Stromal Lysyl Oxidase-Like 1 Increases Extracellular Matrix Stiffness and Enhances Collagen Fibre Alignment in Non-Small Cell Lung Cancer

by

Elena Pasko

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Laboratory Medicine and Pathobiology

University of Toronto

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Abstract

Cancer associated fibroblasts (CAFs) represent a large portion of non-small cell lung cancer (NSCLC) stroma where they mediate extracellular matrix (ECM) remodelling and its interactions with neoplastic cells. A recognized NSCLC CAF marker, fibrillar collagen-binding receptor integrin alpha11 (α11), known to promote lung tumour growth and metastasis, was highly correlated with stromal lysyl oxidase-like 1 (LOXL1) in two NSCLC patient cohorts. Both genes were up-regulated in tumour stroma compared to normal lung tissue and were associated with desmoplasia status in patient tumour samples. Over-expression of α11 resulted in up-regulation of LOXL1 in three in vitro models. When cultured within three-dimensional collagen matrices, cells with over-expressed α11 caused linearization, alignment and contraction of collagen fibres. In the absence of α11 expression, over-expression of LOXL1 rescued this phenotype. LOXL1 expression was directly proportional to tissue stiffness; degree of gel contraction; and collagen fibre linearization in in vitro and xenograft tumour models.
Acknowledgments

First, I would like to thank Dr. Ming Tsao for his supervision. He supported me in my research and other academic endeavours, was always patient and willing to share his immeasurable knowledge. While providing guidance, he gave me freedom to carry out independent research and develop my scientific creativity. I would like to thank my committee members, Dr. Khokha and Dr. Muthuswamy, for invaluable advice that allowed me to achieve my objectives. Additionally, I thank Dr. Hu and Dr. Yeger for agreeing to be part of the examination committee.

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This would not have been possible without my parents and grandparents, who have taught me the value of education, hard work, and perseverance from an early age. I would like to thank my grandmother for her love and continuous support. Delicious Sunday night dinners at her place with my sister will always be some of my most cherished memories.

Finally, I would like to express my deepest gratitude to my one and only. Daniel has been my rock and my number one fan. He stood by me through late nights at the lab and long hours of thesis preparation. He cooked me delicious meals, took me flying, and reminded me that I should not “put my life on a shelf”, as it will not wait for me. His unfailing support and encouragement have helped me pursue my passion and made the completion of this degree possible.
Contributions

I would like to thank our colleagues from the University Health Network, University of Toronto and University of Copenhagen who have provided insight and expertise and have greatly contributed to the completion of this project.

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List of Abbreviations

α11 integrin alpha 11
ADC adenocarcinoma
AFM atomic force microscopy
AJCC American joint committee on cancer
ALK anaplastic lymphoma kinase
ANOVA analysis of variance
α-SMA alpha-smooth muscle actin
ATCC American type culture collection
B2M beta-2-microglobulin
βgal beta-galactosidase
bFGF basic fibroblast growth factor
β-ME beta-mercaptoethanol
BMP1 bone morphogenetic protein 1
BSA bovine serum albumin
CAF cancer-associated fibroblast
CD cluster of differentiation (identification of cell surface molecules)
Colα1 collagen alpha 1
Cq quantification cycle
CRL cytokine receptor-like (domain)
CT computed tomography
CYP cytochrome p450
DDR discoidin domain receptor
DEPC diethylpyrocarbonate
DMEM Dulbecco’s modified Eagle’s medium
DOX doxycycline
DTT dithiothreitol
ECM extracellular matrix
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>EML4</td>
<td>echinoderm microtubule-associated protein-like 4</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem (cells)</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FAPα</td>
<td>fibroblast activation protein alpha</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FLPe</td>
<td>flippase</td>
</tr>
<tr>
<td>FSP-1</td>
<td>fibroblast specific protein 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEM</td>
<td>genetically engineered mouse</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-s-transferases</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>HDA</td>
<td>high desmoplastic area</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor 1alpha</td>
</tr>
<tr>
<td>HSB</td>
<td>hue-saturation-brightness</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>HZ</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IMD</td>
<td>integrin-mediated death</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s DMEM</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>KRAS</td>
<td>kirsten rat sarcoma oncogene homologue</td>
</tr>
<tr>
<td>LAIR</td>
<td>leukocyte-associated Ig-like receptor</td>
</tr>
<tr>
<td>LCC</td>
<td>large-cell carcinoma</td>
</tr>
<tr>
<td>LCM</td>
<td>laser capture microdissection</td>
</tr>
<tr>
<td>LOX</td>
<td>lysyl oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LOXL1-4</td>
<td>LOX-like 1-4</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LTQ</td>
<td>lysyl-tyrosyl-quinone (co-factor)</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCT1/2</td>
<td>monocarboxylate transporter 1/2</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MET</td>
<td>MET proto-oncogene, receptor tyrosine kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>NA</td>
<td>numerical aperture</td>
</tr>
<tr>
<td>NF</td>
<td>normal fibroblast</td>
</tr>
<tr>
<td>NG2</td>
<td>neuronal antigen-2</td>
</tr>
<tr>
<td>NLST</td>
<td>national lung screening trial</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>optimal minimal essential medium</td>
</tr>
<tr>
<td>P2A</td>
<td>2A peptide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PMCC</td>
<td>Princess Margaret Cancer Centre</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RPS13</td>
<td>40S ribosomal protein S13</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time PCR</td>
</tr>
<tr>
<td>rtTA</td>
<td>reverse tetracycline (controlled) transactivator</td>
</tr>
<tr>
<td>SCC</td>
<td>squamous cell carcinoma</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immune deficient</td>
</tr>
<tr>
<td>SCLC</td>
<td>small-cell lung cancer</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SFK</td>
<td>SRK family kinases</td>
</tr>
<tr>
<td>SHG</td>
<td>second harmonic generation</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SPARC</td>
<td>secreted protein, acidic, cysteine-rich</td>
</tr>
<tr>
<td>SRC</td>
<td>Rous sarcoma oncogene</td>
</tr>
<tr>
<td>SRCR</td>
<td>scavenger receptor cysteine-rich (domain)</td>
</tr>
<tr>
<td>STK11/LKB1</td>
<td>serine/threonine kinase 11</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TCGA</td>
<td>the cancer genome atlas</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Thy-1</td>
<td>thymocyte differentiation antigen 1</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>TKI</td>
<td>TK inhibitor</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour, node, and metastasis (method of staging)</td>
</tr>
<tr>
<td>TP53</td>
<td>tumour protein 53</td>
</tr>
<tr>
<td>UHN</td>
<td>university health network</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>YAP</td>
<td>yes-associated protein</td>
</tr>
</tbody>
</table>
Chapter 1
Background

1.1 Lung Cancer

1.1.1 Epidemiology

Lung cancer is one of the most frequently diagnosed cancers and is the leading cause of cancer-related death in Canada and the world (1-3). In 2012, approximately 1.8 million new cases occurred, accounting for ~13% of all cancer diagnoses (3). In Canada, the current incidence rate is 48 to 58 per 100,000 with over half of newly diagnosed cases occurring among people aged 70 years or older (2). The mortality rates in males have been declining since 1980s and in females since mid-2000s and are currently at 46.3 and 35.6 per 100,000, respectively (2). The majority of deaths occur in patients of 70-79 years of age and the changing mortality trends may have resulted due to past differences in tobacco use (2, 3). Despite increasing knowledge in molecular mechanisms of lung cancer and development of several new therapeutic agents, the dismal five-year relative survival rates (17% in Canada) have remained relatively unchanged.

Tobacco and second-hand smoke are the leading determinants of risk, accounting for 80% and 50% of lung cancer burden in males and females, respectively (4-6). Other known risk factors include exposure to radiation or to carcinogens, such as asbestos, radon, arsenic, and polycyclic aromatic hydrocarbons (7-10). Recently, outdoor and indoor air pollution caused by unventilated coal-fueled stoves has been shown to increase the incidence of lung cancer (11-16). Furthermore, lupus and previous lung disease such as asthma, tuberculosis, Chlamydophila pneumoniae infection or chronic obstructive pulmonary disease have been shown to increase the risk of developing this deadly disease in affected individuals (17, 18).

Having a family history of lung cancer increases individual risk by more than two-fold (19). Genetic susceptibility may occur due to allelic variants that modify the risk associated with various carcinogens. For instance, individuals with activating mutations of several cytochrome p450 (CYP) genes, which are known to bio-activate pro-carcinogens within the lung, or null polymorphisms in glutathione-s-transferases (GST), which are known to detoxify carcinogens associated with tobacco smoke, have an elevated risk of lung cancer (20-22). These factors vary
by ethnicity, tumour histology, exposure, and other social factors, making it difficult to predict associated lung cancer risk at individual level (23, 24).

1.1.2 Histopathology

Lung cancer is divided into two broad categories of small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancer cases (25). NSCLC is further subdivided according to histology into three main groups, consisting of adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large-cell carcinoma (LCC) (25). Some of the more rare NSCLC subtypes include adenosquamous and sarcomatoid carcinomas (25).

ADC arises from alveolar and bronchial epithelial cells and is now the most commonly diagnosed subtype, accounting for 50% of NSCLC cases (26, 27). Although it is most commonly seen in smokers, it is the most frequently detected lung cancer subtype in individuals who have never smoked (27). SCC, which accounts for ~40% of NSCLC cases, originates from bronchial epithelia and is more strongly associated with smoking than ADC, as 90% of its cases are diagnosed in smokers (28-30). LCC is an undifferentiated subtype with no clear defining features of SCLC, ADC or SCC, which accounts for 9% of NSCLC (31).

NSCLC is diagnosed according to the internationally accepted tumour, node, and metastasis (TNM) staging system described in the American Joint Committee on Cancer (AJCC) Cancer Staging Manual 7th Edition and summarized in Table 1 (32). To determine prognosis and create a treatment plan, the TNM criteria are then combined to establish the anatomical stage, summarized in Table 2 (32).
Table 1. TNM classification of lung cancer

<table>
<thead>
<tr>
<th>Primary Tumour (T)</th>
<th>Regional Lymph Nodes (N)</th>
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<tbody>
<tr>
<td>TX</td>
<td>NX</td>
</tr>
<tr>
<td>T0</td>
<td>N0</td>
</tr>
<tr>
<td>Tis</td>
<td>N1</td>
</tr>
<tr>
<td>T1</td>
<td>N2</td>
</tr>
<tr>
<td>T1a</td>
<td>N3</td>
</tr>
<tr>
<td>T1b</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td></td>
</tr>
<tr>
<td>T2a</td>
<td>Tumour &gt;3cm but ≤5cm</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumour &gt;5cm but ≤7cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour &gt;7cm or one that invades any of the following:</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>Any size tumour that invades:</td>
</tr>
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</tbody>
</table>

- Parietal pleura, chest wall, diaphragm, phrenic nerve, mediastinal pleura, parietal pericardium, main bronchus, opposite or associated with atelectasis obstructive pneumonitis of whole lung, or separate tumour nodule in same lobe
- Mediastinum, heart, great vessels, trachea, laryngeal nerve, esophagus, carina, or there are separate nodules in a different ipsilateral lobe
Table 2. Anatomic stages of lung cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
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<td>N0</td>
<td>M0</td>
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1.1.3 Clinical Features, Detection, Treatment and Prognosis

In its earliest stages, lung cancer does not cause distinct symptoms, making it difficult to detect early. Thus the majority of the cases are not diagnosed until the disease has already progressed into its late stages, where the five-year survival is only approximately 4% (33). The symptoms in advanced lung cancer may include persistent cough, worsening shortness of breath, chest pain, hoarseness of the voice and recurrent pneumonia or bronchitis. Diagnosis may be established through a variety of diagnostic procedures, which may include medical history and physical examination, chest x-ray, computed tomography (CT) scan, positron emission tomography (PET) scan, sputum test, endoscopic procedures, ultrasound, or biopsy (34).

