.

In normal myofibers with intact sarcolemma, dysferlin is evenly distributed at the plasma membrane and in the cytoplasmic vesicles [143]. During resealing of ruptured membrane, enhanced dysferlin expression is found in the membrane patch and at the damaged site, suggesting that dysferlin is involved in vesicle fusion and membrane repair [127, 144-146]. The mechanism of dysferlin-mediated membrane repair is not fully known, but studies have shown that dysferlin interacts with annexins (A1 and A2) [146, 147], AHNAK [148], Mitsugumin 53 [149], caveolin-3 [150], tubulin [151] and calpain 3 [152]. Each of these dysferlin-interacting proteins, except caveolin-3, has been independently shown to traffic and fuse intracellular vesicles to the cytosolic side of plasma membrane to reseal the ruptured membrane [153-158]. In dysferlin-null myofibers, injured sarcolemma triggers the accumulation of vesicles, but the vesicles fail to fuse together to form a membrane patch to reseal the damaged membrane [127, 159]. Emerging evidence indicates that dysferlin not only traffics vesicles for membrane repair in skeletal muscles, but also transports membrane proteins, such as insulin-like growth factor 1 receptor (IGF1R), as cargo proteins in the vesicles [160]. In addition to membrane repair and protein transport, dysferlin also contributes to the inflammatory response as dysferlin-deficient muscles show a decrease in cytokine and chemokine release along with defects in recruiting neutrophils in cumulative damaged muscles [161-163].

The majority of dysferlin research has focused on understanding the mechanism by

which dysferlin mediates membrane repair in ruptured skeletal muscles. Function of dysferlin in other non-skeletal muscles has been ignored in the past. Emerging evidence shows that dysferlin is also present in the lung, heart, brain, kidney, liver, placenta, pancreas and vascular endothelium [138, 139, 164, 165]. However, the function of dysferlin may differ depending on the tissue type. In human placenta, dysferlin maintains the stability of syncytiotrophoblasts (STBs) [166] and mediates fusion of STB [167]. In vascular endothelium, dysferlin regulates the expression of a membrane-bound protein called platelet endothelial cell adhesion molecule 1 (PECAM-1) as well as mediating cell adhesion and growth, and fusion of lysosomes [168, 169]. In monocytes, dysferlin regulates the expression of fibronectin and fibronetcin-binding integrins for cell adhesion [170]. Although dysferlin has different roles in these non-skeletal muscle cells, the common mechanism seems to involve dysferlin-mediated membrane trafficking. Our previous tracking experiment using green fluorescent protein tagged dysferlin to visualize the intracellular trafficking and assembly of labeled dysferlin in kidney epithelial cells further confirmed the contribution of dysferlin in trafficking membrane vesicles that possibly contain protein cargo [138]. In the same study, we also identified annexin A2 and S100A10, two well-known membrane repair proteins, as dysferlin-interacting partners in human vascular endothelial cells using a pull-down assay [138]. In epithelial cells, annexin A2 and S100A10 together form a complex not only to mediate membrane repair, but also recruit membrane proteins like E-cadherin to form AJs [171]. Another protein, AHNAK, which was previously shown to bind to dysferlin in skeletal muscle cells, interacts with the annexin A2/S100A10 complex to facilitate the organization and structure of the epithelial cell membrane during the formation of intercellular contact and establishment of cell polarity [154]. Taken together, these findings suggest that dysferlin mediates membrane-associated function in epithelial cells.

1.5.5.2. Myoferlin

Myoferlin has an amino acid sequence that is highly homologous to that of dysferlin (Figure 6) [133]. Myoferlin is localized at the plasma membrane for membrane fusion event and at the nuclear membrane for trafficking intracellular vesicles [128]. Although its function in human is not clear, myoferlin has been shown to be responsible for muscle development and repair in mice [172]. During muscle development, high levels of myoferlin expression are found in proliferating myoblasts, fusing myoblasts and newly formed myotubes [128, 173]. In order to respond to external growth signals, myoblasts need to express specific membrane receptors, which, when bound by the appropriate ligands, activate a cascade of intracellular events leading to proliferation. Myoferlin is responsible for transporting IGF1R by regulating the trafficking of membrane vesicles [128, 173]. The mechanism of myoferlin-mediated vesicle trafficking remains unknown. However, myoferlin interacts with Eps15 homology domain containing 2 (EHD2) [174], which plays a role in endocytosis [175], membrane repair [176, 177] and recycling of membrane receptor [178]. As myoblasts continue to proliferate, myoferlin mediates their fusion to form myofibers [174]. Once myofibers are fully mature, expression of myoferlin is substantially decreased [128]. However, skeletal muscle injury can induce expression of myoferlin to mediate muscle repair [179].

With the knowledge of myoferlin function in muscle growth and development, it is believed that myoferlin may rescue the phenotypes of muscular atrophy and dystrophy. Myoferlin messenger RNA (mRNA) levels are also increased in atrophic mouse skeletal muscles [180]. Likewise, dystrophic skeletal muscles isolated from dystrophin mutant mice also demonstrate a 2.7-fold increase in myoferlin protein expression [128]. However, these studies cannot conclude the compensatory role of myoferlin in atrophic and dystrophic muscles. In dysferlin-null myoblasts, overexpression of myoferlin does reverse the

deficiency of membrane fusion, but the dystrophic phenotype remains unchanged [181]. This study suggests that even though myoferlin and dysferlin have similar genetic sequences, their functions seem to be different.

Myoferlin expression is not limited to skeletal muscles, other tissues, including lung, placenta, heart and vasculature, also express myoferlin [217,213]. Previously, our laboratory found that siRNA knockdown of myoferlin decreases the membrane expression of vascular endothelial growth factor receptor 2 (VEGFR-2) in vascular endothelial cells, resulting in reduced VEGF-induced cell proliferation [182]. Within trafficking vesicles targeted to the plasma membrane, myoferlin is believed to form a complex with dynamin-2 (Dyn-2), an endocytic protein, to stabilize the expression of VEGFR-2 at the cell membrane, preventing VEGFR-2 from poly-ubiquitylation and proteasomal degradation [182]. Such myoferlin-mediated vesicle trafficking is likely dependent on caveolae/lipid raft localization of these signaling complexes [183]. In addition to VEGFR-2, another tyrosine kinase receptor called Tie-2 is also regulated by myoferlin in vascular endothelial cells via an unknown trafficking process [184].

Several gene expression microarray studies reported myoferlin gene overexpression in epithelial tumors, such as pancreatic adenocarcinomas and small cell lung carcinomas [185-189]. However, not much attention was given to investigate the role of myoferlin in cancer. It was not until the last 2 years when new compelling evidence provided more insight into the involvement of myoferlin in the development of various types of cancer. In breast cancer, myoferlin regulates the expression of EGFR and human epidermal growth factor receptor (HER), both of which are known to promote tumor growth and metastasis [190-192]. The anti-cancer effect of myoferlin knockdown is also reported in pancreatic adenocarcinoma [193].

1.5.5.3. Other ferlin proteins

Another ferlin protein, otoferlin, is found to transmit auditory information from human auditory inner hair cells to the brain (Figure 6) [194]. Mutations of otoferlin impair the exocytosis of vesicles containing neurotransmitter in auditory inner hair cells and causes profound deafness in both human and mouse [194, 195]. Studies have shown that otoferlin controls the trafficking of vesicles to the basolateral membrane of inner hair cells by interacting with two other vesicle trafficking proteins, Rab8b and myosin 6 [196, 197]. Upon vesicle fusion with the plasma membrane, otoferlin binds to the SNARE proteins syntaxin 1 and SNAP in a calcium-dependent manner [195, 198, 199]. Unlike dysferlin and myoferlin, expression of otoferlin is limited to the inner ear and the brain [200].

Among the remaining ferlin proteins, Fer1L4, Fer1L5 and Fer1L6, only Fer1L5 has been shown to interact with endocytic recycling proteins Eps15 homology domain containing 1 (EHD1) and EHD2 for myoblast fusion (Figure 6) [201]. Although the functions of Fer1L4 and Fer1L6 are not known, non-coding RNA levels of Fer1L4 are substantially decreased in human gastric cancer tissue and plasma [202, 203]. All these findings indicate the role of ferlins in vesicle trafficking and membrane fusion events that are necessary for membrane repair and protein transport to rescue the damaged cells from apoptosis.

1.5.6. Lung cancer is an over-healing wound

Lung cancer frequently develops at the regions where chronic injury to lung epithelium fails to complete the repair process and leads to airway remodeling. As mentioned previously, apoptosis, dedifferentiation, migration, proliferation, and differentiation of epithelial cells are the basic processes needed for optimal repair [204, 205]. The same processes are also found in cancer development. Microarray studies have reported that similar genes are found in both

wound repair and cancer, further supporting the connections between wound repair and cancer [206-209]. However, abnormal repair processes and associated gene expression are often found in lung cancer, leading researchers in the past to consider cancer as an over-healing wound [210]. In light of the linkage between wound healing and cancer, alterations of genes encoding proteins involved in epithelial repair have been observed to predispose susceptible cells to neoplastic transformation [211, 212] and accelerate tumorigenesis [213]. To further support this linkage, lung epithelial cell carcinogenesis is frequently associated with loss of cell polarity, altered expression of polarized proteins, cell detachment, cell morphological changes, enhanced cell motility and increased cell proliferation, phenomena that are also commonly observed in airway epithelial repair [214]. In addition, transformed epithelial cells are able to prolong cell survival by resisting apoptotic death. All these processes are mediated by membrane-associated events that include trafficking of membrane receptors, remodeling of cell membrane, and transducing receptor tyrosine kinase (RTK)-mediated signals. Since previous studies have shown that myoferlin mediates many membrane-associated events in normal and cancer cells, it is possible that myoferlin may play a central role in membrane-associated events in lung cancer.

1.5.6.1. Dysregulation of cell adhesion and epithelial-mesenchymal transition

Cellular contacts with the adjacent cells and with the surrounding ECM greatly influence the fate of a cell. In the 1940s, Coman et al. showed that malignant epithelial cells possess substantially weaker intercellular adhesion compared to normal epithelial cells [215]. The loosely attached cancer cells penetrate into the nearby tissues or enter into the blood circulation and transmigrate to distant organs, a process known as metastasis. Therefore, loss of adhesion becomes a fundamental characteristic of tumor malignancy.

Alterations in tight junctions are a pathological hallmark of lung cancer [216-218]. As the cancer progresses, the epithelial barriers are constantly broken down. Structure of tight junctions in epithelium is altered in neoplasia and it has been shown in the lung cancer [219]. Tight junction components, zonula occludens, in bronchiolo-alveolar carcinoma tissue sections appear as three or more stands that assemble in parallel or polygonal patterns [219]. Tumoour cells proliferate irregularly with the reduction in tight junction strand number when observed using freeze-fracture electron microscopy [219]. Furthermore, reduction of ZO and claudins mRNA expression are evident in lung adenocarcinomas and squamous cell carcinomas [220]. Claudin expression is also linked to the tumor metastasis and it has been shown that metastatic pulmonary tumors have significantly lower expression of certain claudins than the primary pulmonary tumor cells [221]. In addition, the expression of JAM-A is much higher in lung tumor samples than in corresponding non-tumor samples isolated from the NSCLC patients [222]. Contrary to other tight junction proteins, occludin expression remains the same in lung cancer, regardless of the tumor grade, when compared to normal airway epithelium [223]. Furthermore, loss of functional adherens junction is often observed in lung cancer development. Shimoyama et al. reported that loss of cell-cell adhesion is due to the absence of α-catenin while functional expression of E-cadherin remains the same in studies on a particular human lung cancer cell line, PC 9 [224]. Subsequent findings demonstrated that cancer tissues isolated from NSCLC patients express lower E-cadherin and β-catenin levels than the control [225]. The same study showed a correlation between decreased E-cadherin expression and metastasis as well as the correlation between decreased β-catenin with survival rate [225]. In addition to cell-cell contact, alteration in the affinity of NSCLC cells to ECM also facilitates the tumor metastasis [226, 227]. The cell-ECM interaction is mediated by integrins that form heterodimeric

receptors on the cell surface and interact with the ECM molecules including fibronectins and collagens [228]. In NSCLC patients, increased expression of integrins β1 and α5 is associated with lymph node metastasis [229] and shorter survival time [226, 230]. Taken together, cell adhesion provides the metastatic phenotypes of NSCLC cells.

Loss of cell adhesion is essential for the normal plasticity of epithelial cells to dedifferentiate, flatten, migrate and differentiate during repair [113]. During this process, epithelial cells undergo morphological changes to a mesenchymal cell phenotype through a biological process known as epithelial-mesenchymal transition (EMT) [231]. Currently, three EMT subtypes are classified based on their biological role. The first type (type 1 EMT) occurs only during embryonic development and allows the endoderm to differentiate into the mesoderm to form the blastocyst [232]. Inflammation, wound healing, and tissue regeneration trigger type 2 EMT in which fibroblasts and different types of epithelial cells are generated to repair and reconstruct airway epithelium and tissues following injury. Persistent type 2 EMT responses to chronic inflammation in fibrotic organs are detrimental as they can lead to organ destruction [233]. Type 3 EMT is essential for cancer metastasis in which neoplastic epithelial cells obtain an invasive phenotype, allowing tumor cell migration and invasion [234]. These processes are tightly regulated by growth factors and the corresponding downstream signaling events.

For all subtypes of EMT, the process begins with the loss of epithelial cell adhesion by sequentially degrading or down-regulating intercellular junction proteins ZO-1, claudins, occludins, E-cadherin, and demosomal protein (desmoplakin) (Figure 7) [235-238]. Dissociation of intercellular junctions results in the loss of the apical-basal polarity [239]. These transitioning cells reorganize the cytoskeletal architecture to enable cell morphological changes, cell elongation, membrane protrusion and front-back polarity that are important for

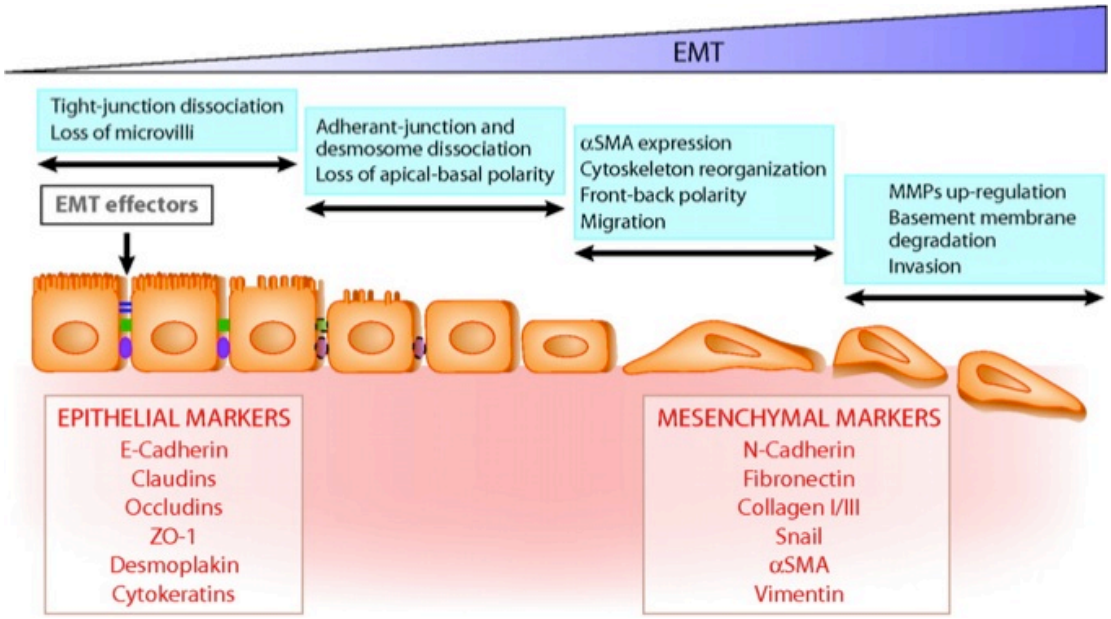


Figure 7. Schematic illustration of important events during epithelial-mesenchymal transition.

Epithelial cells disintegrate intercellular junctions by decreasing the expression of tight junction proteins (claudins, occludins, ZO-1), adherens junction protein (E-cadherin), and desmosomal protein (desmoplakin), resulting in the loss of microvilli and apical-basal polarity. These cells then reorganize the cytoskeletal filaments (cytokeratins) to acquire a front-back polarity. At this point, expression of mesenchymal markers (N-cadherin, fibronectin, collagen I/III, snail, alpha smooth muscle actin, and vimentin) is also upregulated to adopt mesenchymal phenotype and become motile. In the final stage of EMT, cells secrete matrix metalloproteinases to degrade the basement membrane and invade through the degraded basement membrane to the subepithelial layer. Reproduced with permission from *Journal of the American Society of Nephrology* [241].

directed migration [240]. Cells secrete proteases such as MMPs to degrade the cell-ECM interaction that allows the cells to migrate forward to cover the ruptured site and restore the epithelial barrier [112, 242]. The proteases also degrade the basement membrane for the cells to invade through to the subepithelial layer [243-245]. Ultimately, the cells dissociate from the epithelial layer and express mesenchymal proteins that include N-cadherin, fibronectin-EDA, collagen I/III, snail, vimentin, alpha smooth muscle actin, and vimentin to fully attain

the mesenchymal phenotype (Figure 7) [233, 246, 247].

Cell migration is a recurring multi-step process that begins with a morphological polarization to have unique cell front and back [248, 249]. The plasma membrane at the cell front protrudes or extends (membrane protrusion or extension) to form lamellipodia and filopodia that directs the movement. In the protruding membrane, the cytoskeletal actin filaments polymerize to form actin bundles, which subsequently attach to the ECM via adhesive complexes that consist of receptors of β_1 and β_3 integrins, kinases such as RTKs and phosphatidylinositol-3-kinase (PI3K), adaptor proteins and structural molecules [248, 250]. The cell-matrix adhesions become stronger and act as traction forces for the cell to move forward [251]. While the leading edge of the cell moves forward, the actin-myosin contraction at the rear pushes most of the cellular organelles toward the front [252]. Finally, the cell-substrate adhesion at the rear disassembles via the action of proteases, such as MMPs, to retract the membrane for forward movement. Throughout the process, membrane proteins are distributed to the front of migrating cells via exocytosis [253] and then recycled via endocytosis [254]. In addition to single cell migration, cells can migrate in the form of a cluster or sheet. Collective cell migration follows the same steps as single cell migration, except that the traction at the leading edge and the retraction at the rear are now the collaborative work shared among the cells within the cluster.

Type 3 EMT plays an important role in lung cancer progression. During oncogenic EMT, the epithelial cancer cells acquire mesenchymal-like features, becoming motile, invasive and migratory [255]. As the lung cancer progresses, tumor cells migrate and invade to the new site for development. Tumor metastasis is a series of events that begins with tumor cell detachment from the primary site, epithelial-mesenchymal transition, ECM-independent survival, migration out of the primary site and intravasation into new blood

vessels, followed by survival in circulating blood, and finishing with extravasation, migration through the ECM and attachment to distal organs [256, 257].

For tumor cells to detach from the primary sites and acquire motility, it is necessary to break down the cell-cell contacts by down-regulating E-cadherin. Studies have demonstrated that down-regulation of E-cadherin and up-regulation of N-cadherin is the main feature of EMT in lung cancer [258]. These results are consistent with the molecular and morphological changes that are associated with EMT-like phenotypes in human lung cancer cell lines [259]. After the tumor cells obtain the elongated migratory morphology, overproduction and activation of MMPs degrade the ECM and basement membrane [260, 261]. These tumor cells then can escape from the original sites and invade to the neighboring tissues and basement membrane only if they survive from the apoptosis that induced by insufficient interaction between the cells and ECM [262]. Next, the tumor cells intravasate into the blood or lymphatic vessels and circulate in the bloodstream [263, 264]. The circulating tumor cells, once again, are susceptible to apoptosis due to lack of interaction with ECM. Only those surviving cells adhere to the endothelial cells and extravasate into the distant sites to form secondary tumors [257].

1.5.6.2. Evasion of apoptosis

The importance of apoptosis in removing damaged cells and maintaining the integrity of epithelium prevents the lungs from any abnormal activities. Unfortunately, cells can escape from apoptosis that can lead to pre-neoplastic lesions in the lungs over time and such lesions are difficult to detect [265]. As the lesions develop into NSCLC, apoptosis evasion becomes crucial for cancer metastasis and invasion. Tumor cells resist apoptotic death by upregulating anti-apoptotic genes such as Bcl-2, Fas and inhibitor of apoptosis proteins (IAPs), and by

downregulating proapoptotic genes such as p53 and Bax [266-268]. In addition, tumor cells produce excessive amount of MMPs that not only degrade ECM, but also cleave Fas ligands to disrupt the onset of apoptosis in cancer cells [269, 270]. Together, evasion of apoptosis prolongs the survival of tumor cells that is particularly crucial in tumor metastasis and invasion.

The morphological features of apoptosis are characterized by cell shrinkage, nucleus condensation, DNA and cytoskeleton fragmentation, and the subsequent formation of apoptotic bodies before undergoing cell shedding or phagocytosis [271]. Several signals (death ligands, oxidative stress, and radiation) initiate apoptosis through activation of caspases. Cells synthesize inactive caspase zymogens that must undergo cleavage at the aspartic acid residues to become active. The initiator caspase-8 gathers signal from the death receptors (Fas, tumor necrosis factor receptor 1, death receptors 3, 4 and 5) on the cell surface to cleave and activate the effector caspase-3, which then acts at the targeted site in the nucleus and cytosol to execute apoptosis (Figure 8) [271, 272] [273, 274]. Since binding of extracellular signal to the membrane receptors triggers the activation of caspase cascade, this pathway is termed as extrinsic pathway. Alternatively, cell stressors (for example, DNA damage) act directly on cellular organelles, resulting in enhanced mitochondrial membrane permeability followed by mitochondrial release of cytochrome c. By combining with apoptotic protease activating factor 1 (APAF-1), cytochrome c forms a complex that cleaves and activates initiator caspase-9, which in turn, activate the effector caspase-3 to promote apoptosis [275]. Unlike extrinsic pathway, this pathway does not require the cell surface receptors; hence, it is called intrinsic pathway [271].

Although stimulation of extrinsic and intrinsic apoptotic pathways involves different signals, they eventually converge to activate the same effector caspases. The effector

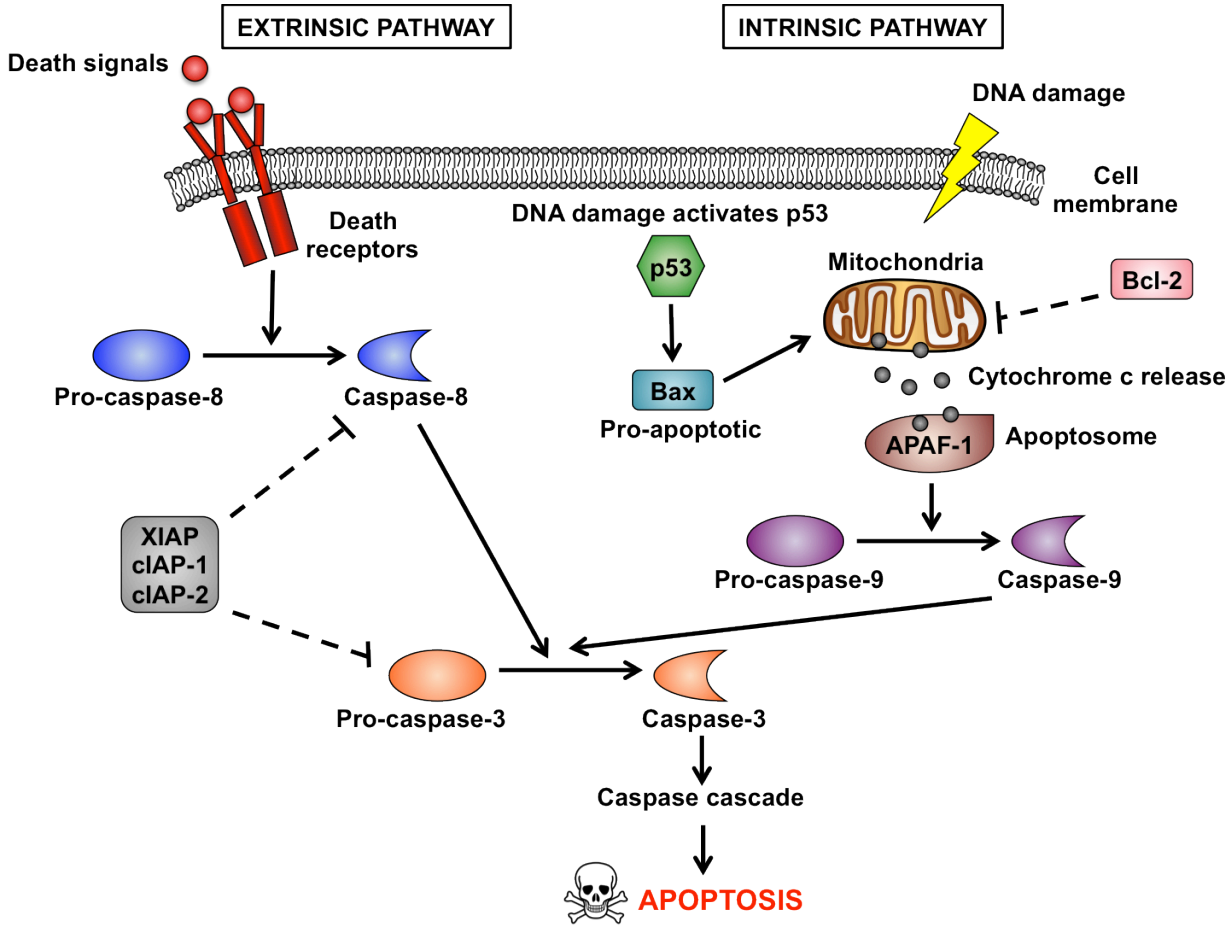


Figure 8. Overview of the two pathways of apoptosis: death receptor (extrinsic) pathway and stress-mediated (intrinsic) pathway.

Apoptosis is activated by the binding of ligands to the death receptors on the cell membrane (extrinsic pathway) or stress such as DNA damage that leads to release of cytochrome c (intrinsic pathway). Binding of death signals to death receptors on the cell membrane leads to the cleavage of pro-caspase-8. The caspase-8 then stimulates the cleavage and activation of caspase-3. On the other hand, stress (e.g. DNA damage) activates p53, which in turn, induces activation of pro-apoptotic proteins (e.g. Bax) and inhibition of anti-apoptotic proteins (e.g. Bcl-2) to stimulate the release of mitochondrial cytochrome c. Presence of cytochrome c leads to the cleavage and activation of caspase-9 which induces the cleavage and activation of caspase-3. Activation of caspase-3 in both extrinsic and intrinsic pathways further stimulates the downstream caspases, resulting in apoptosis.

caspases have been shown to inhibit DNA replication and repair, interfere mRNA splicing, destroy nuclear lamina, condense the chromatin, cleave cytoskeletal proteins, disassemble focal adhesion, and cleave anti-apoptotic proteins that result in apoptosis [276]. Due to the detrimental function of caspases in the cells, both extrinsic and intrinsic apoptotic pathways contain IAPs to tightly regulate the activity of caspases [277]. These IAPs suppress caspase activity by one or several mechanisms that include i) binding to the active site of effector caspases for catalytic reaction, ii) binding to the amino terminus of caspase-9 monomers for caspase-9 dimerization, iii) sequestering mitochondrial proteins for antagonizing other IAPs, and iv) promoting proteasomal degradation and ubiquitylation of procaspases or caspases [278]. Each IAP impedes the activity of its specific caspase targets or interacts with other IAPs to broaden its inhibitory targets. In extrinsic apoptotic pathway, X-linked IAP (XIAP) binds to caspase-3 and -9 directly to impede their activities [279, 280] whereas cellular IAP-1 (cIAP-1) and cIAP-2 interact with other IAPs to inhibit caspase-3 activity [281] but not caspase-9 (Figure 8) [279]. Unlike the IAPs in extrinsic pathway that directly hinder the activity of pro-caspases or caspases, the intrinsic apoptotic pathway contains Bcl-2 family proteins that regulate the release of cytochrome c from mitochondria which indirectly controls the activation of caspases (Figure 8) [282]. Bcl-2 protein family is composed of anti-apoptotic proteins such as Bcl-2, and pro-apoptotic proteins such as Bax [283]. Interestingly, Bcl-2 forms a heterodimer with Bax to prevent the homodimerization of Bax that requires for prompting cell death [284, 285]. In addition, the upstream regulator, tumor suppressor gene p53, decreases gene expression of Bcl-2 while concurrently increases gene expression of Bax to induce apoptosis [286]. Since anti-apoptotic and pro-apoptotic proteins work against each other, the balance between them will determine the cellular susceptibility to the stressors [287].

Apoptosis is seen as a suicide mechanism to remove severely damaged cells. Its beneficial role in balancing cell proliferation and death to maintain epithelial homeostasis is often underestimated. Upon challenge imposed by inhaled substances such as bacteria and cigarette smoke, the challenge triggers the release of inflammatory cytokines and chemokines including IL-6 and IL-8 from airway epithelium [288, 289]. These cytokines and chemokines then stimulate the synthesis of mucin, which in turn, induces the proliferating and pre-existing cells to differentiate into mucus cells [290], resulting in goblet cell metaplasia and hyperplasia at the airway epithelium [291]. After removal of the invading substances, the number of mucus cells will be reduced by apoptosis [292, 293]. Even though the mucus cells are not damaged, apoptosis needs to remove excessive mucus cells to resume the normal cellular composition of the epithelium and epithelial homeostasis. In addition to remove excessive number of cells, apoptosis acts as negative signal in cell-cycle progression to control cell growth and cell division.

1.5.6.3. Uncontrolled cell proliferation

Massive cell growth commonly seen in lung cancer cannot solely be explained by the evasion of apoptosis; instead, cell proliferation is the main driver toward rapid tumor growth. Cell proliferation is triggered by mitogenic activation of RTK signaling pathways. Binding of growth signals, such as epidermal growth factor (EGF) and insulin growth factor (IGF), to their tyrosine kinase receptors on the membrane activates the downstream signaling cascade to stimulate cell division [54, 294, 295]. Cell division is composed of 4 sequential stages, gap 1 (G_1), synthesis (S), gap 2 (G_2) and mitosis (M) [296]. Cells progress through different phases of cells cycle through the guidance of cyclin-dependent kinases (CDKs) and cyclins. CDKs are present all time in the cell cycle where cyclins are only synthesized and degraded

at particular times in the cell cycle. The temporal regulation of cyclin-CDK complexes ensures successful completion of one phase before entering into the next phase of cell cycle [297]. Once cyclins are produced, they bind to CDKs to form multimeric complexes which then stimulate a series of phosphorylation or dephosphorylation at appropriate times [297]. Because of the temporal regulation, different types of cyclins regulate different phase of cell cycle.

