Microenvironmental Control of Sortilin Exosomes in Lung Cancer

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Abstract

Lung cancer, despite medical advancement, is still the most common form of cancer, and contributes to the highest incidence rate of mortality of all cancer types. Sortilin, through the use of exosome-based cell-cell communication may play an important role in the way that cancer progression takes place. Cell invasion assays revealed that in sortilin knockdown cells, invasion rates are reduced significantly, and upon the introduction of exosomes derived from cells overexpressing sortilin, invasion rates are restored to that of the wild type cells. Additionally, sortilin overexpression from exosomes dramatically raised cell proliferation rates compared to standard wild types. The addition of exosomes derived from overexpressing sortilin cells revealed that induced sortilin expression in knockdown cells was likely to be as a result of miRNA cargo from the exosome over transported proteomic sortilin between cells. NF-κB cleavage was observed over time upon treatment of exosomes derived from cells overexpressing sortilin in sortilin knockdown cells. The results indicate that sortilin plays a role in the invasion rate of lung cancer cells, and suggests that exosomal control of sortilin expression is controlled via miRNA cargo and holds influence in the tumorigenic properties of cancer cells in culture.

Introduction

Lung Cancer

The adult human body is composed of billions of cells, with each cell performing its own specific function to keep each individual alive and healthy. In order to maintain normal function, cells have the ability to replicate before they become damaged and ineffective in serving the human body, either from undifferentiated cells such as bone marrow, or as simple cell division in epithelial and epidermal cells. When cells lose their ability to benefit the body, they are destroyed and recycled either by the immune system via macrophages, or as a product of programmed cell death, also known as apoptosis. However, on occasion cell damage does not always result in automatic destruction, and there is a rare chance of replication to take place with the damaged cells (Hanahan and Weinberg, 2011). Even if this takes place, usually the immune system will identify imperfect cells and remove them, although there is also the potential for this to go unnoticed. Carcinogenesis can take place as a result of these hidden self-replicating cells, in which point mutations in the genetic code causes altered gene expression which can alter normal cell function, with cancer cells being capable of self-replicating indefinitely. As cancer progresses, tumours can develop which begin to disrupt functions within the human body, and cancers will begin to spread out away from the initial development site (Qian, Mei and Zhang, 2017). The spread of cancer away from the
original tumour site is known as metastasis, and is not limited to local organs and tissue, being capable of reaching any part of the body. Metastasis is the major cause of cancer mortality, responsible for up to 90% of deaths, as it is within this stage that the growth of cancer has become so considerable that multiple organs are affected. Cancer cells detach from the original tumour and enter the circulatory and lymphatic systems, while evading the immune system, and invade distant organs (Seyfried and Huysentruyt, 2013).

Cancer is one of the most prevalent diseases occurring worldwide, wherein as of 2017 there were an estimated 42 million people suffering from cancer globally (Roser and Ritchie, 2015). Additionally, in 2018 there were over 18 million new cases of cancer recorded, and an estimated 9.5 million cases of mortality due to cancer in that year (Gco.iarc.fr, 2019). Unlike many pathogenic diseases, cancer incidence rates have been increasing over time, with a multitude of factors affecting this problem. Generally, the development of cancer is ultimately due to chance, with anybody having some risk to developing the disease. However, health and lifestyle choices will either reduce or increase the risk of cancer to develop, with different cancer types being affected by different factors. Age is one of the biggest players in the likelihood of cancer prevalence, with the highest incidence rates occurring in individuals aged 75+ in the UK, and more than 50% of all cancer patients being aged between 50-74 years old (Office for National Statistics, 2017). Over time, cells will become damaged, and the body will attempt to rectify this cellular damage by either repairing or destroying the damaged cells. However, as age increases, the likelihood of a build-up of cell damage in the body also increases, which in turn increases the risk of cancer to develop as the body cannot keep up with maintaining healthy cellular function. As age progresses, effective cell function reduces, with DNA damage risk increasing, and impairment of cellular function becoming apparent (Aunan, Cho and Søreide, 2017).

Genetic inheritance is an important factor to consider when identifying the risks of cancer development, as family history can be used as a guideline in diagnosing specific cancer types during general health check-ups. Although not as great a risk as age related cell damage, ancestral genetic deformity still plays a role in cancer development. For example, female individuals with a family history of known instances of breast cancer are also at a higher risk of developing the disease than those without, and as such, for example in the UK, may be tested for the cancer before symptoms occur (Cancer Research UK, 2018). Alongside these risk factors of cancer progression, diseases and lifestyle choices can further increase the possibility of promoting cancer growth, with multiple factors resulting in a potentially high risk. For example, diseases can promote the risk of cancer, depending on the site of the disease and its causes and/or side effects. For example, rheumatoid arthritis, an autoimmune disease affecting the mobility of joints caused by inflammation of the
area, has been found to be associated with a higher occurrence of some cancers such as lymphomas and lung cancer in patients when compared to the healthy population (Simon et al., 2015). Similarly, Crohn’s disease, caused by inflammation in the gastrointestinal tract between the mouth and anus, has been found to increase the risk of developing colorectal cancer (Freeman, 2008).

In addition to genetic alteration and disease prevalence, lifestyle choices play a large part in the incidence rates of cancer, of which these can be avoided but are still prevalent due to the individual’s lack of information or unwillingness to change. The largest lifestyle contributor to cancer is caused by smoking tobacco, with 15% of all cancer incidences resulting from smoking in the UK. This includes direct tobacco smoking, as well as second hand smoking, and can cause the development of a multitude of cancer types, including pancreatic, colorectal, bladder, liver, as well as lung and oesophageal cancers, which are the highest incidences (Cancer Research UK, 2018).

Of all cancer types, lung cancers are the most common, with 11.6% of all cancer incidences being of the lung. Additionally, lung cancer is also responsible for more cancer related deaths than any other, at 18.4% of the total. Incidence rates of lung cancer are twice as likely in men than in women, and in males, lung cancer is the most common cancer type with approximately 1.4 million cases, while in females it is the third most common at approximately 0.7 million cases (Bray et al., 2018). In the UK, lung cancer incidences account for 13% of all cases, and are the third most common type of cancer behind breast and prostate cancer. Lung cancer is the most common cause of cancer related mortality in the UK, contributing to 21% of all cancer deaths (Cancer Research UK, 2018).

Incidence rates by gender in 2016 were similar in the UK, with 22,342 female cases and 25,046 male cases (Cancer Research UK, 2019). Globally, incidence and mortality rates are more prevalent in developed countries, with male incidence being highest in Central, Eastern and Southern Europe, East Asia, and Micronesia, whilst female incidence is highest in North America, Micronesia, Australia and New Zealand, and Western Europe (Wong et al., 2017). Incidence by age is more likely later in life, due to DNA damage likelihood increasing over time, with lung cancer in the USA being most common between the ages of 55 and 74, followed by incidences over 75 years of age (de Groot et al., 2018).

**Risk Factors**

Tobacco smoking is the leading cause of lung cancer by a considerable amount, being responsible for up to 90% of all cases, which includes direct smoking of cigarettes and cigars as well as second hand smoking. Tobacco contains a significant number of chemicals, many of which are carcinogenic, which all increase the risk of developing lung cancer by up to 20 times more than those who do not smoke tobacco (Alberg et al., 2013). Examples of carcinogens found in tobacco
are polycyclic aromatic hydrocarbons (PAH), Nicotine-derived nitrosamine ketone (NNK), Polonium-210 ($^{210}$Po), and various heavy metals such as Arsenic, Cadmium, Chromium and Nickel. Both NNK and PAH have been found to cause mutations in the KRAS gene, and PAH additionally causes mutations in the P53 tumour suppressor gene (Hecht, 2012). Cadmium, Nickel and Arsenic are Class 1 carcinogens classified by the International Agency for Research on Cancer (IARC), and pose significant risk to human health when exposed to regularly (Caruso et al., 2013). $^{210}$Po is a rare naturally occurring radioactive isotope of polonium created by the decay of uranium-238. It is present in trace amount in the earth’s crust and in the air, in which tobacco plants are able to directly take in $^{210}$Po through the roots, as well as collecting $^{210}$Po on the leaves during meteorological events. Additionally, the use of some fertilisers may produce $^{210}$Po from apatite which contains radium-226 that decays to $^{210}$Po, and may be taken up by tobacco if it has been used. Normally, naturally occurring $^{210}$Po poses little threat to human health, as the radiation produced is alpha decay, and while being the most damaging type of radiation it is easily blocked by barriers, including skin. The lethality of $^{210}$Po becomes apparent when it is introduced directly into the body through wounds, ingestion and inhalation, where $^{210}$Po has direct contact with internal organs and the circulatory system where alpha decay can cause potentially fatal damage. The tumour suppressor gene p16 is involved in the prevention of the development of cancer, as its coded p16 protein is used for slowing down cell proliferation when DNA damage has occurred in order for genetic repair to take place. Inactivation of the p16 gene in mice results in a high chance of cancer development, in particular when exposed to carcinogens. Hyper-methylation of p16 results in silencing of the gene, and is common in rat lung tumours after inhalation of radioactive plutonium. Similar silencing of p16 is observed in lung tumours due to tobacco smoke exposure, in which airborne plutonium from tobacco smoke deposits onto the lung in hotspots, emitting high volumes of alpha radiation to the surrounding tissue (Prueitt, Goodman and Valberg, 2009). These compounds all pose health risks individually, and the combination of multiple carcinogens increases the risk of developing cancer by a considerable amount. The inhalation of smoke containing carcinogenic substances allows direct exposure to the lung, where $^{210}$Po radiation for example is normally blocked by the skin in non-smokers, but smoking poses a significant risk of carcinogenesis from alpha decay of $^{210}$Po (Zagà et al., 2011).

Lung cancer, although in the majority of cases caused by smoking tobacco, is also present in a small percentage of patients with no history of smoking, and is mainly onset from other factors. The main cause of lung cancer in never smokers is from secondary tobacco smoke exposure via inhalation, followed by exposure to residential radon, in which radon gas levels are detected at higher indoor concentrations than outside (Yang, 2011). Radon is found naturally in the earth’s crust as a product
of radioactive decay from unstable elements such as uranium and radium, emitted as a gas which enters the air. Outdoors, radon is dilute and poses little threat to general health, but indoors it can become concentrated with potential dangerous levels being achieved. Poorly ventilated flooring allows radon to enter buildings, especially in colder climates where indoor heating is commonly used, which naturally draws radon from the ground caused by a negative pressure from the difference in temperature between the ground and the indoor air. Poor roofing ventilation allows radon to become trapped, increasing the air bound concentration to hazardous levels. Degraded radon products emit alpha radiation which lead to DNA damage, potentially leading to lung cancer when breathed in, and additionally can be found dissolved in water, further increasing the risk of exposure (Garcia-Rodriguez, 2018).

Due to the majority of lung cancer patients having a history of smoking tobacco, it is difficult to identify other potential carcinogenic contributors for lung cancer. Exposure to asbestos is one non-smoking related carcinogen known to cause mesothelioma and more recently lung cancer, through direct exposure by inhalation of asbestos fibres. Asbestos, before its danger to health was discovered, was a commonly used building material, and as such is still found today in roofing and cement. When asbestos fibres are inhaled, it becomes trapped in the lung and broken down by phagocytes during an inflammatory response (Mott, 2012). Asbestos fibres activate the epidermal growth factor receptor (EGFR) located on the cell surface of mesothelial and lung epithelial cells, acting as a ligand when in contact with EGFR. This interaction initiates the activation of protein pathways, with increased expression levels of downstream proteins such as ERK1/2 and c-fos, which are required for normal cell function to take place. Additionally, inhalation of asbestos fibres activates NF-κB and its signalling pathway, which are essential in regulating cell proliferation and apoptosis, as well as being involved in inflammation response to cell damage and disease (Heintz, Janssen-Heininger and Mossman, 2010).