Screening is recommended for individuals at high risk of developing lung cancer, as early detection and treatment of the disease have been shown to increase the five-year survival to 55-75% (33). After the review of the National Lung Screening Trial (NLST) findings, the American Cancer Society now recommends low-dose CT screening for individuals 55-74 years of age, who are in good health, and who currently are or were heavy smokers (30 pack-year smoking history) within the last 15 years (35, 36).

Treatment plan is established based on the stage and resectability of lung cancer, as well as the overall health and lung function. Surgery is used as the first line of treatment in the early-stage NSCLC, which can be completely removed (37). External beam radiation therapy may be used with or without chemotherapy prior to surgery to shrink the tumour mass or as the primary treatment for advanced, non-resectable tumours (37). Some of the prognostic factors for NSCLC include stage, lymph node status, resectability, weight loss, sex, lung function, number and type of metastases (25). The prognosis is more favourable in early stage tumours localized to the lung that can be resected completely (25).

1.1.4 Genomic Alterations

Genomic alterations in NSCLC occur through gene mutations, copy aberrations and rearrangements. Identification of several molecular oncogenic drivers in NSCLC has led to the development of molecular targeted therapies, which are a cornerstone of precision medicine. The Cancer Genome Atlas (TCGA) Research Network recently published results from a comprehensive molecular profiling of lung ADC (38). This study found significant genomic
alterations in 18 genes. Some of the top genes included TP53 (46%), KRAS (33%), EGFR (14%), STK11/LKB1 (17%) and MET (7%).

Inactivating mutations of TP53 tumour suppressor are the most common to NSCLC and other cancers (39). Its physiological functions include cell-cycle regulation, induction of apoptosis, and genome stabilization (40). It is more common in SCC than in the ADC patient population and occurs in 90% of SCC malignancies, accounting for 50% of all NSCLC cases (41). KRAS is an oncogene that is implicated in signal transduction (40). The incidence of its mutations in NSCLC ranges from 8% to 24% and is uncommon in SCC and non-smokers (39, 42). Most of its activating mutations are located on codons 12 or 13 and are mainly reported in ADC tumours (43). They result in constitutive activation of cell proliferation signals through the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (44). STK11/LKB1 is a tumour suppressor that is commonly inactivated in NSCLC. The inactivating mutations have been found to co-exist with KRAS activating mutations in lung cancer samples (45).

Mutations within the epidermal growth factor receptor (EGFR) family of tyrosine kinases (TKs) occur predominantly in ADC, female patients and never smokers with an overall incidence of 27% (39, 46). Activating EGFR mutations tend to be associated with a better prognosis compared to wild-type EGFR, because the mutants exhibit preferential binding to anti-EGFR TK inhibitors (TKIs), such as gefitinib or erlotinib (46, 47). These mutations primarily occur on exons 18 to 21 of the TK domain, 90% of which are deletions on exon 19 and a L858R mutation on exon 21 (48, 49). However, there are other mutations within the same region correlated with primary resistance to EGFR inhibitors. Furthermore, other genetic alterations, such as KRAS and PIK3CA mutations, loss of PTEN tumour suppressor and MET amplification have been shown to confer primary or acquired resistance to EGFR-TKIs (50).

MET is a receptor tyrosine kinase whose natural ligand is hepatocyte growth factor (HGF) (46). Its signalling regulates many downstream pathways involved in cell proliferation and motility, the deregulation of which could result in tumourigenicity, cancer cell invasion, metastasis and epithelial-to-mesenchymal transition (EMT) (51, 52). MET may be altered through genomic amplification, overexpression, mutations or alternative splicing and may be mediated through HGF-dependent or –independent mechanisms (46, 51). MET amplification was found to be
mutually exclusive with EGFR and KRAS mutations, but was identified as a mechanism of acquired resistance in EGFR-TKI treated NSCLC (53).

Another important genomic aberration in NSCLC is the inversion of anaplastic lymphoma kinase (ALK) and its fusion with the N-terminal of the echinoderm microtubule-associated protein-like 4 (EML4) gene, resulting in EML4-ALK rearrangement (54, 55). It is found in 3-5% of NSCLC cases and was determined to be enriched in tumours of patients who are female, never- or light-smokers, Asian, and of ADC subtype (39). This rearrangement appears to be mutually exclusive with EGFR and KRAS mutations and represents a distinct molecular NSCLC subtype (55).

A large number of other genomic alterations and oncogenic driver mutations have been found in NSCLC (56). Understanding underlying mechanisms will further advance the field of targeted therapeutics and personalized medicine.

1.2 Cancer Microenvironment

1.2.1 Tumour Stroma

For a long time cancer was viewed as a disease of transformed epithelial cells, which were the focus of treatment strategies and the targets of therapeutic agents. However, malignant epithelial cells do not act autonomously, but in concert with the surrounding stromal microenvironment (57-61). During the early stages of the disease, proliferating tumour cells are encased within the basement membrane and are separated from the interstitial stromal matrix. As the cancer progresses, the neoplastic cells acquire the ability to degrade and breach the barrier, migrating into the co-evolving stroma (62). The stromal matrix is then remodelled and activated through continuous paracrine and autocrine signaling, making it a supportive environment that promotes tumour growth and metastasis (63-65).

Tumour stroma is phenotypically similar to injured granulation tissue undergoing wound-healing. Both tissue types are associated with an increase in vasculature, consisting of pericytes, endothelial and smooth muscle cells, to promote tissue oxygenation (66); in inflammatory and immune cells, recruited by cytokines and chemokines (67); in proliferation of activated fibroblasts or myofibroblasts (68); and in deposition of extracellular matrix (ECM), consisting of
fibrillar collagens, glycoproteins, fibronectin, and proteoglycans (57). Once the wound healing process is completed, the myofibroblasts are removed from the granulation tissue via apoptosis (69). Activated fibroblasts or cancer-associated fibroblasts (CAFs) in the tumour stroma, on the other hand, continue proliferating and become a major contributing factor to tumour growth and progression (57, 70). Thus, cancer is referred to as a wound that never heals (61).

ECM has been long regarded as passive structural support required in the maintenance of tissue morphology and homeostasis. However, its physical, biochemical, and biomechanical properties are now recognized to play an essential role in normal cellular behaviour and major developmental processes (71). As such many stringent regulatory mechanisms exist to regulate its production, degradation, and remodeling (72). Under pathological conditions, however, the activity of matrix remodeling enzymes, primarily secreted by CAFs, may be deregulated resulting in elevated deposition and abnormal composition and organization of the ECM (73, 74).

The fibrotic reaction observed in the stroma of many cancers, characterized by an excess accumulation of fibrillar collagens type I and III, is referred to as desmoplasia and is considered to be a hallmark of cancer (57, 74, 75). Furthermore, non-oriented, relaxed and morphologically curly fibres within the normal stroma are very different from the highly linearized fibres of intra-tumoural collagen (76, 77). The increased alignment of collagen fibres is associated with increased stromal tissue stiffness and may be attributed to elevated activity of lysyl oxidase (LOX) enzymes, whose primary function is covalent cross-linking of collagen fibres (77-79). Collagen-cell interactions are mediated through several collagen-binding receptors, including integrins, discoidin domain receptors (DDRs), leukocyte-associated Ig-like receptors (LAIRs), mannose receptor family, and glycoprotein VI (80). Integrins are also key regulators of proliferation, migration, and invasion and are highly implicated in cancer progression (81, 82). Interestingly, collagen cross-linking and the resultant collagen fibre linearization and matrix stiffness have been shown to upregulate integrin signaling and promote cancer cell survival, proliferation and migration (77, 83, 84).
1.2.2 Cancer Associated Fibroblasts

1.2.2.1 Origin and Characteristics

Fibroblasts are cells of mesenchymal origin with spindle-shape morphology and are the most abundant cell type in connective tissues (85). In normal tissue homeostasis, they are in inactive quiescent state and their primary role is to synthesize the components of the ECM, such as collagens and fibronectin (86). Within granulation tissue, in order to achieve efficient wound contraction, fibroblasts become phenotypically altered to become highly contractile activated myofibroblast cells (87). This is an intermediate state between fibroblasts and smooth-muscle cells, which is morphologically and functionally different from quiescent fibroblasts (63, 88). Transition to and maintenance of the activated phenotype depends on the combined action of paracrine and autocrine signalling of the transforming growth factor beta (TGFβ) cytokine and mechanical tension, a function of ECM rigidity and the contractility of the cells (89, 90). One of the characteristic features of myofibroblasts is the acquisition of contractile stress fibres and de novo expression of alpha-smooth muscle actin (α-SMA) (70, 86).

Mimicking wound healing, neoplastic cells recruit and activate fibroblasts within tumour stroma through secretion of various factors. For instance, TGFβ and platelet-derived growth factor (PDGF) are recognized as major regulators of desmoplasia and have been shown to activate CAFs and promote their proliferation (91, 92). Other reported cancer cell-secreted CAF activators are basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and lysophosphatidic acid (LPA) (93-95). Moreover, the yes-associated protein (YAP) has been demonstrated to activate stromal fibroblasts in a tension-dependent manner (96).

CAFs are found in most solid tumours, but their abundance and characteristics vary between different sites and types of cancer (97-100). They are most commonly defined by exclusion as non-neoplastic, non-smooth muscle, non-endothelial, non-inflammatory and non-epithelial cells found in tumor stroma (101). CAFs appear to be composed of heterogeneous subpopulations of cells, as no molecular marker common and specific to all CAFs has been identified (101). Some of the previously reported markers include fibroblast specific protein (FSP-1), PDGF, neuronal antigen-2 (NG2), CD90 or Thy-1, fibroblast activation protein alpha (FAPα), integrin alpha11 (α11), and α-SMA (102-107). Various cell types have been demonstrated to transdifferentiate into CAFs where the heterogeneous expression of CAF markers may indicate varying degrees of
activation and different origins of cells (101). They have been shown to originate primarily from resident normal fibroblasts and also from other cell types found within the tumour, such as pericytes, adipocytes, endothelial or epithelial cells (101). Furthermore, CAFs have been shown to arise from recruited bone marrow-derived precursors and resident mesenchymal stem cells (108).

1.2.2.2 Role of CAFs in Cancer Progression

The role of CAFs in epithelial cancer development and progression is multifaceted. They stimulate neoplastic proliferation by secreting various cytokines, chemokines, growth factors and proteases (109). CAFs have been demonstrated to secrete ligands, such as HGF, epidermal growth factor (EGF), insulin-like growth factor (IGF), bFGF, and vascular endothelial derived growth factor (VEGF) that bind to corresponding receptors expressed on the surface of epithelial cells (66, 99, 110-112). CAFs also secrete pro-inflammatory mediators, which include interleukins, interferons and members of the tumour necrosis factor family (86). This leads to an immune cell infiltration and in turn promotes angiogenesis and metastasis (113). As active mediators of the desmoplastic reaction, CAFs secrete ECM components and ECM-remodelling enzymes, such as members of the LOX family and matrix metalloproteinases (MMPs) (72, 81). Cancer cells take advantage of this process, as increased ECM deposition and collagen remodelling promotes their motility and invasive potential.

1.2.2.3 Role of CAFs in NSCLC

Consistent with reported data obtained from other solid tumours, PDGFα/β and TGF-β1 were shown to be secreted by epithelial NSCLC cells, a known set of mediators in activating the CAF phenotype through paracrine signalling (114). Primary NSCLC patient CAFs were able to up-regulate primary tumour growth in vivo and also to induce EMT of co-cultured neoplastic cells in vitro, significantly more compared to matched normal fibroblast (NF) controls (97, 115). Furthermore, a prognostic signature was identified in association with CAF gene expression (97). In another study, a lung cancer CAF marker, α11, has been shown to promote NSCLC tumourigenicity through up-regulation of insulin-like growth factor 2 (IGF2) (111). A recent report has indicated that the binding of this ligand to the IGF receptor found on epithelial cells, may induce Nanog expression to promote stem-like properties in lung cancer cells. The acquired stemness in epithelial cells correlates with poor prognosis in NSCLC patients (116).
Another study demonstrated that CAFs regulate cancer cell glucose metabolism by shifting it towards aerobic glycolysis, which is very distinct from oxidative phosphorylation in normal cells (117). This process induces a build-up of lactate in the ECM, which has been shown to upregulate the expression of LOXL1 and MMP2/MMP9 in metastatic lung cancer sites, both in patients and in an in vivo mouse model (118).