In response to the extracellular growth signals, cell produces cyclin D which binds to CDK4 and CDK6 to activate the G_1 phase of cell cycle [298]. The cell is growing and synthesizing RNAs and proteins to prepare for DNA replication. At the same time, the cell also synthesizes cyclin E to form a complex with CDK2 to further trigger the irreversible expression of genes for transitioning into S phase [299]. Before entering the S phase, the cell undergoes major regulatory checkpoint (G_1 checkpoint) to inspect for size, nutrients, growth factors and intact DNA that are available for DNA replication. If the cell fails to meet the criteria, it will produce CDK inhibitors to inhibit CDK4, CDK6 and CDK2 to arrest the cell cycle at the G_0 phase for repair. In addition, p53 level is also increased to trigger apoptosis. If the cell meets the criteria, it will then enter into S phase. During the S phase, cell generates cyclin A that binds to CDK2 to initiate DNA replication [300]. Following the completion of DNA replication, cell passes into G_2 phase where it continues to grow, synthesize proteins and form cyclin B-CDK1 complex [298]. Prior to the entry into M phase, the cell undertakes a G_2 checkpoint to check for any incomplete DNA replication. Detection of any DNA damage blocks the activation of cyclin B-CDK1 complex that put the cell cycle at a resting phase. As the cell advances to M phase, chromosomes begin to separate by going through prophase, metaphase, anaphase and telophase. A checkpoint at metaphase inspects for proper attachment of kinetochore to spindle fibers to ensure correct chromosome separation. Finally,

the cell cytoplasm splits and forms 2 daughter cells, a process known as cytokinesis.

The physical splitting of a cell requires an increase in membrane surface area to encompass the cytoplasm of 2 daughter cells. For the division to occur, the cell marks the division site by forming a contractile ring [301]. The actomyosin interactions cause the contractile ring to constrict and form a cleavage furrow [302]. At this stage, the cytoplasm of the cells remains connected through an intercellular bridge. The cells then recruit the necessary abscission proteins for the final cleavage of intercellular bridge to form 2 individual daughter cells [303]. Growing evidence indicate that membrane trafficking and remodeling appear at the furrow and abscission sites [304-306]. At the furrow site, secretory vesicles tether and fuse with cell membrane driven by SNAREs to insert new membrane, aiming to increase the membrane surface area that is enough to fully wrap around the cellular contents for two cells [307, 308]. At the abscission site, vesicle trafficking and recycling are also required to recruit proteins for abscission machinery [309, 310]. Thus, membrane trafficking and remodeling are important events for cytokinesis.

In lung cancer, abnormal expression of cyclins, CDKs, or CDK inhibitors accelerates cell-cycle progression and bypasses the cell-cycle checkpoints that result in uncontrolled cell proliferation [300]. NSCLC cells overexpress cyclin D, CDK4 and cyclin E to facilitate the transition of tumor cells into G_1 phase and S phase of cell cycle [311, 312]. At the same time, down-regulation of CDK inhibitors and apoptotic p53 prevents NSCLC cells from entering G_0 phase at the first cell cycle checkpoint [313]. After bypassing the checkpoint, NSCLC cells amplify the cyclin A protein expression to further assist the cells in advancing through the S phase [314, 315]. High levels of cyclin B and CDK1 are also reported in NSCLC cells to expedite the cell cycle progression to G_2 and M phases [316-319]. However, tumor cells are not only capable to alter the expression of cell cycle regulators, but also change the

expression of mitotic proteins that favor cell division. One of the mitotic proteins, dynamin-2, is upregulated in human pancreatic and prostate cancers to facilitate the enhanced demand of membrane-trafficking events during cancer growth [320, 321]. Dynamin-2 has been reported to play an important role in cytokinesis as inhibition of dynamin-2 blocks the tumor cells from undergoing abscission process of cytokinesis, leading to apoptosis [322, 323]. Taken together, tumor cells are capable to alter the expression of cell cycle regulators and mitotic proteins to support the rapid tumor growth.

1.5.6.4. Dysregulation of RTK-mediated PI3K/Akt signaling pathway

NSCLC is a diverse and heterogeneous disease that involves dysregulation of cell adhesion and EMT, evasion of apoptosis, and uncontrolled cell proliferation. These abnormal cellular events are often resulted from hyperactivation of RTK-mediated signaling pathways in cancer cells [324-329]. Currently, more than 60 RTKs have been identified, but all of them share similar structure, in which each of them consists of an extracellular binding domain, a transmembrane domain and an intracellular tyrosine kinase domain [330]. Upon binding of growth factors on the extracellular domain of RTKs, the RTKs form homodimers and/or heterodimers to activate the receptors [331]. Activation of the receptors then triggers the autophosphorylation of cytoplasmic tyrosine kinase domain and creates the docking sites for adaptor proteins and kinases to initiate the downstream signaling pathways, including PI3K/Akt, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase, and signal transducer and activator of transcription pathways [329, 332]. The PI3K/Akt signaling pathway is a crucial molecular pathway that regulates cell survival, growth, proliferation, migration, invasion, adhesion and vesicle trafficking [328, 333]. Study has also demonstrated that PI3K/Akt signaling pathway is associated with the resistance to chemotherapy and

radiation therapy in NSCLC patients [334]. Thus, any modifications on this signaling pathway may result in unwanted outcome. Normally, every single step of signaling transduction is strictly monitored, however, genetic alterations in RTKs and components of the PI3K/Akt signaling pathways lead to excessive signaling output that fosters lung cancer tumorigenesis [328, 330].

Among the RTKs, genetic alterations in epidermal growth factor receptor family proteins, EGFR and HER-2, and IGF1R, have been identified in NSCLC [335, 336]. EGFR mutations are the first molecular alterations found in NSCLC [335, 336]. Binding of ligands, EGF or transforming growth factor alpha (TGFα), to the EGFR, stimulates the homodimerization and heterodimerization with other RTKs to activate the receptors and trigger the autophosphorylation of cytoplasmic tyrosine kinase domain. Mutations at the intracellular tyrosine kinase domain of EGFR frequently cause enhanced activity of tyrosine kinase and result in hyperactivation of downstream PI3K/Akt signaling pathways [337]. Other mutations, such as upregulation of EGFR expression, amplification of chromosomal region 7p12 where EGFR gene situated, and unusual frequent expression of EGFR ligands, also lead to hyperactivity of EGFR in NSCLC [338-341]. EGFR mutations are localized in both normal and cancerous respiratory epithelium within the tumor, but not those tissues that are distant from the tumor, indicating the importance of mutated EGFR in tumor growth [324]. Interestingly, approximately 30% of lung adenocarcinoma cases possess EGFR mutations while only 2% of other NSCLC types (squamous cell carcinomas and large cell carcinomas) have EGFR mutations [342]. Unlike EGFR, mutations of another human EGFR protein, HER-2, are exclusively found in lung adenocarcinoma [343]. In addition, amplification of HER-2 gene and overexpression of HER-2 are predominately observed in lung adenocarcinoma and less frequently in squamous cell carcinomas and large cell

carcinomas [344]. Although the role of HER-2 in cancer is not fully understood, overexpression of HER-2 has been shown to drive the development of lung adenocarcinoma in transgenic mice [345]. Overexpression of HER-2 allows excessive receptor activation through homogenous dimerization of HER-2 monomers or through heterogenous dimerization of HER-2 monomer and another RTK monomer without the presence of ligands [343]. Once HER-2 is activated, it triggers the downstream PI3K/Akt signaling pathway to promote tumor cell survival, proliferation, and metastasis in lung cancer [346].

Another RTK that has been associated with NSCLC is IGF1R. Similar to EGFR, binding of extracellular ligand, in this case, insulin growth factor 1 (IGF1), to the extracellular domain of IGF1R activates the intracellular tyrosine kinase domain and initiates the PI3K/Akt signaling transduction pathway [347]. Patients with NSCLC show a significant increase in the expression of IGF1R [348] and such increase is more common in squamous cell carcinomas than in adenocarcinomas [349, 350]. Furthermore, some NSCLC patients have elevated plasma IGF levels [348]. The increased IGF1R expression and plasma IGF1 levels result in hyperactivation of IGF1R signaling pathway that supports tumorigenesis in NSCLC [348, 351].

The increased activity of RTKs either through overexpression of growth signals or activating mutations and/or amplification of receptors leads to constitutive stimulation of PI3K/Akt signaling [328, 333]. Activation of RTKs recruits PI3K to the cell membrane and phosphorylates phosphatidylinositol lipid substrates, which activate Akt to promote cell proliferation, survival, adhesion, motility and vesicle trafficking [328, 333, 352]. In lung cancer, the PI3K/Akt signaling pathway is frequently dysregulated due to genetic amplification and activating mutations in PI3K as well as activating mutations and overexpression of Akt [328]. Evidence shows that the number of PIK3CA gene encoding the

p110α catalytic subunits of PI3K is increased in squamous cell carcinoma and adenocarcinoma types of NSCLC [353]. Studies have identified the region that frequently amplified in lung cancers is located at chromosome 3q25-27 (PIK3CA is located at chromosome 3q26) [353, 354]. In addition, activating mutations are also found in PIK3CA gene in NSCLC [355]. Both genetic amplification and activating mutations augment the enzymatic activity of PI3K that activates Akt without the presence of growth factors [331]. Furthermore, overexpression of Akt is also found in 19-32% of squamous cell carcinoma and 12-16% of adenocarcinoma of NSCLC patients while genetic mutations of Akt are only found in 1% of NSCLC patients [356, 357]. Since Akt-mediated activation has been shown to be the driver of the lung tumor cell proliferation, survival, and metastasis, the ultimate outcome of Akt overexpression or oncogenic mutation is to facilitate lung cancer growth.

1.6. Current treatments for NSCLC

The classic cancer treatment, including resection, platinum-based adjuvant chemotherapy and radiotherapy, is often the therapeutic options received by lung cancer patients depending on their tumor stage. The heterogeneity of NSCLC cells often gives rise to the resistance to these conventional cancer therapies. In recent years, personalized treatment has become the popular choice in NSCLC patients such that treatments will be given to patients based on the presence of specific tumor markers and treatment response to maximize the therapeutic index and cost-effectiveness. A class of cancer therapeutics called tyrosine kinase inhibitors (TKIs) has been developed to reversibly bind to the adenosine triphosphate-binding cleft of the cytoplasmic kinase domain and ultimately block the activity of RTKs in NSCLC [358]. Currently, TKIs such as gefitinib and erlotinib that block EGFR are already approved to use in a group of NSCLC patients who have activation mutation in EGFR [359, 360]. Although

NSCLC patients initially respond to the drugs, they eventually develop acquired resistance within 2 years of treatment [361-363]. Patients developing resistance to TKIs are either due to the presence of secondary mutations in the targeted RTK which abolishes the inhibitory effect of the TKIs, or due to the mutational activation of signaling components that is downstream of targeted RTK [329]. Because of this, researchers turn their focus on the downstream signaling components, such as PI3K and Akt, as new therapeutic targets for NSCLC. Although inhibitors of PI3K and Akt have promising anti-tumor effects in NSCLC patients in the early stage of clinical trials, further clinical evaluations are needed before they can get approval to use.

The downfall of TKIs indicates that blocking the activation of RTKs is not an effective mechanism to slow down the lung cancer growth. Instead of inhibiting the activity of the RTKs on the membrane surface, reducing the number of RTKs expressed on the membrane surface may be more efficient in slowing down the tumor growth. Aberrant expression and activity of RTKs are frequently observed in NSCLC and contribute to cancer pathogenesis. Since translocation of RTKs from cytosol to the plasma membrane employs protein machinery to traffic vesicles that carry RTKs as protein cargo, the novel idea of modifying the vesicle trafficking machinery may reduce the expression of RTKs on membrane surface. Furthermore, vesicle trafficking also involves in directing intracellular and soluble cargo proteins to their destination that is important for membrane remodeling during cytokinesis [322, 323]. For example, inhibition of dynamin-2 blocks the abscission process of cytokinesis and causes tumor cells to undergo apoptosis [322, 323]. Thus, many studies turn their focus on disrupting the vesicle trafficking machinery as an alternative approach for developing therapeutics to target cancer growth.

Increasing evidence has shown that myoferlin plays a role in regulating receptor expression, cell migration and invasion in breast and pancreatic cancers [190-193, 364], it is possible that ferlin proteins including myoferlin and dysferlin may also have an important role in lung cancer growth. The notion that epithelial membrane trafficking alone is responsible for the development of lung cancer offers new therapeutic options. Future drugs that target on blocking myoferlin and dysferlin function would thus be expected to be beneficial for slowing lung cancer growth and metastasis.

2. Rationale, Hypothesis and Aims

The identification of myoferlin and dysferlin in mediating vesicles trafficking and membrane fusion events in skeletal muscles signifies the importance of these proteins in repairing the "wear and tear" damages occurred in normal physiological activities. Skeletal muscles are not the only tissues susceptible to stress and damages, other tissues such as lung epithelium and vascular endothelium also undergo continuous stress and damages.

Traces of evidence indicate the expression of myoferlin and dysferlin in lung tissues. However, no research has followed up to characterize the functional roles of these ferlins in the lung. Similar to skeletal muscles and vascular endothelium, the lung epithelium is also susceptible to damages caused by inhaled chemicals, pathogens and toxins, which ultimately, lead to the onset of lung diseases, such as cancer. Knowledge of ferlins in the development of lung cancer is very limited, but a gene microarray study demonstrates the upregulation of myoferlin gene in human lung cancer tissues [188, 189]. These findings set up the platform for our laboratory to extend the ferlin research to the normal and neoplastic lung epithelium and tissues.

My overarching hypothesis is that myoferlin and dysferlin have distinct functional roles in normal and neoplastic epithelial cells of the lung. To address my hypothesis, I propose following specific aims:

1. *To examine the expression of myoferlin within the human airway and its role in airway epithelial integrity.* Previous study showed that myoferlin expression is found in lung tissue homogenates [128]; however, they did not characterize the presence of myoferlin in airway epithelium. In addition, the role of myoferlin in maintaining

airway epithelial integrity had not been investigated. The sub-hypothesis in this Aim is that down-regulation of myoferlin in airway epithelial cells leads to abnormal epithelial homeostasis through decreased expression of tight junction molecule, ZO-1, and increased cell detachment and apoptosis.

2. *To investigate the expression of myoferlin in mouse lung cancer cells as well as mouse and human lung tumor tissues.* No study had shown the myoferlin expression in lung tumor although gene microarray study demonstrated the up-regulation of myoferlin gene in SCLC.

   2.1. *To examine the role of myoferlin in proliferation of mouse lung tumor cells and solid lung tumor growth as well as membrane repair*. Myoferlin has been known to mediate membrane-fusion events and membrane trafficking events in skeletal muscles and vascular endothelial cells [172, 174-177, 182-184], its role in highly proliferating tumor cells was never investigated. Although gene microarray studies demonstrated the up-regulation of myoferlin gene in human lung cancer [188], nothing was known about the functional role of myoferlin in lung cancer. The sub-hypothesis in this Aim is that knockdown of myoferlin decreases mouse lung tumor cell proliferation and membrane repair *in vitro* and solid tumor growth *in vivo*.

3. *To identify the molecular mechanisms mediated by myoferlin to regulate human cancer proliferation, migration and invasion.* Our sub-hypothesis is that myoferlin mediates the signaling pathways that are important for tumor metastasis.

3. Methodology

3.1. Subjects and ethics statement

Normal human donor lungs not suitable for transplantation and therefore donated for medical research were obtained with written informed consent through the International Institute for the Advancement of Medicine (Edison, New Jersey). Human tumor sections were approved to use for this study by oversight committee at St. Paul's Hospital James Hogg Research Centre Biobank along with patient consent. Both studies were approved by Research Ethics Board of all institutions that include University of British Columbia (certificate number H0-50110).

3.2. In vitro assays

3.2.1. Isolation of human airway epithelial cells and lung fibroblasts

Primary airway epithelial cells were extracted via protease digestion of human airways as previously described [58, 365]. Briefly, bronchi to the 3rd generation were dissected and then rinsed with cold phosphate buffer saline (PBS) without calcium and magnesium (Thermo Scientific) three times to remove blood and mucus. Epithelial layers on intact segments of bronchi (2-4 cm in length) were dissociated at 4 ^{o}C for 16 hours (h) in 100 mL of minimum essential media (MEM, Fisher Scientific) containing 1.4 mg/mL Pronase and 0.1 mg/mL of DNase (Roche Diagnostics). Dissociated epithelial cells were strained through a 70 μm nylon mesh (BD Biosciences). Cells were then re-suspended and incubated in MEM supplemented with 10% fetal bovine serum (FBS, Life Technologies) for 10 minutes (mins) and washed twice with MEM by centrifugation at 4 ^{o}C to neutralize the pronase. Cells were then seeded

in tissue culture flasks and incubated at 37 ^{o}C in a humidified 5% carbon dioxide (CO_2) atmosphere with bronchial epithelial growth media (Cambrex) until confluent monolayers were formed.

Primary human bronchial and parenchymal fibroblasts were established from the respective bronchial rings and pleura-free lung parenchyma as previously described [366]. Briefly, bronchial and parenchymal tissues from the same lung were dissected into 2 mm^3 size explants. Four to five explants were placed into the well of 6-well tissue culture plates (BD Biosciences) with 0.75 mL of Dulbecco's modified Eagle's medium (DMEM, Life Technologies), containing 10% FBS, 2 mM L-glutamine and 1% antibiotic/antimycotic solution (Life Technologies), and incubated at 37^{o}C with 95% air and 5% CO_2. Media was replaced every other day to remove tissue debris and non-adherent cells. After seven days, the tissue explants were removed and the outgrowth of fibroblasts were maintained until a confluent monolayer was formed. Total protein from both primary epithelial cell and fibroblast cultures was extracted as described under the immunoblot procedures below.

3.2.2. Cell lines and culture condition

The bovine aortic endothelial cells (BAEC) and human fetal lung fibroblast (HFL) were purchased from American Type Culture Collection (ATCC). The well described 16HBE14o-SV40-transformed human bronchial epithelial (16HBE) cell line was obtained from Dr. D. Gruenert (University of Vermont) [367]. Mouse Lewis lung carcinoma (LLC) cell line was a kind gift from Dr. William Sessa (Yale University). Human lung adenocarcinoma cell line A549 was generously given by Dr. Tillie Hackett (University of British Columbia). All cell lines were individually grown in 100 mm x 25 mm cell culture dishes (Corning) in DMEM growth media supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL

streptomycin (Sigma-Aldrich), and incubated at 37 ^{o}C in a humidified 5% CO_2 atmosphere. Human cell lines used in this study were approved by the Providence Health Care Research Ethics Committee of the University of British Columbia; Certificate number H0-50110-1A.

3.2.3. Cell transfection with or without siRNA

Cells were grown to 40% confluency on 6-well tissue culture plates (BD Biosciences) in DMEM growth media with either 5% or 10% FBS depending on the cell lines. The day before treatment, growth media was replaced with serum-free DMEM to synchronize cell cycle. Then, cells were treated with transfection media (containing 800 µL of Opti-Mem-1 media with 5 µL of Oligofectamine, Life Technologies), non-silencing scrambled control siRNA, dysferlin siRNA for both human and mouse (5'-CTCCCTGTTTGCGGCCTTCTA-3') or myoferlin siRNA for both human and mouse (5'-AACCCTGTCTGGAATGAGATT-3') in transfection media at appropriate concentrations for 8 h in the incubator (37^{o}C with 5% CO_2). Then, transfection media were removed and replaced with fresh 5% or 10% FBS-containing DMEM growth media and allowed to grow for 48 h for cell proliferation and 72 h before performing subsequent Western blot analysis, cell detachment assay and apoptosis assays. The knockdown efficiency of the siRNA was first evaluated with Western blot analysis before performing other assays. Both myoferlin and dysferlin siRNA sequences were checked with BLAST search to ensure 100% homology to specifically target myoferlin and to specifically target dysferlin, respectively.

3.2.4. Western blot analysis for protein lysates collected from cell cultures

Proteins were extracted from cell monolayers using protein extraction buffer with protease

and phosphatase inhibitor cocktails (Sigma). The total protein concentration of each cell lysate was determined using the DC protein assay as instructed by the manufacturer (BioRad). Equal amounts of protein lysates (80 μg) were loaded to resolve the proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then transferred to nitrocellulose membranes. The primary antibodies used for immunoblot were dysferlin (NCL-Hamlet, Novocastra), myoferlin (ab76746, Abcam), E-cadherin (sc-8426, Santa Cruz), caveolin-1 (sc-894, Santa Cruz), β-tubulin (16-231, Upstate), VEGFR-2 (sc-504, Santa Cruz), ZO-1 (ab59720, Abcam), claudin-1 (37-4900, Life Technologies Corporation), occludin (71-1500, Life Technologies Corporation), fibronectin-EDA (MAB1940, Chemicon International, Temecula, CA), and heat shock protein 90 (HSP90; 610418, BD Biosciences). Detection was performed with IR700 anti-mouse and IR800 anti-rabbit antibodies (Cell Signaling Technology) and the Odyssey Infrared Imaging System (LI-COR Biotechnology) using the manufacturer's protocol. Band densitometry was analyzed with Odyssey software 1.1 (LI-COR Biotechnology) using two infrared channels independently. The results are expressed as the density ratio of protein of interest relative to the loading controls (β-tubulin or HSP90).

3.2.5. Cell proliferation assays

After cells were transfected with or without non-silencing scrambled control siRNA, dysferlin siRNA or myoferlin siRNA, cells were trypsinized and counted before seeding designated number of cells (2.5×10^3 cells for LLC and 2.5×10^4 cells for A549) into 24-well tissue culture plates. Following serum starvation to synchronize cell cycle, cells were trypsinized and counted at 0, 24, 48 and 72 h timepoints using hemocytometer.

3.2.6. Cell detachment and apoptosis assays

Spent cell culture media for each treatment condition was collected to determine the number of detached and dead cells using trypan blue (Life Technologies) staining and a hemocytometer. To quantify the percentage of cells undergoing apoptosis, we used fluorescein isothiocyanate annexin V and propidium iodide apoptosis detection kit I as per manufacturer's instructions (BD Pharmingen) and cells were analyzed immediately by flow cytometry using a BD LSRII machine (BD Biosciences) and flow cytometry analysis was performed using DiVa software (BD Biosciences).

3.2.7. Immunofluorescent staining and confocal microscopy

16HBE cells were seeded on chamber slides (BD Biosciences), washed in PBS (Sigma-Aldrich) pH 7.4, fixed in 4% paraformaldehyde (Fisher Scientific) for 20 mins and blocked with 10% goat serum (Life Technologies) for 20 mins at room temperature (RT). Cells were stained with either 1:40 dysferlin (HAMLET-CE, Leica Biosystems) or 1:500 myoferlin (ab76746, Abcam) and 1:200 ZO-1 (ab59720, Abcam) or 1:200 GM130 (610822, BD Biosciences) antibodies in PBS with 0.1% saponin overnight at 4 ^{o}C. After removing the primary antibodies and washing the cells 3 times with PBS at RT, a secondary antibody conjugated with 10 µg/mL goat anti-mouse IgG Alexa Fluor 594 (A-11005, Life Technologies) was added and incubated for 2 h at RT. Cells were washed again with PBS and then incubated with 1 µg/mL of 4,6-diamidino-2-phenylindole (DAPI) (D1306, Life Technologies) before visualizing with a Nikon fluorescent microscope incorporated with a C-spot camera (Nikon Instruments). Fluorescent images were captured using a Leica AOBS SP2 laser scanning confocal microscope (Leica Biosystems) with Zeiss LSM 510 software

(version 3.2) and analyzed with Volocity software (Improvision).

3.2.8. Membrane injury assays

After transfecting with or without siRNA, cells were incubated with membrane marker, FM1-43 dye (25 μM, T-35356, Life Technologies), for 5 mins in glass bottom culture plates containing 5% FBS/DMEM. Plates were mounted on a heated Plexiglas chamber (Zeiss) at 37 °C, 5% CO_2 and 95% humidity. Membrane damaged was induced by a full power argon laser on a 5 μm x 5 μm area away from the nucleus. Images were captured before the damage and every 30 seconds after damage for duration of 10 mins using an Olympus FV1000 confocal microscope. The intensity of FM1-43 fluorescence was measured using the Zeiss LSM software.

3.2.9. Caspase-8 activity assays

Following 48 h of transfection, LLC cell lysates were collected for caspase-8 activity using colorimeter assay kit (R&D Systems) according to the manufacturer's manual. Briefly, LLC cells were lysed and caspase-specific peptide conjugated with color reporter molecule p-nitroaniline (pNA) was added to the lysates to test for protease activity. The conjugated peptide was cleaved by the presence of caspase in the lysates to release the chromophore pNA and can be detected at 405 nm using spectrophotometer.

3.2.10. Scratch assays

Human lung tumor cells (A549) were grown to 50% confluency in a 24-well plate with DMEM growth media containing 10% FBS, 100 U/mL penicillin and 100 μg streptomycin.

Cells were starved in serum-free DMEM growth media overnight prior to treatment. Then, cells were treated with transfection media (200 μL of Opti-Mem media with 2 μL of Oligofectamine), non-silencing siRNA or human myoferlin siRNA in transfection media at appropriate concentrations for 8 h in an incubator (37 ^{o}C with 5% CO_2). Then, transfection media was removed and replaced with fresh DMEM growth media. Cells were grown to 100% confluency for 48 h before overnight starvation in serum-free DMEM growth media to synchronize the cell cycle. On the next day, a cross sign scratch was made on the cell monolayer using a sterile yellow plastic pipette tip in each well. Cell monolayers were then washed with DPBS twice to remove the detached cells and fresh serum-containing DMEM growth media was added for the subsequent incubation. Wound healing in each treatment was followed over 3 days and the pictures were taken at 0-, 24-, 48- and 72-h time points. By using Adobe Photoshop, the captured images were segmented by coloring the cell-occupied regions in black and the cell-free regions in white. The segmented regions were then quantified by ImagePro and expressed the relative cell-occupied areas at 24-, 48-, and 72-h to the area at 0 h in percentage.

3.2.11. Cell migration and invasion assays

Tumor cell migration was performed using 24-well Transwell inserts (BD Biosciences, Bedford, MA) containing membrane filters with 8-μm pores. Cells were first treated with growth media, transfection reagent, scrambled and myoferlin siRNA for 48 h in normal DMEM media containing 10% FBS. Then, cells were serum starved overnight. Following trypsinization, cells were resuspended in serum free media and seeded at 5 x 10^4 cells per insert in 200 μL on the upper side of the membrane filter. Cells were allowed to migrate for

24 h at 37 °C. After 24 h of incubation, media was pipetted out and then cotton swabs were used to carefully remove the cells on the upper side of the membrane filters. Cells on the lower side of the membrane filters, on the other hand, were fixed with 3.7% paraformaldehyde in PBS (Sigma-Aldrich) for 30 mins and washed with PBS twice. Following fixation, cells were stained with 0.5% crystal violet for 10 mins and then washed with tap water twice. Membrane filters were allowed to dry overnight before carefully cutting out the filters and mounting on microscope slides. The slides were scanned with an Aperio Scancope XT (Leica Microsystems, Wetzlar) at 40X magnification and images were captured with Aperio ImageScope software. Cells within the eight randomly selected fields on the images were counted and expressed in percentage relative to scrambled siRNA control cells. Experiments were repeated 5 times per condition.

Invasion assay was performed the same way except that the transwell inserts were coated with 50 μL of 50% Matrigel (BD Biosciences) diluted 1:1 with serum free media. The coated inserts were allowed to gel at RT for 2 h. Then, 750 μL of DMEM containing 10% of FBS was added on the bottom wells for overnight incubation. Also, cells were incubated for 40 h before fixed with 3.7% paraformaldehyde.

3.3. In vivo assays

3.3.1. Tumor implantation and growth

Animal experiments were approved by the UBC Animal Care Committee under protocols A12-0136. LLC were cultured in low glucose DMEM with 5% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. Tumor cells were grown to 80% confluence, trypsinized, resuspended in serum free DMEM and subcutaneously injected on the back of 8-

week-old congenic C57BL/6 mice (JAX). After tumors reached a palpable size (approximately week 2), mice were randomized and injected with siRNA/DOTAP mixture (intratumoral; 100 μL) every 3 days. Tumor volume was determined by using a caliper and applying the formula volume (in mm^3) = $width^2$ (in mm^2) x length (in m) x 0.5 to approximate the volume of a spheroid. The experiments were stopped when two animals reached a tumor size of 2500 mm^3.

3.4. Ex vivo assays

3.4.1. Human lung tissue processing and immunohistochemical staining

Surgically removed human lung specimens from 5 different subjects were washed in Custodiol histidine-tryptophan-ketoglutarate solution (Odyssey Pharmaceuticals Inc.) and transported on ice. Each specimen was inflated with Cryomatrix embedding resin (Thermo Scientific) and then cut out the airway sections with 5-8 mm. Donor airway sections (5 μm) were deparaffinized, rehydrated and antigens were retrieved by autoclaving the sections in citrate target retrieval solution (Dako) for 15 mins at 120 ^oC and 30 per square inch (psi). Endogenous peroxidase was quenched with 3% hydrogen peroxide for 20 mins and non-specific binding was blocked with 10% horse serum. Slides were incubated overnight with antibodies against human dysferlin or myoferlin at 4 ^oC in 5% horse serum. Subsequent to three washes in Tris-buffered saline (TBS) (Sigma-Aldrich), sections were then incubated with horse anti-mouse secondary antibody (1:100, Vector Labs) for 2 h, followed by incubation with streptavidin-horse radish peroxidase (Dako) for 20 mins. Staining was visualized using brown chromogen 3,3-diaminobenzidine (Dako) and counterstained with hematoxylin (Sigma-Aldrich). Slides were then dehydrated and mounted with Cytoseal 60

(Richard-Allan Scientific). Negative controls were stained in parallel with mouse IgG isotype control (sc-2025, Santa Cruz) or by omitting the primary antibody.

Human carcinoma sections were from females, approximately 65 years of age. The lobe of the lung with the tumor from each female was removed by surgery, and samples of the tumor and non-tumor tissues were taken and fixed by inflation with formalin. Blocks of fixed samples were randomly selected for paraffin embedding and stored at RT. Immunohistochemical staining was performed as described above.