**Classification**

Lung cancers are classified under two main groups, small-cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), with NSCLC being further subcategorised into squamous cell lung cancer (SQCLC), adenocarcinoma and large cell anaplastic carcinoma (LCAC) (Figure 1). NSCLC accounts for approximately 85% of all lung cancers, with SCLC being the other 15%. Of NSCLC, adenocarcinoma is the most common type at 40% of all lung cancers, followed by SQCLC at 25%, and LCAC at 10% (Lemjabbar-Alaoui et al., 2015).
SCLC is an aggressive form of lung cancer with a high proliferation rate, as well as presenting dissemination in a large number of patient cases. Metastasis occurs relatively quickly with SCLC, with early stages going unnoticed in the majority of cases, as symptoms may not become apparent until the disease has begun to spread, and once developed can be perceived as insignificant, such as a persisting cough or sudden lack of energy. Symptoms of later stage SCLC are more likely to prompt a medical diagnosis as the disease becomes metastatic, and symptoms of SCLC become more severe, including bone pain, weight loss, jaundice and changes to the nervous system (Cancer.org, 2016). Due to the dangers of smoking tobacco being well known in developed countries, incidence rates have been slowly declining each year, although actual survival rates of SCLC patients have not improved by much despite improvements in general cancer therapies. This is largely due to difficulties in early diagnosis of SCLC, as imaging techniques such as chest x-rays
and CT scans are in many cases ineffective in identifying early stage SCLC before the cancer becomes metastatic (Pietanza et al., 2015).

NSCLCs are less aggressive than SCLC, taking much longer to accumulate, making them harder to detect in low stages due to symptoms taking longer to become apparent, with minor symptoms going unnoticed due to slow progression. As such, many cases of NSCLC are diagnosed at later stages when the cancer becomes more developed and even metastatic (Zappa and Mousa, 2016). Adenocarcinoma of the lung is the most prevalent form of lung cancer, originating in the glands of the lung, with 40% of all lung cancer incidences being adenocarcinomas. Adenocarcinomas are classified under two main sub-sections based on the invasiveness of the tumour, adenocarcinoma in situ and minimally invasive adenocarcinoma. Adenocarcinoma in situ is defined as lepidic, with tumour diameter being under 3cm, while minimally invasive adenocarcinoma is classified as being under 3cm in diameter with invasion size being under 5mm, with tumours greater in diameter than 3cm classified as lepidic predominant adenocarcinoma (Inamura, 2017).

Formerly the most common type of lung cancer, SQCLC affects over 400 000 people worldwide, although incidence rates are gradually dropping due to a decrease in the use of smoking tobacco, as the majority of patients suffering from SQCLC are current or former heavy smokers (Gandara et al., 2015). As its name suggests, SQCLC develops from squamous epithelial cells located in the lung, with the greatest risk factor for progression arising from tobacco smoke (Yan et al., 2011). Certain gene mutations may be used in diagnosis for SQCLC, including TP63 (tumour protein 63), with current research being carried out to further investigate potential biomarkers for diagnosing SQCLC and differentiating from other forms of NSCLC (Shoshan-Barmatz et al., 2017).

LCAC is the least common and most poorly classified variant of NSCLC, currently identified as a non-small cell, non-adenomatous and non-squamous cancer of the lung. Diagnosis of LCAC is made by exclusion of all other types of lung cancers, which can prolong any correct diagnosis, especially when additionally needing to rule out other forms of lung disease and infection that mimic similar symptoms (Barbareschi et al., 2011). LCAC is thought to act more aggressively than other forms of NSCLC, with progression of tumour growth and metastases being similar to that of SCLC, and when growth is considerably significant can mimic lung pathologies and inflammatory conditions (Rajdev et al., 2018).

**Current treatments**

Treatment of lung cancer is dependent on its sub-type and staging, in order to most effectively manage and treat the disease. Staging of the cancer is carried out, known as TNM, based on the
size and location of the tumour (T), location of cancer in the surrounding lymph nodes (N), and whether the cancer has become metastatic (M) (*Table 1*) (Lemjabbar-Alaoui et al., 2015).

*Table 1: TNM Staging of NSCLC.*

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*T = Primary tumour size: T1a (≤2cm), T1b (>2-3cm), T2a (>3-5cm), T2b (>5-7cm), T3 (>7cm), T4 (Multiple tumours regardless of size on the same lung but differing lobes).*

*N = Type of lymph node metastasis: N0 (No lymph node metastasis), N1 (Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph and intrapulmonary nodes), N2 (Metastasis in subcarinal lymph node and/or ipsilateral mediastinal nodes), N3 (Metastasis in contralateral hilar, contralateral mediastinal, contralateral or ipsilateral scalene, or supraclavicular lymph nodes).*

*M = Metastasis to other tissues or organs: M1a (Separate tumour nodules and/or pleural or pericardial effusions in the contralateral lung), M1b (Distant metastasis in lymph nodes and organs)*
SCLC is staged under two primary categories, limited and extensive, based on the progression of the primary tumour, in which limited SCLC is localized in the primary tumour and its regional lymph nodes, whereas extensive SCLC encompasses the primary tumour, regional lymph nodes, with additional sites of cancer progression located away from the primary tumour, including distant lymph nodes and metastatic sites (Kalemkerian, 2011).

Modes of Lung cancer treatment

Treatments are available for all stages of lung cancer, and can be used to attempt to remove the tumour all together in low stages, or when the cancer has progressed to a more severe and incurable stage, palliative treatments are used to prolong the patient’s lifespan, and improve the quality of life by reducing symptoms which may cause pain and discomfort. Currently, the three major therapeutic routes are surgery, chemotherapy and radiotherapy, with alternative methods of therapy being currently researched.

Surgical treatment of lung cancer is used to remove the tumour in non-metastatic cases with staging of ≤IIIA and limited SCLC, but is unsuitable at higher staging due to metastasis to other organs and lymph nodes. Imaging and biopsies are carried out to determine whether surgery is a viable option in removing the tumour, and after successful removal, adjuvant therapy of the tumour site by targeted therapy, radiotherapy or chemotherapy to kill any remaining cancer cells left after surgery in stages IIA, IIB and IIIA (Zappa and Mousa, 2016). Advanced stages of lung cancer ≥IIIB and extensive SCLC will have progressed too far to be treated surgically, and require chemotherapy and/or radiotherapy to manage and treat the symptoms of lung cancer.

Radiotherapy and chemotherapy are used as a palliative therapeutic solution to extend the survival time and improve quality of life for cancer patients, as symptoms of late stage lung cancer can be severe and painful, including shortness of breath, coughing, and chronic pain. Chemotherapy involves the use of toxic drugs which are capable of preventing cancer progression, and in some cases may destroy the cancer target (Farbicka and Nowicki, 2013). Chemotherapeutics are, however, also toxic to healthy tissue, and can produce adverse side effects due to the damaging nature of the drugs, including hair loss, damage to the gastrointestinal lining, nausea and vomiting, and a reduction in neutrophils (Nurgali, Jagoe and Abalo, 2018). A reduction in neutrophils can become fatal, as patients undergoing chemotherapy are at a high risk of viral, bacterial and fungal infection, and as such, blood testing of patients after chemotherapy is carried out to monitor potential infection.
Radiotherapy can be used in combination or as an alternative to chemotherapy, depending on the symptoms present in cancer patients. Radiotherapy is effective in reducing symptoms of pain in both the primary tumour site as well as in metastatic sites including bone tissue and the central nervous system, but is less effective in reducing coughing or shortness of breath (Farbicka and Nowicki, 2013). Ionizing radiation is targeted at the cancer site in order to destroy cancer cells, but is also damaging to the surrounding healthy tissues and organs as ionizing radiation damages DNA of all cells in its path. Development of radiotherapy is being carried out to expose tumour sites to optimal levels of radiation while avoiding as much healthy tissue as possible (Baskar et al., 2012).

**Extracellular Vesicles**

Cells of all types release vesicles into the extracellular environment, which are aptly named extracellular vesicles (EVs), which are membrane-contained bodies released into the extracellular environment. EVs are highly conserved through evolution, and are released by simple organisms such as single celled prokaryotes and eukaryotes, as well as in more complex organisms such as animals and plants. EVs are sub-categorised into three groups, microvesicles (or ectosomes), apoptotic bodies, and exosomes. EVs ranging between 40-100nm in diameter are classified as exosomes. Exosomes were originally discovered in 1983 in two separate papers published within a

![General exosome structure](https://www.biorender.com)

*Figure 2: General exosome structure containing genetic and proteomic information, and membrane bound proteins (created using biorender).*
month of one another (Harding, Heuser and Stahl, 1983) and (Pan and Johnstone, 1983), describing small approximately 50nm vesicles being released by blood reticulocytes into the extracellular environment. The term ‘exosome’ was coined by Rose Johnstone a few years after the initial discovery, and since then, research has been carried out on exosomes to highlight their significance in cellular function. Exosomes contain a cargo of genetic material such as miRNA as well as lipids and proteomic content dependent on the parent cell, although certain membrane bound tetraspanin proteins such as CD9, CD63, and Flotillin-2 are found in a large quantity of exosome types, although some variation does exist in certain cell types and diseases, acting as a biological marker for the structure (Raposo and Stoorvogel, 2013). Exosomes are formed within the cell in multivesicular bodies (MVBs), and MVBs are then transported to the cell membrane through a complex biogenesis pathway (Figure 3). Internalization of ligands carried out by endocytosis in the cell membrane forms early endosomes containing membrane proteins and lipids (Alenquer and Amorim, 2015). The early endosome undergoes maturation to form the late endosome/MVB with the use of proteins such as endosomal sorting complex required for transport proteins, tetraspanins, and Alix. Exosomes are formed within the MVB, in which sorting of cargo occurs. Uptake of miRNA into the exosome for communication is carried out, in which KRAS plays a role in sorting specific strands. Additionally, lipid rafts and domains containing cholesterol and glycosphingolipids may be used to introduce proteins such as Flotillin and CD63 into the exosome. MVB transport to the cell membrane for exosome release is then carried out, in which the actin and microtubule cytoskeleton is used. Altering the expression levels of actin changes the rate in which exosomes are released in a direct relationship. Rab proteins, such as Rab27a and Rab27b are involved in the transportation of the MVB to the plasma membrane, with knockdowns of the proteins reducing exosome secretion rates (Ostrowski et al., 2010) Upon contact, the cell membrane and MVB fuse, releasing exosomes into the extracellular environment. Exosome release may be used in long range cell-cell communication as the third variant alongside direct cell-cell contact and hormonal release. Exosome-induced communication is advantageous due to the effective range and large quantity of material that may be passed from one cell to another (Hessvik and Llorente, 2017). The recipient cell is able to internalize exosomes in a number of ways, including simple membrane fusion between the exosome and the cell surface which allows for direct release of material into the cytoplasm, as well as endocytosis and phagocytosis in order to transport exosomes to specific target organelles (Mulcahy, Pink and Carter, 2014). Studies suggest that all cell types can take in exosomes regardless of the parent cell to some extent, although certain cell specific receptor proteins are able to dictate some specificity for exosome uptake, for
example certain cancer cell line derived exosomes undergo selective uptake dependent on the recipient cell based on the type of endocytic pathway mechanism used (Horibe et al., 2018).