There is evidence that CAF inhibit chemo- and radiosensitivity in lung cancer. Secretion of HGF by lung cancer-derived CAFs promoted acquired resistance to EGFR-TKIs by up-regulating MET signalling (119). Finally, while exposure of lung CAFs to radiation inhibited their proliferative, migratory and invasive ability, in a separate study this caused them to promote radioresistance in co-cultured lung cancer cell lines, suggesting a decrease in sensitivity to radiotherapy within clinical setting (120, 121).

1.2.3 Collagen

1.2.3.1 Collagen Structure

Collagens are the most abundant mammalian proteins and are the predominant constituents of ECM. They are essential for tissue scaffolding, cell adhesion and migration, angiogenesis, tissue repair, and cancer progression (122). They are triple-helical in structure, as they each contain three polypeptide α-chains arranged in a right-handed supercoil held together by inter-chain hydrogen bonds (123). Each α-chain contains approximately 338 repeating triplets of Gly-X-Y in its peptide sequence, where glycyl residues occupy every third position and X and Y are often occupied by proline and 4-hydroxyproline, respectively (124). At least 46 distinct polypeptide α-chains have been identified in vertebrates, which assemble into 28 different collagen types (124, 125). Although the majority of collagenas are homotrimeric, some collagens are heterotrimeric, consisting of 2 or 3 different types of α chains. There are 7 fibril-forming collagen proteins in mammals: type I, II, III, V, XI, XXIV and XXVII (126). They are synthesized as procollagens, containing N- and C- propeptides, which are then cleaved off with procollagen proteininases (122). This step is essential to reveal the telopeptides or non-triple helical extensions of the α-chains on each end of the fibre. These regions contain sites for LOX-mediated covalent cross-links, essential for tissue integrity and homeostasis (127).
1.2.3.2 Architecture of Fibrillar Collagen in Cancer

In malignant tissues, ECM remodelling is deregulated and the organization of fibrillar collagen network is severely modified. In normal compliant epithelial tissues, collagen fibres are curly or wavy and randomly oriented, while tumour-associated fibres progressively increase in thickness, linearization and stiffness (77, 128). These aberrant modifications have been implicated in promoting cancer progression through integrin and/or growth factor signalling and in regulating resistance to therapeutics (77, 129, 130).

Collagen fibre linearization within invasive tumours may in part be facilitated by excessive covalent collagen cross-linking, catalyzed by enzymatic function of the LOX family members (77). The function of LOX enzymes will be discussed in a later section. Furthermore, non-enzymatic cross-linking induced by sugars, such as glucose or ribose, may be another contributing factor to collagen stiffness (131). This places type II diabetes patients with impaired glucose metabolism at risk of developing cancer (132). Additionally, two glycoproteins, SPARC and fibronectin, have been shown to bind to and organize fibrillar collagen type I and/or collagen type IV (133, 134).

ECM proteins have been shown to not only promote cancer progression but also inhibit its treatment. For instance, collagens type I and IV can mediate chemoresistance by direct interaction with epithelial integrins (130). Furthermore, increased ECM stiffness and dense fibre network may increase interstitial fluid pressure, which is known to impair drug delivery (135). Collagen can also inhibit drug delivery by binding drug molecules to its components (136).

1.2.4 Integrins

1.2.4.1 Integrin Structure and Function

Integrins are a large family of mammalian transmembrane receptors for cell adhesion to the extracellular matrix. They also play crucial roles in cell proliferation, motility, differentiation, and survival under both normal and tumourigenic conditions (137). Furthermore, integrins control ECM remodelling by regulating the localization and activity of matrix-degrading proteases (83).
There are 18α and 8β subunits, which undergo non-covalent heterodimerization to form at least 24 distinct αβ pairs (80). Each subunit has a large extracellular domain, which interacts with the components of ECM, a single transmembrane helix, and a short cytoplasmic tail, which connects to the cytoskeleton, transducing biochemical or mechanical signals across the cell membrane (138). Different heterodimer combinations exhibit various signaling properties due to differences in ligand binding specificity. Four heterodimers, α1β1, α2β1, α10β1, and α11β1, function as fibrillar collagen receptors. While the expression of α1β1 and α11β1 is restricted to mesenchymal cells, α2β1 and α10β1 are expressed in epithelial cells and chondrocytes, respectively (139-142). Integrins cluster upon adhesion to the ECM, which promotes the recruitment of adhesion adaptor proteins to form focal adhesion complexes. This includes focal adhesion kinases (FAKs), SRK family kinases (SFKs), the p130CAS scaffolding molecule, and proteins such as vinculin, talin, tensin, paxillin, and alpha-actinin (137).

1.2.4.2 Integrin Expression in Cancer

Integrins expressed in neoplastic cells and cancer-associated cells, such as CAFs, play an important role in cancer. Integrins may be involved in both, tumour promotion and suppression. For instance, the expression of epithelial α2β1 gradually diminishes throughout the progression in mammary tumours, resulting in increased malignant cell dissemination (143). Furthermore, environmental cues, such as tissue rigidity may regulate integrin binding affinity to the ECM and cause these receptors to enhance either cell survival or apoptosis. Integrin adhesion has been shown to increase cell survival signalling and to avoid integrin-mediated death (IMD) (144). This process occurs through apoptotic cell death, caused by the recruitment and activation of caspase 8 via unligated integrins on adherent cells.

Desmoplasia has been associated with poor prognosis in a variety of cancers, including breast, skin, pancreas, and lung (145-148). Excessive deposition of collagen type I and III and increased degradation of collagen type IV is a characteristic feature of a desmoplastic reaction in both, primary tumours and their metastases (149-152). The deposited collagen up-regulates integrin signalling through which it increases malignant cell proliferation, metastasis and chemoresistence (130). CAFs are a prominent feature of the desmoplastic reaction and have been shown to over-express integrins implicated in cancer progression, such as integrin α11 (α11) in NSCLC (81, 97, 106, 111, 153).
1.2.4.3 Stromal α11 in NSCLC

Representational differences analysis (RDA) was used to discover that the integrin α11 gene was over-expressed in lung ADC and SCC histological subtypes compared to matched normal tissue (106). These findings were independently confirmed by another research group (153). It has since been shown that α11 was mainly expressed in the stroma of primary NSCLC, specifically by CAFs (97, 111). Furthermore, ablation of stromal α11 in two independent mouse models resulted in significantly attenuated NSCLC tumour growth and dissemination (81, 111). The reduced tumourigenicity was subsequently shown to be mediated by IGF2, whose expression was regulated by α11 (111). Additionally, α11-deficient tumours exhibited disorganization of collagen structure and a decrease in tissue stiffness compared to control (81). Gene expression analysis revealed an association of α11 with a collagen cross-linking gene, LOXL1, whose over-expression has been previously reported in metastatic NSCLS sites (81, 118, 154). However, it remained unclear whether LOXL1 was a mediator of the observed tumour growth attenuation and/or of the stromal collagen structure disruption. These studies highlight the importance of the regulation of growth factor signalling, ECM remodelling and perhaps expression of other secreted factors by stromal integrins in tumour progression.

1.2.5 Lysyl Oxidases

1.2.5.1 Structure and Function

The LOX family consists of five homologous members: LOX, LOXL1, LOXL2, LOXL3, and LOXL4 (78). They are secreted copper-dependent amine oxidases whose primary function is to covalently cross-link collagen and elastin fibres. The C-terminal region is highly conserved within all LOX members containing a copper binding motif and lysyl-tyrosyl-quinone (LTQ) co-factor, which are necessary for protein conformation and catalytic activity, respectively (155). This domain also contains a cytokine receptor-like (CRL) domain. The N-terminal region, on the other hand, is variable in all family members and is thought to confer distinct protein-protein interactions (155). Furthermore, LOX and LOXL1 contain pro-sequences, which allow their secretion as inactive pro-enzymes, while LOXL2, LOXL3, and LOXL4 contain four scavenger receptor cysteine-rich (SRCR) domains, which are believed to be involved in cell adhesion and protein-protein interactions (156, 157). Bone morphogenetic protein 1(BMP1) and BMP1-related
proteinases activate LOX and LOXL1 enzymes through cleavage of the N-terminal pro-peptide region (158).

LOX has been observed to be highly expressed in aorta, lung, kidney and pancreas and was shown to decrease with age (159). LOX knockout mice die soon after birth due to impairment of connective tissues. LOXL1 is abundant in aorta, placenta, skeletal muscle, kidney and pancreas (159). Knockout LOXL1 mice are viable, but females commonly develop rectal prolapse, indicating impaired elastic tissue homeostasis. Furthermore, these mice exhibited enlarged airspaces in the lung, loose skin, and vascular abnormalities (160). The inability to compensate for each other’s function demonstrates the distinct phenotypes of LOX and LOXL1, which are likely mediated by the variable N-terminal regions. By contrast, LOXL2 is found in human reproductive tissues, including the prostate, uterus and placenta (161). LOXL3 and 4 are expressed at much lower levels, where LOXL3 expression is observed in the placenta, uterus, and the heart, while LOXL4 is apparent in the placenta, lung, kidney, pancreas, testis, and ovary (162, 163). No knockout mice have been published for LOXL2, LOXL3, or LOXL4.

1.2.5.2 Role in Cancer

The role of LOX and LOXL2 has been very well characterized in cancer, while very little is known about the function of LOXL1, LOXL3, and LOXL4. LOX was shown to drive cancer cell migration and invasion in several studies. LOX-mediated collagen cross-linking was reported to increase tissue stiffness in breast cancer (77). This biomechanical change was shown to promote integrin-mediated FAK and SRC kinase activity, which was then associated with tumour progression in breast cancer (164). This was later validated in colon cancer (165). LOX and LOXL2 have both been shown to control the expression of E-cadherins, indicating a role in EMT (166). Additionally, high LOX and LOXL2 expression was associated with metastasis and poor survival of breast, colon, esophageal squamous cell carcinoma and lung cancer patients (165, 167-169). Additionally, LOX was the first molecule shown to play an essential role in forming pre-metastatic niche in breast cancer models, creating a more favourable environment for the future colonization of cancer cells at distant sites (170).

Relatively little is known about LOXL1 in cancer. Two NSCLC studies reported it to be upregulated at the metastatic sites in either patient or mouse tissues, but another suggested it has
a tumour suppressive function in bladder cancer. The first NSCLC study demonstrated that metastases, formed in STK11/LKB1 tumour suppressor-deficient mouse models, had increased expression of LOXL1, along with other genes believed to be involved in angiogenesis and/or metastasis (154). A later study, analyzed NSCLC patient samples and found that LOXL1, MMP2/MMP9 and a lactate transporter, MCT1/2, were upregulated in metastatic sites compared to primary sites. MCT1/2 upregulation occurs during aberrant cell glucose metabolism, in the presence of lactate accumulation. LOXL1 over-expression was then able to induce metastasis and invasion in vivo and in vitro (118). By contrast, LOXL1 was reported to be epigenetically silenced in bladder cancer and when it was re-introduced, the colony formation ability of bladder cancer cells was suppressed (171).

1.3 Rationale, Hypothesis, and Objectives

1.3.1 Rationale

This review emphasizes that NSCLC exhibits an immeasurable degree of heterogeneity, conferred not only by vast variations and complexities of aberrant genomic alterations within the epithelium, but also by interactions with its immensely diverse stromal microenvironments. It was shown that tumour stromal constituents, such as CAFs, collagens, and other ECM proteins, exhibit interactions between themselves and with malignant epithelial cells, the understanding of which may allow the development of novel therapeutic agents targeted to disrupt these interactions.