3.4.2. Isolation of xenograft solid tumor and immunohistochemical staining

At the end of tumor growth, mice were euthanized by CO_2 asphyxiation to harvest tumors. Isolated tumors were then cut into three portions (for paraffin, cryo-resin and Western blot applications). The Western blot sections were snap frozen in liquid nitrogen, whereas the paraffin and cyro-resin sections were fixed in 10% formalin for 10 h and transferred to 70% ethanol overnight, followed by embedding. The embedded tissues were sectioned for hematoxylin and eosin staining. For immunohistochemical staining, 3% paraformaldehyde-fixed tumor tissues were frozen in embedding resin (OCT) after overnight dehydration in 30% sucrose in PBS. Sections (6 μm thick) were stained for myoferlin (Atlas Antibodies, Sigma-Aldrich), PECAM-1 (553370, BD Biosciences), Ki-67 (sc-7846, Santa Cruz Biotechnology) and smooth muscle actin (sc-53142, Santa Cruz Biotechnology) were visualized using a Vectastain ABC kit (Vector laboratories), followed by incubation with NovaRed substrate (Vector laboratories).

3.4.3. Apoptosis assays

Cell apoptosis was determined using a Chemicon ApopTag Plus peroxidase *in situ* detection kit (Millipore) and paraformaldehyde-fixed, paraffin-embedded tumor sections (15 μm thick) according to the manufacturer's instructions. Sections were treated with citrate buffer for antigen retrieval and mounted using Vectastain mounting medium (Vector Laboratories). Visualization and quantification were performed on five separate areas per section, with two sections per tumor, and averaged.

3.4.4. Serum vascular endothelial growth factor (VEGF) levels

Serum levels of VEGF 120 and VEGF 164 were quantified using a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Samples were collected through the saphenous vein; blood samples were allowed to clot for 2 h at room temperature before centrifugation for 20 mins at 2000 x g and storage at -20 °C or lower.

3.4.5. Western blot analysis for proteins collected from solid tumor

Individual frozen solid tumor sections were wrapped in aluminum foil and hammered into small pieces before protein extraction with SDS. Protein concentration of each sample was determined with protein assay. Equal amount of extracted protein samples was loaded for SDS-PAGE and transferred to nitrocellulose membrane. Primary antibodies against myoferlin and HSP90 were used. Detection was performed as described previously in section 3.2.4.

3.5. Statistical analysis

Quantitative data are presented as mean ± standard error of mean (SEM) of at least three independent experiments. One-way or two-way analyses of variance with Bonferroni's post-hoc correction were used to compare the treatment groups. Student's t test was used in experiments that contained only two treatment groups. P values of less than 0.05 were considered statistically significant. All statistical analyses were performed using Prism Version 5.0 software (Graphpad Software Inc.).

4. Expression of Myoferlin in Human Airway Epithelium and Its Role in Cell Adhesion and Zonula Occludens-1 Expression[1]

4.1. Introduction

The airway epithelium forms a continuous, highly regulated physical barrier, which lines the airway lumen, separating the underlying tissue from inhaled environmental antigens. Intercellular epithelial junctions form the structural adhesive forces that maintain the airway epithelial barrier, and are comprised of TJs, AJs and desmosomes. AJs mechanically connect adjacent cells and initiate the proliferation and differentiation of cell-cell contacts through homotypic transmembrane E-cadherin adhesions, which are anchored to the actin cytoskeleton and microtubule network by p120 catenin, β-catenin, and α-catenin. TJs are considered the main regulators of paracellular permeability and movement of ions and solutes between cells and are composed of transmembrane proteins such as JAMs, occludin, and claudins 1-20 which anchor to the cytoskeleton by ZO-1, -2, -3 or cingulin. Desmosomes consist of non-classical cadherins that form adhesive bonds between the filament cytoskeleton of epithelial cells, and the lamina propria.

Fusion of vesicles containing adhesion molecules to the plasma membrane is essential for the formation of selectively permeable membrane barriers. As there is an energy barrier to the fusion process, membrane-fusion events generally require molecules that tether and dock membranes into close proximity of one another (<5-10 nm). Lipid vesicle fusion involves numerous factors, including ferlins, SNAREs and synaptotagmins, to facilitate the

[1] A version of figures and text has been published as a research article in *PLoS One*. 2012; 7(7): e40478.

fusion of membrane vesicles [116, 126, 368, 369]. In humans (mammals), six ferlin proteins have been identified to date based on their structural similarities and sequence homologies to fer-1; they are dysferlin (Fer1L1) [132, 135], otoferlin (Fer1L2) [137, 370], myoferlin (Fer1L3) [133, 371], Fer1L4 [372], Fer1L5 [201] and Fer1L6 [372]. Each ferlin protein contains a transmembrane domain at the carboxyl terminal for membrane-anchor and multiple C2 domains with calcium-binding motifs that exhibit numerous cellular functions such as phospholipid binding, signaling and membrane trafficking. The majority of our understanding of mammalian ferlin proteins has come from studies focusing on dysferlin, as mutations in the dysferlin gene lead to LGMD2B, distal myopathy and MM [139, 141, 373]. Patients with dysferlinopathy are often diagnosed with defective membrane fusion events in skeletal muscles and the release of surplus vesicles, the contents of which contribute to inflammation and further damage of skeletal muscle [374]. Although functions of myoferlin are less well known, sequencing of myoferlin gene revealed its homology to dysferlin [133]. Nevertheless, myoferlin has been reported to be responsible for fusion of myoblasts during muscle development and its mutations result in muscle atrophy [172]. Although dysferlin and myoferlin are highly expressed in skeletal and cardiac muscles, studies have confirmed their expression in kidney, placenta, lung, and brain [143, 371, 375-377]. We have previously demonstrated that dysferlin and myoferlin are present in the caveolae-enriched membrane lipid rafts, isolated from vascular endothelial cells [378, 379]. Importantly, this work also demonstrated that targeted knockdown of dysferlin and myoferlin led to decreased cell proliferation and adhesion through down-regulation of PECAM-1 and VEGFR-2, respectively, in vascular endothelial cells [378, 379]. Similar to the vascular endothelium, the airway epithelium also forms a continuous epithelial sheet on top of a basement membrane. Since dysferlin and myoferlin are membrane repair proteins that are crucial for

maintaining membrane integrity, we sort to determine whether these proteins have a functional role in the homeostasis of human airway epithelium. The purpose of this study was thus to examine the expression of dysferlin and myoferlin in human airway and determine the effects of myoferlin and dysferlin knockdown on airway epithelial functions.

4.2. Results

4.2.1. Expression of myoferlin and dysferlin within human airway

To study the expression of myoferlin and dysferlin within the human airway, we performed immunohistochemical staining. As shown in the representative images of human airway (Figure 9), myoferlin and dysferlin (*brown*) were expressed in both basal and ciliated airway

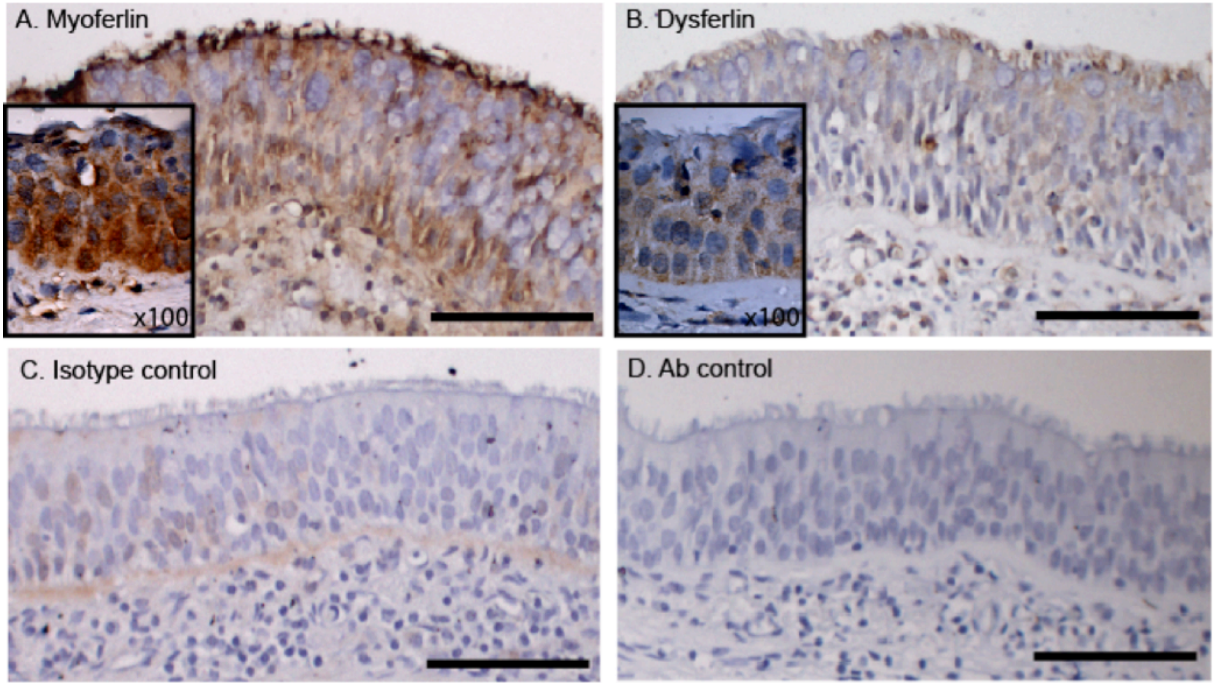


Figure 9. Dysferlin and myoferlin expression in normal human airway epithelium. Airway sections were obtained from non-diseased human subjects (n = 5) and sections were

stained with (A) myoferlin**,** (B) dysferlin, (C) isotype control and (D) secondary antibody (Ab control). The images are representative of the five individuals studied. Scale bar = 100 μM, insert image x100 magnification.

epithelial cells in addition to cells within the lamina propria, which consists mainly of fibroblasts and infiltrating inflammatory cells (Figure 9A and B, respectively). Staining with mouse immunoglobulin control at the same concentration showed minor non-specific staining (Figure 9C), whereas the secondary antibody control alone produced no non-specific staining (Figure 9D).

4.2.2. Expression of dysferlin and myoferlin in human lung cells in vitro

We further confirmed our findings using immunohistochemistry by evaluating the expression of dysferlin and myoferlin by immunoblot in both cell lines and primary cells isolated from human lung. All cells were grown in normal growth conditions to confluency and total protein was isolated to analyze dysferlin and myoferlin expression. Figure 10A showed that dysferlin (~230 kDa) was expressed in both primary airway epithelial cells from 5 healthy donor lungs and the airway epithelial cell line 16HBE, but not in primary bronchial, lung or cell line fibroblasts. In comparison, all airway epithelial cells and fibroblasts expressed myoferlin (~230 kDa) at similar expression levels to that found in BAEC, which we have previously reported to express myoferlin and dysferlin (Figure 10B). To demonstrate the expression of dysferlin and myoferlin in each cell type, the densitometry values normalized to loading control β-tubulin are expressed as a percentage of expression in BAEC (Figure 10C and D).

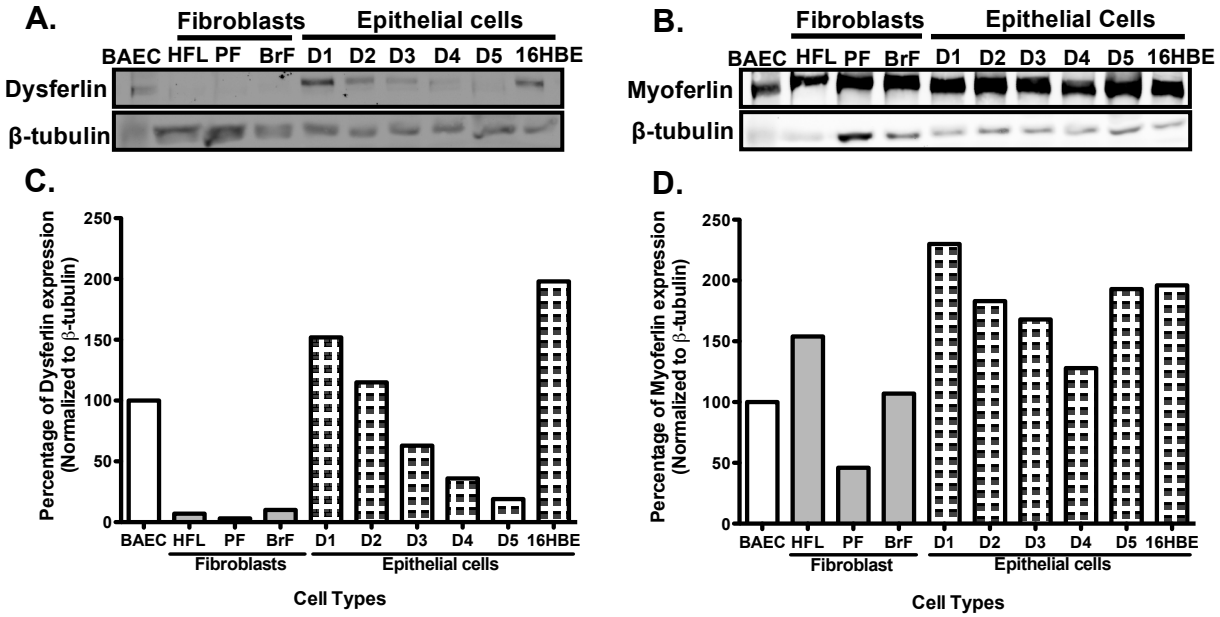


Figure 10. Dysferlin and myoferlin expression in human airway epithelial cells and fibroblasts.

Total cell lysates from primary human lung parenchymal fibroblasts (PF), bronchial fibroblasts (BrF), airway epithelial cells derived from normal donor lung tissues (D1-D5), bovine aortic endothelial cell (BAEC), human fetal lung fibroblasts (HFL) and bronchial epithelial cell (16HBE) lines were analyzed for the expression of (A) dysferlin and (B) myoferlin by immunoblot. The band intensity of (C) dysferlin and (D) myoferlin was normalized to β-tubulin and compared to BAEC to quantify the expression levels of the ferlin proteins by densitometry.

4.2.3. Localization of dysferlin and myoferlin expression in airway epithelial cells

To localize the expression of dysferlin and myoferlin in airway epithelial cells, confluent monolayers of 16HBE cells were analyzed by confocal microscopy using immunofluorescent staining. At low magnification, dysferlin and myoferlin (red staining) were found to be expressed abundantly in all 16HBE cells in monolayer culture, as shown in Figure 11 panels A and B, compared to the isotype control (Figure 11, panel C). Using a 20 X digital zoom

(white boxed areas), dysferlin and myoferlin were observed to be expressed within the cell cytoplasm and cell membrane but not within the nucleus as identified by DAPI (blue straining) (Figure 11, panels A & B), compared to the isotype control antibody (Figure 11, panel C). We also observed dysferlin and myoferlin (red staining) expression within Golgi membranes by co-localization with the Golgi matrix protein, GM-130 (green staining), as shown in Figure 12A and B.

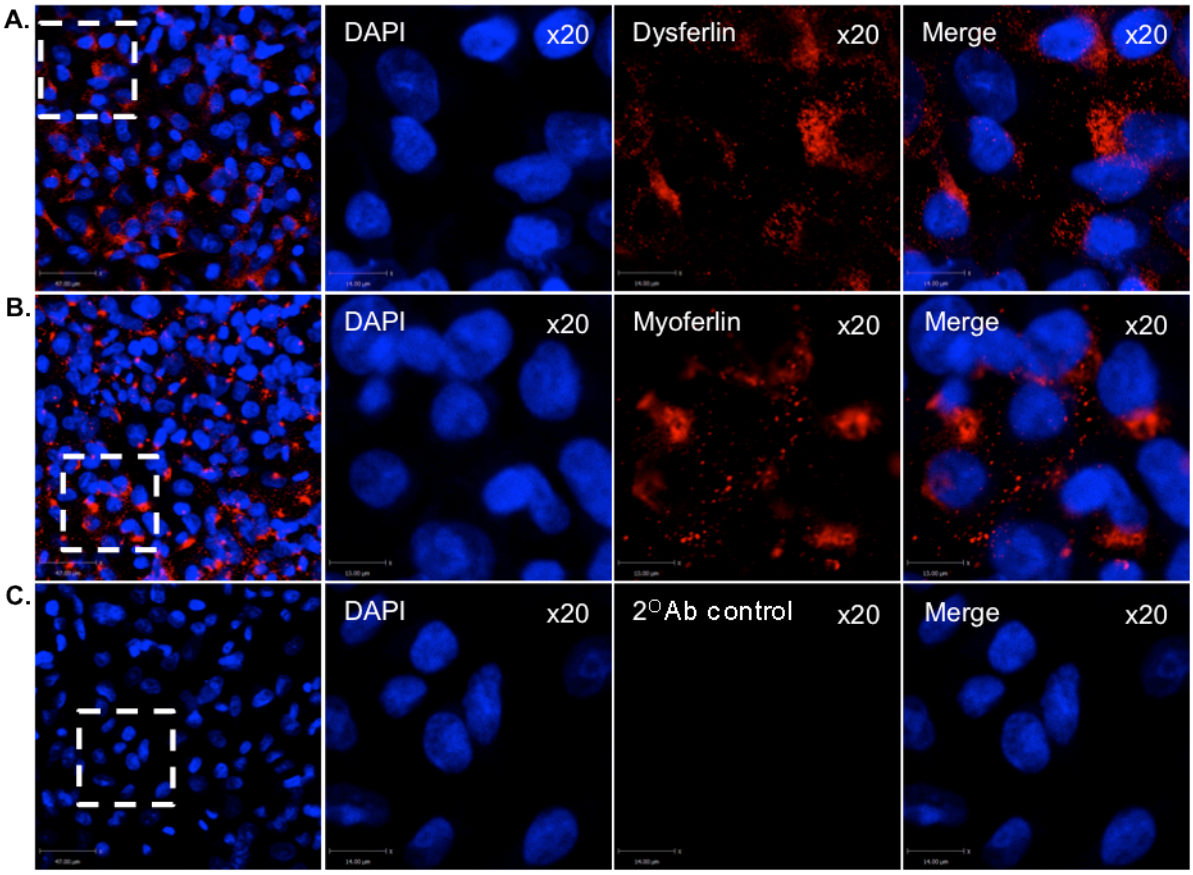


Figure 11. Localization of dysferlin and myoferlin in airway epithelial cells.

16HBE cells were grown to confluency on 8 well chamber slides and fixed for immunofluorescence analysis. Immunofluorescent staining for (A) dysferlin (*red*), (B) myoferlin (*red*), (C) mouse immunoglobulin isotype, and nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI, *blue*), were used to examine the localization of dysferlin and myoferlin. Scale bars are equal to 47 μm and 14 μm on the digital zoomed images.

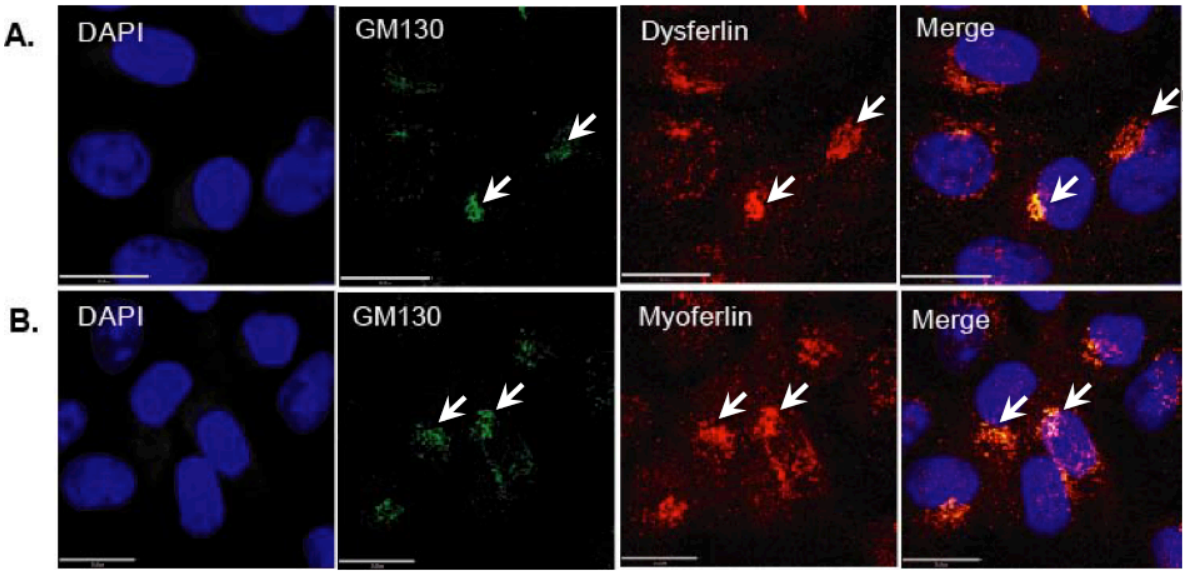


Figure 12. Co-localization of dysferlin and myoferlin with GM130 in human airway epithelial cells.

16HBE cells were grown to confluency on 8 well chamber slides and fixed for immunofluorescence analysis. Immunofluorescent staining for (A) dysferlin *(red)*, (B) myoferlin *(red)*, Golgi matrix protein marker GM130 *(green)*, and nuclei stained with DAPI *(blue)* were used to examine the localization of dysferlin and myoferlin within the Golgi membrane. Scale bars are equal to 20 μm.

4.2.4. Down-regulation of dysferlin and myoferlin by gene silencing in airway epithelial cells

To identify the potential roles of dysferlin and myoferlin in airway epithelial cells, previously designed dysferlin and myoferlin specific siRNAs were used to knock down their gene expression [168, 379]. 16HBE cells were transfected with siRNA sequences specific to dysferlin, myoferlin and a scrambled siRNA control at concentrations of 50 nM, 75 nM and 100 nM for 72 h. As demonstrated by the representative immunoblot in Figure 13A and the densitometry analysis normalized to HSP90 in Figure 13B, dysferlin specific siRNA significantly knocked down dysferlin expression by 72% at 50 nM, 68% at 75 nM and 74%

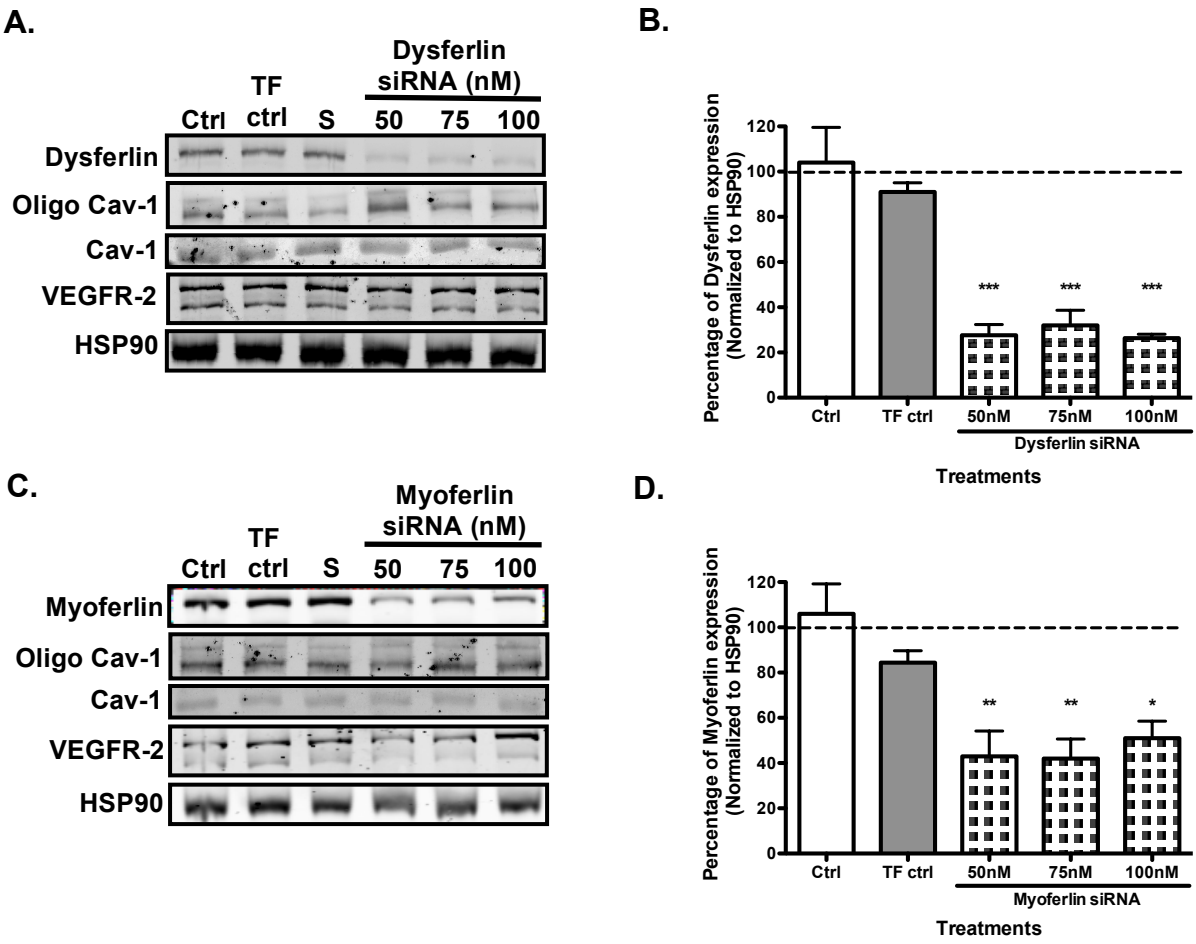


Figure 13. Knockdown of dysferlin or myoferlin has no effect on VEGFR-2 and caveolin-1 expression.

16HBE cells were transfected with control media (Ctrl), oligofectamine transfection reagent (TF con), scrambled siRNA (S), dysferlin or myoferlin siRNA at 50 nM, 75 nM and 100 nM for 72 h. The representative immunoblot of three independent experiments demonstrates that siRNA specific for (A) dysferlin and (C) myoferlin had no effect on caveolin 1 (Cav-1) or VEGFR-2 protein expression. Densitometry analysis of band intensities for (B) dysferlin and (D) myoferlin normalized to HSP90 compared to the scrambled siRNA control (dotted line), indicates knockdown of 50% or more for each ferlin protein. Values are represented as mean ± SEM of three independent experiments. A significant difference between the treatment groups with scrambled siRNA is indicated by asterisk (*$p < 0.05$; **$p < 0.01$, ***$p < 0.005$).

at 100 nM compared to the non-targeting scrambled siRNA control. Likewise, myoferlin siRNA also significantly knocked down myoferlin expression by 57% at 50 nM, 58% at 75 nM and 49% at 100 nM compared to the scrambled siRNA control (Figure 13C and D).

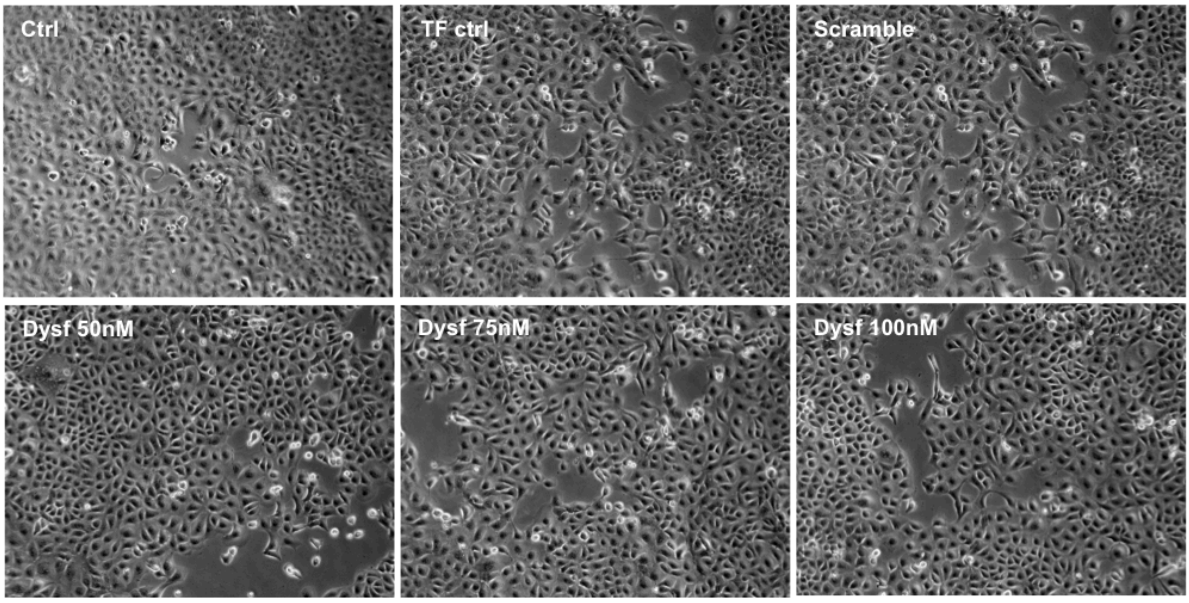
4.2.5. Silencing of dysferlin and myoferlin genes does not change the expressions of caveolin-1 and VEGFR-2

Silencing of dysferlin and myoferlin genes may invoke a compensatory response to resume normal protein expression of dysferlin and myoferlin. Caveolin-1 has been shown to regulate the expressions of dysferlin and interact with myoferlin in membrane resealing events [380, 381]. Airway epithelial cells express high levels of caveolin-1, however as demonstrated by the representative immunoblots we observed no change in caveolin-1 monomer or oligomer expression in 16HBE cells treated with dysferlin or myoferlin siRNA (Figure 13A & C, respectively). Furthermore we have previously demonstrated that knockdown of myoferlin, but not dysferlin, leads to decreased expression of VEGFR-2 in BAEC [168, 379]. However in 16HBE cells, we observed no changes in VEGFR-2 expression following myoferlin or dysferlin knockdown, indicating these ferlin proteins may have cell-specific roles in membrane fusion and trafficking events (Figure 13A & C).