The function and mechanisms of exosome release and uptake is currently not fully understood, although research suggests that they play an important role in transmitting biological information between cells. Cancer cells are able to produce and release exosomes into the extracellular environment, and it is thought that the uptake of cancerous exosomes may be involved in the spread of cancer and tumour development. Compared to healthy cell types, exosome release from
cancer cells is enhanced, as proteins involved in the regulation of exosome release are disrupted, and stress to the cell as a result of cancer progression may also increase exosome biogenesis (Whiteside, 2016). Through horizontal gene transfer, genetic material from cancer exosomes may be coded by healthy cells, causing reprogramming of the cell to become cancerous. For example, glioblastoma tumour cells were found by Al-Nedawi et al to transfer an oncogenic form of EGFR (EGFRvIII) via microvesicles to cells lacking EGFRvIII. This in turn allows for the activation of certain protein pathways such as the MAPK signaling pathway to aid in tumour progression in order to advance oncogenic activity within the tumour (Al-Nedawi et al., 2008). This data was replicated by Skog et al, who identified oncogenic mRNA and miRNA, as well as angiogenic proteins contained within exosomes derived from glioblastomas for transfer of information, including EGFRvIII (Skog et al., 2008). Alongside genetic material, enzymes and protein mutations may be released into the healthy cell causing degradation and inducing the development of cancer. Additionally, tumour-derived exosomes may be used to reduce the effectiveness of immune cells in destroying cancer cells by suppressing natural killer cell and T-cell activity (Tai et al., 2018). Additionally, exosome release may play a role in metastasis to distant tissue and organs from the tumour site, through the transport of oncogenic factors such as genetic and proteomic cargo to distant organs, providing the necessary conditions for metastasis to take place (Kaplan, Rafii and Lyden, 2006). Due to the small size of exosomes, they are capable of being transported to the bloodstream by passing through blood vessels such as capillaries, with brain-derived exosomes even being capable of passing through the blood-brain barrier. Release of cancer-derived information to distant cell types by exosomes may induce pre-metastatic conditions in order for cancer cells to more easily spread to further parts of the body. Additionally, cancer-derived exosomes are capable of disrupting the function of the blood-brain barrier allowing for metastasis of cancer to the brain (Tai et al., 2018, Rajagopal and Harikumar, 2018).

Despite their role in transmitting cancer throughout the body, exosomes also have potential in the use of alternative therapeutics in both detecting and treating cancers. Due to the specificity of exosome content and to the cell origin, exosomes can be used as biomarkers in diagnosing specific cancer types. Zhang et al identified the miRNA cluster miR-17-92 as a potential diagnostic biomarker, in which the expression of miR-17-5p, one of the miRNAs within the cluster, was significantly upregulated in NSCLC patients compared to the control. This miRNA upregulation, alongside NSCLC markers carcinoembryonic antigen, cytokeratin 19 fragment and squamous cell carcinoma antigen were combined to create a 4-molecule diagnostic marker for potential future use in diagnosing NSCLC (Zhang et al., 2019). Additionally, Zhang et al investigated the relationship between chemotherapeutic resistance of temozolomide and long non-coding RNA SBF2 antisense
RNA 1 (IncRNA SBF2-AS1), and found that IncRNA SBF2-AS1 was upregulated in glioblastoma cells resistant to temozolomide. Additionally, it was found that exosomes derived from IncRNA SBF2-AS1 upregulated glioblastoma cells contained high levels of IncRNA SBF2-AS1, and were capable of transferring temozolomide resistance to chemoresponsive glioblastoma cells (Zhang et al., 2019). This method would be less expensive than some currently used techniques such as MRI and other imaging processes, while also being less invasive than biopsies which require sampling. Due to exosome release into the blood stream being raised from tumour progression, available cancer-derived exosomes are highly available, and by making comparisons to exosomes released by cancer cell lines against healthy cell types, early diagnosis can be aided, allowing for further diagnostic testing to be carried out for specific cancer targets (Huang and Deng, 2019).

In addition to their potential in cancer diagnosis, exosomes may also one day be used in treating cancer as transporters for chemotherapeutic drugs. A current issue in chemotherapy is in the non-specific binding of the drug, as they both target cancer cells and healthy tissue. This can in some cases lead to a reduced quality of life if chemotherapeutics begin to cause damage to healthy tissue without completely removing the cancer, and as such high specificity of chemotherapeutics towards cancer needs to be developed. Due to their ability to be naturally taken in by cells to deliver cargo directly to the target, exosomes show obvious potential to be used in delivering chemotherapeutic drugs. Their selectivity depending on the parent cell allows for specific targeting of cancer cells, which subsequently requires a lower amount of the drug (Gilligan and Dwyer, 2017). One study by Kim et al investigated the effectiveness of paclitaxel (PTX)-loaded exosomes (exoPTX) in treating MDR cancer. In order to do so, exosomes derived from macrophages were isolated and loaded with PTX using various methods. PTX loading via mild sonication proved to be most successful, and exoPTX treatment was carried out by exposure of MDR to exoPTX over 48 hours. It was found that exoPTX treatment increased cytotoxicity of the drug in MDR by more than 50 times in resistant cell types, showing an effective potential in the use of exosomes and their selectivity of cancer cell types over health cells (Kim et al., 2016).

Sortilin

Sortilin (SORT1), also known as neurotensin receptor 3 is a membrane glycoprotein belonging to the vacuolar protein sorting 10 protein (Vps10p) family, involved in the sorting and trafficking of proteins from the cell membrane and organelles such as the Golgi apparatus and endoplasmic reticulum (Van Puyenbroeck et al., 2016). In sortilin, the Vps10p domain encompasses the extracellular portion of the protein, forming a hollow 10-bladed beta propeller structure (Quistgaard et al., 2014). Here, ligands bind to sortilin in the tunnel of the Vps10p domain including
neurotensin (Quistgaard et al., 2009, Nykjaer and Willnow, 2012). Additionally, sortilin has been plays an important role in the sorting and trafficking of Brain-derived neurotrophic factor to the regulated secretory pathway (Chen et al., 2005). Sortilin is highly expressed in neuronal tissue such as the central and peripheral nervous system, as well as adipose, male and female, and lung tissue (Proteinatlas.org, n.d.). In the nervous system, sortilin plays a role in the induction of neuronal death as a receptor for pro-nerve growth factor (proNGF) and pro-brain-derived neurotrophic factor. Sortilin is one of three receptors involved in proNGF mediated neuronal death alongside P75NTR and tropomyosin receptor kinase A (TrkA), which requires a balance of expression within these receptors to control cell death (Nykjaer et al., 2004). Although its function in neuronal death is understood, our understanding of the role of sortilin in other cell types, with any pathway interactions involving sortilin currently unclear. For example, sortilin is involved in cholesterol metabolism as a component for very-low-density lipoprotein degradation, as well as a transporter for low-density lipoproteins from the cell membrane to the endosome (Genome.jp, 2017).

Sortilin is also present in the lysosome as a minor lysosomal membrane protein, involved in trafficking progranulin (Zhou et al., 2018, Genome.jp, 2017). Sortilin’s interaction with P75NTR plays a role in the neurotrophin signalling pathway, which mediates other cellular functions and pathways such as cell survival and differentiation, apoptosis, regulation of the actin cytoskeleton and the mitogen-activated protein kinase pathway, although some interactions within this pathway are not fully understood, such as links between sortilin and cell survival and apoptosis (Genome.jp, 2018). One interaction of interest involves the regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via P75NTR. NF-κB-dependent cell survival can be induced

\[ \text{NF-κB} \rightarrow \text{Cell Survival} \]

Figure 4: Cholesterol metabolism pathway and the role of sortilin on the degradation of VLDLs (Genome.jp, 2017).
through P75\textsuperscript{NTR} in response to NGF, although due to the interactions between sortilin and P75\textsuperscript{NTR}, cell survival may also be mediated by sortilin expression (Elshaer and El-Remessy, 2016). NF-\kappa B is of great interest to understanding cancer, as it is implicated in the formation and metastasis of tumours. One of the main roles of NF-\kappa B is in the promotion of inflammatory response, in which under normal activity is used as a response to cell damage and pathogenic invasion, aiding in the immune response. However, NF-\kappa B expression may also be responsible for the release of pro-tumorigenic agents such as cytokines, and in certain inflammatory diseases this may increase the risk of localised cancer development, such as irritable bowel disease increasing the risk of colon cancer (Xia, Shen and Verma, 2014). Sortilin is expressed in the majority of cancer types to some degree, with differing expression levels of genetic and proteomic sortilin for different cancer types. RNA expression of sortilin is highest in gliomas, ovarian cancer and melanomas, measured at Fragments Per Kilobase of transcript per Million mapped reads (FPKM), with sortilin expression levels in healthy brain tissue are similar to glioma levels at approximately 40-50 RPKM (Proteinatlas.org, n.d.). Expression levels in tumorous ovarian and skin derived sortilin, however, are significantly raised compared to healthy cell types, with ovarian levels increasing from 3.3 to 25 FPKM (Proteinatlas.org, n.d.), and skin levels increasing from 11.4 to 43 FPKM (Proteinatlas.org, n.d.). In lung tissue, sortilin is moderately expressed at approximately 28 RPKM, although in lung cancers, average expression is reduced to 12 RPKM (Proteinatlas.org, n.d., Proteinatlas.org, n.d.). Specific cell lines within lung tissue, both normal and diseased, show varying levels of sortilin expression. A549 adenocarcinoma cell lines, a form of NSCLC, express sortilin at 13.7 Transcripts Per Kilobase Million (TPM), although expression levels in SCLC-21H, a variant of small cell lung cancer, are much higher at 44.8 TPM (Proteinatlas.org, n.d.).

Due to the functions of sortilin, and the progression of cancer through the release and uptake of exosomes, the potential role that sortilin plays in the advancement of cancer through exosome-based communication would prove valuable in understanding cancer metastasis. In this study we investigate the involvement of sortilin on the invasiveness of CALU-1 lung cancer cells, and the physiological and proteomic effects of varying sortilin expressed cell derived-exosome treatment on CALU-1 cells as a potential biomarker for rate of cancer progression.
Methodologies:

Database Consultations:

Databases containing known data of lung cancer, exosomes and sortilin were identified and consulted to gather preliminary knowledge for relevant research methods. PubMed (National Center for Biotechnology Information) was used to identify prior research of exosomes in cancers, with specificity towards lung cancers where possible.

Proteomic pathway and interaction databases STRING 11.0 and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to identify known and hypothesised interactions of sortilin within specific pathways and through to additional distant related pathways.

Cancer proteomic and genetic information was obtained using the Human Protein Atlas and the Catalogue Of Somatic Mutations In Cancer (COSMIC) were used to identify protein and gene expression levels in different cancer types, making comparisons between lung cancer and other distant cancers, as well as differences between lung cancer sub-types.