Stromal CAFs consist of heterogeneous cell sub-populations, indicated by lack of specific markers that are common to all CAFs. Lung cancer-derived CAFs express a stromal marker, α11, which is implicated in NSCLC cancer progression. As previously discussed, in vivo ablation of its expression significantly suppressed primary tumour growth, disrupted intra-tumoural fibrillar collagen structure, decreased tumour stromal stiffness, and down-regulated the stromal expression of collagen cross-linking LOXL1 enzyme. It was not elucidated whether the effect on primary tumour growth and/or on collagen organization was mediated directly through α11 or indirectly via the down-regulation of LOXL1 in the tumour stroma. Previous reports indicate that collagen cross-linking performed by other LOX family members, results in increased cell
proliferation, linearization of collagen fibres and stromal tissue stiffness. This suggests that collagen cross-linking mediated by LOXL1 may be responsible for the observed xenograft tumour features.

1.3.2 Hypothesis

Stromal α11 regulates the expression of LOXL1 in stromal fibroblasts. As a result, LOXL1 promotes NSCLC primary tumour growth, stromal tissue stiffness, and organization of fibrillar collagen architecture.

1.3.3 Objectives

The study aims to confirm the role of α11 as a regulator of LOXL1 expression and to investigate the role of LOXL1 in tumour growth and collagen remodelling.

The overall objectives are:

1) *In silico* analysis of expression and correlation of α11 and LOXL1 in NSCLC patient cohorts

2) Perform gain-of-function experiments to assess α11 as a regulator of LOXL1 expression

3) Evaluate individual effects of α11 and LOXL1 expression on collagen fibre remodelling and the effect of LOXL1 expression on tissue stiffness

4) Investigate the effect of LOXL1 expression on primary tumour growth and intra-tumoural collagen remodelling
Chapter 2
Methods and Materials

2.1 Patient Cohorts

Two publically available data sets were obtained from the University Health Network (UHN) and TCGA. The UHN study (UHN181) consisted of 181 stage I-II NSCLC cases with mixed histological subtypes, while TCGA included 230 stage I-IV lung adenocarcinomas and gene expression analyses were done using Affymetrix U133A Plus 2 microarray and RNASeq Version 2 RSEM, respectively (38, 172). Patient characteristics are listed in Appendix 1.

2.2 Laser Capture Microdissection

Previously, tumour and corresponding normal tissues were resected from 15 NSCLC patients at the University Health Network (UHN) (97). Patient characteristics are listed in Appendix 2. The samples were snap-frozen, sectioned at 5-7μm thickness with a Leica CM1950 cryostat, stained with hematoxylin and eosin (H&E), and mounted onto a mmi MembraneSlide for laser capture microdissection (LCM). A CellCut Plus LCM microscope was used to isolate stromal tissue from the surrounding and bordering epithelium for RNA extraction. The isolated RNA was amplified using the WTA-Ovation Pico RNA Amplification Kit (NuGEN). Gene expression analyses were performed at the UHN Microarray Centre using the Affymetrix Human Exon 1.0 ST v2 microarrays (97).

2.3 Histological Classification of Desmoplasia

NSCLC patient samples (n = 172) were obtained from UHN and the corresponding H&E sections were blindly and independently classified by two pathologists based on the degree of desmoplasia. High desmoplastic areas (HDA) were defined as (1) abundant in fibroblasts, (2) having more fibroblasts than lymphocytes, (3) the nucleus of fibroblasts being bigger than that of lymphocytes and (4) having high cellularity of fibroblasts, where fibroblasts nuclei clustered with one another. Tumours where HDAs accounted for ≥50% of total stroma were defined as
high desmoplasia tumours, while samples where total stroma consisted of <50% HDAs, were classified as tissues with low desmoplasia. Those cases, on which the two pathologists disagreed, were blindly and independently re-scored by both. If the independent scores still did not reach consensus, the cases were then discussed and reviewed together.

2.4 Cell Culture

Previously, primary carcinoma-associated fibroblasts (CAF094) and matched normal fibroblasts (NF094) were isolated from a NSCLC resection specimen and a matched grossly normal lung tissue sample, respectively (97). To immortalize these cells, early passage cultures were infected with a lentivirus expressing human telomerase reverse transcriptase (hTERT) (97). SV40-transformed mouse embryonic fibroblasts (MEF) and murine C2C12 myoblast cells were obtained from Dr. Donald Gullberg (University of Bergen, Norway) (140, 173). MEF cells were isolated from wild type and α11-deficient embryos at 14.5 embryonic days (173). C2C12 cells were originally obtained from the American Type Culture Collection (ATCC). Unless stated otherwise, these cells were all cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Lung adenocarcinoma A549 cell line was obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% FBS.

2.5 Lentiviral Transduction

All cells were transduced using a 3rd generation lentiviral system, as described (174, 175). To produce an infectious transgenic lentivirus, human embryonic kidney, HEK293T, cells were transfected with four plasmids, consisting of two packaging vectors (pMDLg/pRRE and pRSV-Rev), one envelope vector (pCMV-VSV-G) and a vector containing the gene of interest. First, the HEK293T cells were plated at a density of 1.5x10^5/mL in a 10cm culture dish for each condition. The following day, 1750ng of pCMV-VSV-G, 3250ng of pMDLg/pRRE, 1250ng of pRSV-Rev and 5000ng of the plasmid containing the gene of interest were mixed and Opti-MEM media was added to make up the volume to 30µL per condition. In parallel, 30µL of Fugene (Promega, Madison, WI) was added to 420µL of Opti-MEM drop-wise and was incubated for 5 minutes at room temperature. Then the plasmid mixture was added drop-wise to
the Fugene mixture and incubated for 30 minutes at room temperature. Meanwhile, HEK293T DMEM media was changed to 5mL Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FBS. The transfection mixture was added drop-wise to the cells and incubated for 18 hours. The media was then replaced with 5mL of fresh IMDM with 30% FBS and incubated for 48 hours. After the virus was harvested and filtered through a 0.45µm membrane to remove cell debris, 40µg of Polybrene® was added to enhance its infection efficiency. Cells of interest cultured at 60-70% confluence were trypsinized, resuspended and centrifuged at 400g for 5 minutes. The cell pellet was resuspended and plated in the viral media, which was replaced 18 hours later with the standard media appropriate for the cell type.

CAF094<sup>YFPhTERT</sup> cells were transduced with shLOXL1, full length LOXL1, full length α11, or control vectors; NF094<sup>YFPhTERT</sup> cells with full length α11; and C2C12 cells with full length LOXL1 and α11. All short hairpin RNA (shRNA) constructs were obtained from the RNAi Consortium and contain a puromycin selection marker. LOXL1 was stably knocked down by the shLOXL1 vector (TRCN0000234356) with a target sequence 5’-CGCTACGTTTCTGCAACAAAC-3’. Human full length LOXL1 cDNA plasmid, pOTB7, was purchased from Thermo Scientific and was subcloned into a pLD.puro lentiviral expression vector, containing a puromycin resistance gene. The shGFP vector (TRCN0000072179) with a target sequence 5’-CGACCA CATGAAGCAGCACGA-3’ was used for control. Cells transduced with these constructs were selected by 1µg/mL puromycin. Full length human α11 was previously subcloned into a pLKO.hygro.DEST lentiviral expression vector, containing a hygromycin selection marker. Transduced cells were selected by 50µg/mL hygromycin.

### 2.6 RNA Procedures

#### 2.6.1 RNA Isolation

Total RNA was isolated from cultured cells using the Qiagen RNEasy Kit (Qiagen, Venlo, Netherlands). First, β-Mercaptoethanol (β-ME) was added to the RLT lysis buffer (10µL/1mL) in order to inactivate any potential RNases. Then, 350µL of this solution was added to cells plated on a 10cm culture dish. They were scraped down, harvested into a QIAshredder spin column for cell lysate homogenization, and centrifuged at full speed for 2 minutes. One volume
of 70% ethanol in diethylpyrocarbonate (DEPC)-treated water was added to the homogenized lysate and the entire mixture was transferred to an RNeasy spin column. It was centrifuged for 30 seconds at 10,000g and washed once with 700µL of buffer RW1 and then twice with 500 µL of buffer RPE. Total RNA was eluted with 30µL of RNase-free water and kept on ice. RNA concentration was measured with the Nanodrop1000 (ND1000) machine.

### 2.6.2 cDNA Synthesis

Total RNA was reverse-transcribed to synthesize 1 to 3µg of cDNA. RNA was mixed with 1µL Oligo dT (Thermo Scientific, Waltham, USA), 1µL of 10mM dNTP, and RNase-free water for a total volume of 13µL. This reaction mixture was incubated in a Biometra T3 thermocycler (Biometra, Göttingen, Germany) for 5 minutes at 65°C and was then placed on ice for at least 1 minute. Next, the cDNA synthesis mixture was added, which consisted of 4µL of 5X first-strand buffer, 2µL of 0.1M dithiothreitol (DTT), and 1µL of Superscript III® (Life Technologies). The final mixture was incubated for 1 hour at 50°C. The reaction was terminated by heating for 15 minutes at 70°C.

### 2.6.3 Quantitative Real-Time PCR

The synthesized cDNA was diluted to a concentration of 2ng/µL. RT-PCR was set up in a 96-well plate and each reaction consisted of 1µL of 10µM forward primer, 1µL of 10µM reverse primer, 5.5µL of RNase-free water, 12.5µL of 2X SYBR Green master mix (Life Technologies) and 5µL of 2ng/µL cDNA for a total volume of 25µL. The plate was placed into the CFX96 Touch™ RT-PCR Detection System (BioRad, Hercules, USA). The thermal protocol consisted of 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C and 1 minute at 60°C. Cq values of target genes were normalized to housekeeping genes, and ΔCq values were normalized to ΔCq values of control samples. Fold change was calculated with Equation 1.

\[
\text{Fold Change} = 2^{-\Delta\Delta C_q}
\]

Equation 1. Calculation of fold change in relative mRNA expression.
2.6.4 Primer Design and Selection

All primers were designed with the NCBI Primer –BLAST tool for each gene of interest. For successful amplification of target genes in both, genomic DNA (gDNA) and cDNA, primers were designed to avoid exon-exon junctions. To ensure that the primer pairs were specific to genes and species of interest, BLAST was used to search for the designed nucleotide sequences in the NCBI Genbank database. Additionally, an Oligo Analysis tool (Eurofins Genomics) was used to predict and exclude primer pairs with primer-dimer formation.

All new primers were tested for amplification efficiency by constructing standard curves using gDNA of known concentrations. Two-fold serial dilutions (40-2.5 ng/reaction) of gDNA and water as non-template control were used. Cq values were plotted versus the initial gDNA amount and the slope of the standard curve was calculated. Amplification efficiency was determined by Equation 2.

\[
\text{Efficiency} = 10^{(-1/\text{slope})}
\]

Equation 2. Calculation of primer efficiency

Furthermore, dissociation curves were run to determine primer specificity. Primers with efficiency of 90 to 110% were used for further quantification (sequences listed in Appendix 3), while non-specific and/or inefficient primer pairs were excluded.

2.7 Western Blot Analysis

For detection of secreted LOXL1, cells were grown to confluence in 2 x 15 cm culture plates and incubated in serum- and phenol red-free DMEM medium for 48 hours. The conditioned media was then collected, passed through a 0.45µm filter, frozen at -80°C overnight and concentrated through lyophilization. Secreted protein powder was reconstituted in 200µL of water. Protein samples were quantified with Bradford protein assay (BioRad), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes using the Trans-Blot® Turbo™ Transfer System (BioRad). Membranes were blocked with 5% non-fat dry milk for 1 hour at room temperature and then
incubated with primary antibodies to rabbit anti-human/mouse LOXL1 (Santa Cruz, Dallas, TX, USA) and goat anti-human hepatocyte growth factor (HGF) (Santa Cruz) at 1:1000 dilution at 4°C overnight (Appendix 4). Membranes were washed with Tris- buffered saline with Tween (TBST) wash buffer and incubated in respective horseradish peroxidase-conjugated secondary antibodies (anti-rabbit at 1:10,000 and anti-goat at 1:2000 dilutions) for 1 hour at room temperature. All primary antibodies were diluted in TBST with 5% bovine serum albumin (BSA) and secondary antibodies in TBST with 5% non-fat dry milk. Membranes were washed again and 1mL of enhanced chemiluminescent (Roche Diagnostics Canada) reagent was applied for 5 minutes. Protein bands were detected by exposing these membranes to autoradiography film and developing it on a Konica Minolta (Tokyo, Japan) SRX-100A film processor.