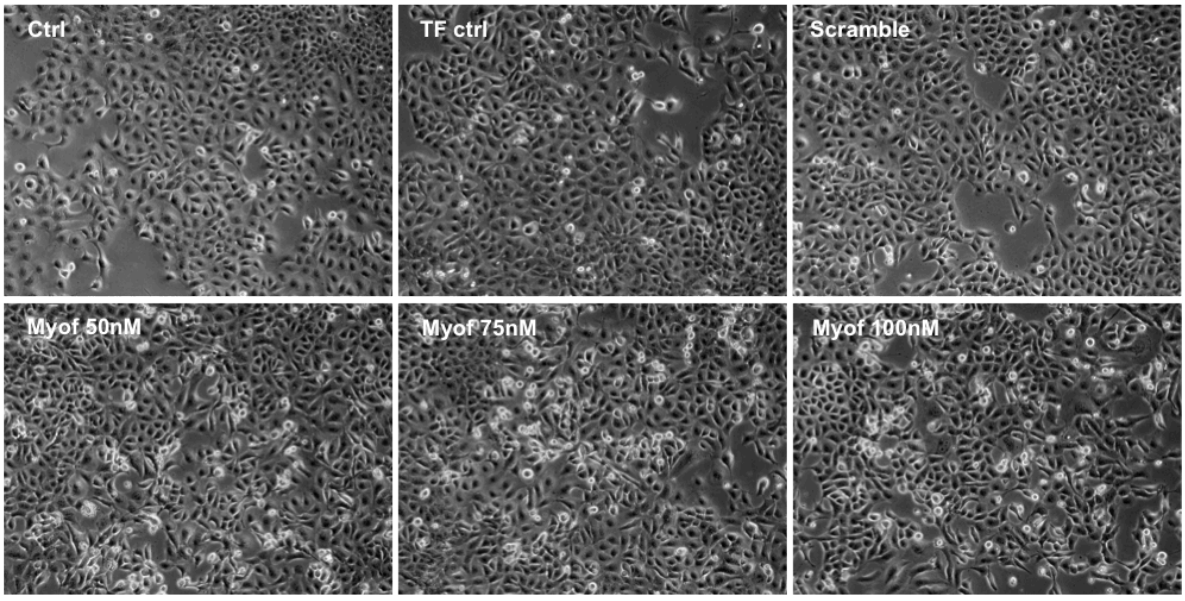
4.2.6. Knockdown of myoferlin changes airway cell appearance and cell detachment

We next examined the effects of knocking down dysferlin and myoferlin using siRNA transfection on cell appearance and attachment. As demonstrated in the phase contrast images of 16HBE cell monolayers (Figure 14A) following 72 h of dysferlin siRNA transfection, we observed no significant changes in cell appearance. In contrast, knockdown of myoferlin led to a loss of normal epithelial cuboidal appearance to a spindle-shaped phenotype at all siRNA concentrations and increased numbers of rounded and detached cells (Figure 14B). To verify our observations of cell detachment, following 72 h of siRNA

A.


Ctrl
TF ctrl
Scramble
Dysf 50nM
Dysf 75nM
Dysf 100nM

B.


Ctrl
TF ctrl
Scramble
Myof 50nM
Myof 75nM
Myof 100nM

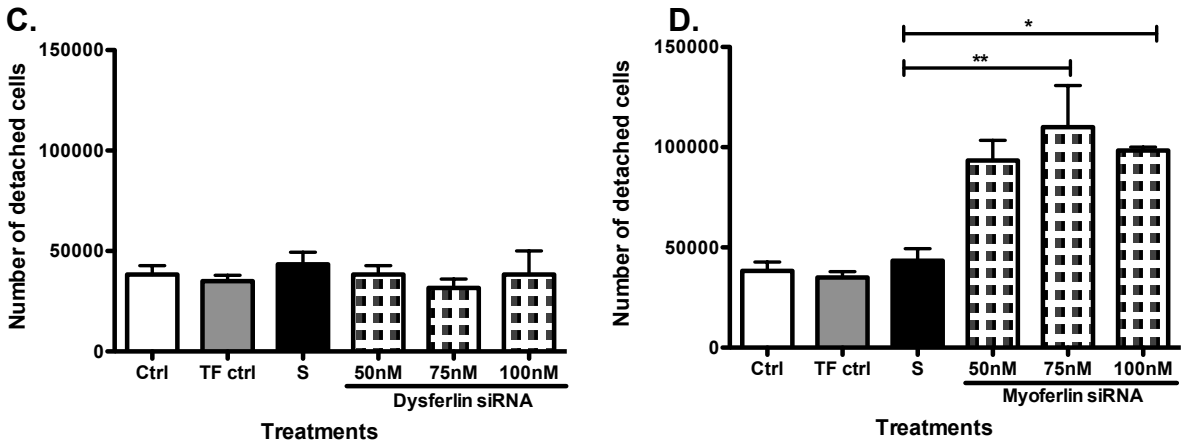


Figure 14. Knockdown of myoferlin but not dysferlin alters cell appearance and cell detachment.

Phase contrast images captured at 10 X magnification of 16HBE cells treated with control media (Ctrl), transfection control (TF ctrl), scrambled siRNA (S), and (A) dysferlin (Dysf) siRNA or (B) myoferlin (Myof) siRNA at 50 nM, 75 nM and 100 nM for 72 h. Detached cells within cell culture supernatants were counted in each treatment group following (C) dysferlin or (D) myoferlin siRNA treatment using a hemocytometer. Data are presented as mean ± SEM of three independent experiments. A significant difference between the treatment groups and scrambled siRNA control is indicated by asterisk (* $p < 0.05$; ** $p < 0.01$).

treatment the cell culture media were harvested and cell counts conducted. We found no significant difference between the number of detached 16HBE cells treated with dysferlin siRNA and the scrambled siRNA control (Figure 14C). In comparison, cells treated with myoferlin siRNA at 75 nM and 100 nM concentrations showed significantly greater numbers of detached 16HBE cells compared to the scrambled siRNA control (Figure 14D).

4.2.7. Myoferlin knockdown induces changes in cell detachment resulting in cell death but not inflammation

As the changes in cell appearance and cell detachment observed in cells treated with

myoferlin siRNA could be an indication of cell death, we evaluated the viability of detached cells within the culture media using Trypan blue dye. We found that the number of dead cells following myoferin knockdown increased at all siRNA concentrations (Figure 15A). Cell apoptosis was evaluated after 72 h of myoferlin siRNA treatment by flow cytometry analysis using Annexin-V and propidium iodide (PI) staining (Figure 15B). During the early stages of apoptosis, translocation of phosphatidylserine from the inner to outer plasma membrane can be detected using Annexin-V and PI staining. The results showed that the percentage of apoptosis was 12.3% in cells treated with 75 nM scrambled siRNA control compared to 46.8% in 16HBE cells treated with 75 nM myoferlin siRNA (Figure 15C and D, respectively). We next examined if knockdown of myoferlin resulted in elevated release of innate pro-inflammatory cytokines IL-6 and IL-8. As demonstrated in Figure 15E and F, the concentration of IL-6 and IL-8 released by 16HBE cells did not alter following treatment of myoferlin specific siRNA compared to transfection controls.

4.2.8. Myoferlin knockdown in airway epithelial cells results in loss of ZO-1 expression

We hypothesized that the appearance change and cell detachment observed in epithelial cells treated with myoferlin siRNA is an indication of cell junction loss. To test this hypothesis, we evaluated the expression of adherens junction molecule, E-cadherin, as well as tight junction molecules occludin, claudin-1, ZO-1 and ECM adhesion receptor integrin β1 following knockdown of dysferlin and myoferlin. Dysferlin knockdown did not result in cell detachment; and as expected, we did not detect any difference in the expression of any of the adhesion proteins measured (Figure 16A). In comparison, myoferlin knockdown led to a decrease in the expression of ZO-1 at all myoferlin siRNA concentrations, but not E-cadherin,

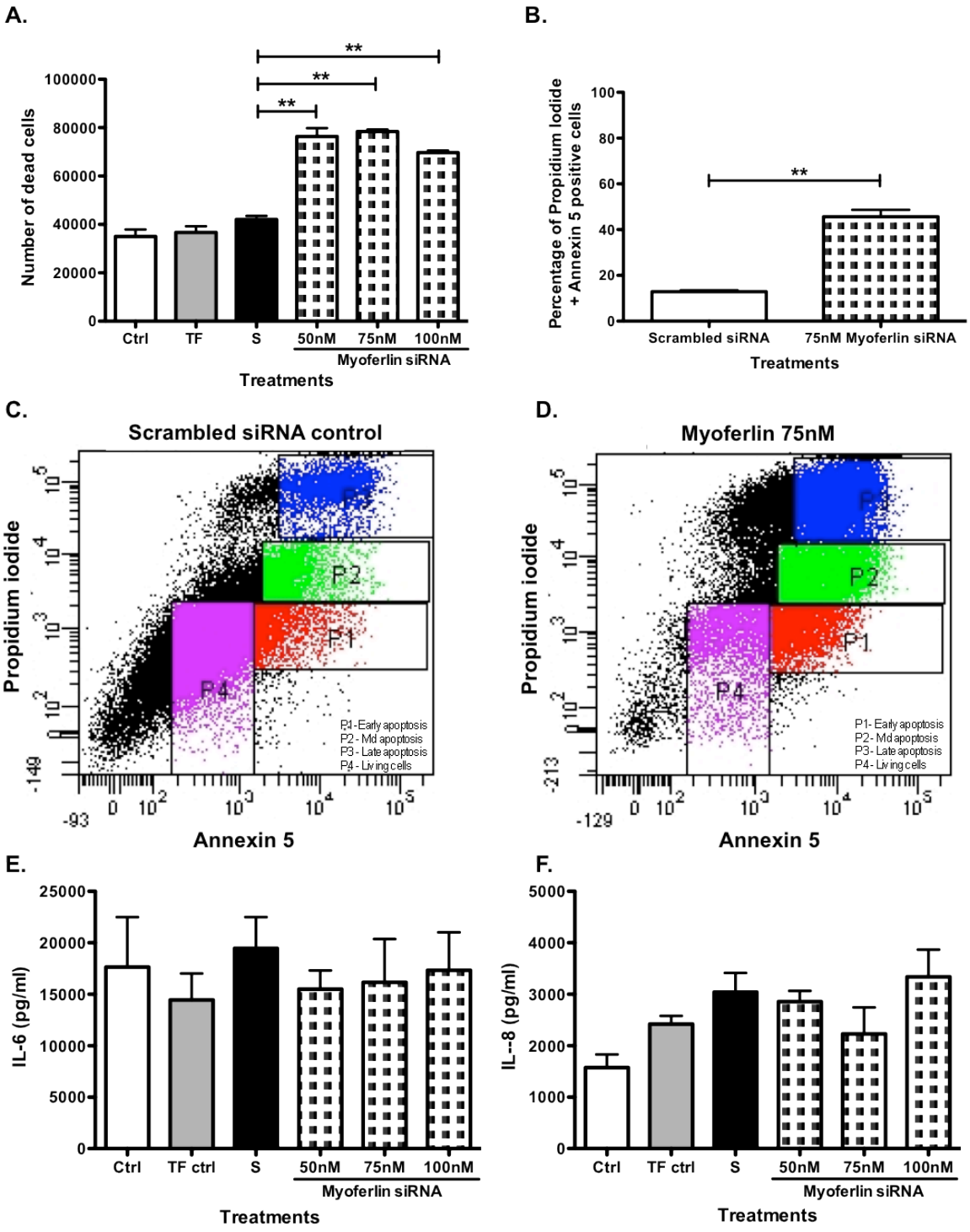


Figure 15. Myoferlin knockdown induces changes in cell detachment and apoptosis but not inflammation.

16HBE cells were treated with control media (Ctrl), transfection control (TF ctrl), scrambled siRNA (S) or myoferlin siRNA at 50 nM, 75 nM and 100 nM for 72 h. Cell culture supernatants were removed and analyzed for (A) dead cells using trypan blue assay and (B)

percentage of cells positive for annexin 5 and propidium iodide by FACS analysis, which are surrogate markers of apoptosis. Representative FACS plots of 16HBE cells treated with (C) scrambled siRNA control compared to (D) myoferlin siRNA at 75nM concentration indicate elevated numbers of cells positive for propidium iodide and annexin 5 (gated in P3). Cell culture supernatants were also analyzed for inflammatory cytokines (E) IL-6 and (F) IL-8. Data are presented as mean ± SEM of six independent experiments. A significant difference between the treatment groups and scrambled siRNA control is indicated by asterisk (** $p <$ 0.01).

occludin, claudin-1, or integrin β1 when compared to the transfection control conditions (Figure 16B and C). Others and we have previously shown that loss of cell-cell contacts and acquisition of a spindle-shaped cell appearance are two of the initial morphological changes associated with EMT. To confirm that loss of ZO-1 is not associated with EMT, we analyzed the expression of E-cadherin, and mesenchymal marker, fibronectin-EDA by immunoblot. As shown by the densitometry analysis in Figure 17, we observed no differences in the expression of E-cadherin or fibronectin-EDA.

4.2.9. Myoferlin and ZO-1 co-localize in airway epithelial cells

To understand the association between myoferlin and ZO-1, we looked at the distribution patterns of myoferlin and ZO-1 in 16HBE cells using confocal microscopy. The distribution pattern of ZO-1 staining (*green*) was co-localized to that of myoferlin staining (*red*), as evidenced by the overlay images, which demonstrate co-localized proteins in yellow (Figure 18). Furthermore, when we knocked down myoferlin with siRNA (75 nM), we observed reduced expression of both myoferlin and ZO-1, as demonstrated by loss of total (*green and red*) and co-localized staining (*yellow*) compared to the transfection control conditions.

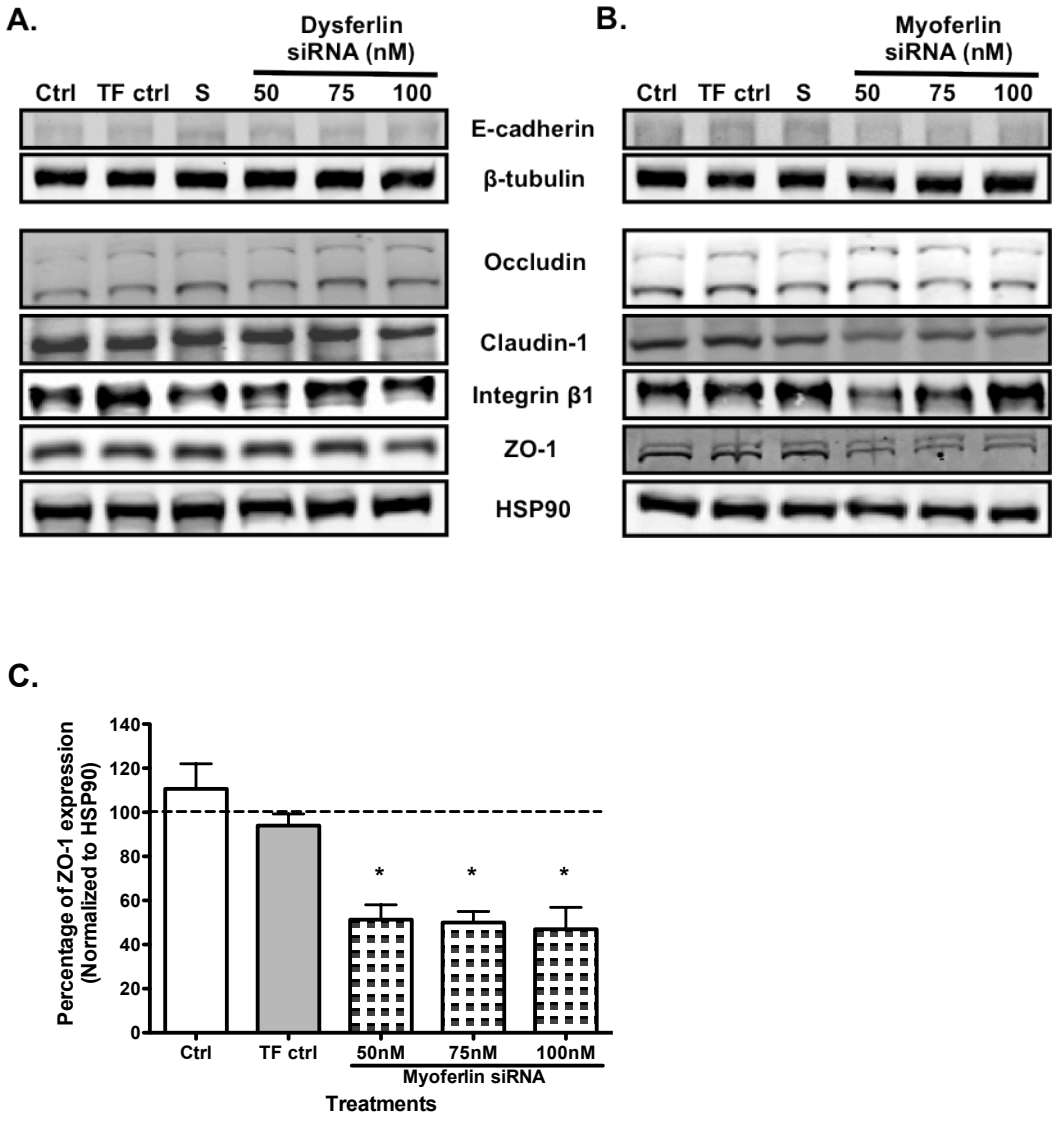


Figure 16. Myoferlin knockdown decreases the expression of tight junction molecule ZO-1.

16HBE cells were treated with control media (Ctrl), transfection control (TF ctrl), scrambled siRNA (S), and (A) dysferlin or (B) myoferlin siRNA at 50 nM, 75 nM and 100 nM for 72 h. Total cell lysates were prepared to analyze the expressions of adherens junction molecule, E-cadherin, and tight junction molecules, occludin, claudin 1, ZO-1, and integrin β1. Representative immunoblots from three independent experiments are presented. β-tubulin and HSP90 served as loading controls. (C) Densitometry analysis of band intensities for ZO-1 normalized to HSP90 compared to the scrambled siRNA control (dotted line). Values are presented as mean ± SEM of three independent experiments. A significant difference between the treatment groups and scrambled siRNA control is indicated by asterisk (* $p <$ 0.05).

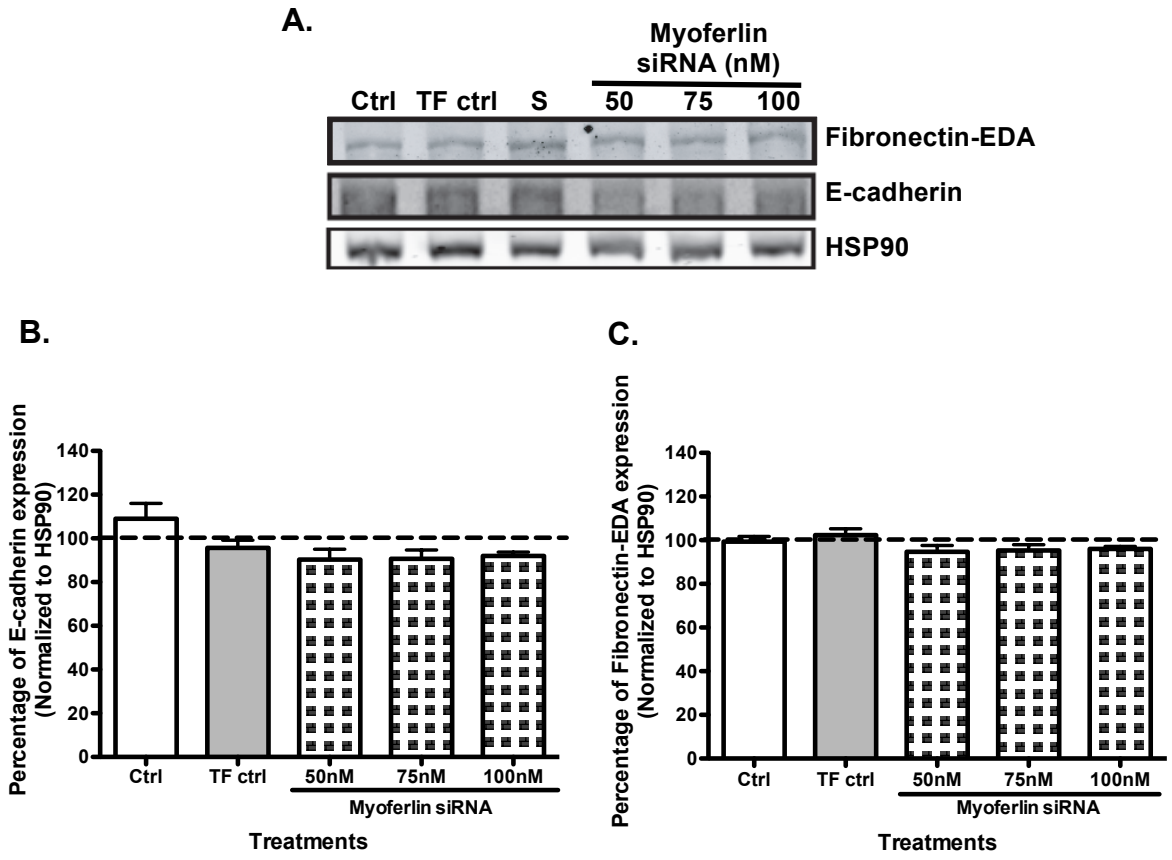


Figure 17. Myoferlin knockdown does not induce features of epithelial-mesenchymal transition.

(A) 16HBE cells were treated with control media (Ctrl), transfection control (TF ctrl), scrambled siRNA (S), and myoferlin siRNA at 50 nM, 75 nM and 100 nM for 72 h. Total cell lysates were prepared to analyze the expressions of adherens junction molecule E-cadherin and fibronectin-EDA. Representative immunoblots from three independent experiments are presented. HSP90 served as a loading control. (B) Densitometry analysis of band intensities for E-cadherin and (C) fibronectin-EDA normalized to HSP90 compared to the scrambled siRNA control (dotted line). Values are represented as mean ± SEM of three independent experiments.

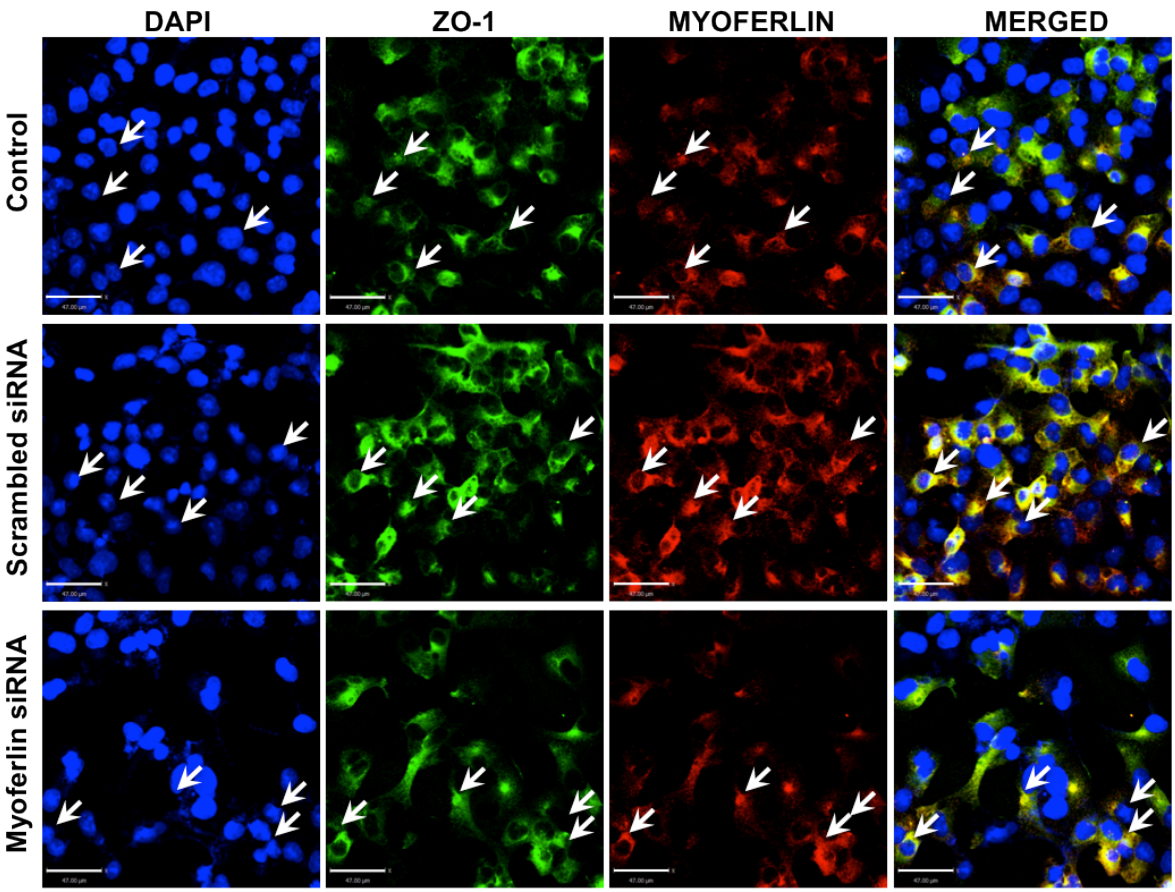


Figure 18. Co-localization of myoferlin and ZO-1.

16HBE cells were grown on 8-well chamber slides and treated with transfection control, scrambled siRNA or myoferlin siRNA both at 75 nM concentrations for 72 h then fixed for immunofluorescence analysis. Immunofluorescent staining for myoferlin (*green*), ZO- 1 (*red*), and nuclei stained with DAPI (*blue*), were used to examine the localization of ZO-1 and myoferlin following siRNA treatments. Scale bars are equal to 24 μm on all images.

4.3. Discussion

Under normal circumstances, the polarized airway epithelium forms a highly regulated and semi-permeable barrier through the formation of cell-cell adhesions. The formation of these cell-cell adhesion junctions requires the coordinated fusion and exocytosis of vesicles containing adhesion molecules at targeted areas within the cell membrane. In this study, we demonstrate for the first time that membrane fusion proteins of the ferlin family, dysferlin

and myoferlin, are expressed within airway epithelial cells of the human lung. Furthermore, we demonstrate that knockdown of myoferlin, but not dysferlin using siRNA, results in loss of epithelial TJ protein ZO-1 expression, cell detachment and apoptosis.

Sorting of cell membrane-associated proteins occurs predominantly in the *trans*-Golgi network in both polarized epithelia and "non-polarized" fibroblasts through well-defined cytoplasmic domain-sorting signals [382-384] and specialized organization of actin and microtubule cytoskeleton beneath the plasma membrane [385]. During membrane fusion, a membrane bound structure fuses with the plasma membrane and expels its contents to the exterior. The fusion mechanisms involved include aggregation of the membranes, resulting in close bilayer apposition; destabilization and rupture of the membrane; and merging of the aqueous contents and membrane components [386]. This fusion of vesicles within a target plasma membrane is regulated by organelle-specific Rab GTPases, vesicle-tethering complexes [34], and SNAREs [35]. Ferlin family members are known to mediate membrane fusion events upon membrane damage, endocytosis and exocytosis [381, 387]. Ferlins anchor to the plasma membrane via a single C-terminal transmembrane domain and contain C2 domains that mediate lipid and protein binding. Both dysferlin and myoferlin contain C2 domains which can coordinate calcium ions within a negatively charged binding pocket to regulate calcium-activated events, akin to the well characterized classical mediators of vesicle fusion, the synaptotagmins, which contain two C2 domains [388, 389].

The roles of dysferlin and myoferlin in membrane fusion events have primarily focused on skeletal muscles, as mutations in the dysferlin gene are associated with the development of muscular dystrophy, a muscle wasting disease. Since then, dysferlin and myoferlin have also been demonstrated to be expressed in a number of other cell types, including myoblasts, inflammatory cells, endothelial cells and specialized epithelium of the

placenta, renal tubular and glomerular structures [375, 390]. Here, we report that dysferlin and myoferlin are expressed in the airway epithelium at similar levels to vascular endothelial cells whereas bronchial and lung fibroblasts only express myoferlin. We observed that the subcellular distribution of dysferlin and myoferlin within airway epithelial cells is localized to the Golgi-membrane, cytoplasm and plasma membrane. Furthermore, this staining was punctate in appearance, suggesting expression was localized to vesicles. Previous studies have reported low expression levels of dysferlin and myoferlin in human lung [143, 371], but these studies did not investigate the expression of these proteins in specific cell types.

To determine the function of dysferlin and myoferlin within the airway epithelial cells, we used specific siRNAs to knock down gene expression. Downregulation of myoferlin, but not dysferlin, resulted in loss of cuboidal epithelium and acquisition of a spindle-shape cell appearance. In addition, the numbers of detached cells within culture media also significantly increased following myoferlin knockdown when compared to dysferlin and control conditions. We have previously demonstrated dysferlin knockdown impairs the proliferation, migration and adhesion of sub-confluent vascular endothelial cells through loss of the adhesion molecule, PECAM-1 [378]. On the other hand, myoferlin knockdown led to a decrease in vascular endothelial cell proliferation and migration associated with decreased expression of VEGFR-2, the receptor of vascular endothelial growth factor that is important for vasculogenesis and angiogenesis [379]. In addition, dysferlin and myoferlin have been shown to interact with caveolin in lipid rafts of endothelial cells, skeletal muscles and fibroblasts [381, 391]. As PECAM-1 is not expressed on epithelial cells, we quantified the expression of VEGFR-2 and caveolin in airway epithelial cells. We expected that knockdown of dysferlin and myoferlin may stimulate a compensatory response on caveolin in airway epithelial cells. However, we did not see any difference in the

expression of caveolin-1, suggesting that dysferlin and myoferlin have tissue- or cell-specific functions. Although we observed that downregulation of myoferlin in airway epithelial cells was not associated with VEGFR-2 expression, it was associated with the loss of TJ protein, ZO-1. This finding further suggests that dysferlin and myoferlin may have tissue-specific regulatory roles of membrane-associated proteins. Interestingly, myoferlin associated downregulation of ZO-1 and cell detachment were not linked to expression changes in the other transmembrane TJ and AJ proteins analyzed, which included occludin, claudin 1 and E-cadherin. In support of our findings, it has recently been shown in the mouse jejunum epithelium that the process of cell shedding requires redistribution of ZO-1 prior to cell extrusion from the epithelium and that shed epithelial cells undergo nuclear chromatin condensation, a hallmark of apoptosis, after cells leave the monolayer [392]. This study and others suggest that TJs rearrange beneath dividing and shedding cells to maintain epithelial barrier function by connecting neighboring cells surrounding an extruding cell [393-396]. Thus, loss of ZO-1 expression via downregulation of myoferlin expression may be an active participant in catalyzing cell extrusion. This would support our findings of increased numbers of epithelial cells positive for annexin V and PI staining, which are markers for apoptosis, and no alterations in inflammatory cytokines IL-6 and IL-8 being released within cultures treated with myoferlin siRNA. As calcium-dependent intercellular adhesion is required for ZO-1 localization to the TJ [397, 398], we propose that in epithelial homeostasis, the multiple C2-calcium binding domains of myoferlin may play an important role in regulating the graduated fusion in response to gradients of calcium release for vesicle trafficking. In support of our hypothesis, recent studies have shown that myoferlin forms a protein complex with Dyn-2 that is essential for the fission of endocytic vesicles [399, 400]. TJ proteins mislocalize and cell polarity is lost upon Dyn-2 depletion. These results together

suggest a Dyn-2-myoferlin mediated endocytic pathway may be involved in spatial regulation and distribution of TJ proteins and their binding partners.