Cell Culture:

Following standard cell culture protocols and applying sterile techniques throughout:

Before carrying out the cell passage, current cell populations were ensured to have reached >80% confluency using a light microscope at low magnification, and additionally healthy individual cell growth was observed at higher magnifications. CALU-1 cells were cultured in T-75 flasks for optimal population count to be used in further testing. Once confluency had been reached, old media from each flask was discarded, and cell cultures were washed through with 5ml Phosphate Buffered Saline (PBS) (pH 7.4, -CaCl₂, -MgCl₂) (Gibco) twice to remove all media from the flasks. Per flask, 1ml of Trypsin-EDTA (0.25%) was added and incubated at room temperate for 5 minutes to detach cells from the flask. Detached cells were re-suspended in ~5ml of 1X Dulbecco’s Modified Eagle Medium (+4.5g/L D-Glucose, +0.11gL Sodium Pyruvate, -L-Glutamine, +10ml/L penicillin/streptomycin, +100ml/L fetal bovine serum) (Gibco) and centrifuged at 1500rpm (360 x g) for 5 minutes to pellet the cell population. Upon addition of media, Trypsin-EDTA was deactivated to prevent further interaction with cell samples. Supernatant was discarded, and cell pellets were resuspended in fresh media, and ¼ of the total population passaged into a new T-75 flask with between 10-15ml of fresh media, and incubated at 37°C with 5% CO₂ for 2-3 days, after which the next cell passage was carried out. Cell populations were left to incubate for longer
periods of time when necessary, splitting a lower ratio of cells into the next passage, for example a 7 day incubation period requiring a 1:10 split.

**Cell Counting:**

Once cells had been passaged into new flasks, remaining cells were counted to determine the total population. 10µl of suspended cells were added to 10µl of Trypan Blue (0.4%), and observed under the light microscope using a haemocytometer. Living cells were differentiated from dead cells based on the uptake of Trypan Blue into the cytoplasm, as living cells are less permeable and do not present the dye inside the cell, while dead cells are permeable and become blue due to dye uptake. Cell counts were made on the haemocytometer by counting total cell numbers in the four quadrants and taking the average. Total cell number of the population was calculated by multiplying the average cell number by 2 to correct the 1:2 dye dilution, and then multiplied by $10^4$ to achieve the cells/ml.

**Cell Immunofluorescence:**

Well plates were suitable for the growth and preparation of samples, as they allow for multiple sample types to be grown under the exact same conditions, or for the use of producing replicates under the same conditions. It was found that 12 well plates proved most useful, as they allow for a large number of samples whilst being spacious enough to house round microscope cover slips. Per well, a single cover slip was added, along with ~60,000 cells and 1ml of fresh media, and was incubated at 37°C for 2 days until good confluency was reached. Media was discarded from each well, and cover slips were washed twice with 0.5ml PBS to remove all media. Fixation of cells was achieved by incubating cover slips in methanol and incubated at -20°C for 10 minutes, and washed with PBS. Non-specific antibody binding was blocked by incubating cover slips with PBS containing normal goat serum for 60 minutes. The primary antibody Beta-Actin (Sigma-Aldrich) was diluted to 1:2000 in PBS solution and coated on each cover slip, and incubated at room temperature for 60 minutes. Cover slips were washed with PBS, followed by the addition of 1:1000 goat anti-mouse secondary antibody Alexa Fluor 488 (ThermoFisher) diluted in PBS and incubated at room temperature for 60 minutes in the dark. Cover slips were washed with PBS, and coated with 4’, 6’-Di- Amidino-2-phenylindole diluted to 1µg/ml and incubated in the dark for 5 minutes, and washed in PBS. Slides were prepared with a single drop of mounting media placed per cover slip, and cover slips were dried completely and placed on the slide, cell side onto the mounting media. Slides were left to dry completely before observing under fluorescent microscopy.
Resazurin Assay:

1000X stock (44mM) was made up by dissolving 0.5g resazurin powder in 50ml of PBS, and stored at -20°C. A 10X stock (44nM) was made up by adding 150µl of 1000X resazurin to 15ml (-150µl) PBS. After initial cell passage during cell culture, remaining cells were counted and dispensed into a 96 well plate at 180µl per sample. 20µl of dimethyl sulfoxide (DMSO) was added to negative control groups, and 20µl of 10X resazurin was added to target measurement groups. Plate readings were taken using the FLUOstar Omega (Bmg Labtech) at 0, 15, 30, 45, 60, 90, 120, 180 and 240 minutes, and results were recorded in Microsoft Excel.

Laemmli Sample Buffer Preparation:

Laemmli sample buffer was produced following standard protocols (4ml 10% SDS, 2ml Glycerol, 1.2ml 1M Tris-Cl (pH 6.8), 2.8ml dH2O)

Exosome Extraction:

Following standard Polyethylene Glycol (PEG) extraction techniques:

Upon high cell confluency in culture (≥80%), old growth media containing extracellular vesicles was dispensed into 50ml falcon tubes and stored at -20°C. Additional media was further added to the falcon tubes until the full 50ml was achieved. A 2X stock of 16% PEG was prepared by dissolving 32g of crystalline PEG into 200ml of distilled water, ensuring that the mixture had fully dissolved before storing at 4°C in a sealed container to prevent airborne contamination. In fresh 50ml falcon tubes, media and 2X PEG are combined at a 1:1 ratio to produce 1X PEG/media, and were spun at 3260 x g for 60 minutes to pellet extracellular vesicles. The supernatant was discarded, and the pellet was resuspended in 100µl of either PBS or Laemmli sample buffer and stored at -20°C for further analysis.

Exosome Quantification:

Following standard qNano gold protocols:

Exosome extraction and isolation was carried out as previously described, although the final exosome pellet was resuspended in 200µl of PBS. Samples were diluted in PBS, with optimisations made with each individual exosome type to prevent exosome overloading. Samples were counted
through a 40-400nm column, and data was collected in report form using the qNano complimentary software (iZon), and raw data additionally inputted through Microsoft Excel.

**Preparation of Polyacrylamide gel:**

Following standard polyacrylamide gel preparation protocols:

0.75mm plates were prepared (Biorad) with the standard recipe providing material for two polyacrylamide gels. 10% resolving gel was prepared by combining 5.32ml polyacrylamide (30% protogel (National Diagnostics)), 4ml 1.5M Tris pH8.8 (Fisher Scientific), 6.52ml dH$_2$O, 160µl Ammonium persulphate (APS) (Fisher Scientific), 160µ SDS (Fisher Scientific) and 8µl Tetramethylethylenediamine (TEMED) (Sigma-Aldrich). The resolving gel was dispensed into each plate until approximately 80% full, and topped off with isopropanol and left to set for 30 minutes. Once set, the isopropanol was removed and a 3% stacking gel consisting of 0.64ml 30% Protogel, 1.6ml 1M Tris pH6.8 (Fisher Scientific), 4.16ml dH$_2$O, 40µl (APS), 40µ SDS, 10µ TEMED was added, with the addition of a 0.75ml well comb and left to set for a further 30 minutes. Gels were stored in moisture at 4°C for future use.

**SDS-PAGE Sample Run:**

Following standard SDS-PAGE protocols:

TGS running buffer (30g Tris-base (Fisher Scientific), 144g Glycine (SciChem), 10g SDS in 1L dH$_2$O at pH 8.3) was diluted to a 1X solution of 10% 10X TGS + 90% dH$_2$O. Two polyacrylamide gels were combined with an SDS-PAGE electrode cassette and placed into an electrophoresis cell (Biorad), which was filled with 1X TGS to submerge the lower quarter of the polyacrylamide gels. Additionally, the cassette was filled with 1X TGS, and combs were carefully removed from the gels. Loading buffer was prepared from 940µl Laemmli buffer, 50µl 1,4-Dithiothreitol (Sigma-Aldrich) and 10µl bromophenol blue (Sigma-Aldrich). A 1:2 ratio of loading buffer and suspended sample was combined and incubated at 95°C for 5 minutes and centrifuged at 12,000 x g for 1 minute. 3µl of EZ-Run protein ladder (Fisher Scientific) was dispensed into the first well for protein size reference, and 15-20µl of sample was loaded into each well (depending on well size). Gels were initially ran at 120V until proteins had passed through the stacking gel, followed by 150V until loading dye had completely passed through the gel. Once electrophoresis was completed, gels were removed from their plates for further analysis.
Coomassie gel staining:

Staining of gels was performed immediately after SDS-PAGE or after protein transfer to blotting membrane. Gels were placed into containers and submerged in Coomassie brilliant blue stain (Biorad), and left to incubate under constant agitation via rocking table for 30 minutes. Coomassie stain was removed and gels were submerged in destaining solution (25% methanol (Fisher Scientific), 5% acetic acid (Fisher Scientific), 70% dH₂O) and incubated under agitation overnight. Destaining solution was removed and replaced, then incubated for a further 60 minutes. Gels were imaged using the ChemiDoc™ Touch Imaging System (Biorad) under coomassie staining settings, and processed using Image Lab software 6.0.1.

Western Blot:

Standard Western blot protocols were followed:

Once SDS-PAGE was completed, the separated proteins were transferred to a blot membrane via semi-dry transfer using the Trans-Blot® Turbo™ transfer system (Biorad) at 25V and 1.0A for 60 minutes. Once completed, the membrane was cut based on the target proteins for analysis using the protein ladder for reference and placed into separate 50ml falcon tubes. 10X Tris-buffered saline (TBS) (80g NaCl (Fisher Scientific), 2g KCl (Fisher Scientific) and 20g Tris-base in 1L dH₂O and adjusted to pH7.4) was diluted to a 1X TBS stock with the addition of 1ml TWEEN®20 (Sigma-Aldrich). 10ml blocking buffer of 2.5% milk powder (Marvel) in 1X TBS was added to each tube to prevent non-specific binding of antibodies, and incubated on a rolling platform at room temperature for 60 minutes. Blocking buffer was discarded, and 1ml of primary antibodies diluted in fresh blocking buffer at 1:1000 dilution were added to each tube, and incubated on a rolling platform overnight at room temperature. Primary antibodies were discarded, and membranes were washed three times in 1X TBS in 5 minute incubation periods at room temperature. 1ml of secondary antibodies diluted in fresh blocking buffer at 1:2000 dilution were added to each tube, and incubated on a rolling platform for 60 minutes at room temperature. Secondary HRP antibodies were discarded, and membranes were washed three times in 1X TBS in 5 minute incubation periods at room temperature. Chemiluminescent stain was prepared by combining a 1:1 ratio of HRP Substrate Peroxidase Solution and HRP Substrate Luminol Reagent (Sigma-Aldrich) at 1ml total solution per gel transfer. Chemiluminescent stain was added to the membrane, and imaged using the ChemiDoc™ Touch Imaging System under chemiluminescent settings, and images were processed using Image Lab software 6.0.1.
**Scratch-Wound Migration Assay:**

Standard scratch-wound migration assay protocols were followed with some modification:

12 well plates were prepared, with approximately 60,000 cells added per well. 2ml of fresh media was added, or with treatment assays a combination of 1ml old media containing vesicles and 1ml of fresh media to each well, and were incubated at 37°C until ≥95% confluency had been achieved. Using a P200 pipette tip, a single vertical scratch was made in each well to produce a wound devoid of cells. Images of each well were taken at time points: 0Hr, 4Hr and 24Hr to measure growth using the CMOS C-Mount Microscope Camera (AmScope), and recorded on the AmScope software suite v3.7.13522 (AmScope).