2.8 Fibroblast-Populated Collagen Lattice Culture

Collagen gels were prepared and populated with CAF094_shGFP, _shLOXL1, or _LOXL1 cells or with C2C12, C2C12_α11, or C2C12_LOXL1 myoblast cells. A collagen solution was made by mixing 9 volumes of 4mg/ml rat tail type I collagen (Advanced Biomatrix, San Diego, CA, USA), 1 volume of neutralization solution (Advanced Biomatrix), and 12 volumes of 2X serum-free DMEM medium to obtain a final collagen concentration of 1.64 mg/mL. The solution was kept on ice to prevent premature collagen polymerization. Cells were trypsinized, resuspended in equal volume trypsin inhibitor and phosphate-buffered saline (PBS), and then counted. Once the correct number of cells was centrifuged, the cell pellet was resuspended in the collagen solution for a final density of 2.5x10^5 cells/mL. Then, 500μl of cell/collagen mixture was added into each well of a 24-well plate and allowed to polymerize for 30-60 minutes at 37°C. Next, DMEM supplemented with 10% FBS was carefully added on top of each gel, which were incubated at 37°C until contraction. Media was changed every 2-3 days. To visualize collagen fibres with second harmonic generation (SHG) microscopy, gels were lifted out of the 24-well plate onto glass slides and were lightly covered with glass coverslips.
2.9 Second-Harmonic Generation Microscopy

Collagen second harmonic generation (SHG) images were taken with a Zeiss LSM 710 NLO two-photon microscope, equipped with a Coherent Chameleon Ti:Sapphire femtosecond pulsed laser. A 20x (NA=1.0) water immersion objective lens was used. An excitation wavelength of 840nm was used so that the SHG signal would be generated and detected at exactly one-half of the excitation wavelength (i.e. 420nm). Unstained fixed and deparaffinized 10µm tissue sections or whole collagen gels were visualized and images of 425µm by 425 µm regions were taken.

2.10 Fibril Orientation Distribution Analysis

To measure the degree of waviness or alignment and orientation of collagen, distribution of local collagen fibre orientation within images was assessed based on methodology published by Rezakhaniha et al. in (176). Briefly, an ImageJ plug-in, OrientationJ, was used. The local orientation and isotropic properties of pixels were derived from structure tensors, which are commonly used in the field of image processing. Tensors were evaluated for each pixel of an input image by computing the continuous spatial derivatives in x and y directions using a cubic B-spline interpolation to obtain the local predominant orientation (177). Graphical outputs show a hue-saturation-brightness (HSB) colour-coded map indicating the angles of the oriented structures within the image. Orientation distribution peaks were then aligned and normalized before unpaired two-tailed non-parametric testing (Mann-Whitney U) for statistical significance across distributions. The shape of the distribution indicated the degree of alignment within the image, where wide and broad shapes suggested little coherency in alignment and tight peaks with small standard deviations implied aligned structures.

2.11 Development of Transgenic Mice

Transgenic mice were developed based on methodology published by Beard et al in (178). This transgene integration strategy is mediated by site-specific recombination, which allows embryonic stem (ES) cells to carry tetracycline-inducible genes targeted to a specific locus, to
ensure predictable temporal expression in ES cells and mice, as well as spatial expression in mice (Appendix 5 and 6) (178).

Mouse embryonic stem (ES) cells were cultured on top of feeder MEF cells attached to 0.2% gelatin-coated plastic culture plates to maintain ES cell proliferation, pluripotency and differentiation potential. MEF were initially cultured in Knockout DMEM (Gibco®), supplemented with 10% FBS, 1x MEM non-essential amino acid, 2mM L-glutamine, 0.1mM 2-mercaptoethanol, and 1x penicillin/streptomycin. Once ES cells were plated on top of MEF cells, both were cultured in Knockout DMEM media supplemented with 15% FBS, 1x MEM non-essential amino acid, 2mM L-glutamine, 0.1mM 2-mercaptoethanol, and 1x penicillin/streptomycin. Additionally, ESGRO® Leukemia Inhibitory Factor (LIF, 1000 units/mL; EMD Millipore) was added to prevent spontaneous differentiation of ES cells. Media was changed daily.

Pre-modified ES cells were used. They contained a region just downstream of Colα1 locus that was altered with homologous recombination to insert an flt homing site into which tetracycline-inducible transgenes (i.e. LOXL1) would be efficiently integrated in the presence of flippase (FLPe) recombinase (178).

Full-length LOXL1 cDNA was inserted into the pgkATGfrt vector to create a flip-in construct that was later used for integration into the modified Colα1 locus. LacZ gene was used as a reporter and was inserted upstream of the LOXL1 sequence to avoid inactivating its catalytic site, which is encoded at the 3’-end of its DNA sequence. As an additional safeguard, a 2A peptide (P2A) sequence was inserted between LacZ and LOXL1, serving as a cleavage site during translation. This design was put in place to avoid LOXL1 post-translational modification errors, especially in the cleavage of its signal peptide located at the N-terminus, which initiates its secretion into the ECM. Using LacZ gene product name, β-galactosidase (βgal), the final vector and the strain of resultant transgenic mice were referred to as “βgal-P2A-LOXL1”.

ES cells (~1.5x10⁷) were electroporated in a BioRad Gene Pulser at 400V and 125µF with 50µg of target βgal-P2A-LOXL1 DNA construct and 25µg of flippase (pCAGGS-FLPe-puro) plasmid. These cells were re-plated onto feeder MEF culture and were selected with Hygromycin (300µg/mL) the next day. ES clones that subsequently formed were passaged twice to confirm successful selection.
Hygromycin-resistant clones were expanded to verify expression of the target vector. For each clone, half of the cells were treated with Doxycycline (DOX, 1:1000) and the untreated half was used as negative control. Conditioned media was collected for detecting secreted LOXL1 and the cells were stained for βgal. Successful clones exhibited LOXL1 protein bands and blue staining only when treated with DOX, on western blot and in βgal staining, respectively (Appendix 7). The successful clones were expanded, frozen and sent to Toronto Centre for Phenogenomics for a tetraploid embryo complementation procedure. The chimera mice were generated in a mixed C57BL/6, 129SvJ and FVB background. These mice were subsequently bred with severe combined immune deficient (SCID) mice for 5 generations, to date.

The genotyping was determined by PCR for rtTA and Colα1 alleles. Genomic DNA was isolated from mouse tail tissue and two sets of primers were used to genotype the animals in two separate PCR reactions. Common primer for the rtTA allele was 5’-AAAGTCGCTCTGAGTTGTAT-3’, wild type primer was 5’-GGAGCGGGAGAAATGGATATG-3’ and mutant primer was 5’-GCGAAGAGTTTGTCCCTCAACC-3’. The PCR conditions for this locus were: 94°C for 6 minutes, followed by 35 cycles of 94°C for 30sec, 58°C for 45sec, 72°C for 1 minute, and the final extension for at 72°C for 5 minutes. Wild type and mutant rtTA alleles were predicted to be 650bp and 340bp in length, respectively. Both bands were present in heterozygous samples. Heterozygous (rtTA+/−) or mutant (rtTA++/−) samples were then assessed for the Colα1 allele. Common primer for the Colα1 locus was 5’-CCCTCCATGTGTGACCAAGG-3’, wild type primer was 5’-GCACAGCATTGCGGACATGC-3’ and mutant primer was 5’-GCGAAGAGCCGGCGGCGTCTGG-3’. The PCR conditions for this allele were: 95°C for 6 minutes, followed by 40 cycles of 95°C for 30sec, 65°C for 45sec, 72°C for 1 minute, and the final extension at 72°C for 5 minutes. Wild type and mutant Colα1 alleles were predicted to be 331bp and 551bp in length, respectively. Both bands were present in heterozygous samples. Heterozygous (Colα1+/−) samples were selected for further breeding. The mutant (Colα1++/−) genotype was not observed, suggesting that the insertion of the target gene into both Colα1 alleles may be embryonically lethal. Thus, the observed genotypes required for the Tet-On system to function were rtTA+/−/Colα1+/− or rtTA+++/Colα1++/−.
2.12 β-Galactosidase Assay

ES cells were fixed with 0.5% glutaraldehyde in PBS for 15 minutes and rinsed three times with PBS. The staining solution consists of 1mg/mL X-Gal, 2mM magnesium chloride, 5mM potassium ferricyanide, and 5mM potassium ferrocyanide in PBS (final concentrations). Cells were incubated in the staining solution in the dark for 2 hours at 37°C. ES cells, in which the expression of βgal-P2A-LOXL1 vector was successfully induced, were identified by blue colour.

2.13 Atomic Force Microscopy

Atomic force microscopy (AFM) was used to determine LOXL1-mediated stiffness within βgal-P2A-LOXL1 mouse tissues. Paraffin blocks of skin tissue from ±DOX (Week 2 and 8) mouse groups were cut into 10μm sections and stained with H&E to define stromal regions. Two sections from each group were measured with AFM to determine Young’s elastic modulus, during which the samples were maintained in PBS. AFM indentations were performed using an MFP-3D AFM (Asylum Research, Santa Barbara, CA, USA) equipped with silicon nitride cantilevers (Bruker, Goleta, CA, USA) with a nominal spring constant of 0.06 N/m. Accurate spring constant was determined using thermal noise method before each measurement (179). Samples were then indented at a 1μm/s loading rate, ramp size of 5μm, with a maximum force of 500pN. The Young’s modulus was determined by considering load indentation dependence for a paraboloidal tip shape. The determination of the elastic modulus of the tissue (E) from force-distance curves was performed using the Hertz model (Equation 3) (180, 181).

\[ F = \frac{4\sqrt{R}}{3(1-v^2)} E \delta^{3/2} \]

Equation 3. Calculation of Young’s elastic modulus

Where \( F \) is the loading force (N), \( E \) is the Young’s modulus (Pa), \( v \) is the Poisson ratio, \( R \) is the radius of curvature of the tip (m) and \( \delta \) is the indentation depth (m). Tissue samples were assumed to be incompressible and a Poisson’s ratio of 0.5 was used in the calculation of the Young’s elastic modulus.
2.14 Subcutaneous Tumourigenicity Assay

All animal work was approved by the Ontario Cancer Institute animal facility and carried out under sterile conditions in a laminar flow hood. Male SCID mice aged 4 to 6 weeks were used for in vivo experiments. A549 adenocarcinoma cells were injected subcutaneously alone or together with CAF094 cells in a 1:1 ratio (2x10⁶ cells each) or in a 1:4 ratio (1x10⁶ cancer cells to 4x10⁶ CAFs) into mouse abdominal flanks. Tumour growth was assessed and recorded twice each week by measuring tumour length and width with calipers. Tumour volume was calculated using Equation 4.

\[
\text{Volume} = \frac{\pi}{6} (\text{Length} \times \text{Width}^2)
\]

Equation 4. Calculation of tumour volume

Mice were sacrificed once the tumour size reached approximately 1.5cm in diameter. At sacrifice, tumour mass was recorded and portions of tumours were either snap-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin for histological processing.
Chapter 3
Results

3.1 Expression of LOXL1 and α11 in NSCLC Patient Cohorts

3.1.1 Correlation of LOXL1 and α11 mRNA Expression

Previous experimental evidence indicates that stromal α11 increases primary tumour growth and metastasis of lung cancer. This effect may be mediated through downstream up-regulation of stromal LOXL1 expression (81). To explore whether the association between α11 and LOXL1 expression exists in clinical setting, their correlation was assessed in two NSCLC patient cohorts (Appendix 1).