TJs are responsible for regulating paracellular permeability and maintaining cell polarity, which are often referred to as "barrier" and "fence" function, respectively. TJs also play a role in signaling pathways involved in epithelial proliferation, gene expression and differentiation [82, 83]. ZO-1 via its Src homology 3 domain, binds ZONAB (ZO-1-associated nucleic-acid-binding protein), a Y-box transcription factor, sequestering it in the cytoplasm and inhibiting its transcriptional activity [82, 83]. Following disassembly of ZO-1 from the tight junction, ZONAB is able to interact with the cell cycle kinase, CDK4, to regulate the transcription of cell cycle genes including cyclin D1 and proliferating cell nuclear antigen (PCNA), which lead to the proliferation of epithelial cells in culture [401-403]. Thus ZO-1 expression has been shown to be localized both at the cell membrane, within the cell cytoplasm and nucleus in dividing cells. These data support our co-localization of ZO-1 and myoferlin results taken from confocal microscopy as both proteins were found in the cytoplasm and cell membrane. Previously, myoferlin expression has been shown in developing skeletal muscles, cardiac muscles, placenta [371], and caveolae-enriched buoyant lipid rafts in endothelial cells [379]. Interestingly, myoferlin shows developmental ‘switching’ in skeletal muscles due to the fact that it is highly expressed in proliferating mononuclear cells and becomes downregulated with myogenic maturation [404, 405]. In contrast, low level of dysferlin is expressed in proliferative myoblasts and becomes robustly expressed in fused myotubes and in mature muscles [404]. This suggests that myoferlin and dysferlin may possess both overlapping and specialized roles, with functional specialization related to distinct requirements of proliferative and differentiated cells. In our study, we found that myoferlin and, to a limited extent, dysferlin were expressed

ubiquitously in basal, ciliated and mucus expressing cells within normal human airway epithelium. However, our study did not investigate the expression and localization of myoferlin and dysferlin following epithelial proliferation, repair or the compensatory responses by other multiple C2-domain protein family members, in the absence of either protein. Future studies in our laboratory are aimed at unraveling the mechanisms by which myoferlin regulates ZO-1 expression during epithelial damage.

In conclusion, our study provides evidence that dysferlin and myoferlin are present in several cell types within the human airway. Furthermore, knockdown of myoferlin in airway epithelial cells triggers loss of ZO-1 expression and cell adhesion. These *in vivo* and *in vitro* data thus demonstrate that myoferlin in airway epithelial cells may play a role in maintaining epithelial homeostasis through trafficking of TJ protein ZO-1. Future studies are required to understand the potential roles of myoferlin during epithelial repair.

5. Expression of Myoferlin in Human and Murine Carcinoma Tumors: Role in Membrane Repair, Cell Proliferation and Tumorigenesis[2]

5.1. Introduction

The plasmalemma serves as far more than just a simple barrier between the extracellular space and cytoplasm. Mammalian cells require transmembrane signaling, as well as continuous endocytosis and exocytosis of a wide range of soluble molecules and factors, to support cellular homeostasis and growth [406, 407]. A labile and fully functional plasma membrane is therefore an ubiquitous requirement for these processes to take place, suggesting that the plasmalemma relies on sophisticated but essential membrane maintenance and repair mechanisms, to ensure its plasticity, integrity, and continuous remodeling [408]. Conversely, one could assume that loss of plasma membrane integrity or blunted membrane repair signaling might directly interfere with cellular homeostasis and other basic cellular functions, such as proliferation.

Some of the most salient examples of active membrane repair events are those regulated by the ferlin family of proteins. The first ferlin gene, *fer-1*, was described in *Caenorhabditis elegans*, and blunted *fer-1* expression resulted in the absence of calcium–mediated membrane vesicle fusion at the plasmalemma and accumulation of intracellular vesicles in the submembrane space [130, 368, 409]. Three human *fer-1* homolog genes have been identified: dysferlin, myoferlin, and otoferlin [128, 133, 195]. Otoferlin is expressed

[2] All figures and text have been published as a research article in *American Journal of Pathology*. 2013 May; 182(5): 1900-1909.

exclusively in the inner ear, whereas both dysferlin and myoferlin were originally described in muscle tissues. The first direct evidence of ferlin-dependent plasma membrane repair and remodeling in mammalian cells was shown in muscle fibers, the cell type exposed to the highest levels of physical stress and membrane remodeling [127, 410]. In dysferlin-null skeletal myocytes, repair of exercise- or laser-induced membrane damage was profoundly attenuated, leading to an aberrant influx of calcium at the injured sites and abnormal growth and progressive loss of muscle mass and function [127]. Ferlin-dependent membrane repair is akin to the fusion of lipid vesicles to the plasma membrane, resulting in the patching of damaged sites in a calcium-dependent process via the concerted actions of numerous lipid-protein complexes involving ferlins, annexins, neuroblast differentiation-associated protein AHNAK, caveolin, and various other proteins, as yet unidentified [138, 147, 148, 195, 411]. Both myoferlin and dysferlin have been linked to cardiomyopathies [128, 412]. Aberrant expression of dysferlin is known to correlate with MM and LGMD2B [127, 410], and aberrant expression of otoferlin has been correlated with deafness [195].

Although the expression of myoferlin and dysferlin was initially believed to be restricted to muscle cells, a growing number of studies have reported their expression in other tissues and cell types [166, 182, 413, 414]. Indeed, we have recently shown that vascular endothelial cells express high levels of myoferlin and dysferlin, and that they regulate basic endothelial functions, such as proliferation, adhesion, and endocytosis [165, 182, 183], lending credence to the theory that ferlin-dependent membrane integrity and remodeling are essential to homeostasis of both muscle and non-muscle cells. One class of non-muscle cells known to proliferate at critically high rates that likely require sustained membrane remodeling and repair is cancer cells. Cancer cells routinely circumvent cell-cycle checkpoints via genetic instability or DNA mutations that prevent cell-cycle arrest and

regulated mitosis [415]. Such a state of unregulated proliferation not only increases cellular needs for extracellular nutrient uptake, but also dictates intense plasma membrane fusion and fission events during cell division and cytokinesis [416]. The idea thus arises that interfering with tumor cell membrane remodeling and repair could result in a concomitant decrease in cell proliferation and tumor burden.

Gene arrays and protein chip assays reported by others have suggested that myoferlin is indeed one of the many genes upregulated in some cancer cell lines [185]. Here, we report myoferlin expression across a wide range of human and murine cancer lung tumor cell line and skin melanoma cell line, as well as solid animal and human carcinoma tumors. Myoferlin gene silencing caused decreased cell proliferation *in vitro*, and decreased tumor burden in a mouse xenograft model, a consequence of myoferlin-dependent membrane repair during accelerated cell proliferation. Thus, the data reported here further support the expression and biological significance of ferlins in non-contractile cells and suggest an anti-proliferative effect associated with attenuated myoferlin expression *in vitro* and *in vivo*.

5.2. Results

5.2.1. Human and mouse epithelial tumor cell lines endogenously express myoferlin

To investigate the potential expression of myoferlin by tumor cells, we performed Western blot analyses on a range of mouse and human cancer cell lines, as previously described [417-419]. Positive myoferlin expression was detected in LLC cells (a well-characterized mouse lung epithelial cancer line) and in B16-BL6 cells (a metastatic, epithelial-like mouse melanoma cell line) (Figure 19) Overexpression of a hemagglutinin-tagged version of

myoferlin (HA-myoferlin) in COS-7 cells confirmed positive myoferlin detection, compared with untransfected cells (Figure 19) [182].

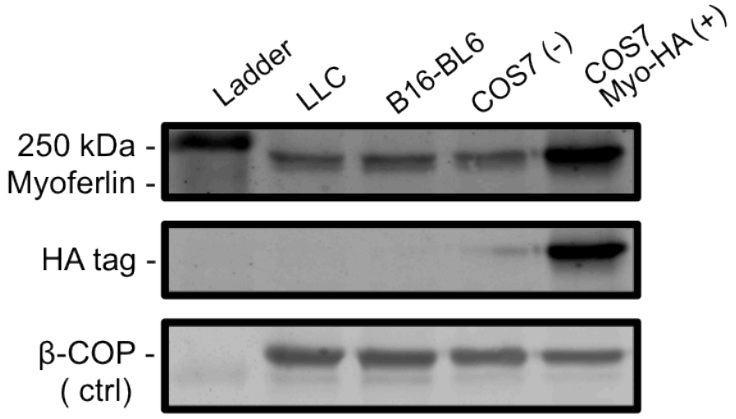


Figure 19. Myoferlin is expressed in two cultured mouse tumor cell lines, LLC and B16-BL6.

Proteins extracted from two cultured mouse tumor cell lines, LLC and B16-BL6, as well as control and Myoferlin-HA plasmid-transfected COS-7 cells were immunoblotted against Myoferlin (top) and showed positive staining for the 250 kDA full length Myoferlin protein and HA-tag (centre). β-COP was used as a loading control.

Western blotting using anti-dysferlin antiserum revealed that both LLC and B16-BL6 cells express a second ferlin, dysferlin (Figure 20A), providing evidence of the complexity of the membrane repair machinery in epithelial cancer cells. Myoferlin localization by immunofluorescence in cultured LLC cells was similar to what we have reported in vascular endothelial cells [148], with robust detection around the perinuclear region and cytoplasm, with a predominant localization in proximity of the Golgi apparatus (Figure 20B). Nuclei were stained with DAPI.

In solid xenografted LLC mouse tumors, myoferlin staining was observed in the periphery of the tumor using an anti-myoferlin antibody, with abundant staining surrounding

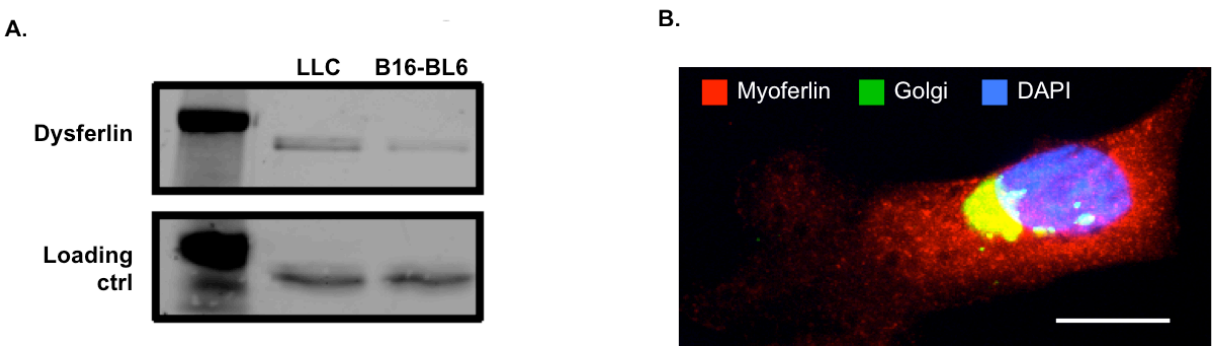


Figure 20. Myoferlin is expressed in human and mouse carcinoma cell lines.

(A) LLC and B16-BL6 cells express low levels of dysferlin. (B) Myoferlin (*red*) showed robust staining in closed proximity to the Golgi apparatus (*green*; GM130) and the rest of peri-nuclear region (*blue*; DAPI) by IF. Scale bar = 5 μm.

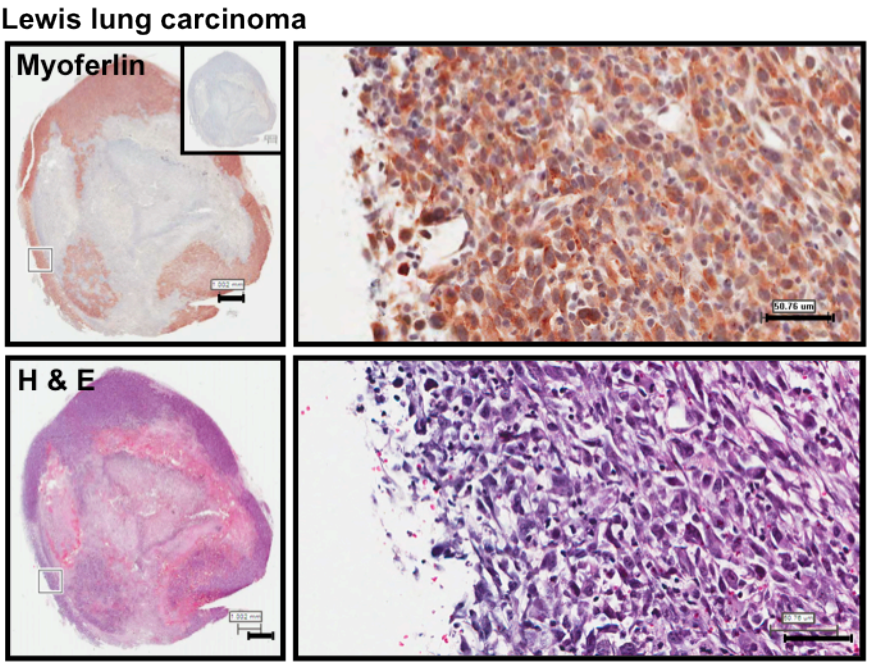
a necrotic core (Figure 21A). H&E staining was used to illustrate tumor morphology. Similarly, human metastatic carcinoma tumors removed from lungs of patients showed broad myoferlin staining throughout the non-necrotic sections examined (Figure 21B), confirming broad expression of myoferlin in a range of murine and human solid tumors and cell lines.

5.2.2. Myoferlin knockdown decreases tumorigenesis

To assess the role of myoferlin in tumor pathogenesis, loss-of-function studies were performed using a siRNA-based myoferlin gene knockdown approach previously demonstrated by our research group to inhibit myoferlin expression *in vivo* and to require 100% template homology [182]. Initial experiments in cultured LLC cells showed that transfection of myoferlin siRNA sequences (75 nM)/DOTAP resulted in a 78% and 87% decrease in myoferlin expression *in vitro* at 48 and 72 h, respectively, compared with a scrambled sequence (identical to control siRNA) (Figure 22A).

In vivo, intratumoral injections of myoferlin/DOTAP mixture were performed every 3

A.



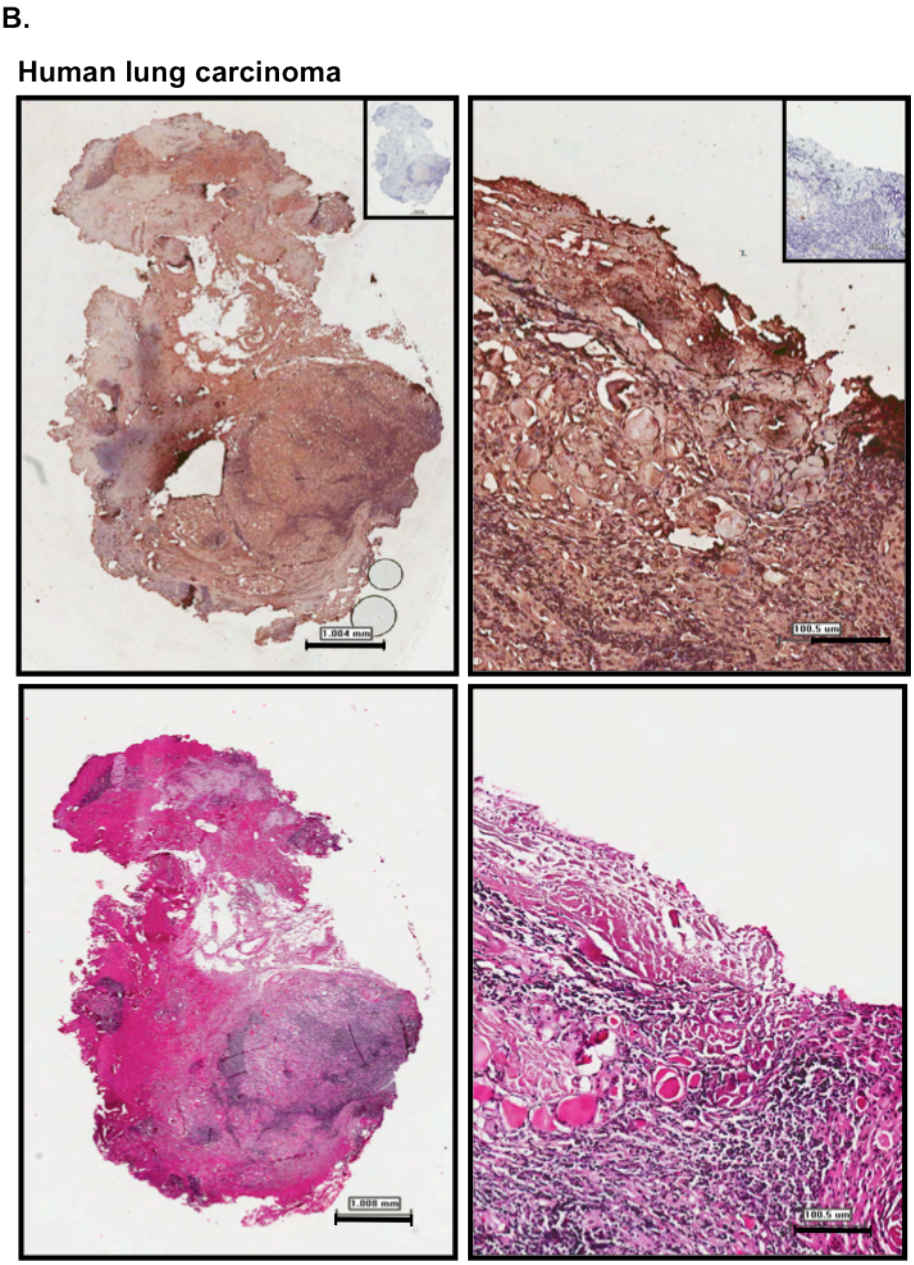


Figure 21. Myoferlin is expressed in mouse and human solid lung tumors.

(A) Positive myoferlin detection (*brown*) in solid LLC tumors (top panel), along with H&E stain of an adjacent section (bottom panel) reveals tumor morphology. The necrotic core is indicated by white and light blue. Inset: control IgG condition. (B) Positive myoferlin detection (*brown*) in human lung carcinoma tissue sections (top panel), along with H&E staining of an adjacent section (bottom panel). Inset: control IgG condition. Scale bars = 1 μm (A and B, top panels) and 100 μm (A and B, bottom panels).

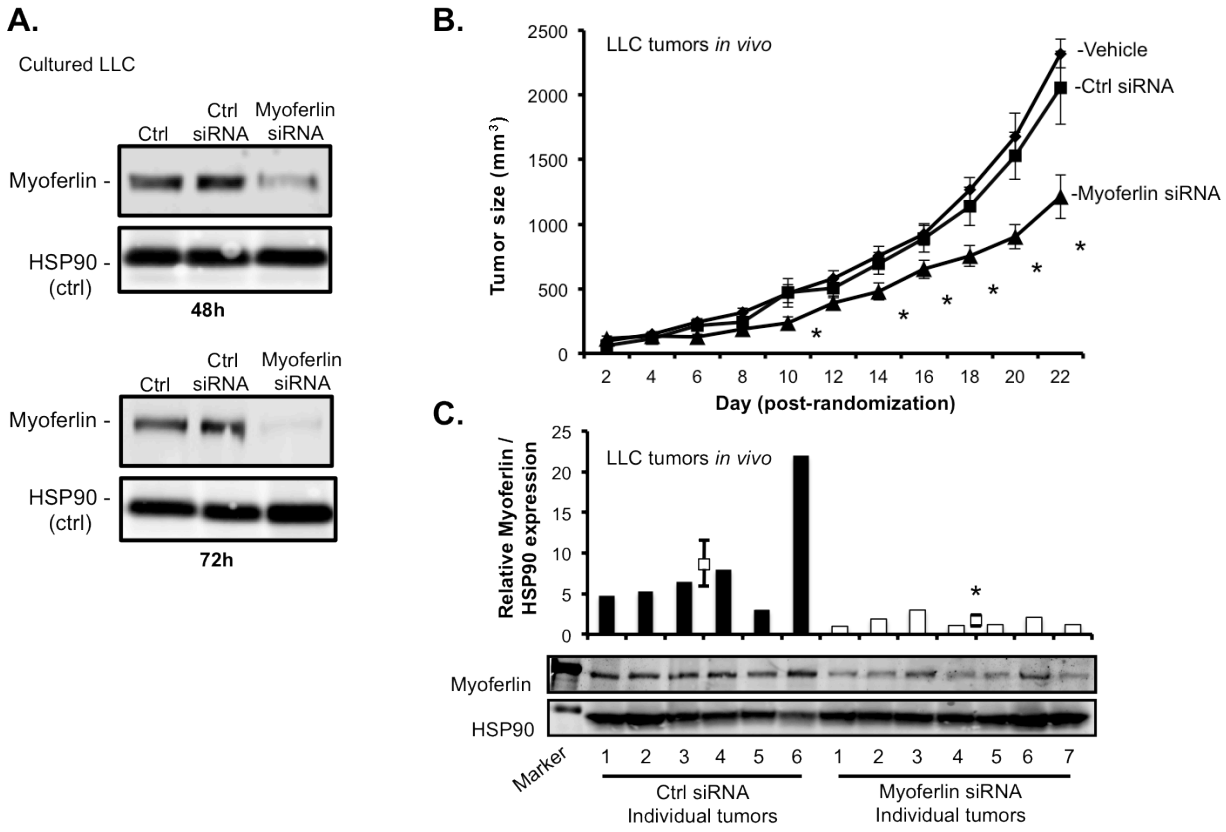


Figure 22. Myoferlin knockdown decreases tumor growth *in vivo*.

(A) Cultured LLC cells were pretreated with vehicle (Ctrl), control siRNA or Myoferlin siRNA (75 nM) for 48 or 72 h, proteins were isolated and probed for Myoferlin or HSP90. (B) *In vivo* knockdown of myoferlin expression decreases tumor burden. Following initial implantation and randomization, mice were intratumorally injected with Dotap/siRNA solution every three days in a blinded fashion. Average size ± SEM as a function of day is shown; n = 6-7. Experiments were performed in duplicate. * $p < 0.05$ by unpaired Student's t test for orthogonal comparisons. (C) Before tumor reaching 2500mm^3, mice were euthanized, tumors isolated and proteins solubilized and immunoblotted against Myoferlin and HSP90. Bar graph shows individual (bar) and average (empty square) relative Myoferlin/HSP90 ratio quantification ± SEM, in which the lowest value was set to '1'. * $p < 0.05$.

days after a 2-week initial implantation period and randomization (day 0). At day 10, myoferlin siRNA-treated mice showed a significant 73% decrease in tumor burden, compared with scrambled siRNA treatments ($p < 0.01$) (Figure 22B), and 50% and 41% at

day 22, compared with vehicle (saline/DOTAP) and scrambled siRNA, respectively. After euthanasia at day 22, tumors were isolated. Quantification of myoferlin expression by the ratio of myoferlin to the HSP90 loading control in individual tumors revealed an average of 71% decrease in myoferlin expression in myoferlin siRNA-treated tumors, compared with control siRNA-treated tumors ($p < 0.001$) (Figure 22C). This finding indicates that loss of myoferlin expression decreases tumorigenesis in solid LLC tumors.

Because we have previously shown that endothelial cells express myoferlin [182], anti-PECAM-1/CD31 and myoferlin immunefluorescence experiments were performed in individual LLC tumors, revealing a relatively homogeneous myoferlin expression (Figure 23A). This finding was as expected, given that both LLC and endothelial cells express myoferlin, although slightly greater myoferlin staining was detected around blood vessels (Figure 23A). Surprisingly, however, quantification of vessel density (vessels per area) and maturity (ratio of PECAM-1/CD31-positive vessels smooth muscle cell to actin-positive vessels) at day 22 by immunohistochemistry, and likewise immunofluorescence, revealed no difference between control siRNA and myoferlin siRNA-treated tumors (Figure 23B & C). This finding indicates that decreased vessel perfusion per tissue area is unlikely the predominant mechanism behind decreased tumorigenesis in myoferlin siRNA-treated animals (although the extrapolated average total number of vessels per tumor will likely be lower in myoferlin siRNA-treated tumors, because of smaller tumor size). In addition, both myoferlin and scrambled siRNA groups showed similar serum levels of the angiogenic factor VEGF throughout the first 14 days of the experiments (Figure 23D), indicating normal activation of the angiogenic switch.

To quantify the state of poliferation in siRNA-treated tumors, KI-67 staining was performed on tumor sections; this revealed a significant 52% and 46% decrease in KI-67-

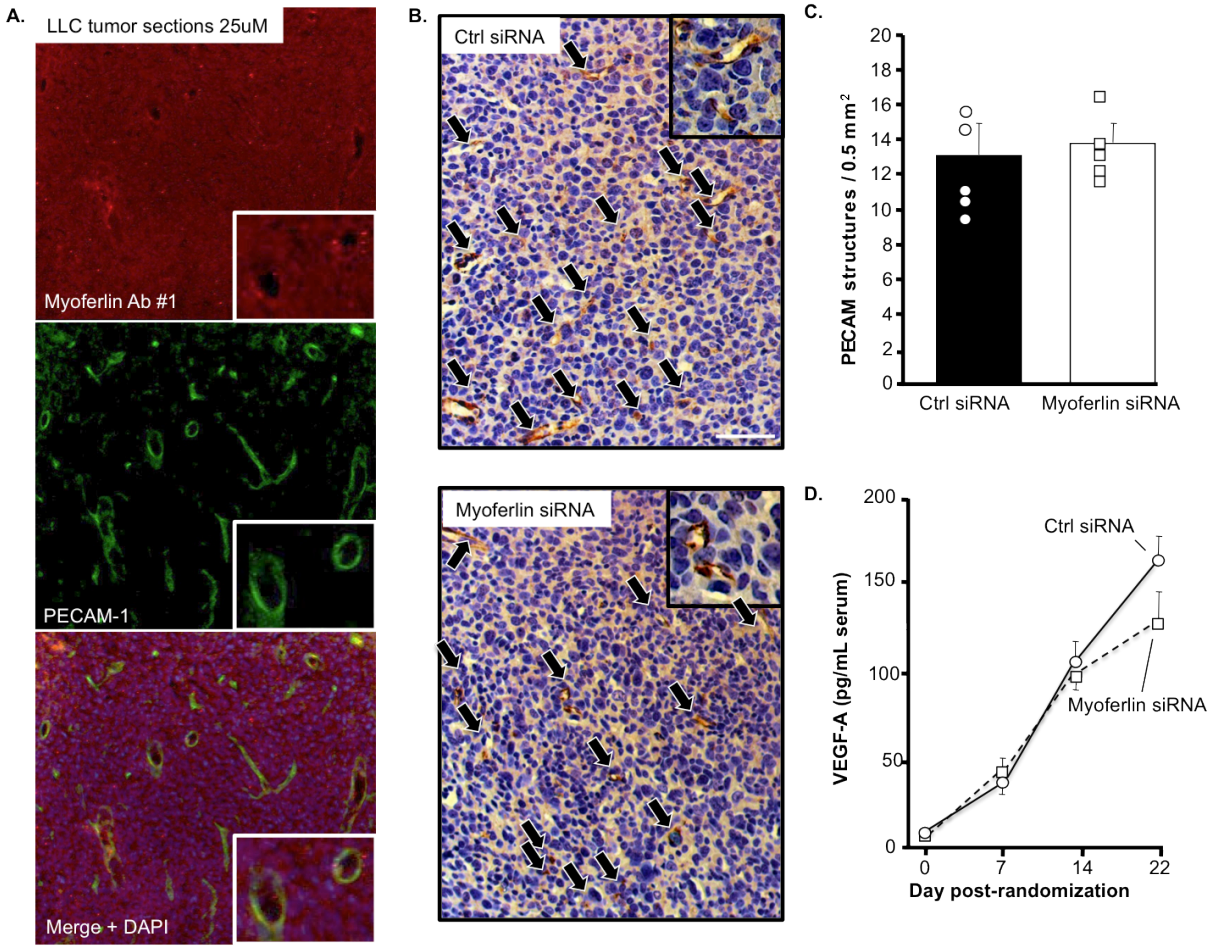


Figure 23. Myoferlin knockdown does not decrease blood vessel density in tumors.

(A) Immunofluorescence images from 25 μm tumor sections treated with anti-PECAM/CD31 antibodies coupled to Alexa-488 secondary antibodies (green) and Myoferlin antibody coupled to Alexa-568 antibodies (red). The merge image (right) contains DAPI stain (nucleus; blue). Inset shows magnified images. Scale bar = 100 μm. (B) Immunohistochemistry against CD31 and Mayer's haematoxilin counterstain using 6 μm thick tumor sections treated with control or Myoferlin siRNA sequences. Inset and arrows show magnified CD31-positive structures, scale bar = 75 μm. (C) Average (bars) and individual (empty circles/squares) quantifications of vessel density (CD31-positive structures) in tumors with control or Myoferlin siRNA sequences in a typical experiment, n = 6. (D) Myoferlin siRNA-treated animals showed near-identical serum VEGF levels (pg/mL) as control siRNA treated mice at days 0, 7, and 14, but slightly lower at day 22. Data represent mean ± SEM, n = 6.

positive cells (total tumor cells/area) at days 14 and 22, respectively, in tumors treated with myoferlin siRNA, compared with control siRNA ($p < 0.01$) (Figure 24A and B). To compensate for any changes in cell density as a result of blunted proliferation or tissue processing, we quantified the ratio of KI-67-positive versus KI-67-negative cells in individual tumors and observed average ratios of 3.6 and 1.2 at day 14 in control siRNA versus myoferlin siRNA-treated tumors, and 3.5 and 1.4, at day 22, representing a respective 66% and 60% decrease in proliferation for tumors treated with myoferlin siRNA (Figure 24C). In contrast, TUNEL staining at days 14 and 22 revealed low but similar levels of apoptosis in control and myoferlin siRNA-treated tumors (Figure 24D), suggesting that myoferlin silencing decreases tumor burden *in vivo* by decreasing the rate of proliferation.