**Cell Proliferation Assay:**

Cell samples were deposited into 12 well plates at 100,000 cells per well and either 2ml of fresh media was added, or with treatment assays a combination of 1ml old media containing vesicles and 1ml of fresh media to each well, and were incubated at 37°C for 72 hours. Once incubated, 10μl of media from each well was added to 10μl of Trypan Blue (0.4%), and remaining media from each well was discarded. Wells were washed twice with PBS, and 100μl of trypsin was pipetted to each well and incubated at room temperature for 5 minutes. Detached cells and trypsin was resuspended in 900μl of fresh media, of which 10μl of suspended cells were added to 10μl of Trypan Blue (0.4%). All samples were counted under light microscopy using cell counting techniques, with living and dead cell counts from media and cell suspensions from each well were combined to calculate total cell proliferation in each well, normalised by cell death percentage.

**Exosomal Cell Treatment and Extraction:**

Cell samples were deposited into 12 well plates at 100,000 cells per well and either 2ml of fresh media was added, or with treatment assays a combination of 1ml old media containing vesicles and 1ml of fresh media to each well, and were incubated at 37°C for 72 hours. For timed cell treatments, cell isolates were extracted at intervals after treatment of 30 minutes, 4 hours, 24 hours and 48 hours. After incubation, media from each well was discarded, and 100μl of Laemmli buffer was added to each well, and incubated at room temperature for 2 minutes to break down cell membranes. Laemmli buffer containing cell content was pipetted into micro-centrifuge tubes. Cell samples were sonicated at 20 kHz for 10 x 2 second intervals to ensure good cell lysis was
achieved. Cell samples were stored at -20°C, and proteomic analysis of cell samples was carried out by SDS-PAGE/Western blot.

**Statistical Analysis of Results:**

Once data was collected, statistical analysis was carried out, with different techniques being used depending on the type of data collected. Scratch wound assay images were analysed using ImageJ 1.8.0_112 by measuring the width of each wound at four random points across each image. Four replicates per sample and treatment were used, with four measurements per replicate, and were recorded in SPSS Statistics 23.0.0.3 (IBM). Data sets were checked for normality, and analysed by one-way analysis of variance (ANOVA) for significance. Tukey’s post-hoc test was carried out to identify specific significance between each testing group. Graphical figures were obtained using GraphPad Prism 5 statistical software.

Western blot images were analysed for differences in band saturation using ImageJ 1.8.0_112 software with reduction of background noise due to excess chemiluminescent stain. Data was inputted to Microsoft Excel to produce averages of replicates, and histograms were obtained GraphPad Prism 5 statistical software.

Cell proliferation assays were analysed through Microsoft Excel with calculations being made by taking averages of repetitions per experiment, and normalising cell death rate by calculating percentages based on the difference between living and dead cell counts. Results were displayed as histograms, which were obtained GraphPad Prism 5 statistical software.
Results

Calu-1 Cell Culture and Morphology:

Calu-1 cell lines, an epidermoid carcinoma expressing moderate levels of sortilin, cultured at a relatively stable pace, with high confluency and nutrient uptake being achieved after 3-4 days. Upon observation by light microscopy, each cell line variant shows variation in morphology to the other (Figure 5). Wild type colonies were closely packed together, with each cell being somewhat cuboidal in shape. Sortilin knockdown cells showed similarities to wild type cell colonies with tight packing, although individual cells were more rounded compared to wild types. Scrambled siRNA cell shape and colony formation showed the most difference, with cell growth being more randomised than wild type or sortilin knockdown, and cell shape was significantly elongated. Under fluorescent microscopy, individual cell shape and behaviour was observed at high magnification. Wild type cells were shown to group closely together in tight formation, and cell shape was somewhat cuboidal, presenting clear straight lines between neighbouring cells. The cytoskeleton protein \( \alpha \)-actin, a highly conserved structural protein involved in cytoskeletal structure, cell motility and intercellular signalling expression (Uniprot.org, 2019), shown in green, was strongly presented throughout the cells, with higher concentrations surrounding the nuclei (shown in blue DAPI stain), as well as in network-like structures throughout the cell (Figure 6). Scrambled siRNA cells were significantly different to wild type cells in terms of structure and behaviour. Individual cell structure was greatly elongated, with cell bodies surrounding the nucleus being relatively small when compared to the wild type. Cell connection was achieved through elongated sections of the cell.
body, with a lack of clear boundaries between neighbouring cell membranes. Overall actin expression was similar to wild type cells, although expression surrounding the nucleus was less apparent in scrambled siRNA cells.

Sortilin knockdown cells were shown to colonise similarly to wild type cells, with close formation of neighbouring cells in small colonies. Individual cell shape was more rounded than wild type cells and cell membrane boundaries were less profound in sortilin knockdown cells. In some individual cell examples, small microvilli-like protrusions were observed across the cell membrane. Actin expression was higher than that of wild type cells, with the addition of highly concentrated deposits of actin within the cytoplasm and surrounding the nucleus. Organisation of the nuclei appeared to be somewhat random, with some cell nuclei being towards the cell membrane. In terms of cell size, wild type cells were similar with deviations of approximately 200μm from the average diameter, while scrambled siRNA and sortilin knockdown cells had a high variance from the average, with scrambled siRNA cells deviating by approximately 400μm from the average, and in sortilin knockdown cells, diameter was observed in some examples to have been over twice the average.

<table>
<thead>
<tr>
<th>Calu-1 Cell Lines</th>
<th>Wild Type</th>
<th>Scrambled siRNA</th>
<th>Sortilin Knockdown</th>
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<tbody>
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<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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Figure 6: Fluorescence microscopy images of Calu-1 wild type cells (left), scrambled siRNA cells (middle), and sortilin knockdown cells (right). Average cell diameters taken at the longest point were 57.3μm for wild type, 105.5μm for scrambled siRNA, and 75.7μm for sortilin knockdown. Actin cytoskeleton staining is shown in green, and DAPI staining of the nucleus is shown in blue.
The resazurin assay is used to determine cell viability by measuring cell proliferation over time. Growth rates of each cell line showed variation from one another, measured by the metabolism of resazurin to resorufin and reading the change in fluorescence at 570 nm excitation / 595 nm emission. Wild type cell metabolism was highest, followed by scrambled siRNA, and sortilin knockdown cell metabolism was lowest with a significance of \( p = <0.01 \) against wild type and scrambled siRNA cells, suggesting that the knockdown of sortilin has a negative impact on the proliferation rate of lung cancer cells. (Figure 7).

**Resazurin Proliferation Assay of CALU-1 Cell Lines**

![Graph showing resazurin proliferation assay of CALU-1 cell lines](image)

*Figure 7: Growth curve analysis of Calu-1 wild type, scrambled siRNA and sortilin knockdown cell lines. Averages were taken from 3 replicates in wild type and sortilin knockdown groups, and 2 replicates for scrambled siRNA. A significance of \( p = <0.01 \) was found for each cell type.*

**Exosome Characterisation:**

Initial isolation of exosomes from cell culture media proved difficult, with concentrations being too low for detection by Western blot. Ultracentrifugation techniques were unable to process large quantities of media, and as such the PEG exosome isolation method was used instead. Analysis of exosomes by particle analysis revealed differences in apparent exosome release from cells into the culture media (Figure 8). Exosome count was lowest in wild type cells at \( 6.8 \times 10^5 \) exosomes/ml, while exosome numbers from scrambled siRNA cells were almost four times greater, and in sortilin knockdown cells exosome count was almost three times greater than in wild type (Figure 8 and Table 2). Average exosome diameter was relatively similar between cell types, with wild type exosome average being the highest at 93nm, and scrambled siRNA average at the lowest with
84nm. However, the most common diameter size in wild type and scrambled siRNA exosomes was equal at 71nm, and the mode sortilin knockdown exosome diameter was at 65nm (Table 2).

Proteomic analysis of cell lines and exosomes released by cells showed variance in expression levels between groups. In cell lysates, band intensity of flotillin 2, EGFR and calnexin was unchanged between groups, although expression levels of tetraspanins CD9 and CD63 were reduced in sortilin knockdown cells compared to wild type and scrambled siRNA (Figure 9). Sortilin levels, as expected, were lowest in sortilin knockdown cells, expressing low levels of residual sortilin. Sortilin expression was slightly lower in scrambled siRNA compared to wild type cells, but was still clearly expressing. In exosome samples, calnexin, a negative control in exosomes, and CD63 was absent in all cell lines,

<table>
<thead>
<tr>
<th>Table 2: Exosome count and diameter of Calu-1 cell lines</th>
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<tr>
<td>Average Exosome Diameter (nm)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Mode Exosome Diameter (nm)</td>
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<td>Exosome Count/ml</td>
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</table>

Figure 8: qNano gold analysis of CALU-1 exosomes from wild type (WT), scrambled siRNA (SER) and sortilin knockdown (Kd) cells.
and low expression of EGFR was observed. Interestingly, sortilin expression was also low in all cell types, with faint bands present in each exosome sample type, suggesting that sortilin protein is not concentrated in the exosome and instead largely remains in the cell. Flotilllin expression was high in all exosome types, and CD9 expression was highest in wild type-derived exosomes, with moderate expression being observed in scrambled siRNA and sortilin knockdown cells.

Figure 9: Western blot analysis of whole cell lysate (WCL) and exosome samples (EVs) from CALU-1 wild type (CALU1), scrambled siRNA (CALU1 SCR) and sortilin knockdown (CALU1 SORT KD) cell lines. Antibodies for CD9, Flotillin 2, EGFR, Sortilin, CD63 and Calnexin were used.
The Physiological Role of Sortilin EVs

The scratch wound cell invasion assay is a simple procedure to measure the migration of cells into a freshly made wound in the culture, with measurements taken over time in which a direct comparison can be made between cell lines (Figure 10). The scratch wound migration assays
displayed differing cell line behaviours and their ability to close artificially made wounds within colonies.

Observations were made during a 24 hour period, with measurements being taken at 0 hours on initial wound formation, 4 hours, and 24 hours (Figure 11). Normalisation of results were made by calculating the percentage difference between the initial wound size and final wound size at 24 hours. Each cell line showed differences in average percentage wound closure, with scrambled siRNA cells showing the greatest percentage difference in wound size at over 60%, followed by wild type cells at approximately 36% and sortilin knockdown cells at 28% (Figure 11). A significant difference in wound closure percentage was found between each cell line, with the significance between wild type and sortilin knockdown cells being at >95% confidence, and a strong significance
between scrambled siRNA and both wild type and sortilin knockdown cells with a confidence of >99.9%.