Plotting expression levels of α11 against LOXL1 revealed that there is a strong correlation between the two variables in both datasets (Figure 1).

![Figure 1. Correlation of LOXL1 and α11 mRNA expression in two NSCLC patient cohorts.](image-url)

mRNA expression analyses were performed on whole tumour patient samples. Expression of LOXL1 correlated with α11 in (A) UHN181 (n = 181) and (B) TCGA lung adenocarcinoma (n = 230) datasets. Statistics performed by Spearman correlation.
3.1.2 Association with Desmoplasia

From the UHN181 dataset, 172 samples were scored for desmoplasia. In total, 110 samples were characterized as low- and 62 as high-desmoplasia tumours. Plotting mRNA expression values of LOXL1 and α11, stratified based on the degree of desmoplasia, revealed that both are significantly up-regulated within highly desmoplastic tissues (Figure 2).

![Figure 2. mRNA expression of LOXL1 and α11 in high versus low desmoplasia. Tumour (A) LOXL1 and (B) α11 mRNA expression was greater in NSCLC patient samples with highly desmoplastic stromal tissue, compared to those with low desmoplasia. Statistics performed by unpaired, two-tailed t-test. N = 110 in low desmoplasia, n = 62 in high desmoplasia. Error bars represent standard deviation.](image-url)
3.2 mRNA Expression of LOXL1 and α11 in Cancer Stroma Versus Normal Lung

Previously, tumour and matched normal tissues were resected and collected from 15 NSCLC patients at UHN (Appendix 2) (97). Stromal tissue was isolated from adjacent epithelial cells and was analyzed for mRNA expression changes. To determine whether LOXL1 and α11 were differentially expressed in tumour stroma compared to normal lung tissues, both variables were plotted and sub-grouped based on the type of stroma. Data analysis revealed that mRNA expression of both LOXL1 and α11 was significantly up-regulated in tumour stroma compared to normal lung tissues of the 15 NSCLC patients (Figure 3).

![Figure 3](image)

**Figure 3.** mRNA expression of LOXL1 and α11 in tumour stroma versus normal lung tissue. A significant difference in (A) LOXL1 and (B) α11 mRNA expression was observed in stroma derived from tumour or corresponding normal lung parenchyma. Statistics performed by paired, two-tailed t-tests. N = 15. Each data point represents each patient and error bars represent standard deviation within each group.

Next, the same microarray dataset was used to investigate whether other LOX family members were differentially expressed in the lung tumour stroma. Expression of LOX, LOXL2, LOXL3, and LOXL4 mRNA was assessed. The only other LOX family member that was differentially expressed was LOXL3 (Figure 4).
Figure 4. mRNA expression of LOX, LOXL2, LOXL3, and LOXL4 in tumour stroma versus normal lung tissue. (A, B, D) There was no significant difference in LOX, LOXL2 or LOXL4 mRNA expression between two tissue types. (C) LOXL3 mRNA expression was up-regulated in the tumour stroma. Statistical comparisons were performed by paired, two-tailed t-tests. N = 15. Each data point represents each patient and error bars represent standard deviation within each group.
3.3 mRNA Expression of LOXL1 in Stromal Versus Epithelial Cells

To confirm stromal specificity of LOXL1 mRNA expression, RT-PCR was performed on RNA isolated from 26 patient-derived CAFs and 41 NSCLC cell lines obtained from ATCC. Due to the lack of normal epithelial cells for normalization of cancer cells, fold change value could not be calculated. Instead, delta Cq (ΔCq) values were used to compare LOXL1 expression levels between CAFs and cancer cells. Lower ΔCq values represent greater gene expression and vice versa. Data analysis revealed that LOXL1 mRNA expression was significantly greater in CAFs compared to cancer cells, suggesting that its expression within tumours originates from the stromal moieties (Figure 5) (81).

![Figure 5. mRNA expression of LOXL1 in CAFs and tumour cell lines.](image)

RT-PCR showed significantly greater expression of LOXL1 in CAFs (n =26) compared to NSCLC (n = 41) cell lines. Results were normalized to RPS13 and B2M expression. Statistical comparison was performed by unpaired, two-tail t-test between the ΔCq values of both groups. Each data point represents one sample and error bars represent standard deviation within each group.
3.4 Role of $\alpha_{11}$ as a Regulator of LOXL1 *In Vitro*

To investigate the potential role of $\alpha_{11}$ as a regulator of LOXL1 mRNA expression, human full-length $\alpha_{11}$ was over-expressed in three *in vitro* models. They consisted of MEFs isolated from $\alpha_{11}$-deficient mice (MEF KO) (173), mouse C2C12 myoblast cells, (140) and immortalized human normal lung fibroblasts (NF094$^{YFP;\text{TERT}}$) (97).

RT-PCR analysis demonstrated that over-expression of full-length $\alpha_{11}$ resulted in significant up-regulation of LOXL1 mRNA expression in all three cell lines. Furthermore, its mRNA expression was significantly down-regulated in MEF KO cells compared to MEF isolated from wild type mice (MEF WT) (Figure 6).
Figure 6. mRNA expression of LOXL1 in three cell lines with over-expressed α11. RT-PCR results showed that over-expression of full-length human α11 in (A) MEF KO, (B) C2C12, and (C) NF094YPF/TER cells resulted in significant up-regulation of LOXL1 mRNA levels. Results were normalized to GAPDH and RPS13/B2M in mouse and human cell lines, respectively. Statistical comparisons were performed by unpaired, two-tail t-test between fold change values. Error bars represent standard error.
mRNA expression of all LOX family members was compared in C2C12 and C2C12_α11 cells. While LOXL1 expression experienced the most up-regulation, α11 also increased the expression of LOXL2 and LOXL3 (Figure 7).

Figure 7. mRNA expression of all LOX family members in C2C12 and C2C12_α11 cell lines. RT-PCR results showed that over-expression of full-length human α11 in C2C12 resulted in significant up-regulation of LOXL1, LOXL2, and LOXL3, but not LOX or LOXL4. Results were normalized to GAPDH. Statistical comparisons were performed by unpaired, two-tail t-test between fold change values. Error bars represent standard deviation. N.S., not significant; *** Indicates p-value < 0.0001.
3.5 Role of LOXL1 Expression in Collagen Fibre Architecture *In Vitro*

3.5.1 C2C12-Populated Collagen Gel Assay

Cells were embedded and cultured within collagen matrices to study the role of α11 and LOXL1 expression in collagen remodelling. This was measured in terms of collagen gel contraction and collagen fibre alignment. SHG microscopy was used to image collagen fibres and fibril orientation distribution analysis was used to quantify their alignment. C2C12 cells and their derivatives with over-expressed human α11 or LOXL1 (C2C12_α11 or _LOXL1) were used first (Figure 8).

![Figure 8](image_url)  

**Figure 8.** mRNA expression of over-expressed human α11 and LOXL1 in C2C12 cells. RT-PCR results showed stable over-expression of (A) α11 or (B) LOXL1 in C2C12 cells. Statistical comparisons were performed by unpaired, two-tail t-test between fold change values. Error bars represent standard deviation.

Gels were found to exhibit various levels of contraction. In comparison to the C2C12 group, collagen gels embedded with C2C12_α11 or C2C12_LOXL1 cells exhibited much greater contraction. Moreover, the colour overlay and the distribution analysis demonstrated that collagen fibres containing C2C12_α11 or C2C12_LOXL1 cells had a dramatically greater degree of alignment compared to C2C12 control, but were not significantly different from each other (Figure 9 and Table 3).
Figure 9. Effect of α11 and LOXL1 expression on collagen remodelling in C2C12-populated matrices. (A) Collagen gel contraction. C2C12_α11 and C2C12_LOXL1-populated gels showed greater
contraction than C2C12 control. Representative images of collagen gels are shown. (B) Representative images of graphical output indicating directional orientation of fibres within the image. Dominant colour represents dominant alignment direction. (C) Quantitative representation of collagen fibre alignment. C2C12_\(\alpha_1\) and C2C12_LOXL1-populated gels were significantly more aligned compared to C2C12 control, but exhibited similar alignment to each other. SHG images were taken on 3 to 4 large regions per gel, with 3 gels per group. Each line in the graph represented a mean of 10-12 distributions. Statistics were performed by unpaired, two-tailed Mann-Whitney U test (summarized in Table 3).

Table 3. Effect of \(\alpha_1\) and LOXL1 expression on collagen alignment in C2C12-populated matrices

<table>
<thead>
<tr>
<th>Groups Compared</th>
<th>P-value</th>
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<tr>
<td>C2C12 vs. _(\alpha_1)</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>C2C12 vs. _LOXL1</td>
<td>0.0002**</td>
</tr>
<tr>
<td>C2C12_(\alpha_1) vs. _LOXL1</td>
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</table>

Statistics by unpaired, two-tailed Mann-Whitney U test.

** *** Statistically significant
3.5.2 Fibroblast-Populated Collagen Gel Assay

Next, CAF cells were cultured in collagen matrices to assess the influence of varying LOXL1 expression levels on the degree of collagen gel contraction and fibre alignment. CAFs with low, intermediate or high expression of LOXL1 were used (Figure 10).

![Graphs showing relative LOXL1 mRNA gene expression and protein levels with statistical comparisons](image)

**Figure 10.** Stable knockdown and over-expression of human LOXL1 in CAF094YFPlenti cells. RT-PCR and WB analysis of LOXL1 (A) knockdown (_shLOXL1; construct #2 was used in the experiments) and (B) over-expression (_LOXL1). Statistical comparisons were performed by unpaired, two-tail t-test between fold change values.
After a 12-15 day incubation period, gels began exhibiting contraction that was directly proportional to the expression level of LOXL1. Gels populated with _LOXL1_ cells exhibited significant contraction as compared to control _shGFP_ cells. Gels embedded with lowest LOXL1 levels experienced virtually no change. Collagen gel diameters were measured (Figure 11) to quantify the degree of LOXL1-mediated contraction (Table 4).

**Figure 11.** Effect of LOXL1 expression on collagen gel contraction in CAF-populated matrices. (A) Representative images of collagen gels. (B) Quantification of collagen gel diameters, for which statistical analysis is summarized in Table 4. Statistical comparisons were performed by ANOVA, followed by Bonferroni post-test (summarized in Table 4). N = 12 for all CAF094-populated gels, n = 3 for gels without embedded cells. Error bars represent standard deviation.
Table 4. Effect of LOXL1 expression on collagen gel contraction in CAF-populated matrices

<table>
<thead>
<tr>
<th>Comparison Of Means</th>
<th>Mean Difference</th>
<th>95% Confidence Intervals</th>
<th>Adjusted P-value</th>
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<td>3.557</td>
<td>4.402</td>
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<tr>
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<td>-1.243</td>
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<td>3.836</td>
<td>3.586</td>
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</table>

Statistics by ANOVA with Bonferroni post-test.

*** Statistically significant

The colour overlay and fibril orientation distribution demonstrated that collagen fibres in _shGFP and _LOXL1 groups were highly linearized and oriented in a single direction. In contrast, the fibres in the collagen gels containing the lowest levels of LOXL1 were wavy, disorganized and not oriented in any specific direction. Despite the visually evident differences in collagen fibre densities, there was no significant difference in the degree of fibre alignment between _shGFP and _LOXL1 (Figure 12 and Table 5).
Figure 12. Effect of LOXL1 expression on collagen alignment in CAF-populated matrices. (A) Representative images of graphical output indicating directional orientation of fibres within the image. Collagen fibres were highly aligned in both, _shGFP and _LOXL1 groups, resulting in dominant overlay colours. (B) Comparison of alignment distributions quantitatively demonstrated more fibre alignment in collagen matrices populated with _shGFP or _LOXL1 in comparison to _shLOXL1. SHG images were taken on 2 large regions per gel, with 2 gels per group. Each line in the graph represented a mean of 4 distributions. Statistics were performed by unpaired, two-tailed Mann-Whitney U test (summarized in Table 5).
Table 5. Effect of LOXL1 expression on collagen alignment in CAF-populated matrices

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<tr>
<td>CAF094_shGFP vs. _shLOXL1</td>
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<td>CAF094_shGFP vs. _LOXL1</td>
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<td>CAF094_shLOXL1 vs. _LOXL1</td>
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Statistics by unpaired, two-tailed Mann-Whitney U test.