5.2.3. Myoferlin regulates LLC cell proliferation in vitro

The changes we observed in KI-67-positive cells led us to treat cultured LLC cells with siRNA sequences, followed by starvation for G_0 cell-cycle synchronization and growth for 48 hours and 72 hours using 5% FBS proliferation medium. LLC cell proliferation was not affected by pre-treatment with non-silencing siRNA, compared with non-pre-treated cells, whereas myoferlin silencing (compared with non-silencing siRNA treatment) decreased LLC cell proliferation by up to 67% and 74% at 48 and 72 hours, respectively (Figure 25A). Furthermore, the specificity of our myoferlin siRNA approach was also evidenced by lack of anti-proliferative effect (data not shown) when LLC cells were treated with verified siRNA sequences designed against mouse dysferlin mRNA [165]. Attenuated proliferation with myoferlin siRNA was not a result of increased apoptosis, because 48 hours of pre-treatment of LLC cells with 75 nM myoferlin siRNA did not increase caspase-8 activity compared with control conditions, in contrast to the effect of two known inducers of apoptosis, Fas ligand

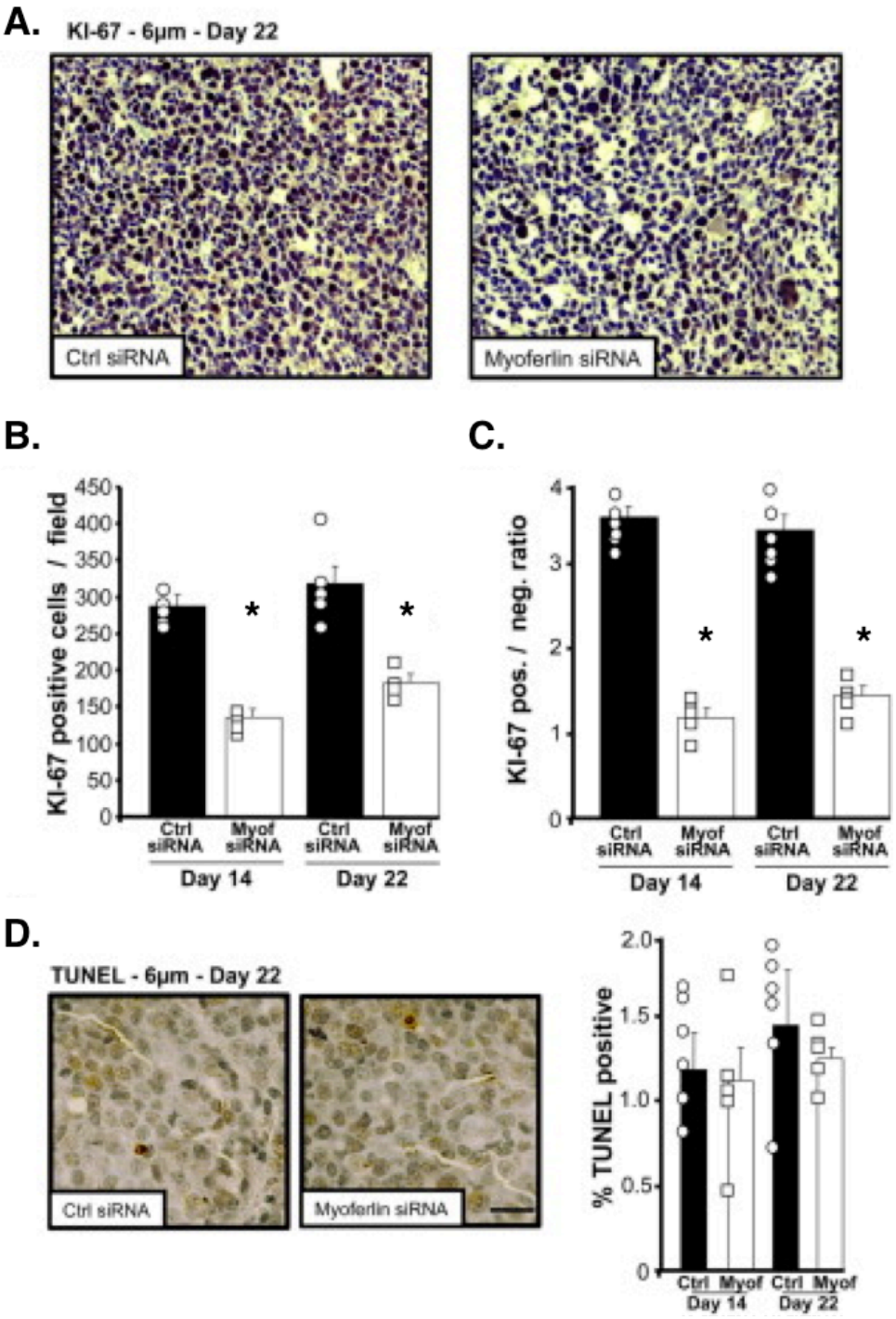
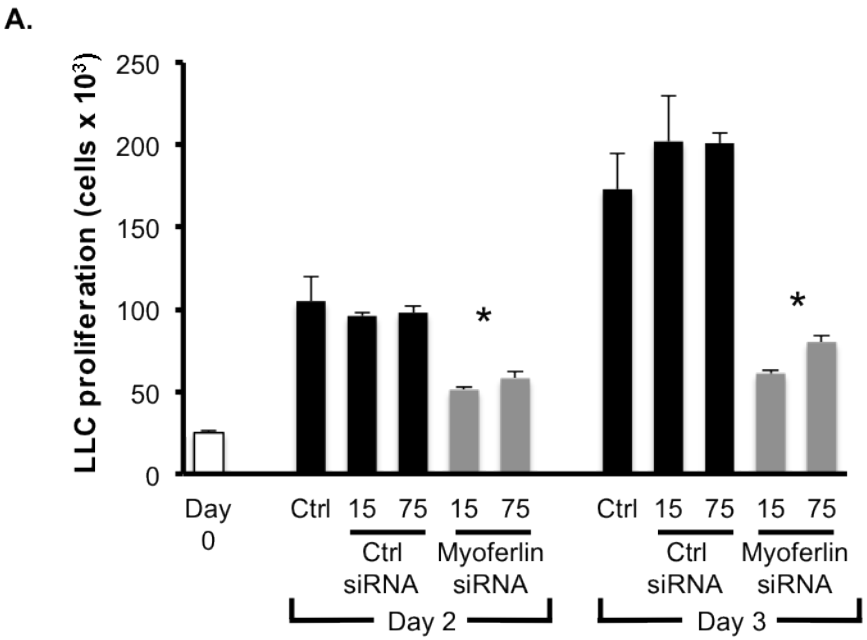


Figure 24. Myoferlin knockdown decreases proliferation in vivo.

(A) Representative tumor section stained by immunhistochemistry against KI-67 expression, a proliferation marker, in tumor sections following control or Myoferlin siRNA sequences treatment. Scale bar = 75 µm. (B-C) Individual (empty circles) and average (bars) quantification of total KI-67-positive cells (B) in tumors sections shown in panel A, and KI-67 positive / negative cell ratio (C) at days 14 and 22. (D) Left panel: Representative histological sections of tumors with control or myoferlin siRNA treatment subjected to

TUNEL assay after isolation at day 22. TUNEL-positive cells show robust brown staining. Right panel: Average (bars) and individual (symbols) quantification of TUNEL-positive cells at days 14 and 22. Data are expressed as means ± SEM and are representative of two independent experiments with similar results. N = 5 (myoferlin); N=6 (control). $p < 0.05$. Original magnification, x20 μm (A and D).

and ceramide (Figure 25B). This finding confirms that myoferlin plays an active role in tumor cell proliferation, rather than in apoptosis. Myoferlin expression levels were not affected by cell-cycle progression, because G_0 synchronization followed by FBS-induced growth did not modulate total myoferlin, as detected by Western blotting (Figure 25C).



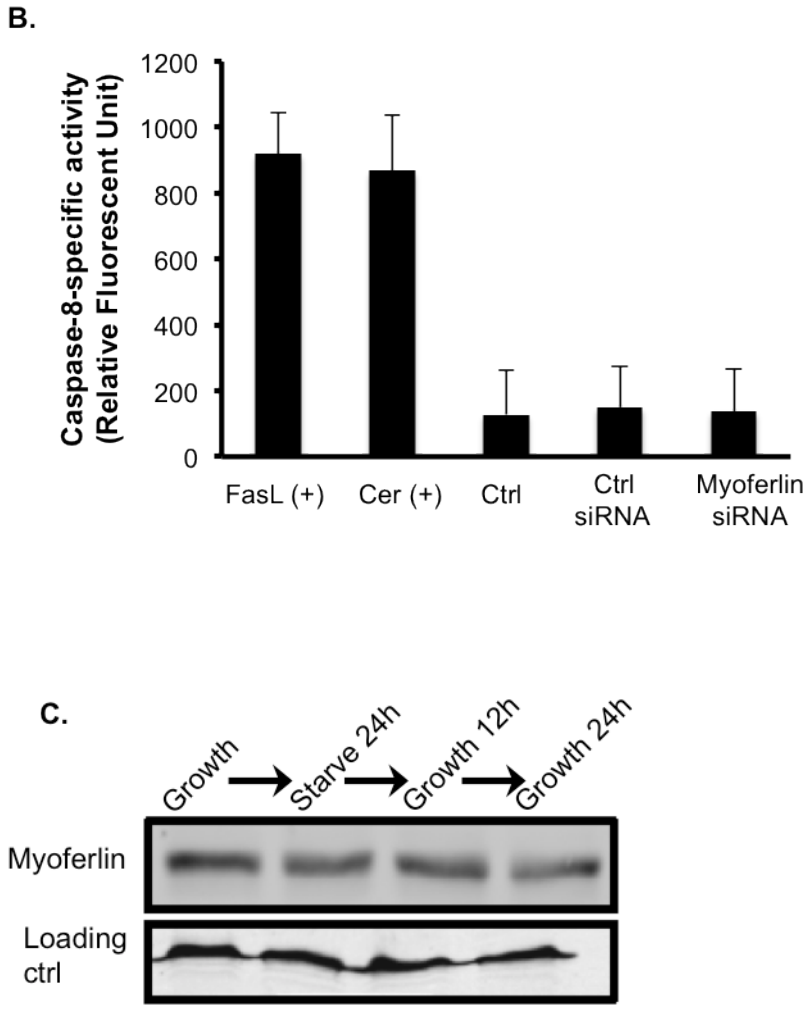


Figure 25. Myoferlin knockdown decreases proliferation *in vitro*.

(A) LLCs were seeded into 12-well plates and transfected with control or Myoferlin siRNA sequences at 15 and 75nM. Following starvation, cells were allowed to proliferate for 48 or 72h and cell number was counted using a hemocytometer. * $p < 0.05$. (B) The anti-proliferative effect by myoferlin knockdown is not triggered by apoptosis. Treatment of LLC with control or myoferlin siRNA does not increase caspase-8 activation, a marker of apoptosis. Stimulation with Fas ligand and ceramide was used as positive controls. N = 6 in triplicate. Average relative fluorescent units ± SEM. (C) Starvation/G_0 cycle synchronization followed by proliferation does not modulate myoferlin/loading control levels. Actively growing LLC cells were starved in serum-free medium for 24 h, followed by active growth (10% FBS) for 12 and 24 h. Cells were lysed and Western blot analysis was performed.

5.2.4. Myoferlin plays an active role in proliferation-induced membrane repair in tumor cells

To determine how myoferlin regulates LLC cell proliferation *in vitro*, we hypothesized that active plasma membrane resealing and remodeling would occur primarily in a setting of high proliferation, and that normal myoferlin expression is required for membrane homeostasis. Cultured LLC cells were treated with siRNA sequences for 48 hours and then subjected to laser damage in the presence of FM1-43 dye, an established model of physical membrane injury that results in local fluorescence (because of membrane permeability), followed by active remodeling and resealing. In LLC cells treated with control siRNA sequences and then incubated in low-FBS conditions (starvation for G_0 synchronization), myoferlin knockdown caused a near-threefold increase in local FM1-43 fluorescence (Figure 26A), indicating that myoferlin is required for optimal membrane integrity in LLC cells under conditions of low proliferation. Most interestingly, loss of myoferlin in LLC cells under high-growth conditions (2.5% FBS) caused a drastic increase in local damage, far beyond that observed in control siRNA–treated LLC cells (Figure 26A). The fluorescence of the damage site in growing myoferlin siRNA–treated LLC tumors was far more intense and greater in size than in control siRNA–treated cells, and was located more toward the cytoplasm of cells. Furthermore, disrupted cell shape and membrane, an indication of far greater damage, were often observed (Figure 26B). Together, these data indicate that the high growth rate of LLC cells requires additional membrane maintenance signaling and, most importantly, that myoferlin is especially required for rapid LLC cell proliferation, providing mechanistic evidence of the role of myoferlin in tumorigenesis.

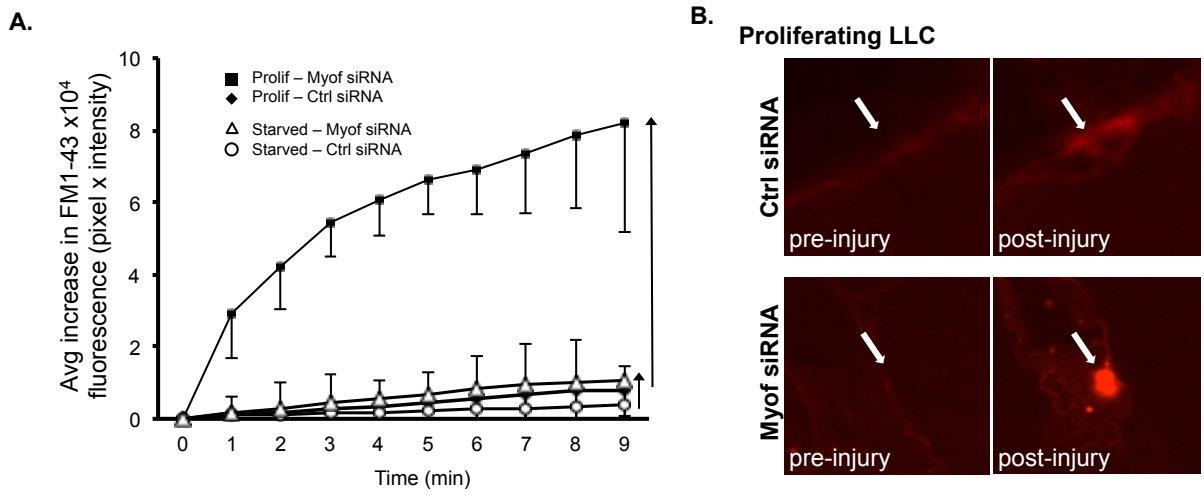


Figure 26. Myoferlin knockdown decreases membrane repair.

Loss of Myoferlin drastically increases membrane damage following laser injury in proliferating LLC cells. A small (3μm x 3μm) area of membrane damage was induced by confocal laser in the presence of FM1-43, a dye that fluoresces only in lipid membrane structures, and images were captured every minute from time 0 (before injury) to 9 minutes. Quantification (A) and visualization (B) of local fluorescence was performed in LLC after siRNA treatment (48h), under starvation or proliferation condition. The effect of myoferlin gene knockdown on the damaged area in starved LLC cells (A) is indicated by a short arrow and in proliferating LLC cells by a long arrow. FM1-43 fluorescence (B) increased in both control and myoferlin siRNA-treated LLC cells under proliferating conditions. The injury site before and 9 minutes after damage is indicated by a white arrow in each image. Data are expressed as means ± SEM. Original magnification, 63 X.

5.3. Discussion

Here we have described the stable and near-ubiquitous expression of myoferlin in human and animal epithelial cancer cell lines and in solid carcinoma tumors. Myoferlin silencing decreased LLC tumorigenesis and proliferation, while leaving the angiogenic switch unaffected. Mechanistically, we observed that LLC cells rely on myoferlin expression for membrane resealing/remodeling, a process that appears to be highly up-regulated in fast-growing tumor cells versus slow-growing cells. Our data further document the functional

presence of a ferlin protein family member in tumors and suggest that interfering with myoferlin expression or plasma membrane integrity could lead to therapeutically relevant anti-proliferative effects.

Our data also further document the expression of ferlin proteins in a growing number of non-contractile cells. Although initially characterized in muscle cells and linked directly to specific forms of muscular dystrophy and cardiomyopathy [135, 373, 420, 421], ferlins have subsequently been linked to endothelial cell angiogenesis and blood vessel permeability, nephropathies, Alzheimer disease, multiple sclerosis, inflammatory diseases, and preeclampsia [166, 377, 412, 422-424]. These discoveries suggest that the nature of ferlins is yet to be fully characterized, and that their roles in non-muscle cells must be evaluated through open-minded approaches. This was clearly evidenced by a recent article reporting that the well-recognized predominant localization of ferlins at the plasma membrane stems from a fixation artifact [425]; our present finding, that the bulk of ferlins are located in the cytosol, further illustrates the limits of our knowledge about ferlins. On another note, past microarray data have suggested that myoferlin might be up-regulated in certain breast cancer cell lines, although no biological significance for such overexpression was reported, apart from mathematical modeling of invasion [426, 427]. The present data, however, show relatively homogeneous myoferlin expression in nontransformed, oncogene-transformed, and metastatic cell lines, suggesting that myoferlin does not participate in malignant transformation.

For decades, many established anticancer therapies, including cisplatin [428], taxol [429], doxorubicin [430, 431], and cyclophosphamide [432], have targeted cancer cell proliferation through a direct or indirect fashion, resulting in cytostasis. The present data suggest that myoferlin functions to control tumor cell proliferation through its role in

regulating plasma membrane integrity, thereby supporting the idea that impairing myoferlin activity and/or plasma membrane integrity could provide unique antitumor effects. It remains to be determined, however, how to interfere with myoferlin activity or, more likely, how to block the endogenous outer membrane remodeling and resealing capabilities of a tumor cell. For the present study, we made use of *in vivo* siRNA-based gene silencing in tumors, a technique previously shown by our research group to be highly RNA template specific [182]. Other researchers have used similar approaches to study the role of different signaling molecules in tumorigenesis [433, 434], thereby confirming the feasibility of knocking down specific genes in tumors to study their biological function. Although our data show that loss of myoferlin in tumors drastically decreases the proliferation rate of cancer cells, myoferlin-dependent angiogenesis appears to be modulated to a lesser extent, because the total number of blood vessels of notoriously hypoxic tumor tissues was decreased, but not their density. Nonetheless, if and how myoferlin-dependent membrane remodeling and turnover regulate other aspects of tumor cells or other cell types found in tumor stroma warrants further investigation.

Although here we have provided direct evidence in support of the role of myoferlin in adapting tumor cells to conditions of high proliferation through its ability to promote membrane resealing, it remains possible that the ability of myoferlin to regulate cargo protein trafficking may also be important for tumor cell proliferation. If and how the calcium-sensing C2 domains of myoferlin help to regulate its activity in the context of the tumor microenvironment also remains to be determined. For example, one complex but profoundly interesting experiment would focus on visualizing in settings of membrane damage not only lipid metabolism (FM1-43 dye), but also its dynamic colocalization with fluorescently tagged versions of myoferlin and calcium entry (Fura-2 probe). Recent evidence suggests that

myoferlin-positive vesicles and/or lipid patches might contain cargo proteins (e.g, transmembrane proteins or neurotransmitters) [160, 165, 182, 184, 195]. Whether the cargo trafficking function of myoferlin in tumors is essential for the high-proliferative aspect of cancer cells, or whether instead its resealing and repair activities are sufficient, remains to be determined. On the whole, it remains enigmatic whether these two distinct types of events (remodeling versus trafficking) can be dissociated, an uncertainty that further stresses the importance of developing biochemical approaches to isolate ferlin-positive intracellular patches of membrane and to characterize their protein cargo content.

Although tumors are not considered to be mechanically active, we provide evidence that tumors rely on a physical membrane resealing and/or remodeling mechanism to maintain their high growth rate. In addition to proliferation, myoferlin-dependent membrane remodeling may also help tumors adapt to the physical constraints of their microenvironment [435] and, according to recent *in vitro* evidence [364], may contribute to their capacity to progress to invasion and even metastasis. As a consequence of unchecked proliferation, tumors also tend to undergo accelerated cellular apoptosis and necrosis, because of a combination of factors such as insufficient angiogenesis, lowered oxygen and nutrient levels, and highly acidic microenvironments. This ultimately leads to the formation of the well-documented necrotic core, a step that could potentially be delayed in tumor cells with more sustained repair signaling. This raises the possibility that a selection process favoring cells with more active membrane repair systems capable of coping with toxic tumor environments may take place in certain tumors, leading to greater tumorigenesis. Conversely, interfering with membrane repair signaling might represent a novel therapeutic strategy for rapidly dividing cancers.

6. Myoferlin Regulates Human Non-Small Cell Lung Cancer Cell Proliferation and Invasion through Akt Signaling Pathway

6.1. Introduction

Tumor metastasis is considered the most lethal aspect of cancer, accounting for 90% of cancer-related deaths [436]. Among all cancers, lung cancer is the second most common malignant cancer, only after skin cancer [437]. The highly vascularized nature of the lungs provides excellent channels for cancer cells to migrate out of to the rest of the body [438]. Lack of early diagnosis of lung cancer normally means it is often found at advanced stages, usually when the lung cancer has already become metastatic. Another challenge encountered by patients with lung cancer is the development of drug resistance, causing chemotherapy and radiation therapy ineffective in some patients. The combination of these factors makes lung cancer the primary cause for cancer-related deaths worldwide.

Research efforts in NSCLCs identified several genetic alterations in the components involved in the receptor tyrosine kinase (RTK)-mediated phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway [334, 439]. Since RTK signal transduction regulates tumor cell proliferation, differentiation, migration, apoptosis and adhesion, which are imperative for tumorigenesis, any aberrant activity or expression of RTKs, or the downstream proteins, PI3K and Akt, could promote lung tumor growth and metastasis [440, 441]. Given this, one therapeutic approach is to inhibit the RTK signaling pathway. A class of drugs known as tyrosine kinase inhibitors (TKIs) has demonstrated some success in NSCLC patients with genetic alterations in RTK signaling proteins. TKI treatment prolongs the overall survival time for NSCLC patients from 10 to 12 months in clinical trials [442].

Subsequent studies using TKIs, such as gefitinib and erlotinib, which inhibit EGFR activity, have shown survival benefits in NSCLC patients with gain-of-function mutations or amplifications of the EGFR gene [443, 444]. Patients who have activation of downstream signaling protein Akt in their tumors are more sensitive to gefitinib than those who do not [445]. Although many patients initially respond to EGFR-TKI treatment, a significant fraction develops secondary resistance while receiving treatment [446]. A likely cause for such resistance is the presence of secondary genetic mutations of EGFR that decreases the potency of TKIs or upregulation of phosphorylated Akt that results in RTK-independent activation of Akt pathway [447, 448]. Both mechanisms coincidently increase Akt activity.

Activation of Akt is mediated by the phosphorylation of PI3K triggered through the binding of extracellular signals such as growth factors to their specific RTKs on the cell membrane. Following phosphorylation, PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate [$PI(3,4,5)P_3$] at the cell membrane [449], which then recruits Akt to the inner leaflet of the cell membrane to be phosphorylated [449]. Activation of Akt promotes cell survival, proliferation, growth, differentiation, migration and apoptosis [450, 451]. Interestingly, little or no Akt activity is found in normal and hyperplastic bronchial epithelia while metaplastic or dysplastic bronchial epithelia show enhanced Akt activity [452]. In addition, NSCLC cells transfected with wild-type Akt plasmids become more invasive than those transfected with green fluorescent protein expression vector or dominant negative Akt vector in the presence of serum or growth factor [452]. On the other hand, dominant-negative Akt plasmids inhibit the invasion of NSCLC cells even when serum or growth factor is used as a chemoattractant [452]. These findings suggest that Akt activation is essential for the malignant progression of bronchial epithelial lesions to lung tumors as well as lung cancer metastasis. Thus, it is possible that modulating the activity of

Akt may suppress the development and progression of lung cancer.

Gene microarray studies performed in lung cancer samples revealed that the expression of numerous genes is upregulated and one of these genes is myoferlin [188, 189]. However, no further research was pursued to study the role of myoferlin in lung cancer. Previously, myoferlin has been shown to regulate the expression of RTKs in various cell types, including VEGFR-2 and Tie-2 receptor in vascular endothelial cells [182, 184] and IGF1-R in myoblast [453]. It is believed that myoferlin mediates trafficking of vesicles that carry RTKs as protein cargos. This is supported by the structural characteristics of myoferlin in which it contains seven C2 domains, a transmembrane domain and a short extracellular domain [133]. Since expression of RTKs is associated with tumorigenesis, myoferlin is likely to have a functional role in lung cancer. In normal bronchial epithelial cells, we previously found that silencing myoferlin in normal bronchial epithelial cells decreases the expression of tight junction protein ZO-1 and increases apoptosis and cell detachment [164]. Myoferlin knockdown decreases both mouse lung cancer cell proliferation *in vitro* and solid lung tumor growth in a mouse xenograft model [454]. However, the underlying molecular mechanism of the anti-proliferative effect of myoferlin knockdown in lung cancer has not been investigated. Recent studies using breast cancer cells shed light on myoferlin as a regulator for the expression of EGFR and HER [190, 191]. These studies examined the effect of myoferlin depletion on the expression of EGFR and HER after pre-incubating the cells with EGF. Such pre-treatment of EGF upregulates the expression of EGFR and HER receptors on the cell membrane, the overall effect of myoferlin depletion on the expression of other membrane receptors may have been ignored under this condition. Furthermore, it is possible that myoferlin also regulates the translocation of cytoplasmic protein Akt during signal transduction via intracellular vesicle trafficking. Since Akt is a downstream mediator of the

RTK-mediated signaling cascade, examining the activity of Akt will provide some insight of overall RTK activity in lung cancer tumorigenesis. Herein we present evidence for myoferlin mediating human lung cancer cell proliferation and invasion being mediated by the activation of Akt.

6.2 Results

6.2.1. Myoferlin siRNA causes appearance changes and inhibits the proliferation and adhesion of A549 cells

To study the effect of myoferlin knockdown on metastatic human NSCLC, we used the A549 cell line and performed myoferlin knockdown assays. The A549 cells were treated with or without transfection reagent, scrambled siRNA or myoferlin siRNA for 72 h. After transfection, we observed that myoferlin siRNA-treated cells lost their normal cuboidal shape, a phenotype observed in epithelial-mesenchymal transitional cells, at all three concentrations (50, 75 and 100 nM) compared to cells treated with scrambled siRNA (Figure 27A). Also, the number of myoferlin siRNA-treated cells detached from the culture plates was about 3-4 times that of scrambled siRNA control at 72 h (Figure 27B). Following protein isolation at 72 h post-transfection, we performed Western blot analysis to examine the expression of myoferlin in cells treated with the conditions described above. As shown in Figure 27C, myoferlin expression levels were decreased by 66%, 73% and 78% compared with scrambled control when myoferlin siRNA sequences were used at 50 nM, 75 nM and 100 nM, respectively, indicating a concentration-dependent effect. These results support the role of myoferlin in maintaining the normal phenotype of A549 cells as well as their adhesion to their growth substrate.

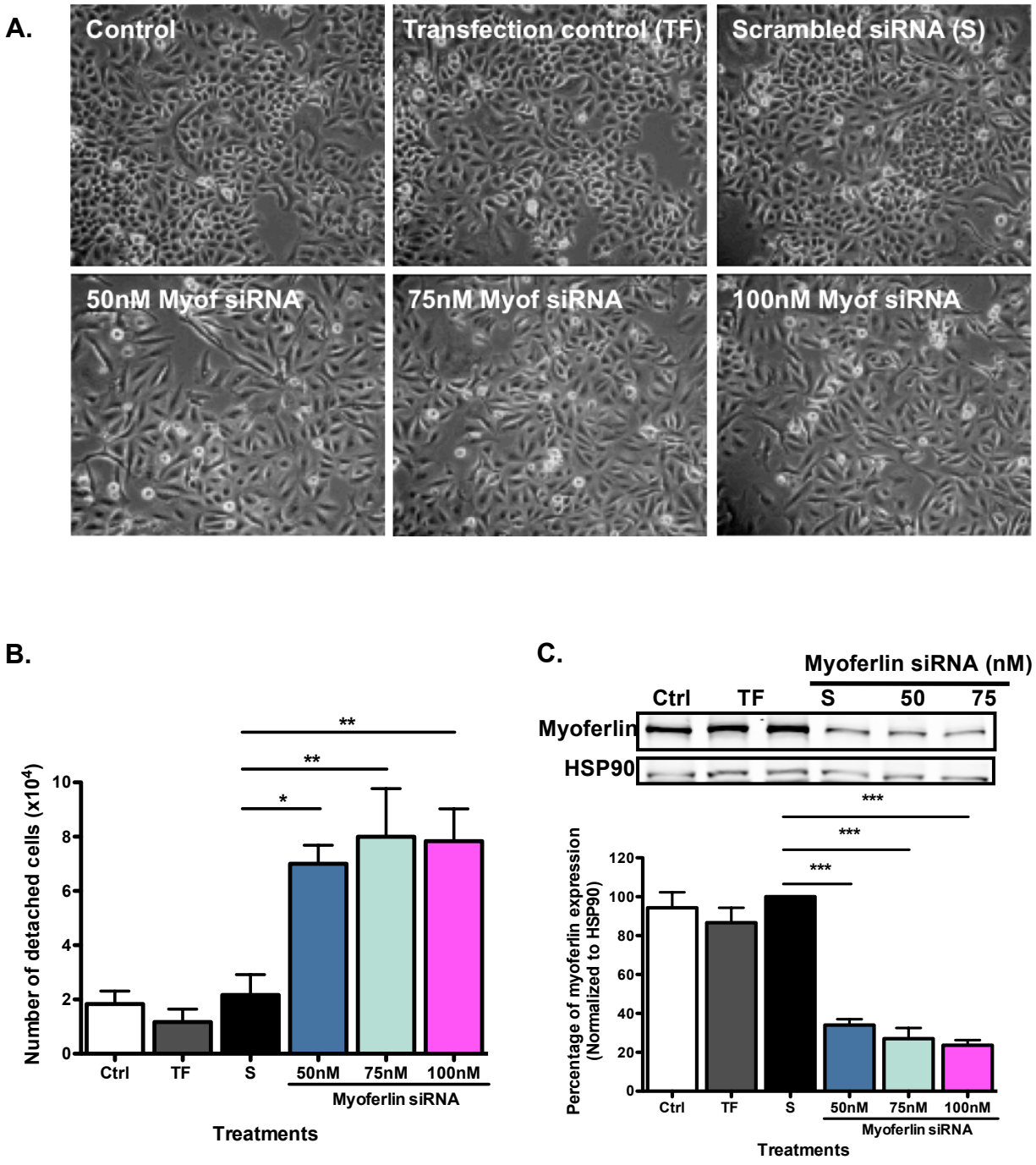


Figure 27. Knockdown of myoferlin with targeted small-interfering RNA sequences induces cell appearance changes and cell detachment of cultured human lung adenocarcinoma cells.