Following cell line migration observations, cell lines were treated with exosomes derived from SK-MES lung cancer cells (squamous cell carcinoma) stably overexpressing sortilin (pSORT). Initial troubleshooting of treatment incubation times before wound formation found that incubation for one hour at 37°C was sufficient to allow for exosome uptake, with longer incubation times having little effect on the difference in cell migration rates. Controls were used to make comparisons with each cell line treated with pSORT exosomes, and percentage difference of wound size over 24 hours was calculated (Figure 12). No significance between wound size in wild type cell controls and wild type cells treated with pSORT exosomes, and similarly there was no significance found between scrambled siRNA cells and scrambled siRNA cells treated with pSORT exosomes. However, a

![Figure 12: Average percentage difference in wound diameter after 24 hours from initial wound formation. One way analysis of variance was carried out with Tukey’s post-hoc test to reveal significance of p=<0.05 between knockdown cell controls and knockdown cells treated with pSORT exosomes, and no significance between wild type cells and wild type cells treated with pSORT exosomes, and scrambled siRNA cells and scrambled siRNA cells treated with pSORT exosomes. Error bars of standard deviation (SD) were used.](image-url)
significance of $p=<0.05$ was found between sortilin knockdown cells and sortilin knockdown cells treated with pSORT exosomes, in which an increase of approximately 10% in wound closure was observed in sortilin knockdown cells treated with pSORT exosomes. Additionally, the total wound closure of sortilin knockdown cells treated with pSORT exosomes (37.4%) was similar to that of the wild type cell control (34.9%) and wild type cells treated with pSORT exosomes (34.3) (Figure 12).

Wild type cells were treated with exosomes derived from multiple versions of SK-MES lung cancer cell lines, alongside controls of CALU-1 wild type, scrambled siRNA and sortilin knockdown cells. Wild type SK-MES naturally lacks sortilin, scrambled siRNA SK-MES, and pSORT SK-MES exosomes were isolated and used as a treatment for wild type CALU-1 cells. No significant difference was observed in percentage wound reduction between wild type cells and those treated with wild type SK-MES or pSORT exosomes, although a significant difference was calculated between wild type cells and wild type cells treated with scrambled siRNA SK-MES exosomes (Figure 13). Scrambled

![Graph showing percentage change in wound diameter after 24 hours from initial wound formation. One way analysis of variance was carried out with Tukey's post-hoc test to reveal significance of $p=<0.05$ between wild type CALU-1 cells and wild type CALU-1 cells treated with scrambled siRNA SK-MES exosomes, and no significance between wild type CALU-1 cells and wild type cells treated with wild type SK-MES or pSORT exosomes. Error bars of standard deviation (SD) were used.](image-url)
siRNA treatment produced an increase in wound closure rate which mimicked that of CALU-1 scrambled siRNA control cells with a percentage difference of 54.9%.

**Cell proliferation assay:**

Due to the difference in invasion rates between cell and treatment types, the growth and death rates of each cell type were investigated to identify the effects of sortilin introduced through exosome uptake on proliferation. Cell proliferation assays had large variation in results, with multiple repetitions producing different death percentages throughout testing. Culture conditions were altered throughout the testing period when carrying out the proliferation assays, with a combination of faults in incubation equipment and changes to media glucose levels, resulting in a drastic reduction in cell numbers and growth rates (Figure 14). Initial results obtained before alterations in cell culture conditions presented a difference in proliferation rate between cell types.
and treatments. In wild type cells, proliferation was reduced by approximately half when treated with sortilin knockdown CALU-1 exosomes when compared to wild type control, while proliferation was almost doubled when treated with pSORT exosomes. Sortilin knockdown cells showed little difference in cell proliferation between sortilin knockdown control cells and sortilin knockdown exosome treatments, while pSORT exosome treatment proliferation was reduced by almost half.

**Western Blot of Cell Treatments:**

Proteomic analysis of CALU-1 wild type and sortilin knockdown cells treated with exosomes of varying sortilin levels was carried out. Sortilin knockdown exosomes were used as a low level sortilin treatment, CALU-1 wild type exosomes were used as a moderate level sortilin treatment,
and SK-MES pSORT exosomes were used as a high level sortilin treatment. Cells were treated with exosomes under two sub-groups, one of which was treated immediately after being deposited into well plates, while the second group was left to incubate over a 48 hour period at 37°C before being treated with.

Cells were treated with exosomes under two sub-groups, one of which was treated immediately after being deposited into well plates, while the second group was left to incubate over a 48 hour period at 37°C before being treated with.

Both groups were left to incubate in exosome treatments for 24 hours. Antibodies were used for calnexin, β-Actin, EGFR, sortilin and flotilin-2, with sortilin as the target protein for observed change in expression levels (Figures 15 and 16). In the 0 hour treatment group, sortilin expression was relatively stable, although some background signal was additionally present which caused some complication in taking accurate measurements (Figures 15 and 16). Visually, sortilin expression in 0Hr wild type cells was relatively moderate, and in 0Hr sortilin knockdown cells was extremely low. In the 48Hr wild type cells, sortilin expression was raised, significantly so in the pSORT treatment. Sortilin expression was also higher in the sortilin knockdown treatment than in the wild type treatment in the 48Hr wild type cells. Expression of sortilin was observable in all 48Hr sortilin knockdown cells, with higher expression in the pSORT treatment, and moderate expression in wild type and sortilin knockdown treatments. The result for sortilin expression was repeated in

![Graph showing relative band intensity of sortilin expression levels for different cell types and exosome treatments.](image)

**Figure 16:** Sortilin expression levels of wild type and sortilin knockdown cells treated with CALU-1 wild type and sortilin knockdown, and SK-MES pSORT exosomes at 0Hr and 48Hr treatments (see Fig. 15).
figure 17, which was additionally tested for β-Actin with an antibody diluted to 1:3000, producing readable bands in each treatment group, although some saturation is present, shown by the difference in shape of the bands compared to sortilin and EGFR. This was likely due to a much faster reaction to the chemiluminescent stain in β-Actin compared to other antibodies.

Sortilin knockdown cells were treated with exosomes of varying sortilin levels (sortilin knockdown CALU-1 (low), wild type CALU-1 (moderate), pSORT SK-MES (high)) and incubated over varying times to identify rate of uptake and expression of sortilin in cells. Incubation periods of 30 minutes, 4 hours, 24 hours and 48 hours were used, and cells were isolated and analysed by Western blot. Sortilin levels at 30 minutes and 4 hours showed little increase in all cell treatments, apart from at the 4 hour point in pSORT treatment in which a small band could be faintly seen. (Figure 18) At 24
hours, the band in the pSORT treatment was stronger, and a faint band for wild type treatment could be seen. At 48 hours, a strong band for both pSORT treatments was visible at a much higher intensity than lower treatment times, with saturation of the bands occurring. Sortilin expression for wild type treatment at 48 hours was moderate, with a slightly more intense band than lower incubation times (Figures 18 and 19). Treatment of sortilin knockdown exosomes produced no visible sortilin expression in all incubation treatments. Cell lysates of sortilin knockdown and wild type CALU-1 were used as sortilin positive and negative controls.

<table>
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<th>24 hour</th>
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<td>Sortilin Knockdown × pSORT</td>
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<tr>
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**Figure 18:** Western blot sortilin knockdown cells treated with CALU-1 wild type and sortilin knockdown, and SK-MES pSORT exosomes. Cells were incubated for varying periods of time with treatments (30 minutes, 4 hours, 24 hours and 48 hours) before proteins were extracted. Antibodies for Calnexin, β-Actin and Sortilin were used.
Figure 19: Sortilin expression levels of sortilin knockdown cells treated with CALU-1 wild type and sortilin knockdown, and SKMES pSORT exosomes, incubated at 30 minutes, 4 hours, 24 hours and 48 hours before proteins were extracted. Untreated cell lysates of CALU-1 wild type and sortilin knockdown were additionally measured for comparison.
Additionally, NF-κB antibodies for the full sized 105 kD (p105) and cleaved 50 kD (p50) variant of the protein were analysed by Western blot for each treatment. Treatment incubation times of 4 hours, 24 hours and 48 hours were used for comparison of NF-κB cleavage rates. Measurement of band intensity shows a gradual increase in cleaved NF-κB, although background signal on the blot membrane may affect gathered data (Figures 20 and 21).

<table>
<thead>
<tr>
<th>Treatment Incubation Periods:</th>
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<th>24 hour</th>
<th>48 hour</th>
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<tr>
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<tr>
<td>Sortilin Knockdown + pSORT</td>
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Figure 20: Western blot sortilin knockdown cells treated with CALU-1 wild type and sortilin knockdown, and SK-MES pSORT exosomes. Cells were incubated for varying periods of time with treatments (4 hours, 24 hours and 48 hours) before proteins were extracted. Antibodies for whole 105 kD NF-κB and cleaved 50 kD NF-κB were used.
Figure 21: cleaved 50 kD NF-κB expression levels of sortilin knockdown cells treated with CALU-1 wild type and sortilin knockdown, and SK-MES pSORT exosomes, incubated at 4 hours, 24 hours and 48 hours before proteins were extracted.
Discussion

Cell Morphology and Fluorescence Imaging

General cell shape and colony behaviour showed variation throughout each cell type. The randomisation in directional growth in scrambled siRNA CALU-1 cells was interesting to observe, as the cell membrane had no normal shape to follow, with each cell having long outcroppings which seemed to reach out to one another. Through these outcroppings, colonies of scrambled siRNA cells were able to fuse membranes, rather than simply coming into direct contact with one another. Subtle differences between morphology of wild type and sortilin knockdown cells show variation in shape between the two cell types. Wild type cells, which express sortilin at a moderate level, were closely colonised with one another in the majority of cases, with few isolated examples, and cell membrane boundaries were almost cuboid in shape with somewhat straight edges. Sortilin knockdown cells were more rounded in shape when in colonies compared to wild type cells, and membrane boundaries were additionally less profound. Kim et al (Kim et al., 2018) found that silencing of sortilin in neuroendocrine tumours, in which sortilin expression is raised, reduced the adhesion and migration ability of neuroendocrine tumour cells. In comparison, sortilin knockdown CALU-1 cells still present the ability to colonise, although a less obvious boundary between cells may be due to differences in membrane bound proteins causing slight fusion between membranes. Through sortilin’s interaction with p75<sup>NTR</sup> in the neurotrophin signalling pathway (Genome.jp, 2018) pathway interactions lead to the regulation of the actin cytoskeleton through both activation and inhibition, and leads further to focal adhesion and its assembly (Genome.jp, 2019). It is possible that a knockdown of sortilin in CALU-1 cells may alter the cells ability for correct focal adhesion, of which cell-cell adhesion is carried out through the extracellular matrix via focal adhesion (Frantz, Stewart and Weaver, 2010). Alterations in the extracellular matrix may result in sortilin knockdown membranes becoming ‘sticky’, increasing adhesion properties between cells and creating stronger binding. During exosome quantification, we found that there was more difficulty in correctly measuring the sortilin knockdown exosomes, in which the qNano gold performs real time one by one counts. It was thought that the sortilin knockdown exosomes were becoming aggregated, as the measuring apparatus of the qNano gold was becoming repeatedly blocked, while in contrast wild type and scrambled siRNA exosomes posed no issue in measurement. It seems that membrane properties of sortilin knockdown CALU-1 cells and their released exosomes may be altered as a result of the removal of sortilin. Additionally, some isolated sortilin knockdown cells identified through fluorescence microscopy displayed multiple outcroppings of the cell membrane. As shown in figure 22, a large sortilin knockdown cell with a cell diameter of approximately 160µm was
identified displaying a significant difference in membrane structure to wild type cells. Surrounded by the cell membrane, microvilli-like structures were seen across the cell. This difference in structure was not observed in all sortilin knockdown cells, in particular in colonies of sortilin knockdown cells, which display almost smooth membranes. As microvilli function partly in cell-cell communication as well as adhesion, it may be explained that a lack of microvilli in sortilin knockdown colonies may be due to achieved adhesion between cells, reducing the need for producing microvilli for further cell adhesion (Lange, 2010). Additionally, Hoa et al identified the use of microvilli in gliomas as a method of preventing cytolytic effector lymphocytes from reaching the main cell body, effectively pushing away the immune response in order to prolong survival (Hoa et al., 2010). However, low sortilin expression may not explain this as a potential direct result, as gliomas express sortilin at a high level (Proteinatlas.org, n.d.), whereas sortilin knockdown CALU-1 cells express sortilin at an undetectable level. It may be more feasible that the function of these microvilli would be as a method of locating and adhering to neighbouring cells in order to form colony cells, similarly to that of the greatly exaggerated scrambled siRNA cells, in which a reduction of sortilin may reduce the ability to moderate the production of microvilli in sortilin knockdown cells compared to wild type.