** *** Statistically significant
3.6 LOXL1-Mediated Influence on Tissue Stiffness *In Vivo*

To assess the role of LOXL1 expression in mediating matrix stiffness, 16 transgenic βgal-P2A-LOXL1 chimera mice were used. They were divided into two groups, where mice were fed with either DOX-enriched diet (+DOX) to induce human LOXL1 expression, or regular irradiated mouse chow (-DOX) for control. Every two weeks for eight weeks, two mice were sacrificed from each group and tissues were processed to paraffin blocks (Figure 13). Normal skin from the earliest (Week 2) and latest (Week 8) time points were used for AFM measurements. Skin tissues were used due to their high collagen content.

![Schematic representation of experimental design](image)

Figure 13. Schematic representation of experimental design.
AFM measurement and analysis of elastic modulus distributions, revealed that skin tissues obtained from mice fed with DOX-enriched diet were significantly more rigid compared to -DOX control (Week 2, $p = 0.0002$; Week 8, $p = 0.0054$). No significant change was observed between the two time points.

![Figure 14](Image)

**Figure 14. Effect of induced LOXL1 expression on normal mouse skin tissue stiffness.** Elastic modulus distributions of mouse skin tissues were significantly greater in the +DOX groups compared to the –DOX control groups at Week 2 ($p = 0.0002$) and Week 8 ($p = 0.0054$), as measured by AFM. No significant difference was observed between Weeks 2 and 8 in the –DOX ($p = 0.1223$) or the +DOX ($p = 0.0560$) groups. Normal skin tissues of 2 mice per group were measured at 40 regions along each sample. Statistics were performed by comparing the means of 40 elastic modulus values of each group by unpaired, two-tailed Mann-Whitney U.
3.7 Role of LOXL1 Expression in Cancer

3.7.1 Primary Tumour Growth

Lung adenocarcinoma A549 cells were injected subcutaneously alone or together with CAF094_shGFP, _shLOXL1 or _LOXL1 in order to assess the effect of stromal LOXL1 expression on primary tumour growth. Two independent experiments were performed. The A549:CAF ratio was 1:1 (2x10^6 cells each) and 1:4 (1x10^6 cancer cells to 4x10^6 CAFs) in first and second experiments, respectively. The _shLOXL1 group was not investigated in the last experiment. Final tumour mass was used to assess tumour growth differences between groups.

In experiment #1, the tumour weight was significantly lower in mice injected with _shLOXL1 compared to the mice injected with _shGFP (p = 0.027). No significant differences were observed in Experiment #2 (Figure 15).

![Figure 15. Effects of stromal LOXL1 expression on subcutaneous primary tumour mass.](image)

(A) Cells were injected with an A549:CAF ratio of 1:1 (2x10^6 cells each). As compared by Mann-Whitney test, there was a significant difference between _shGFP and _shLOXL1 groups (p = 0.027). No other significant differences were observed. (B) Cells were injected with an A549:CAF ratio of 1:4 (1x10^6 cancer cells to 4x10^6 CAFs) and no significant differences were detected within this experiment. Each point represents the final tumour mass of each mouse, where n = 4 per group. Error bars represent standard deviation.
The mRNA levels of human LOXL1 were confirmed with RT-PCR in tissues from Experiment #1. There was no significant difference between any of the groups (Figure 16).

![Relative LOXL1 mRNA gene expression](image)

**Figure 16. mRNA expression of human LOXL1 in xenograft tumours.** RT-PCR demonstrated that there were no significant differences observed between the groups. Statistical comparisons were performed by ANOVA, followed by Bonferroni post-test (not shown). Error bars represent standard deviation.

### 3.7.2 Intra-Tumoural Collagen Organization

Next, the effect of stromal LOXL1 expression was evaluated on collagen organization within tumour tissues of Experiment #1. SHG microscopy was performed and fibre alignment was quantified using fibril orientation distribution analysis.

Consistent with findings in the *in vitro* models, image analysis demonstrated that collagen fibres in the tumours in the _LOXL1_ group had the greatest degree of fibre alignment as it was significantly different from _A549 alone_, _shGFP_ and _shLOXL1_ groups. By contrast, fibres within _shLOXL1_ samples were very disorganized and were similar in appearance to the _A549 alone_ group, where no human stromal LOXL1 was expressed. Although no statistical significance was detected between the _shGFP_ and _shLOXL1_ groups, the data trended towards greater alignment in the _shGFP_ group (Figure 17 and Table 6).
Figure 17. Effect of LOXL1 expression on collagen alignment in xenograft tumours. (A) Representative images of graphical output indicating directional orientation of fibres within the image. Collagen fibres were highly aligned in the _LOXL1 group, resulting in a dominant overlay colour. (B) Collagen fibres were observed to have the greatest degree of alignment in the _LOXL1 group. SHG images were taken on samples from *in vivo* Experiment #1, where 8-12 regions were analyzed per mouse, with 4 mice per group. Each line represented a mean of 40 individual distributions. Statistics were performed by unpaired, two-tailed Mann-Whitney U test (summarized in Table 6).
Table 6. Effect of LOXL1 expression on collagen alignment in xenograft tumours

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<td><strong>CAF094_shLOXL1 vs. _LOXL1</strong></td>
<td><strong>0.0048</strong>**</td>
</tr>
</tbody>
</table>

Statistics by unpaired, two-tailed Mann-Whitney U test

* ** Statistically significant
Chapter 4
Discussion

4.1 *In Silico* Analysis of LOXL1 and α11 Expression and Correlation in NSCLC Patient Cohorts

A correlation was established in LOXL1 and α11 mRNA expression, using mRNA expression data from two NSCLC patient cohorts (Figure 1) (38, 172). Two publically available datasets were obtained from UHN and TCGA, which provided data on tumour samples, primarily consisting of stage I-II lung adenocarcinomas (Appendix 1). These results indicate that the co-expression of LOXL1 and α11 observed within xenograft tumours in the earlier *in vivo* study may also present in the patient population. This suggests that there may be a biologically relevant interaction between the two genes.

Furthermore, LOXL1 and α11 mRNA levels were found to be significantly correlated with the patient tumour desmoplastic status, which was scored and classified as either low or high. Broadly, desmoplasia is characterized as an excess deposition of fibrillar collagen (57). Moreover, α11 is a known collagen-binding receptor exhibiting the highest affinity for collagen type I, while LOXL1 catalyzes covalent cross-linking of fibrillar collagens. Taken together, this data indicates that LOXL1 and α11 may be implicated in promoting the desmoplastic phenotype in NSCLC.

Further, LOXL1 and α11 mRNA expression was analyzed in micro-dissected tumour stroma in comparison to matched normal lung tissue. A previously established in-house RNA microarray dataset was used, which consisted of matched samples obtained from 15 NSCLC patients (Appendix 2). Both, LOXL1 and α11 mRNA levels were found to be up-regulated in the tumour stroma compared to the matched normal parenchyma (Figure 3). This finding suggests that the stromal expression of both genes may have a role in promoting NSCLC. This is consistent with previous reports that have demonstrated that both α11 and LOXL1 played a role in promoting NSCLC tumour growth and metastasis (81, 111, 118, 154). However, compared to some of the other LOX family members, the role of LOXL1 in cancer has not been well characterized.

The expression of the other LOX family members was assessed in this microarray dataset. There was no significant difference in the mRNA expression of LOX, LOXL2, or LOXL4 in tumour
stroma compared to matched normal lung. Although LOX has been previously reported to be up-regulated in lung cancer, its expression was not significantly up-regulated in this dataset (182, 183). This may be explained by the fact that LOX is primarily up-regulated in epithelial cells during cancer progression. However, LOXL1 is stromal in origin, which was confirmed with RT-PCR by comparing its mRNA expression between panels of primary CAFs and NSCLC cell lines (Figure 5). LOXL3 exhibited borderline significance ($p = 0.0468$). However, there was one outlier, excluding which would result in non-significant difference in LOXL3 mRNA expression. Furthermore, the role of LOXL3 is not well characterized in cancer and has been shown to be expressed at much lower levels than LOX, LOXL1, and LOXL2 (162).

In conclusion, demonstrating significant LOXL1 and $\alpha_{11}$ mRNA expression correlation and up-regulation in tumour microenvironment was the first step towards confirming previous *in vivo* findings and demonstrating their relevance in a patient population.

### 4.2 Assessing $\alpha_{11}$ as a Mediator of LOXL1 Expression

Once the correlation of LOXL1 and $\alpha_{11}$ mRNA expression was found to be significant in NSCLC patient samples, the next step was to assess the potential role of $\alpha_{11}$ as a mediator of LOXL1 expression. To accomplish this, full-length human $\alpha_{11}$ was over-expressed in 3 different cell types-MEF KO, C2C12, NF - after which LOXL1 mRNA expression was confirmed with RT-PCR.

The advantage of using MEF cells, isolated from either wild-type or $\alpha_{11}$-deficient mice (MEF WT, MEF KO) was the capability to completely ablate mouse $\alpha_{11}$ and to rescue its phenotype by over-expressing the human equivalent. Furthermore, this allowed for low, intermediate or high expression of $\alpha_{11}$ and to assess the corresponding expression of LOXL1. The benefit of using C2C12 cells was their lack of endogenous collagen receptors – $\alpha_1$, $\alpha_2$, $\alpha_{10}$ and $\alpha_{11}$ (140). This allowed for evaluation of $\alpha_{11}$ independently from the other $\alpha$-chain integrins, which may have overlapping functions. Additionally, NF cells were used, because LOXL1 is primarily expressed in stromal fibroblasts.
LOXL1 was found to be significantly up-regulated in all cell types and was also decreased in MEF cells deficient in α11 compared to wild type control (Figure 6). Assessing mRNA expression of the other LOX family members in C2C12 compared to C2C12_α11 cells revealed that LOXL2 and LOXL3 expression was significantly up-regulated (~3 and ~4 fold, respectively) (Figure 7). However, LOXL1 experienced the highest degree of increase (~6 fold).

This is a seemingly conflicting result, because LOXL2 and LOXL3 expression was not found to be down-regulated as a result of α11 deficiency in vivo. It may be explained by the fact that the only collagen receptor that was expressed in C2C12_α11 cells was the human α11 subunit, expressing no other endogenous collagen receptors. By contrast, all four collagen binding subunits are present in mouse tissues. There is a possibility that these integrins may exhibit functional compensation and mediate LOX family expression, resulting in a complex signalling network.

4.3 Evaluating the Effect of LOXL1 and α11 Expression on Collagen Remodelling and Tissue Stiffness

Previous experimental findings demonstrated that subcutaneous tumours isolated from mice deficient in α11 had disrupted networks of collagen fibres (81). However, it was unclear whether this was directly as a result of α11 deficiency or due to the down-regulation of mouse LOXL1. To recapitulate this phenomenon in vitro, C2C12 or CAF cells were cultured in collagen gel matrices. Rat tail type I collagen was used as it was isolated with acid, rather than pepsin. This allowed its telopeptides to remain intact, making LOX-dependent collagen cross-linking possible in vitro.

First, C2C12 and C2C12_α11 cells were compared (Figure 8). Interestingly, after being embedded for approximately a week, they began contracting. The group with high α11 expression contracted collagen gels to a much greater degree than did the C2C12 control group. Next, images of collagen fibres were taken using SHG microscopy and analyzed using fibril orientation distribution analysis. It was found that collagen fibres containing C2C12_α11 cells were dramatically more aligned compared to those populated with control C2C12 cells, lacking endogenous collagen binding integrins (Figure 9 and Table 3). This confirmed that α11 has an
important function in collagen gel remodelling. However, because C2C12_α11 cells were found to have higher levels of LOXL1, it wasn’t clear if this was a direct effect of α11.