(A) Representative images of A549 cells treated with media (Ctrl), transfection reagent (TF), scrambled siRNA (S), and myoferlin siRNA (Myof siRNA) at 50 nM, 75 nM, and 100 nM concentrations were captured by a phase-contrast microscope (n=3). (B) Quantification of cells detached from the cultured plates in function of treatments. Cell numbers were counted using a hemocytometer at 72 h post-transfection and compared to scrambled siRNA control.

(C) Top, representative immunoblots from 3 independent experiments showing the expression of myoferlin from protein lysates collected at 72 h after treatments. Bottom, bar graph represents densitometry analysis of the band intensities normalized to the loading control, HSP90, and expressed as percentage relative to the scrambled siRNA controls. Data are expressed as mean ± SEM of three independent experiments and significant differences between the treatment groups and scrambled siRNA are denoted with asterisk (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

After confirming the successful knockdown of myoferlin expression using our siRNA sequences by western blots, we examined the effect of myoferlin siRNA on the proliferation of human lung cancer cells. Knockdown of myoferlin did not induce any significant changes in cell proliferation at 24 h. At 48 and 72 h, myoferlin knockdown decreased A549 cell proliferation by up to 54% and 48%, respectively, when compared to scrambled siRNA control (Figure 28). These data indicate that myoferlin plays a crucial role in promoting tumor cell proliferation at all three concentrations used.

6.2.2. Myoferlin siRNA attenuates wound closure and cell invasion, but not migration, of human lung adenocarcinoma cells

Tumor cell proliferation is the main contributor to the growth of solid tumors, whereas cell migration and invasion are key events for tumor metastasis. Since myoferlin mediates vesicle trafficking and membrane fusion events, myoferlin may be involved in intensive cell membrane remodeling, migration and invasion, where endocytosis and exocytosis occur on a frequent basis. To evaluate the anti-metastatic effect of myoferlin knockdown on A549 cells, scratch assays, transwell cell migration experiments and matrigel cell invasion assays were performed.

For scratch assays, images of the scratches were taken by phase-contrast microscopy

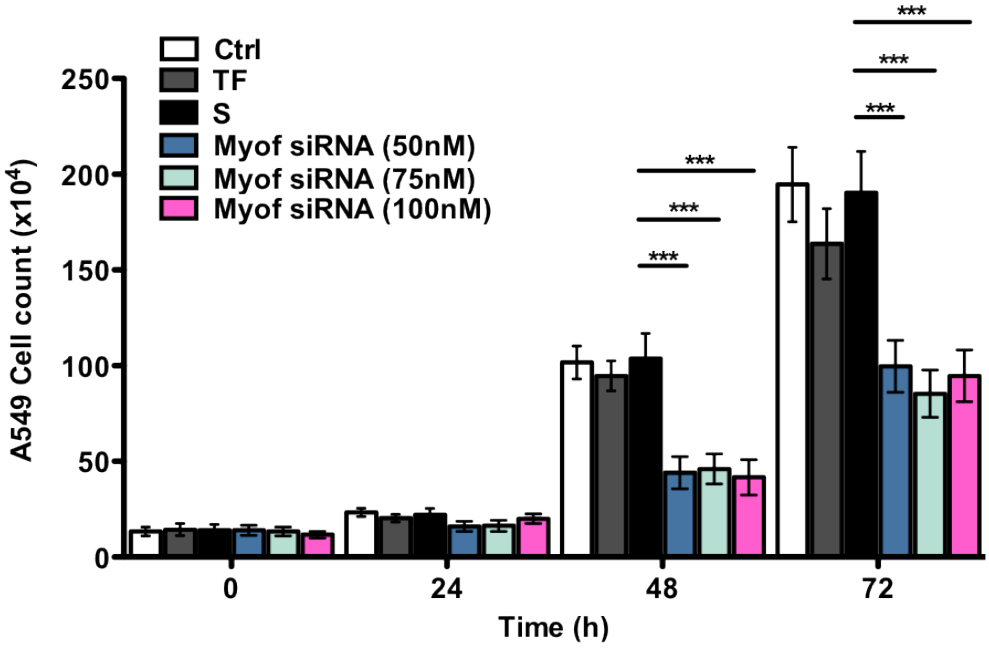
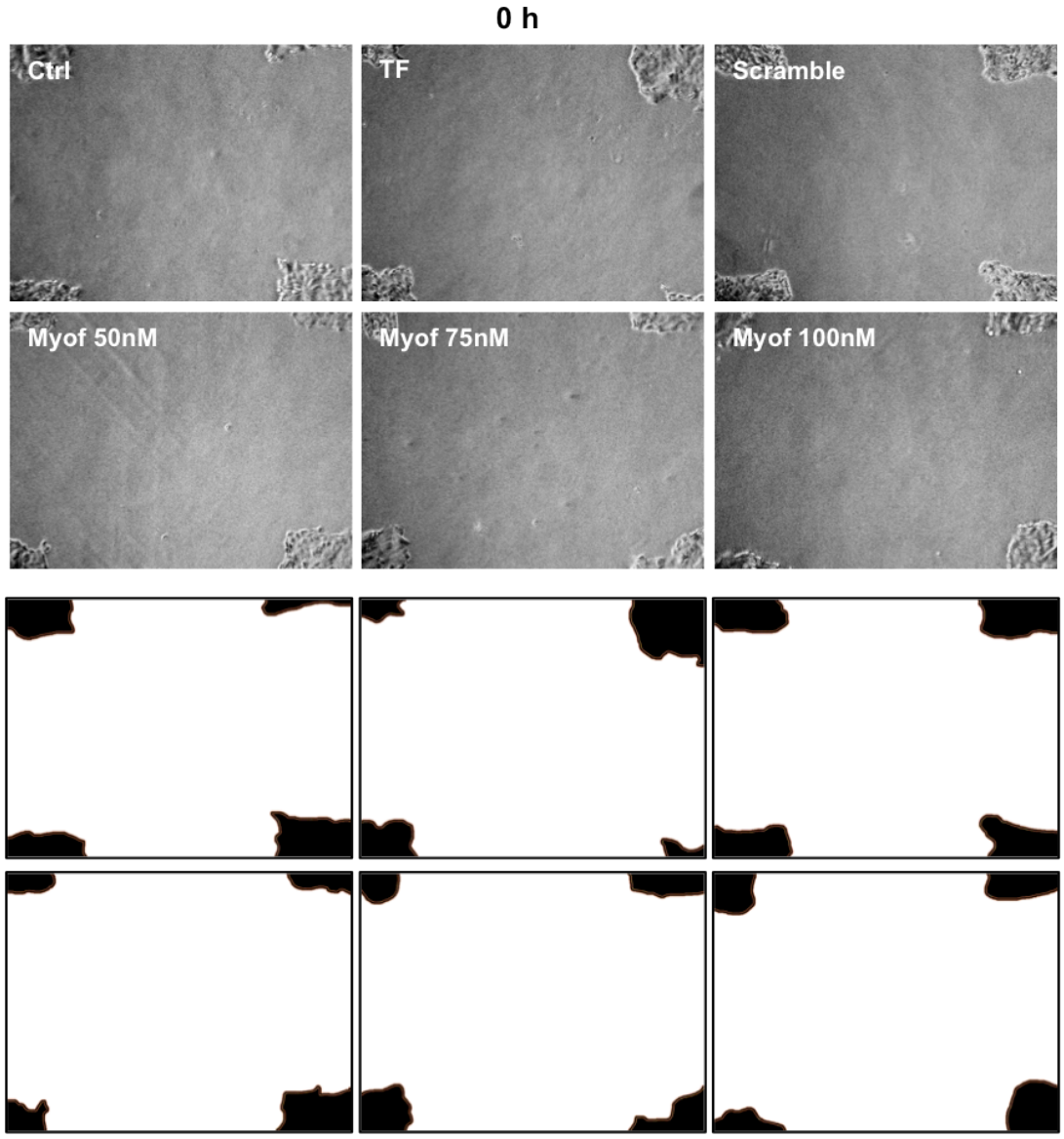


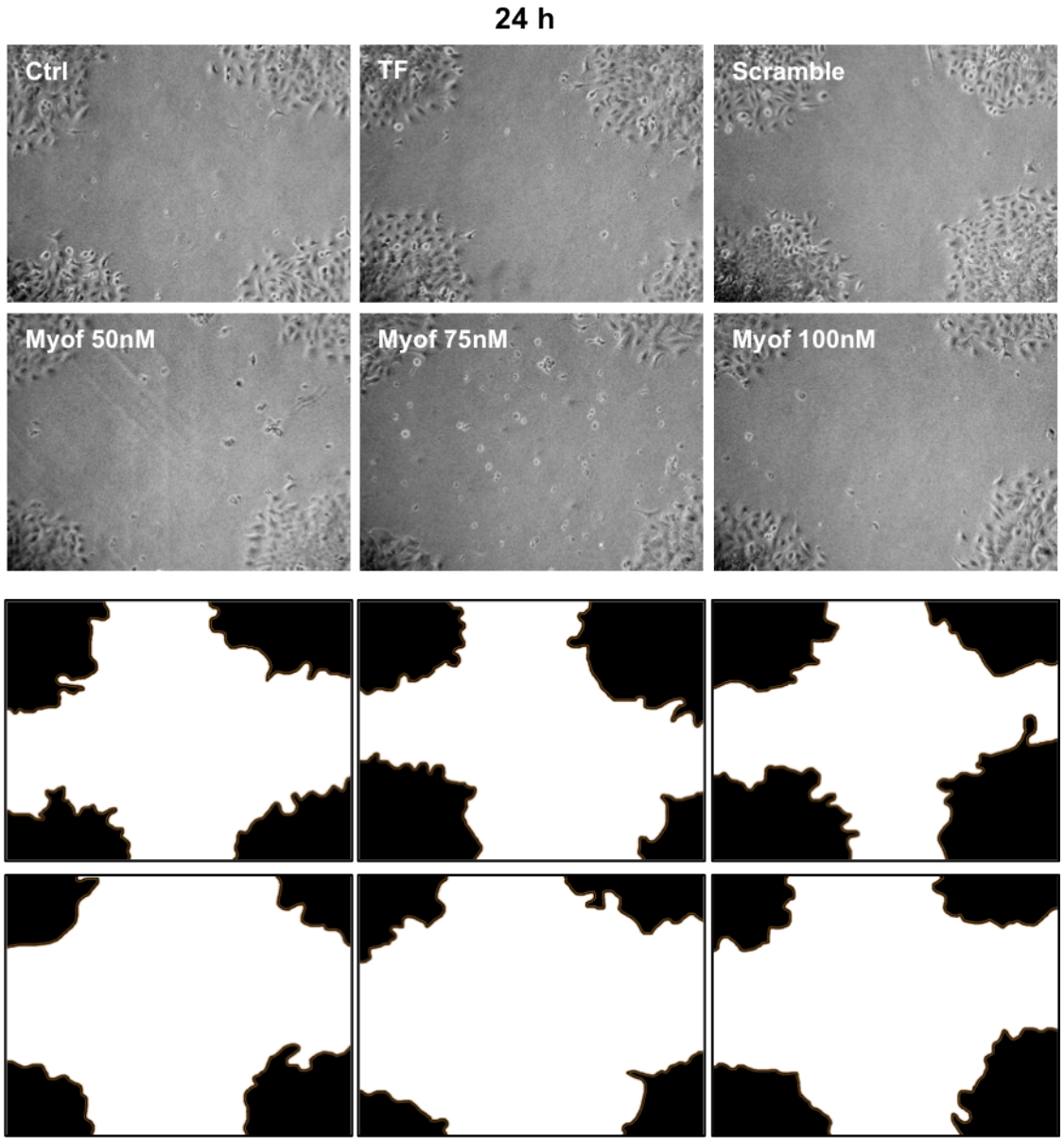
Figure 28. Myoferlin siRNA decreases cell proliferation of cultured human lung adenocarcinoma cells.

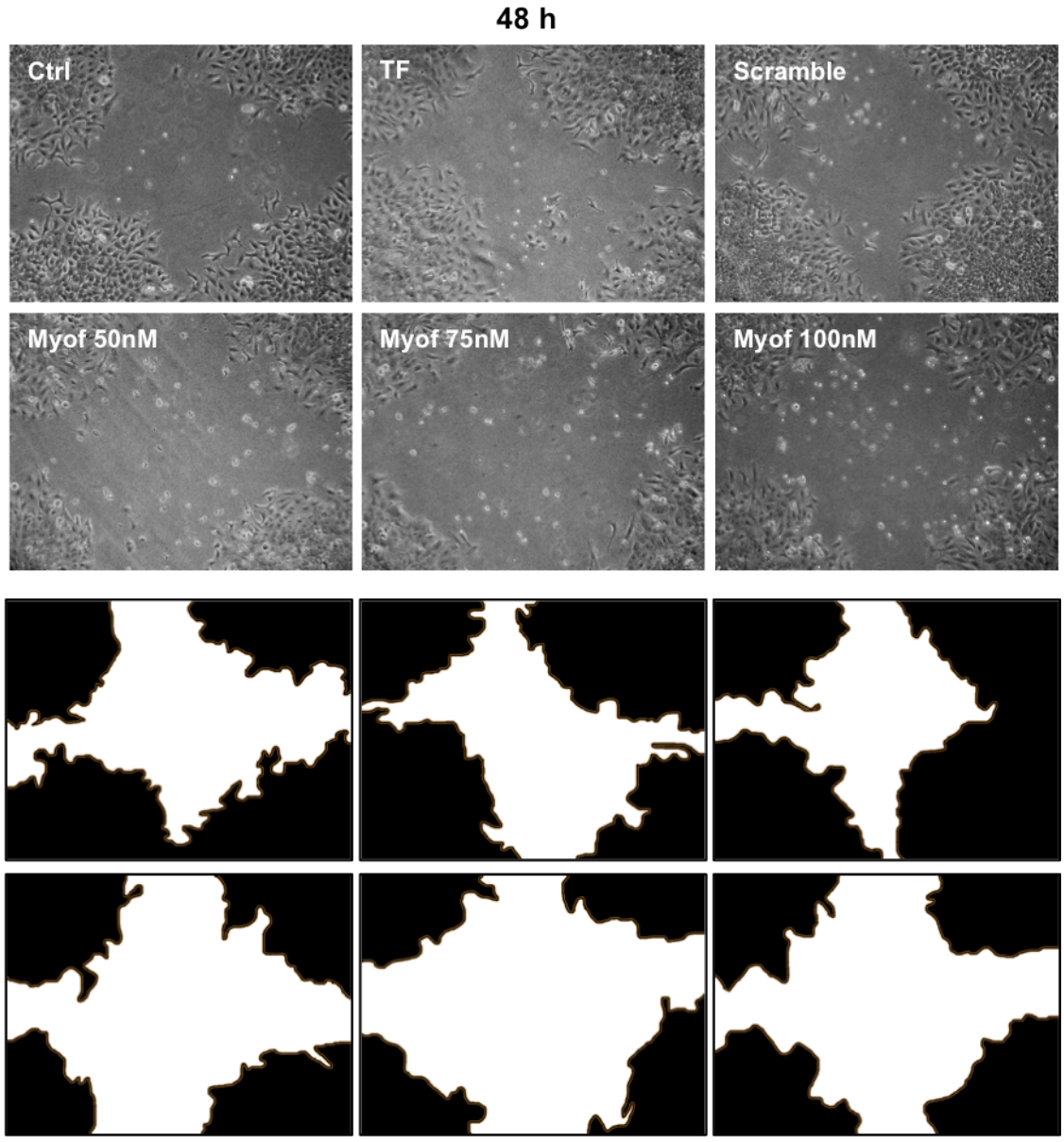
A549 cells were treated with media control (Ctrl), transfection reagent (TF), scrambled siRNA (S), and myoferlin siRNA (Myof siRNA) for 48 h before separately seeding $5x10^4$ cells into 24-well plates. Next day, growth media were replaced with serum-free media to synchronize the cell cycle overnight. Number of cells in each treatment group was counted using hemocytometer at 0, 24, 48 and 72 h time points. Values are expressed as the mean ± SEM of three independent experiments (***$p < 0.001$ versus scrambled siRNA control).

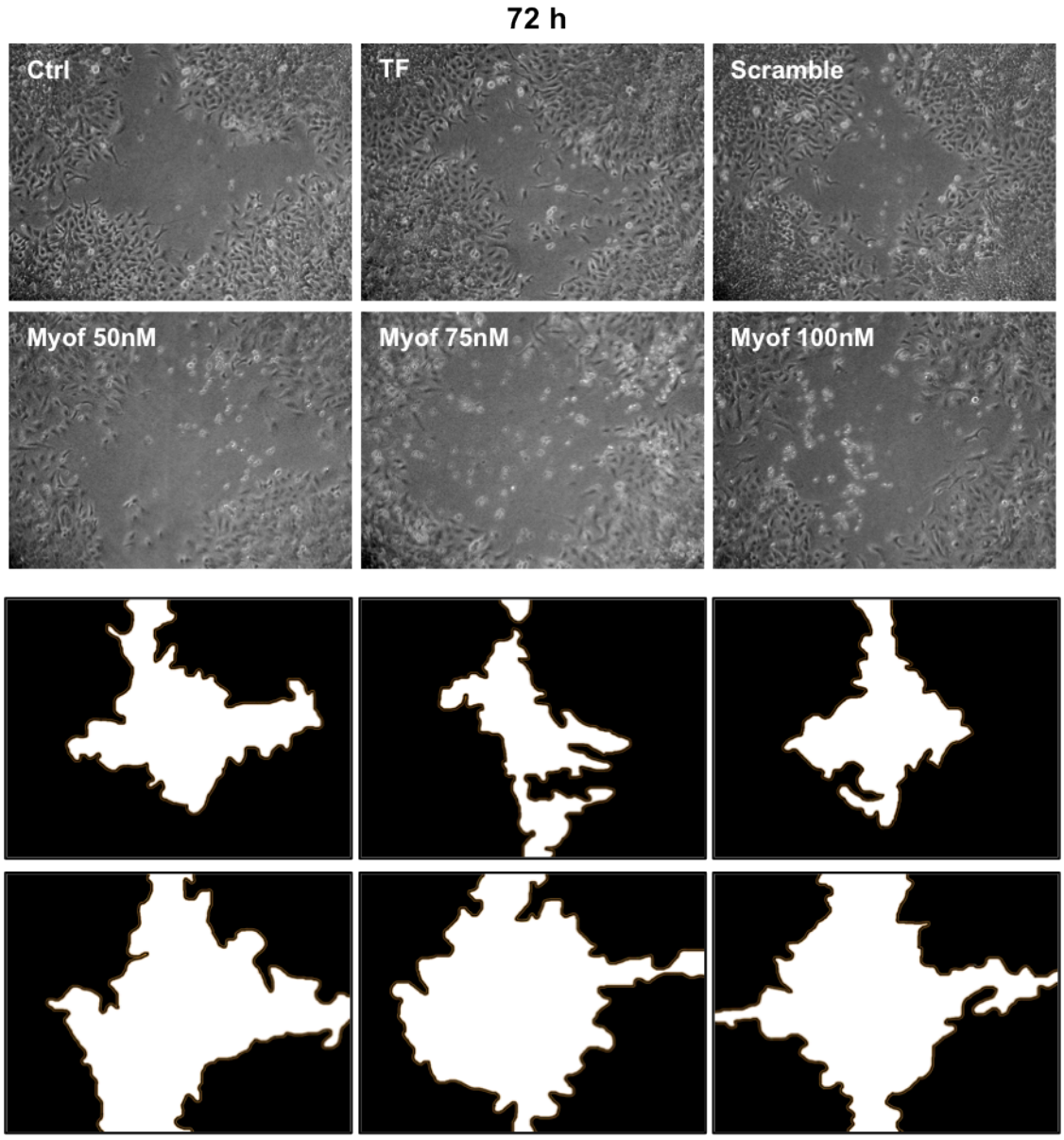
every 24 h. As depicted in Figure 29A, we observed that A549 cells treated with myoferlin siRNA had impaired wound healing process at 24, 48 and 72 h after the scratch (at 0 h). Quantification of wound closures of the myoferlin siRNA treatment groups showed a 22% and 19% reduction in wound closure at 48 and 72 h, respectively, when compared to scrambled siRNA control. Myoferlin knockdown did not have a significant effect on wound closure at 24 h (Figure 29B).

A.




24 h
Ctrl
TF
Scramble
Myof 50nM
Myof 75nM
Myof 100nM


48 h
Ctrl
TF
Scramble
Myof 50nM
Myof 75nM
Myof 100nM


72 h
Ctrl
TF
Scramble
Myof 50nM
Myof 75nM
Myof 100nM

B.

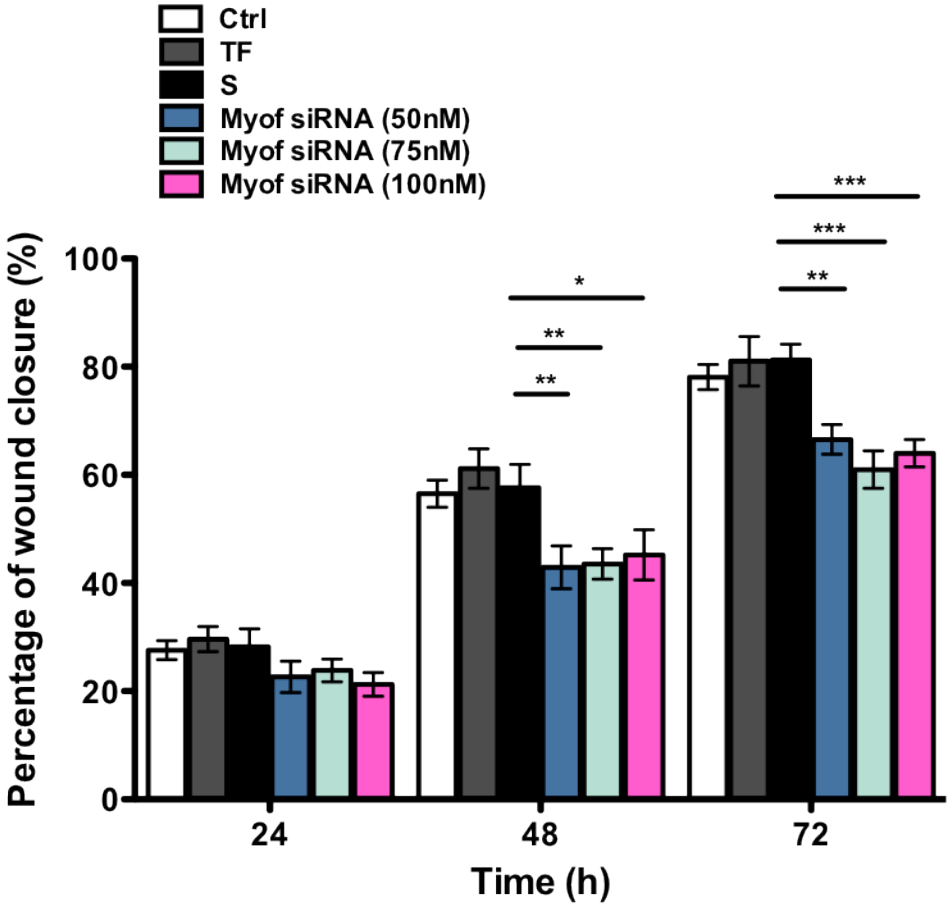


Figure 29. Myoferlin knockdown impairs wound healing of A549 cells at 48 h and 72 h following the scratch.

(A) Subconfluent A549 cells were transfected with media control (ctrl), transfection reagent (TF), scrambled siRNA (S) or myoferlin siRNA (Myof siRNA) and allowed to grow to full confluence before making a cross-sign scratch in the confluent monolayer (at time 0 h). Images were taken under phase-contrast microscope at 24, 48 and 72 h post scratch to monitor the change in wound closure. Representative images of each treatment group illustrate the progress of wound-closure at 0, 24, 48 and 72 h. Color-segmentation was implemented on the captured images to quantify the area of the wound. White regions indicated the wounded area and black regions indicated the wound-closure process. (B) Areas of the wound closure were calculated for each treatment group and expressed as a percentage of wound closure at the given time point compared to the initial wound at 0 h. Data are represented as mean ± SEM of 5 independent experiments. A significant difference between the treatment groups and scrambled siRNA control is denoted with asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

To further characterize the potential role of myoferlin in A549 cell migration, we performed transwell migration assay. We found that human lung adenocarcinoma cells treated with or without myoferlin siRNA were capable of migrating through the membrane filters at 24 h at similar levels (Figure 30A). Thus, myoferlin knockdown did not affect the migration of A549 cells when serum was used as a chemoattractant (Figure 30B).

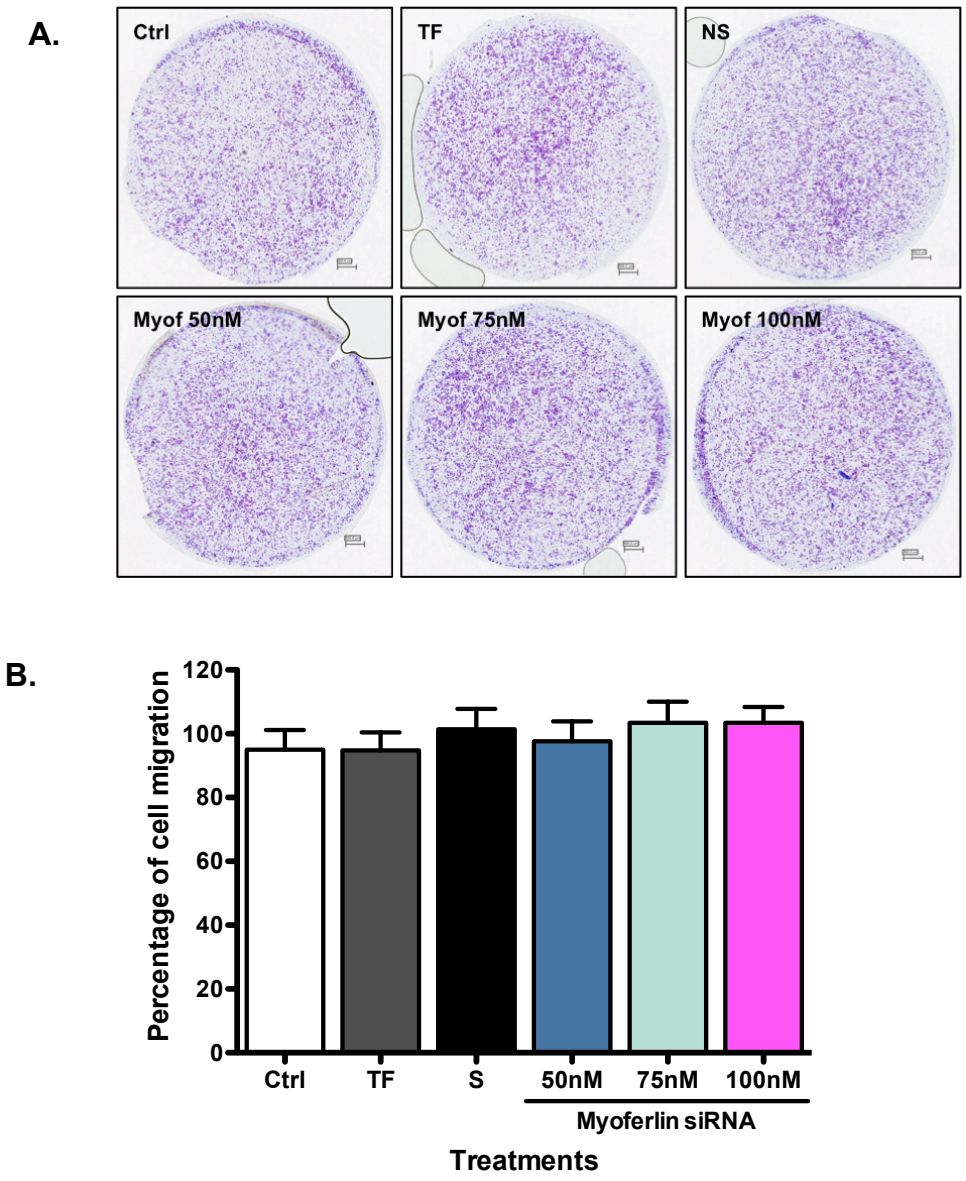


Figure 30. Knockdown of Myoferlin in human A549 cells does not affect Transwell cell migration.

(A) Media control (ctrl)-, transfection reagent (TF)-, scrambled siRNA (S)-, or myoferlin siRNA (Myof)-transfected A549 cells. Cells migrated to the lower side of the membrane were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Representative images obtained from 5 independent experiments are shown. (B) Migrated cells were counted by taking the total number of cells contained in 8 randomly selected fields on the images captured using an Aperio ImageScope at 40X magnification. The total numbers of migrated cells were expressed in percentage relative to the scrambled siRNA control cells. Migration of scrambled siRNA cells was set at 100%. Values are represented as means ± SEM of 5 independent experiments.

As lung cancer cells acquire an invasive phenotype as cancer progresses and becomes a metastatic tumor, we also assessed whether myoferlin contributes to the invasiveness of A549 cells by coating a thin layer of matrigel on the upper side of Transwell inserts. As shown in Figure 31A, the invasiveness of A549 cells through the matrigel was impeded by myoferlin siRNA treatments. Cells treated with 50 nM, 75 nM or 100 nM of myoferlin siRNA showed a significant decrease in invasion by 73%, 74% and 76%, respectively, when compared to the scrambled siRNA control (Figure 31B). These data suggest that myoferlin is essential for the wound healing process at longer time points as well as invasion of lung cancer cells.

6.2.3. Myoferlin regulates the activation of the Akt signaling pathway

To investigate the molecular mechanism of how myoferlin mediates cell proliferation, wound healing and invasion of human lung cancer cells, we evaluated the Akt signaling pathway. We found that myoferlin knockdown showed a minor increase in total Akt (Figure 32A). On the other hand, phosphorylation of Akt in A549 cells was decreased by 44%, 56% and 44% at 50 nM, 75 nM and 100 nM of myoferlin siRNA, respectively (Figure 32B). These results

indicate that loss of myoferlin decreases Akt phosphorylation, which in turn might rationalize the decrease in tumor cell proliferation, wound healing and tumor cell invasion observed in Figure 28, Figure 29 and Figure 31.

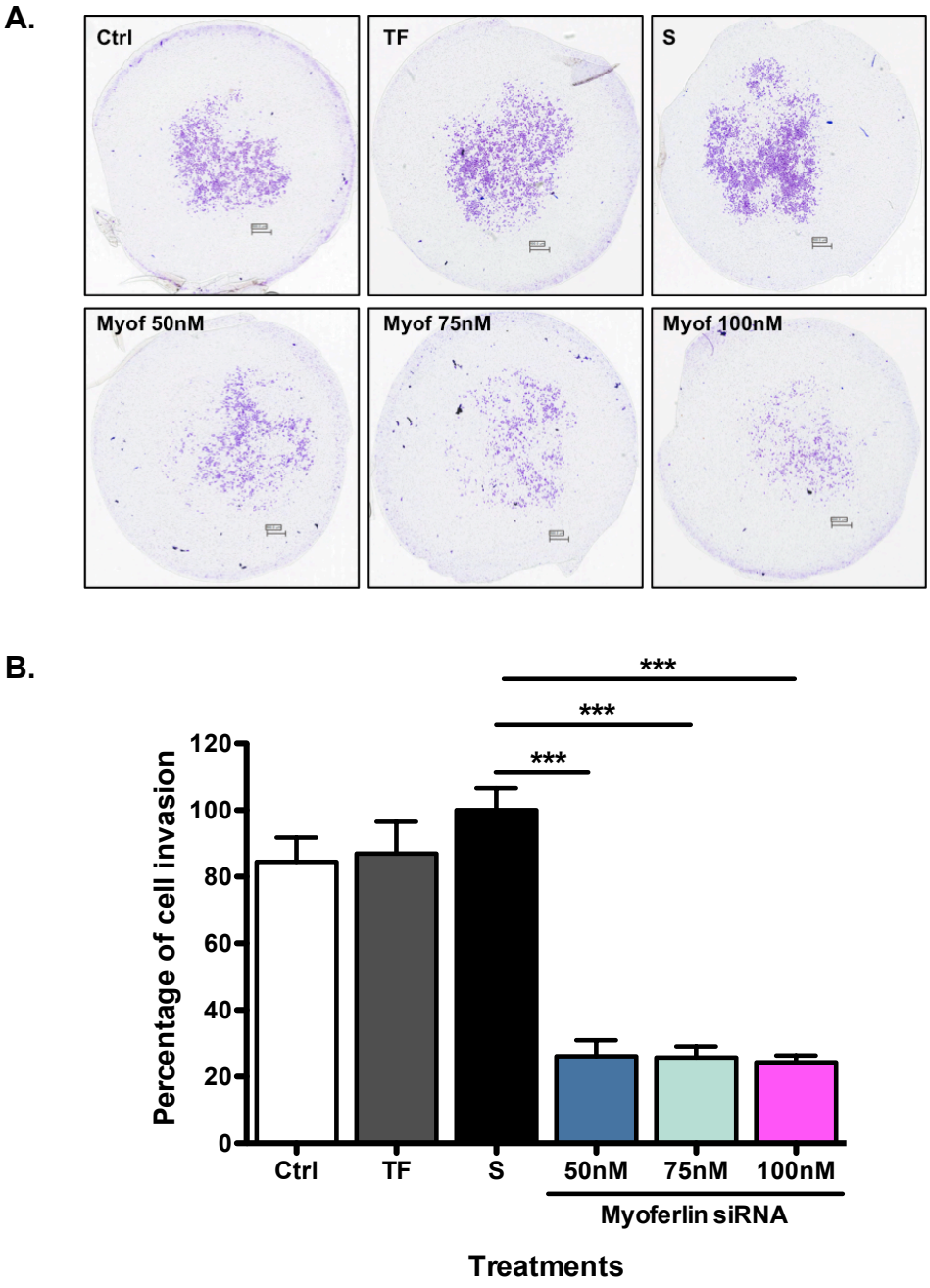


Figure 31. Knockdown of Myoferlin decreases invasiveness of A549 cells in vitro.

(A) Representative images of membrane filters containing invaded cells treated with media control (Ctrl)-, transfection reagent (TF)-, scrambled siRNA (S)-, or myoferlin siRNA (Myof). Cells first invaded through the Matrigel on the upper side of the membrane filters before reaching to the lower side. (B) The numbers of invaded cells contained in 8 randomly selected fields on the captured images were counted. Percentages of invaded cells were expressed relative to the scrambled siRNA control. Values are represented as mean ± SEM (n = 5). A significant difference between the treatment groups and scrambled siRNA control is indicated by an asterisk (*** $p < 0.001$).

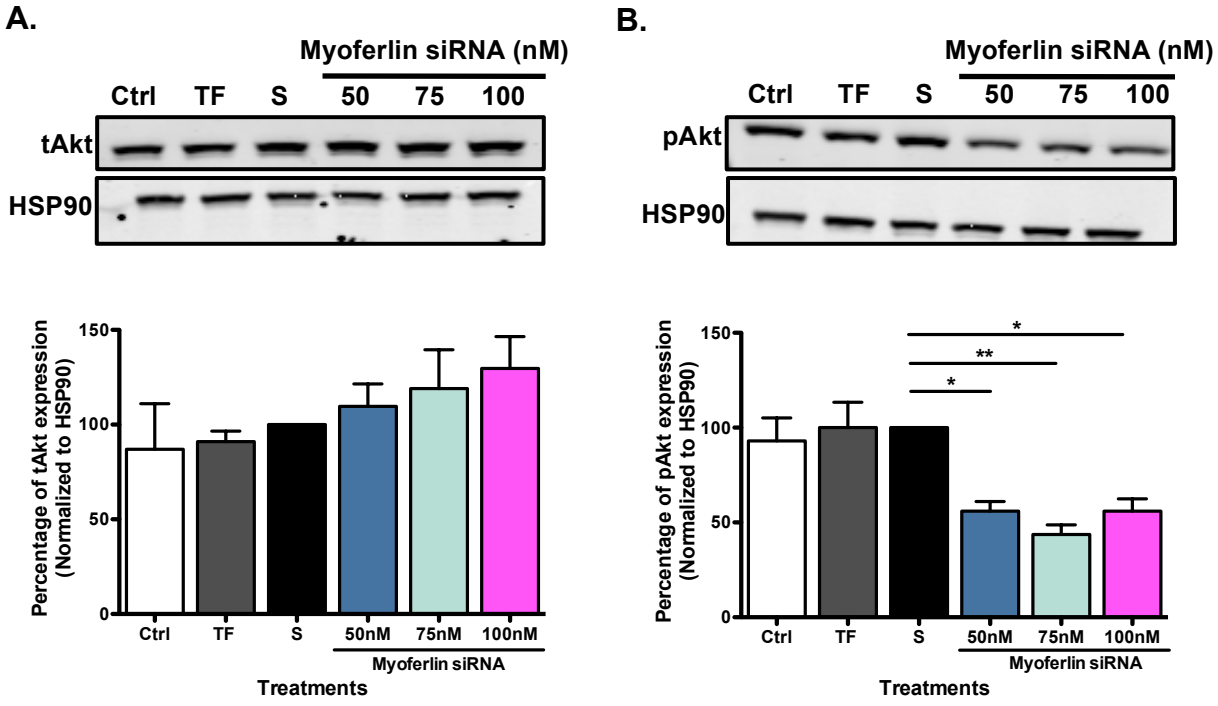


Figure 32. Myoferlin siRNA attenuates the expression of phosphorylated Akt in A549 cells.

A549 cells were transfected with or without myoferlin siRNA before collecting the cell lysates for western blot analysis. Eighty micrograms of proteins from each treatment condition was used to probe for (A) total Akt (tAkt) and (B) phosphorylated Akt (pAkt) with HSP90 as a loading control. Expression levels of indicated proteins were quantified by linear densitometry. Data are represented as mean ± SEM of 3 independent experiments (*$p < 0.05$; **$p < 0.01$ versus scrambled siRNA control).

6.3. Discussion

Our previous work revealed the crucial role of myoferlin in tumor cell proliferation using both cultured mouse lung carcinoma cells and mouse solid lung tumor xenograft model [454]. In addition, evidence from breast and pancreatic adenocarcinomas further supports the importance of myoferlin in tumor growth and metastasis [192, 193]. However, the effect of myoferlin depletion on tumor cell invasion and migration, the two key players in driving tumor metastasis, has not been examined in lung adenocarcinomas. Despite myoferlin knockdown demonstrating an anti-proliferative effect in lung tumor growth, the mechanistic pathway leading to such effect is not known. In this study, we investigated (i) if myoferlin regulates cancer cell proliferation, migration and invasion in the A549 human lung adenocarcinoma cell line and (ii) whether myoferlin regulates cellular events through Akt signaling pathways.

By using gene-silencing technology, we demonstrated that proliferation of A549 cells requires myoferlin at 48 h and 72 h, but not at 24 h, post-treatment. These results not only are consistent with our previous findings in which myoferlin is required in the proliferation of mouse Lewis lung carcinoma cells, but also validate our work from an animal to a human model. Currently, no other study has reported the importance of myoferlin in lung adenocarcinoma cell proliferation. Nevertheless, another group of researchers has noted that myoferlin siRNA decreases the proliferation of human pancreatic adenocarcinoma cells and their findings are consistent with our results using human lung adenocarcinoma cells [193]. The same study also showed that myoferlin knockdown induces cell cycle arrest at S phase and decreases the number of human pancreatic adenocarcinoma cells in G_0/G_1 and G_2/M phases [193]. Since cell undergoes DNA replication at the S phase, knockdown of myoferlin may inhibit DNA synthesis and prevent the cells from completing DNA replication in order

to advance to the G_2 phase. Although the molecular mechanism of how myoferlin contributes to DNA synthesis is not known, it is possible that myoferlin mediates the trafficking of cyclins A or E to the binding sites on CDK to trigger DNA synthesis. By arresting the cancer cells at S phase of cell cycle, it will block cancer cell proliferation [455]. In this study, the inhibitory effects on lung adenocarcinoma cell proliferation upon myoferlin depletion suggest that disrupting the expression of myoferlin might slow down lung tumor growth. However, we did not study whether myoferlin knockdown causes cell cycle arrest that contributes to the decreased proliferation of lung adenocarcinoma cells. Unlike in lung and pancreatic cancers, myoferlin depletion does not affect the proliferation of human breast cancer cells [190, 364]. Although the breast adenocarcinoma cell lines are also derived from epithelial cells, like the lung and pancreatic adenocarcinoma cell lines, myoferlin does not seem to regulate the proliferation of all epithelial-derived cancer cell types. Such discrepancy suggests that myoferlin has distinct function depending on the tissue type.

The relatively low survival rate in lung cancer is mainly due to metastasis which accounts for more than 90% of lung cancer deaths [456]. Solid metastatic lung adenocarcinoma may spread to other organs such as liver, brain, bones and adrenal gland [457]. Hence, managing the extent of lung cancer metastasis can prolong the survival of the patients. In the current study, we observed that knockdown of myoferlin changes the appearance of A549 cells from cuboidal shape to spindle shape, a typical phenomenon of EMT. Often, but not always, the immediate outcome of EMT is to enhance the migration capabilities of cancer cells to facilitate tumor metastasis. The appearance change in A549 cells after myoferlin knockdown suggests that these cells might be more motile compared to the control cells. To evaluate the migration of A549 cells, we employed the 2-dimensional scratch assay and found that myoferlin siRNA had no effect on wound closure at 24 h, but

significantly decreased the wound closure at 48 h and 72 h. Coincidentally, the decrease in wound closure occurred at the same time point as the reduction in cell proliferation. We suspected that cell proliferation might pose some effect on the wound closure that did not truly reflect the motility of A549 cells after myoferlin knockdown. To address this potential problem, we evaluated the motility of A549 cells using a transwell migration assay. We revealed that myoferlin knockdown does not affect the motility of A549 cells when compared to non-silencing control. These results are not consistent with the data we obtained through scratch assays, indicating that the effect of myoferlin knockdown on wound closure is possibly caused by the decreased cell proliferation. Similar to our findings from transwell migration assay, Li et al. also observed no significant changes in cell migration after depleting myoferlin expression in breast adenocarcinoma cell line MDA-231 [364]. In contrast, a decrease in cell migration is reported in pancreatic adenocarcinoma cells depleted of myoferlin [193]. In addition, two other groups of researchers revealed that myoferlin depletion in breast adenocarcinoma cell lines MDA-231 and MDA-MB-468 decreases cell migration [190, 192]. The decrease in cell migration was accompanied by a change in MDA-231 cells, which underwent a transformation from a mesenchymal phenotype to an epithelial phenotype, which is a less motile [192]. In another study, using the same MDA-231 cell line, depletion of myoferlin did not affect cell migration; instead, cell invasion was significantly reduced [364]. Interestingly, our study demonstrated that loss of myoferlin changed the appearance of A549 cells to a more motile mesenchymal phenotype, but we did not observe an increase in cell invasion, rather, cell invasion was decreased. Since myoferlin depletion has been shown to alter the cytoskeletal structure in breast adenocarcinoma cell lines, it is possible that myoferlin knockdown also disrupted the cytoskeletal arrangement that is required to maintain the cell appearance in lung adenocarcinoma cells. This would suggest

that the appearance change observed in A549 cells is a consequence of abnormal cytoskeletal structure, independent of EMT. Further investigation is necessary to confirm whether myoferlin knockdown disrupts cytoskeletal structure in lung cancer cells. Nevertheless, the decrease in A549 cell invasion upon myoferlin knockdown indicates the importance of myoferlin in facilitating the metastasis of lung adenocarcinoma.

The mechanistic pathway that leads to the changes in cell proliferation and invasion was also investigated in the current study. Tumor progression and malignant conversion frequently require cellular processes, such as cell proliferation, migration, invasion and survival, to occur in an uncontrollable way. The Akt signaling pathway is responsible for the regulation of cell proliferation, apoptosis, migration and invasion that implicate in tumorigenesis [458]. There has been increased attention focused on finding ways to inhibit Akt activity in an attempt to inhibit tumorigenesis. Without pre-incubating the cells with any growth factors, we showed that myoferlin knockdown significantly inhibited the activation of Akt in A549 cells. Activation of Akt is initiated by RTKs which has been shown to be regulated by myoferlin. For example, myoferlin depletion suppresses tumor growth and metastasis by decreasing the expression of EGFR and HER involved in Akt signaling in breast adenocarcinoma cells [190, 191]. Hence, it is likely that the decrease in cell proliferation and invasion observed in A549 cells is due to the decrease of Akt activity following myoferlin knockdown. However, we did not identify the specific RTKs that are responsible for the decrease in Akt activation. With the role of myoferlin in mediating the vesicle trafficking process, we speculate that myoferlin regulates the expression of many RTKs on the plasma membrane of A549 cells. To support our speculation, study in breast cancer cells showed downregulation of several RTKs, including IGF1R, VEGFR-2, ephrin type-B receptor 4, and fibroblast growth factor receptor 2, in myoferlin-knockdown breast

cancer cells [459]. Since Akt is downstream of RTKs, the decrease in Akt activity in A549 cells is likely the overall effect of myoferlin knockdown on the expression of RTKs as well as other membrane receptors. Another aspect of Akt signaling that we did not examine thoroughly in this study is apoptosis. Although we did observe increased cell detachment after myoferlin knockdown from the phase-contrast images, future investigation on cell apoptosis will provide information on whether inhibiting Akt phosphorylation has any apoptotic effect on lung tumor cells.

In conclusion, our data demonstrate that myoferlin mediates the proliferation and invasion of cultured human NSCLC cells. Moreover, myoferlin is required for the phosphorylation of Akt in human lung adenocarcinoma cells. Based on the role of Akt in tumor cell proliferation and invasion, myoferlin likely regulates lung tumor cell proliferation and invasion through activation of the Akt signaling pathway. Myoferlin inhibition in NSCLC could be an exciting therapeutic target for prolonging the survival of NSCLC patients.

7. General Discussion and Conclusion

7.1. Introduction

Lung cancer is the number one killer of all cancers in the world. This devastating disease develops over a long period of time through a sequence of morphological and molecular changes that afflict normal epithelium. The lung epithelium acts as the first line of defense against inhaled toxins and pathogens. Once inside the lung, inhaled toxins and pathogens disrupt the lung epithelium by forming pores directly on the epithelial cell membrane and decreasing the expression of junction proteins that normally maintain structural integrity [100-102] [104]. Disruption in lung epithelium triggers a repair process that involves a series of cell spreading, dedifferentiation, migration, proliferation and redifferentiation [110]. Restoration of epithelial integrity requires cells to reassemble intercellular junctions by trafficking in vesicles the newly synthesized or previously recycled junctional proteins to the cell membrane. Interestingly, membrane repair also uses the same vesicle trafficking, docking and fusion events to reseal the ruptured membrane. Regulation of these membrane-associated cellular events often requires the coordinated interaction between cytoplasmic proteins and the inner leaflet of plasma membrane. Among the membrane-associated proteins, two members of ferlin proteins, myoferlin and dysferlin, were first identified in skeletal muscles to mediate membrane repair processes [127, 128, 179, 410]. Subsequent studies revealed that myoferlin and dysferlin also regulate the expression of membrane receptors such as insulin-like growth factor 1 receptor (IGF1R) on the sarcolemma via vesicle trafficking [160, 453]. Although the majority of the knowledge we have on myoferlin and dysferlin came from studies performed on skeletal muscles, emerging evidence indicates that

these proteins are also expressed in other non-skeletal muscles, such as vascular endothelium [165, 182] and lung epithelium and mesenchymal cells [128, 460, 461]. Chronic exposure to inhaled toxins and incomplete epithelial repair often resulted in pre-neoplastic changes that set up the foundation of multi-step carcinogenesis in the lung. As cancer continues to grow and progress, there is an increasing demand in membrane remodeling and repair to support the rapid tumor cell proliferation, migration and invasion [462-465]. Thus, proteins that mediate the membrane remodeling and repair processes are frequently overexpressed in lung cancer [466, 467]. Genetic upregulation of a membrane repair protein, myoferlin, has been identified in lung cancer [188, 189]. However, the functional role of myoferlin in lung cancer remains unknown.

My thesis answered the above two questions by investigating the distinctive role of myoferlin and dysferlin in regulating normal airway epithelial expression of tight junctional proteins and neoplastic lung epithelial cancer growth and invasion.

7.2. Myoferlin is critical for maintenance of epithelial phenotype in normal human lung epithelial and non-small cell lung carcinoma (NSCLC) cells

Previous studies have shown the expression of myoferlin and dysferlin in lung tissue homogenates [128, 460, 461], however they did not identify which cell types are responsible for the expression of these proteins. Myoferlin and dysferlin are expressed by many cell types, including vascular endothelial cells and inflammatory cells [468], which can also be found in the lung. As demonstrated in Chapter 4, we provide the first evidence that both myoferlin and dysferlin are expressed in primary and cultured human airway epithelial cells. In addition, myoferlin and dysferlin are also present in cultured mouse and human lung

cancer cell lines, Lewis lung carcinoma (LLC) and human lung adenocarcinoma (A549), respectively (Chapters 5 and 6). Although we did not compare the protein expression levels of myoferlin and dysferlin among the cell lines we used in our studies, different cell lines seem to express myoferlin and dysferlin at different levels. Nevertheless, we demonstrate that myoferlin expression, but not that of dysferlin, is essential for maintaining the epithelial appearance in normal and neoplastic human lung epithelial cells (Chapters 4 and 6). Such appearance change does not resemble that of the classical epithelial-mesenchymal transition, as depletion of myoferlin had no effect on the expression of classical epithelial-mesenchymal transition (EMT) markers, E-cadherin and fibronectin (Chapter 4). The fact that myoferlin knockdown does not increase the motility of A549 cells further supports that the appearance changes induced by myoferlin knockdown are not associated with EMT (Chapter 6). Similar to A549 cells, Volakis et al. showed that depletion of myoferlin also changes the morphology of human breast cancer cells [192]. They found that the morphological changes of myoferlin-depleted breast cancer cells are associated with the disruption of cytoskeletal architecture, but does not result in EMT [192]. Many proteins have been shown to regulate the organization of cytoskeletal actin filaments and microtubules that are crucial for maintaining the shape of mammalian cells [469, 470]. One may hypothesize that myoferlin contributes to cytoskeletal organization that regulates the shape and appearance of 16HBE and A549 cells. However, future studies that investigate the effect of myoferlin knockdown on cytoskeletal architecture in 16HBE and A540 cells need to be performed to validate the proposed explanation.

7.3. Myoferlin is important for NSCLC tumor growth and cell invasion

Several studies have demonstrated that myoferlin gene expression is upregulated in human lung cancer biopsies [188, 189]. These findings indicate that myoferlin may involve in lung

cancer development. However, the functional role of myoferlin in lung cancer has not been investigated. Studies in skeletal myoblasts and vascular endothelial cells revealed that loss of myoferlin decreases cell proliferation and impairs the corresponding skeletal muscle growth and angiogenesis [179, 182-184, 453]. Since cell proliferation plays a key role in supporting uncontrolled tumor growth, depletion of myoferlin may have anti-proliferative effects that can block lung tumor growth. We found that knockdown of myoferlin decreases the proliferation of LLC and A549 cells *in vitro* (Chapters 5 and 6). The anti-proliferative effect was also observed in a mouse tumor xenograft model as intra-tumoral injection of myoferlin gene small interfering RNA (siRNA) effectively represses solid lung tumor growth by almost 50% compared with the control groups (Chapter 5). Consistent with our findings, Wang et al. showed that loss of myoferlin decreases pancreatic tumor cell proliferation and solid pancreatic tumor growth [193]. They found that the myoferlin knockdown induces cell cycle arrest at S phase, which reduces cell cycle progression. Ultimately, myoferlin-depleted pancreatic tumor cells become less proliferative and inhibit solid tumor growth. Although we did not examine the effect of myoferlin knockdown on the cell cycle of lung tumor cells, we cannot exclude the possibility that myoferlin-depleted lung tumor cells are arrested at S phase of the cell cycle. In some tumor types, cell cycle arrest can cause apoptosis, which can also impact on cell proliferation. However, no apoptotic effect was observed in our mouse lung tumor cells after myoferlin knockdown (Chapter 5). On the other hand, myoferlin-depleted human lung tumor cells showed an increase in cell detachment (Chapter 6), a phenomenon that often occurs as a result of apoptotic cell death. Unfortunately, we did not perform any experiments to verify whether the detached cells are apoptotic or not. Thus, further investigation on apoptosis together with cell cycle distribution needs to be evaluated by flow cytometry in the future.

Uncontrolled tumor cell proliferation is one of the features of tumor development. As tumors progress, some tumor cells acquire the ability to migrate and invade the surrounding tissues by becoming metastatic. Tumor metastasis is the most life-threatening feature of cancer that accounts for 90% of cancer-related deaths [471]. Thus inhibiting tumor metastasis could potentially prolong survival time of cancer patients. Among all cancer types, lung cancer is the second most common malignant tumor and it is also the deadliest cancer in the world [1, 472]. Hence, identification of myoferlin that potentially regulates lung tumor cell migration and invasion may provide a new therapeutic strategy to target lung tumor metastasis. By using the traditional scratch assay to evaluate cell migration, our data suggested that myoferlin knockdown decreases the motility of A549 cells at 48 h and 72 h, but not at 24 h time points (Chapter 6). When we used transwell migration assays with serum as a chemoattractant, we did not see any inhibitory effect on cell migration at 24 h time point. These time-point-dependent findings immediately revealed the caveat of scratch assay, that is, the effect of myoferlin knockdown on cell proliferation at 48 h and 72 h time points seems to interfere with the wound closure. Therefore, we excluded the results at 48 h and 72 h time points and concluded that myoferlin depletion does not affect motility of A549 cells based on the results at 24 h time point. Using a modified transwell assay coated with Matrigel matrix, we were able to demonstrate that myoferlin knockdown decreases the invasiveness of A549 cells (Chapter 6). Although our two-dimensional (2D) *in vitro* data suggest that myoferlin plays a crucial role in tumor cell invasion, we do not know how these findings relate to the *in vivo* condition. Future studies using lung-specific myoferlin conditional knockout mice that are exposed to lung carcinogens will provide more information on the role of myoferlin in lung tumor growth and metastasis. Furthermore, this animal model will also allow us to study the functional role of myoferlin in the tumor microenvironment.

7.4. Myoferlin participates in membrane-associated cellular processes

The involvement of myoferlin in many membrane-associated cellular processes in skeletal muscles and vascular endothelia prompted us to investigate the role of myoferlin in regulating these processes in the lung. We found that myoferlin knockdown decreases the attachment of human airway epithelial cells and lung adenocarcinoma epithelial cells to their neighboring cells or extracellular matrices (ECMs). Cell detachment usually reflects the loss of cell adhesion that can be determined by evaluating the expression of cell adhesion proteins. In human airway epithelial cells, myoferlin knockdown only decreases the tight junction (TJ) protein component zonular occludens 1 (ZO-1), but not occludins, claudin-1, ECM adhesion receptor, integrin β1, and adherens junction (AJ) protein, E-cadherin, indicating that cell detachment is not due to the loss of cell adhesion. Instead, the increased cell detachment after myoferlin depletion may be the consequence of disrupted epithelial homeostasis. Since dividing and shedding epithelial cells require redistribution of ZO-1 to build new cell-cell and/or cell-ECM contacts for the neighboring cells to preserve epithelial barrier preceding cell extrusion [392], knockdown of myoferlin disrupts the trafficking and distribution of ZO-1 which causes the neighboring cells to be more susceptible for cell detachment due to the loss of tight junctions. Following cell detachment, myoferlin-depleted human airway epithelial cells are more likely to undergo apoptotic cell death than control cells. The increase in cell apoptosis due to the loss of myoferlin-mediated membrane remodeling and signal transduction does not appear to be influenced by inflammatory cytokines (interleukins 6 or 8) (Chapter 4). However, we demonstrated that myoferlin depletion disrupts membrane resealing in neoplastic lung epithelial cells (Chapter 5). Myoferlin has been shown to mediate trafficking and fusion of vesicles that are required for tissue growth and repair [179, 183, 201, 453, 473]. Although we did not investigate the effect of myoferlin knockdown on vesicle

trafficking and fusion, we speculate that membrane repair defects in lung cancer cells might be due to reduced trafficking and fusion of vesicles to the plasma membrane. Trafficking and fusion of vesicles are important not only for membrane repair, but also for delivering membrane receptors. Vesicles carry newly synthesized membrane proteins from the Golgi apparatus to the cell membrane through vesicle transport and then deliver the membrane proteins to the cell membrane through vesicle fusion [474]. Myoferlin is known to mediate trafficking of vesicles that carry receptor tyrosine kinases, such as IGF1R in myoblasts, vascular endothelial growth factor receptor 2 (VEGFR-2) and Tie2 receptor in vascular endothelial cells, and epidermal growth factor receptor (EGFR) in breast cancer cells [160, 182] [184, 190]. Since lung cancer cells express many RTKs, it is possible that myoferlin regulates the expression of several RTKs. Protein kinase B (Akt) is a major downstream effector of many RTKs that regulates a variety of cellular processes, including cell proliferation, migration, invasion, and apoptosis. Thus, dysregulation of Akt can contribute to cancer development and progression. Hyperactivation of Akt has been reported in pre-neoplastic lung lesions and NSCLC [452] and we observed myoferlin knockdown decreases lung tumor cell proliferation and invasion; it is likely that myoferlin governs Akt activity in lung tumor cells. By evaluating the expression of phosphorylated and total Akt, we found that myoferlin knockdown does substantially reduce Akt phosphorylation in A549 cells (Chapter 6). Currently, we do not know which RTKs are being affected in myoferlin-depleted lung tumor cells that lead to the decreased Akt activity. Future studies using protein microarray may be able to fill out this missing information.

7.5. Study limitations

The work in this thesis provides new insights into the functional role of myoferlin and

dysferlin in normal and neoplastic lung epithelium. However, there are some limitations to our studies. First of all, we only studied the role of myoferlin and dysferlin in one cell line for both normal and neoplastic human lung epithelia as well as mouse neoplastic lung epithelium. Since there are several different types of epithelial cells in human and mouse lungs, the functional role of myoferlin and dysferlin observed in 16HBE cells may not apply to other lung epithelial cell lines such as ciliated cells, goblet cells and alveolar type II cells. Secondly, the use of LLC and A549 cell lines as *in vitro* models raises questions on reliability and relevance of these cell lines to resemble the actual human lung tumor development. Although these cultured tumor cells allow us to understand how they behave *in vivo*, the absence of stromal and inflammatory cells may not truly reflect the actual solid tumor development in human lungs. In addition, LLC is a cell line from the mouse that does not represent any form of human lung tumors; the anti-tumor effect we observed in the mouse tumor xenograft model may have limited clinical relevance in human lung tumors. Thirdly, we did not compare the expression level and function of myoferlin and dysferlin between normal and lung tumor cells. Since several normal mouse lung epithelial cell lines are available, we could have performed knockdown studies to gain better knowledge on the role of myoferlin and dysferlin under normal physiological condition. In comparison, such studies are not possible in normal human alveolar type II cells due to the difficulty to isolate this cell type and to maintain it in culture. Next, the heterogeneity of lung adenocarcinoma often involves multiple mutations and not a single signaling pathway can solely drive the oncogenic behavior of tumors. However, our study just focused on the Akt signaling pathway and did not look at other signaling pathways such as K-Ras. Finally, funding for lung cancer research is very limited even though lung cancer has a high mortality rate. This funding situation reduces the progress of identifying new diagnostic and therapeutic targets that can

improve the survival of lung cancer patients.

7.6. Conclusion

Research on the functional role of myoferlin and dysferlin remains in its infancy. The work that is presented in this dissertation reveals the distinctive roles of myoferlin and dysferlin in normal and neoplastic lung epithelia. We identified that myoferlin and dysferlin do not share the same function in the lung. Myoferlin, but not dysferlin, contributes in maintaining the appearance of human bronchial epithelial and lung adenocarcinoma cells. In normal bronchial epithelial cells, myoferlin regulates the expression of TJ component, ZO-1, and facilitates cell adhesion and survival. On the other hand, in mouse lung tumor cells, myoferlin mediates the repair of ruptured plasma membrane that frequently occurred in highly proliferating tumor cells. In addition, myoferlin participates in phosphorylation of Akt, an important signaling pathway that regulates tumor cell proliferation and invasion. All these findings extend the knowledge of myoferlin function into normal and neoplastic lung epithelia. The latter role of myoferlin in lung tumor growth and invasion marks the new direction of investigating the potential of myoferlin as a diagnostic marker or therapeutic target for lung cancer patients.

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