The next step was to determine whether the expression of LOXL1 could result in the same phenotype in the absence of α11. To accomplish this, C2C12 cells were compared to those with over-expressed human LOXL1 (C2C12_LOXL1), which also lacked collagen-binding integrin expression (Figure 8). Gels populated with these cells began to contract after long-term culture, in a LOXL1-dependent manner. They showed a similar degree of contraction to the C2C12_α11 group and much greater contraction compared to control. Fibre alignment distribution analysis showed that the contracted gels also had an increased fibre alignment, which was significantly different from control, but not from the C2C12_α11 group (Figure 9 and Table 3). This finding suggests that LOXL1 is an effective collagen-remodelling enzyme that can independently increase collagen fibre alignment and increase gel tension to cause contraction.

To validate this finding, CAF cells with low, intermediate, or high levels of LOXL1 expression were embedded in collagen matrices (Figure 10). These collagen gels were also found to exhibit collagen contraction and fibre alignment in a LOXL1-dependent manner (Figure 11, Figure 12, Table 4, and Table 5). Although no statistical difference was observed between the _shGFP and _LOXL1 groups, it is important to note that fibre alignment distribution analysis does not take into account the difference in collagen fibre densities, but the fibre directionality only. Thus, while the degree of alignment in the two groups was similar, the fibres were more densely packed in the _LOXL1 group.

Furthermore, experimental evidence suggests that collagen cross-linking is associated with tissue rigidity (74, 77, 84, 122, 184). To assess the effect of LOXL1 on tissue stiffness, transgenic mice with inducible human LOXL1 expression system were developed (Figure 13). AFM measurements showed that collagen-rich skin tissues obtained from mice fed with DOX-enriched diet had a significantly higher elastic modulus distribution compared to control (Figure 14).

Together, these results suggest that LOXL1-mediated collagen cross-linking can effectively re-organize fibrillar collagen architecture and increase tissue stiffness. Other LOX family members, LOX and LOXL2, have been shown to have similar functions in tumour-associated microenvironments, which were shown to increase cancer progression. This may be an indication that collagen cross-linking mediated by LOXL1 may play an important role in NSCLC.
4.4 Investigating the Effect of LOXL1 Expression on Primary Tumour Growth and Collagen Remodelling

Next, the effect of LOXL1 on cancer was evaluated in the context of *in vivo* primary tumour growth. A549 cells were subcutaneously injected alone or with CAFs expressing low (_shLOXL1), intermediate (_shGFP), or high (_LOXL1) levels of LOXL1 into SCID mice. Initially, an equal number of CAF and A549 cells were injected (Experiment #1). Knock down of LOXL1 resulted in significant suppression of xenograft tumour size compared to _shGFP control group. However, over-expression of LOXL1 was not significantly different (Figure 15).

Next, intra-tumoural collagen fibres were imaged with SHG microscopy and analyzed for alignment distribution (Figure 17 and Table 6). The fibres in the _shLOXL1 group were highly disorganized and appeared very similar to those in the A549 alone group. Interestingly, despite not being significantly different in tumour size, the _LOXL1 group exhibited the highest level of collagen fibre alignment. The _shGFP control group appeared to have an intermediate level of fibre orientation, although it wasn’t statistically different from the _shLOXL1 or A549 alone groups.

The fact that there was no significant difference in tumour size between the _LOXL1 and _shGFP groups may be explained by a small sample size (n =4). It may also be accounted for by unexpected mRNA expression levels of the human LOXL1 within the tumour tissues. There was no significant difference in LOXL1 expression between the different groups (Figure 16). This can perhaps be explained by the fact that tissues were collected for mRNA analysis after the majority of human CAFs were replaced by the host stroma, a phenomenon which has been previously observed in our laboratory. Furthermore, as evidenced in the *in vitro* model, collagen cross-linking is not an immediate reaction, but is a continuous process by which collagen accumulates cross-links over time, in proportion to the LOX activity. Thus, if the human CAFs were replaced by mouse stroma before sufficient collagen cross-linking had occurred, the tumour growth may not be affected. Thus although there is a significantly greater alignment observed in the group with over-expressed LOXL1, perhaps it was still not enough to increase tumour size.

To speed up the CAF-mediated collagen cross-linking process, more CAFs were injected in relation to A549 cells in the next experiment (Experiment #2) (Figure 15). Results from this assay showed a larger difference between _LOXL1 and _shGFP groups, but the differences were
still non-significant. This trend taken together with the results from Experiment #1, showing tumour growth suppression in the _shLOXL1 group, suggest that perhaps LOXL1 does play a role in regulating tumour size, but requires better models to investigate it.

The current animal model is not ideal. As mentioned, collagen cross-linking occurs over an extended period of time. The endogenous mouse stroma has fairly high levels of mouse LOXL1 and if it replaces CAFs before human LOXL1 can sufficiently remodel collagen fibres, it may nullify its effect. It should also be noted that in the studies where LOX was shown to promote primary tumour growth, it was not expressed by stromal cells, but by the malignant ones (165). Thus other mouse models should be considered for the study of stromal LOXL1.

### 4.5 Future Directions

There is still a great deal to discover about LOXL1 and its role in cancer. The fact that it exhibits the same catalytic function as the other LOX family members, suggests that it may also play an important role in cancer progression. Because LOXL1 is stromally expressed and would most likely promote cancer progression indirectly through ECM remodelling, new models must be developed, which would allow creating specific ECM modifications by regulating stromal gene expression and studying their interactions with cancer cells.

We are currently in the initial stages of developing two new mouse models. The first model is a null Loxl1 mouse purchased from the Jackson Lab (Appendix 8). These mice were generated in a C57Bl/6 background and are currently being crossed into a SCID strain to allow for injection of human NSCLC cells. We have also generated transgenic mice with a tetracycline-inducible human LOXL1 expression system (Appendix 6, Appendix 7). The chimera mice were generated in a mixed C57BL/6, 129SvJ and FVB background and are also being crossed into a SCID background. Since lung tumour stroma is associated with an increased expression of LOXL1, this gain-of-function model would better recapitulate the pathological microenvironment.

Using these models would eliminate the need to use human CAFs for modulating LOXL1 expression. In the future they may be used to assess the effect of LOXL1 on primary tumour growth, metastasis and associated collagen remodelling and tissue stiffness.
4.6 Conclusions

Despite extensive research, the outcomes for lung cancer have not significantly improved. However, the concept of tumour cell-centred view of cancer has become outdated and the paradigm has shifted towards recognizing tumour microenvironment as a key element of cancer progression. Tumour stroma has been found to not only act as a physical barrier to therapeutic agents, but also as a dynamic microenvironment that actively promotes cancer growth, migration, and invasion.

This study found that over-expression of stromally expressed LOXL1 results in highly linearized collagen architecture and tissue stiffness, the very properties that characterize desmoplasia. However, new models are required to conclusively assess its role in cancer progression. Elucidating the complex interactions between extracellular matrix, stromal and cancer cells may allow for the development of new therapies and to improve the outcome of lung cancer.
References


## Appendices

### Appendix 1. Demographic summary of UHN181 and TCGA cohorts

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<th>TCGA Lung ADC (n=230)</th>
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<td></td>
</tr>
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<td>Median (Range)</td>
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<td>83 (46%)</td>
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<tr>
<td>carcinoma</td>
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<td>Others</td>
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<td>I</td>
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### Appendix 2. Patient demographics, tumour stage, and pathological diagnosis for tumours used for LCM stroma isolation

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## Appendix 3. RT-PCR primer sequences

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Reverse 5’- TGGGTAAGGTGCTTTTCAGGC -3’ |
| Loxl1 | Forward 5’- TAGAGTAGTGGGTCTGGAGGC -3’  
Reverse 5’- GGGAGAGGAGCAAAGAAGTGG -3’ |
| Loxl2 | Forward 5’- AAAGTATGACAGCAGAGGAGGT -3’  
Reverse 5’- TCTGGAGTTGAAGACGGGAG -3’ |
| Loxl3 | Forward 5’- AGTTGGTGCTCCTCACTACG -3’  
Reverse 5’- GTGGTGTTCTGTCTTCTTGC -3’ |
| Loxl4 | Forward 5’- TCAGGGATGAACTGTGACCAAG -3’  
Reverse 5’- GTCCCTTTTGCCTGAGACGTAT -3’ |
| Itga11 | Forward 5’- CAGTAACCACCAACCCACTCAA -3’  
Reverse 5’- ACGGAGAGCCACGGATTTTA -3’ |
| Gapdh | Forward 5’- GCAAGGACACTGAGCAAGAGA -3’  
Reverse 5’- ATTATGGGGGTCTGGGATGGA -3’ |
| **Human** | |
| LOXL1 | Forward 5’- GTCGCTACGTTTCTGCAACA -3’  
Reverse 5’- GCTTTGGAAGGGGAGAGATT -3’ |
| ITGA11 | Forward 5’- TGCCCTTTTCTCTCAACCCATC -3’  
Reverse 5’- CTTTCTTCATCCCTGGCTTG -3’ |
| RPS13 | Forward 5’- GGTGGCTGGTAAAGCATCTTG -3’  
Reverse 5’- AATATCGAGCAAAAACGGTGAA -3’ |
| B2M | Forward 5’- GAGTGCTGTCTCCATGTTTAGTG -3’  
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## Appendix 4. Antibodies used in western blot analysis

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<tbody>
<tr>
<td>LOXL1</td>
<td>Rabbit polyclonal H-165</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>HGF</td>
<td>Goat polyclonal N-17</td>
<td>1:1000</td>
<td>Santa Cruz</td>
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</table>
Appendix 5. Diagram of transgene integration strategy to generate single-copy transgenic mice. Diagram adapted from methodology published by Beard et al. in (178). (A) Schematic representation of the Colα1 locus and downstream region. (B) A homing cassette was inserted downstream of Colα1 locus. A vector containing a gene of interest is targeted to the modified Colα1 locus by co-electroporation with a flippase transient expression vector. Exons and 3’ UTR are represented as black and grey boxes, respectively. pA, polyadenylation signal. P, PstI; S, SpeI; X, XhoI; E, EcoRI; XbaI.
Appendix 6. Diagram of Tet-On gene expression system in transgenic mice. Tetracycline-controlled transcriptional activation is a method of inducible gene expression, where the Tet-On system activates expression in the presence of doxycycline (DOX, a tetracycline analogue). The reverse tetracycline-controlled transactivator (rtTA) protein must bind to the tetracycline response element (TRE) in order to increase transcriptional activation of the downstream genes. The rtTA protein is capable of binding to the TRE only in the presence of DOX. This system was implemented in the transgenic mice, where expression of the βgal-P2A-LOXL1 vector may be induced by DOX-enriched diet.
Appendix 7. Identification of ES cells positive for doxycycline-mediated βgal-P2A-LOXL1 expression. (A) βgal staining and (B) Western blot of secreted LOXL1 in two ES cell clones.

A

ES Clone #1  ES Clone #2

-DOX

+DOX

B

<table>
<thead>
<tr>
<th>DOX</th>
<th>ES Clone #1</th>
<th>ES Clone #2</th>
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</thead>
<tbody>
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<td></td>
<td>-</td>
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LOXL1 (43kDa)
Appendix 8. Knockout LOXL1 mouse strain. (A) Schematic representation of VelociGene null allele design. The expression of the target gene, mouse LOXL1, is ablated by replacing its coding exons with an expression-selection cassette, from a BacVec targeting vector through homologous recombination. (B) Genotyping of WT and KO mice results in single bands of 189bp and 700bp, respectively, while heterozygous (Hz) mice exhibit both of these bands. (C) Validation of mouse LOXL1 genotypes with RT-PCR of the lung tissue.
Appendix 8 continued.

C