Actin expression was observed in all cell types under fluorescence microscopy, in which the actin appeared in green throughout the cell. In wild type cells, actin expression was clear, with obvious differentiation from the cytoplasm and the actin cytoskeleton, in which networks running...
throughout the cell body from the nucleus were observed (Figure 23(a)). Actin appears to be concentrated surrounding the nucleus, most likely due to the behaviour of actin networks leading towards the nucleus from elsewhere in the cell. These actin networks may potentially be actin stress fibres, responsible for roles such as cell migration and non-muscular contraction (Pellegrin and Mellor, 2007).

In sortilin knockdown cells, overall actin was less profound than in wild type cells, although still present throughout the cytoplasm. The main observable difference in actin expression between wild type and sortilin knockdown cells was in the lack of stress fibres in sortilin knockdown cells. In
isolated sortilin knockdown cells, actin expression was present in microvilli as areas of intense green, possibly contributing to the formation of microvilli in order to seek out neighbouring cells. Sortilin knockdown cells additionally presented cases of high concentrations of actin located beside the nucleus in some cells. Identified in figure 23(b), these deposits present themselves against the nucleus, and are highly likely to be the formation of actin aggresomes. Aggresomes are formed as a response to high levels of misfolded proteins, in which the cellular protein degradation process is overwhelmed (Johnston, Ward and Kopito, 1998). In sortilin knockdown cells, it appears that actin aggresome formation may occur as a result of misfolded actin as a result of a reduction of sortilin in the cell. Kazami, Usui and Osada identified that upon the introduction of actin toxins, actin stress fibres retract and form actin aggresomes over the course of 24 hours (Kazami, Usui and Osada, 2011). This response seems to compare somewhat to the difference in sortilin levels between wild type and sortilin knockdown cells, in which moderate sortilin expression produces actin stress fibres, and an elimination of sortilin from the cell may retract stress fibres to form aggresomes, simulating actin toxicity.

Metabolism of cell lines showed variance between one another with scrambled siRNA metabolising resazurin at the highest rate at approximately 70,000 RFU after 6 hours. This was followed by wild type cells at approximately 50,000 RFU, and finally sortilin knockdown cells at just under 18,000 RFU. Due to the clear decrease in metabolic activity of the mitochondria in sortilin knockdown cells versus wild type cells through reduced levels of resazurin converted to resorufin, it can be assumed that sortilin plays some role in the process. Because there is still metabolic activity present, it is clear that sortilin expression is not entirely critical, but may still play a role in the activity of the mitochondria. Due to the major role of sortilin as a sorting and tracking protein in the cell membrane, a knockout of sortilin would certainly disrupt the transportation of some proteins into the cell. Sortilin’s role in lipid metabolism may give reason to a reduction in mitochondrial activity. Tsai, Rainey and Bollag discuss the use of very low-density lipoproteins (VLDL) by cells to mediate aldosterone production, which is biosynthesized in the mitochondria (Tsai, Rainey and Bollag, 2017). Additionally, Ibarrola et al found that aldosterone impairs the function of human cardiac fibroblast mitochondria through down regulation of A-kinase anchor protein-12 (Ibarrola et al., 2018). Sortilin, when partnered with apolipoprotein B, transports VLDL’s to the lysosome for degradation. It is possible that due to a knockdown in sortilin, the levels of VLDL’s in the cell may be increased as their ability to be transported to the lysosome for degradation would be impaired, leading to a potential reduced activity in the mitochondria.
Exosome Release and Proteomics

Exosomes released from each cell type were measured for size and concentration to identify any differences based on the knockdown of sortilin. In terms of overall exosome diameter, the most common size in wild type exosomes was just over 9% greater than that of sortilin knockdown exosomes. However, total exosome count per ml was much higher in sortilin knockdown cells than in wild type cells. Wilson et al found that sortilin, along with TrkB and EGFR, mediates the release of exosomes in A549 lung cancer cells (Wilson et al., 2014). It may be possible that exosome release is controlled by specific levels of sortilin in the cell as a method of limiting exosome release, creating a balance in expression levels with TrkB and EGFR. Through the knockdown of sortilin, the balance in protein expression levels would be offset, potentially allowing for an increased rate of exosomes being released from the cell. Alternatively, an increase in exosome release in sortilin knockdown cells may be as a result of a stress response. Exosomes are released at an increased level when cells undergo stressful conditions, such as change in temperature, environmental pH and exposure to toxins, and release exosomes containing information to other cells as a form of communication (Vulpis et al., 2019). As discussed earlier, observations of sortilin knockdown cell actin distribution compared to wild type cells display similarities to an actin toxin response, and as such sortilin knockdown cells may in turn release raised levels of exosomes in response to these conditions.

β-actin levels in exosome sample were identified to show differences in expression levels between cell types. Wild type cells expressed high levels of β-actin, with roughly 20% reduction in sortilin knockdown cells (Figure 24). This may be as a result of the interactions of p75NTR mediated by sortilin leading to the regulation of the actin cytoskeleton, reducing the amount of actin produced within sortilin knockdown cells. Additionally the role of sortilin in exosome release may alter the levels of β-actin entering the exosome as a result of a knockdown of sortilin.
Cell Migration Assays

Cell migration measurements were taken from each cell line to analyse the invasion rates of CALU-1 cells based on sortilin levels. In each case, scrambled siRNA cells presented the greatest percentage change in wound closure. Based on visual observations of scrambled siRNA cells, their tendency to migrate into empty spaces makes sense. The exaggerated tendrils formed from each cell, along with their recorded high metabolism rates, display the ability for scrambled siRNA cells to expand over considerable distances in order to extend colonies. In comparison, wild type and sortilin knockdown cells were less invasive, decreasing the scratch wound diameter by approximately 36% and 28% respectively. Each wound closure percentage was significantly different between each groups, in which scrambled siRNA cells presented a high significance between wild type and sortilin knockdown cells. Reasoning for the large difference in migration between scrambled siRNA cells and both wild type and sortilin knockdown cells may be due to general cell morphology over metabolic activity. Wild type and sortilin knockdown cells were both generally compacted, lacking the long outcroppings observed in scrambled siRNA cells. Zhong and

![Bar graph showing relative band absorption for wild type, scrambled siRNA, and sortilin knockdown CALU-1 cells.](image_url)
Ji investigated the effects of overall shape on the migratory behaviour of cells, and found that polarity and focal adhesion play a role in the cells ability to move. Additionally, it was found that rounded cells, such as keratocytes, were much more successful in migration than elongated cells such as fibroblasts, as the rounded shape generates a greater focal adhesion complex instability towards the posterior side of the cell (in terms of directional movement), allowing for greater focal adhesion polarity towards the front of the cell (Zhong and Ji, 2013). However, the scrambled siRNA cells were significantly elongated compared to wild type and sortilin knockdown cells, and as such it cannot be assumed that cell shape was responsible for the difference in migration rate. Focal adhesion polarity may be the driving force in the difference in cell migration between CALU-1 cell types instead, in which scrambled siRNA cells are capable of generating enough polarity to transport into the wound, along with their general size compared to wild type and sortilin knockdown cells allowing for less cell division required to eventually reach high confluency within the wound. The difference in actin distribution between wild type and sortilin knockdown cells may be responsible for the variation in cell migration rates, as focal adhesion polarity may be different.

To gain a better understanding on the role of sortilin in the rate of migration of cell samples, and the potential delivery system in the use of exosomes, cell samples were treated with exosomes in media. Treatment of cell lines with exosomes containing high sortilin levels was used, and measurements were taken to identify any significance against untreated cell lines. The addition of excess sortilin in wild type cells had little effect in the rate of migration into the wound, similarly with scrambled siRNA cells, in which although a slight increase was seen, but there was no statistical significance. However, treatment of sortilin knockdown cells with exosomes containing excess sortilin did increase the rate of invasions, with untreated cells decreasing wound size by 27%, and treated cells decreasing wound size by 37% on average. This increase in sortilin appears to have restored the growth rate of sortilin knockdown cells to invade at a similar rate to the wild type cell. In order to investigate the opposite effect of treating sortilin-expressing cells with sortilin-lacking exosomes, wild type cells were treated with multiple variants of SK-MES-derived exosomes. Wild type SK-MES cells do not express sortilin, and were used in conjunction with SK-MES scrambled siRNA exosomes and pSORT exosomes. There was no significant change in the rate of invasion in wild type cells when treated with either upregulated or downregulated sortilin exosomes. However, invasion rates were increased in wild type cells treated with scrambled siRNA SK-MES exosomes, which displayed a similar rate of invasion to scrambled siRNA CALU-1 cells. It may be possible that the introduction of genetic material from scrambled siRNA-derived exosomes to act more randomised, changing the morphology of the wild type cell to behave similarly to the scrambled siRNA cells. The results gathered when comparing sortilin treatments in the different
cell types are of great interest. It appears that sortilin does play a role in the behaviour of cell migration, but it is not a simple relationship in which an increase of sortilin also increases cell invasion indefinitely, and instead suggests that sortilin levels influence migration to a certain limit, but excess sortilin levels will not accelerate the rate any further. This is seen when comparing wild type and sortilin knockdown cells, of which both cell types have a base sortilin expression level. Moderate levels of sortilin in wild type cells give a standard invasion rate, while reduced sortilin levels in sortilin knockdown cells display a reduction in migration rates. Upon the introduction of excess sortilin from exosomes, sortilin knockdown cell invasion rate is ‘restored’ to that of wild type cells. However, the introduction of excess sortilin to wild type cells already expressing sortilin show no improvement to the rate of invasion. Similarly, treatment of wild type cells with exosomes lacking sortilin has no effect on migration rates either, suggesting that the levels of sortilin currently expressed in wild type cells are sufficient to maintain normal migration rates. Through the neurotrophin regulation pathway, sortilin’s involvement through its interaction with p75NTR leads to a number of different pathways. It is possible that in sortilin knockdown cells, the regulation of the actin cytoskeleton is somewhat disrupted, and although sortilin is not the sole interactor leading to the actin cytoskeleton, it may have some involvement. As the actin cytoskeleton is involved in non-muscular contraction and relaxation of the cell, a disruption of sortilin may lead to altered expression of actin, as described earlier. A re-introduction of sortilin to knockdown cells may restore the regulation of the actin cytoskeleton, as sortilin contained within exosomes would be readily available once inside the sortilin knockdown cells. Rho GTPases are crucial in cell migration, and are involved in protrusion, polarisation and adhesion, with three proteins in the Rho GTPase family having been extensively researched: Rac, RhoA and Cdc42. RhoA is regulated by p75NTR through the neurotrophin signalling pathway, in which p75NTR interacts both directly with RhoA and with RhoGDI, which in itself is an inhibitor of RhoA (Devreotes and Horwitz, 2015). The role of p75NTR in cell migration is partly in the regulation of RhoA in order to correctly polarise and adhere the cell, with signalling of p75NTR inducing RhoA activation. Low sortilin levels resulting in reduced signalling of p75NTR would therefore result in a reduced activity of RhoA, in turn reducing the migration rate of cells by altered polarity and adhesion properties (Yamashita and Tohyama, 2003). Due to the use of multiple Rho GTPases in cell adhesion and polarity for cellular migration, the reduction in just RhoA would have a slowing effect rather than a complete prevention of the cells ability to migrate.
Cell Proliferation:

Measurements of cell death rates revealed that sortilin-contained exosomes play a role in the mediation of apoptosis when taken into the cell. Wild type cells treated with exosomes of varying sortilin levels displayed a relationship between the levels of sortilin taken in and death percentage rates, with low sortilin treatment resulting in a lower death rate at 2.1%, moderate sortilin at 4.5%, and high sortilin at 8.9%. In neurons, it has been established that sortilin plays an important role in a cell death mediation complex, in which sortilin binds to p75NTR and pro-nerve growth factor (proNGF), the precursor to nerve growth factor (NGF), to regulate neuronal apoptosis. Nykjaer et al identified that in order to create this cell death complex, both sortilin and p75NTR were required to cooperate with one another to successfully bind to proNGF, as binding of proNGF was unsuccessful in cells lacking either sortilin or p75NTR. In the absence of sortilin, however, mediated apoptosis is carried out by the binding of mature NGF and p75NTR, with sortilin having little effect on the process (Nykjaer et al., 2004). In CALU-1 wild type cells, the altered levels of sortilin uptake by exosomes would have an impact on the cells ability to induce apoptosis through this complex, as an increase in sortilin would therefore increase the occurrence of the formation of the proNGF-p75NTR-sortilin complex, raising the rate of cell death observed.

In sortilin knockdown cells, the relationship between sortilin treatment levels and measured cell death identified with wild type cells was not observed. Instead, the opposite effect was seen, in which raised sortilin levels through exosome treatment appeared to reduce cell death, with pSORT treatment presenting a death rate of 4.3% compared to sortilin knockdown CALU-1 treatment death rate of 8.5%. The treatment of sortilin knockdown cells with wild type CALU-1 exosomes did show a slight reduction in cell death rate at 7.9%. Treatment of pSORT exosomes in sortilin knockdown cells effectively restored the cell death rate to the observed rate in wild type cells treated with wild type CALU-1 exosomes. Due to the absence of sortilin in sortilin knockdown cells, apoptosis would be mediated predominantly by NGF induced death over the proNGF-p75NTR-sortilin complex, with NGF interacting with TrkA to control cell death and survival. Upon the introduction of excess sortilin to sortilin knockdown cells, the formation of the proNGF-p75NTR-sortilin complex can occur, with proNGF binding being used and reducing the amount of mature NGF being produced. Additionally, p75NTR is involved in cell survival through the interactions of NF-kB, which suppresses programmed cell death (Luo, Kamata and Karin, 2005). Through the introduction of exosomal sortilin to cells, cell survival may be induced when initial sortilin expression levels are low, although a high total expression level of sortilin may instead induce cell death due to the formation of the proNGF-p75NTR-sortilin. It appears that sortilin may play a role in
both the induced apoptosis and survival of the cell, and as such a moderate expression level may be required in order to balance the rate of proliferation, with low levels of sortilin reducing cell survivability through NF-κB, and high levels inducing apoptosis through the proNGF-p75NTR-sortilin complex.

Interestingly, although cell death rate in wild type cells exposed to high sortilin levels was raised, the growth rate was also high. Wild type cells treated with exosomes containing low and moderate sortilin levels displayed a similar total living cell count to untreated wild type cells, with wild type cells at approximately 100,000 cells, wild type cells treated with wild type CALU-1 exosomes at approximately 80,000 cells, and wild type cells treated with sortilin knockdown CALU-1 exosomes at approximately 90,000 cells. Wild type cell treated with pSORT exosomes had a cell count of approximately 300,000, which is triple that of the other treatments. Al-akhrass et al reported that sortilin is a regulator of the internalisation of EGFR into the cell, and in tumour cells this process is involved in the rate at which cells proliferate. When sortilin is knocked out, EGFR signalling is sustained at the surface of the cell, and allows for the rapid progression of the cancer (Al-Akhrass et al., 2017). These findings contrast the findings of this study, as the introduction of raised sortilin levels to CALU-1 wild type cells appears to have raised the growth rate. However, our findings do not necessarily conflict with those of Al-akhrass et al, as the test parameters were different. In Al-akhrass et al’s study, observations on the reduction of sortilin were made, but they did not investigate the opposite conditions when sortilin levels were raised. It may be possible that raising the levels of sortilin past a certain point allows for raised internalisation of EGFR in order to promote proliferation, but in turn additionally increases the rate of apoptosis due to the formation of other cell death complexes.

**Proteomic Analysis of Exosome Treatment on Cells**

Due to the physiological effect shown in cells treated with varying levels of sortilin through exosome uptake, western blots were carried out to identify any change in protein expression as a result of the addition of sortilin to the cell system. Additionally, a comparison on the pre-incubation times before treatment addition was carried out to identify any difference in sortilin expression. In the 0 hour pre-incubation group, sortilin levels were similar in wild type cells, and undetectable in sortilin knockdown cells, with treatment types having little effect on expression level. However, when a pre-incubation period of 48 hours was used, sortilin expression level was higher, with differences in expression level being observable between treatment types. This may be due to the differences in growth rates of cells during treatment, as treatments were made at different phases of cell culture. 0 hour pre-treatment cells would have been entering the exponential growth phase,
as replication and migration of cultured cells would be prioritised. Additionally, during the incubation period, exosome uptake would be carried out gradually throughout, with the loss of some measurable protein resulting from cell death during the incubation period. During the exponential growth phase, proliferation of cells is raised and as such a faster turnover of resources occurs (Robador et al., 2018), in which it is possible that exosome uptake is additionally raised as a result of this. In comparison, 48 hour pre-treatment cells were already at high confluency prior to treatment, and as such proliferation rates would have slowed, reducing the rate of exosome uptake. Additionally, the time between treatment and cell extraction differed between pre-incubation groups, with 0 hour pre-incubation groups being incubated for 48 hours after treatment, while 48 hour pre-incubation groups being incubated for 24 hours after treatment. This difference in treatment incubation may give additional reason to the difference in observed sortilin expression, as an increase in time would result in the reduction of available treatment exosomes in media. As shown in figures 15 and 16, the treatment of exosomes with varying sortilin levels has an effect on the expression of sortilin in the target cell in the 48 hour pre-incubation group. Measurable sortilin was observed in sortilin knockdown cells after treatment, with a relationship between sortilin levels in exosome types and cellular sortilin output with wild type and pSORT exosomes. Interestingly, sortilin levels were observed in sortilin knockdown cells treated with sortilin knockdown exosomes, with achieved repetitions, despite a lack of sortilin protein content in the exosomes. This result was unexpected as sortilin expression levels should be negligible, although this may be due to miRNA control of gene expression (Catalanotto, Cogoni and Zardo, 2016) when introduced to the nucleus from the exosome, causing the production of sortilin due to the presence of knockdown exosomes. However, this type of result is unheard of, and as such further repetitions of new cell lines and exosomes would be required to ensure that this result is due to genetic control from exosomes of the upregulation of sortilin in sortilin knockdown cells, and not some form of error during the experimental process.

The effect of incubation periods after treatments was investigated to identify the rate in uptake and expression of sortilin from exosomes, with a pre-incubation growth period of 48 hours being used to allow for cells to reach a high confluency. Measurements of sortilin expression by western blot identified a difference in sortilin expression compared to the previous experiment for sortilin knockdown exosome treatment, although the wild type and pSORT treatments did produce observable sortilin expression. Shown in figure 18, sortilin expression increases over time, with the 48 hour incubation period expressing the highest levels in each treatment type. Franzen et al found that exosome uptake occurs within a 24 hour period from initial exposure, and begins from approximately one hour when analysing human bladder cancer cells (Franzen et al., 2014).
Additionally, Horibe et al. identified that exosome uptake rate is dependent on the cell type, with some cell types being more efficient than others (Horibe et al., 2018). However, our results indicate a slow rise in expression of sortilin, and one would expect that even after 4 hours of incubation some measurable sortilin would be observed, as upon exosome uptake, contained proteomic cargo would be available in the cytoplasm of the cell. It may be possible, however, that exosomes derived from each cell type used do not contain sortilin in the protein form, but may instead carry genetic information involved in the production and expression of sortilin by the cell. As exosomes contain genetic material, it is possible that exosomes derived from cancer cells expressing varying levels of sortilin contain miRNA, mRNA and DNA for the regulation of sortilin in other cell types. This would give reasoning to the apparent gradual rise in sortilin expression in treatment groups, as the turnover duration in time for exosome uptake, transport to the nucleus, transcription and/or translation of genetic information, and finally protein expression would take far longer than the direct uptake of proteomic sortilin into the cell. MiRNAs are involved in the post-transcriptional regulation of genes, playing an important role in the way that protein expression is performed within cells (Catalanotto, Cogoni and Zardo, 2016). Selective packing of miRNA into exosomes for intentional influence of sortilin expression in sortilin knockdown cells would give reason for the expression of proteomic sortilin after 24 hours. Exosome-derived miRNA has been found to aid in the progression of cancer metastasis, and the observed increase in invasion rate of sortilin knockdown cells treated with pSORT exosomes would be explained by an intake of cancerous miRNA to progress the cancer (Bhome et al., 2018).

**Conclusion**

In addition to sortilin, NF-κB 105 kD precursor (p105), and its cleaved p50 kD forms were measured for expression levels over time. The expression of p50 increased over time, with the largest change in expression seen in sortilin knockdown cells treated with wild type CALU-1 exosomes, with expression levels almost doubling between 4 hour and 48 hour treatment incubation periods. In terms of promoting cancer survival and metastasis, NF-κB plays an important role. Cancer cell migration is promoted through NF-κB by raised proliferation and reduced apoptosis, angiogenesis to allow for invasion to distant tissues, and inducing inflammation to promote metastasis (Xia, Shen and Verma, 2014). Mediation of the ubiquitination of p105 generates p50 through KPC1, in which silencing of KPC1 reduces the generation of p50 from p105. There is an inverse relationship in expression levels of NF-κB subunits p50 and p65, in which p50 expression downregulates p65, seen in p50 overexpressing cells which express low levels of p65 (Campagnolo et al., 2014). The link between inflammation and carcinogenesis is well established, with p105 suppressing inflammation.
and p50 promoting, with an increase in sortilin raising the expression of p50 from experimental observation (Yu, Wan and Huang, 2009).

This study carried out provides a wide range of implications for further research in regards to the effect of sortilin uptake through exosomes in lung cancer. The results collected indicate that sortilin plays a role in the invasive behaviour of CALU-1 cells, through the knockdown of sortilin reducing invasiveness compared to the wild type, and a restoration of invasion rate when exposed to exosomes originating from cells overexpressing sortilin. Future research would be required to uncover the mechanisms of this relationship, although it appears to be involved with the way that actin distributes itself within the cell, and additionally with protein pathways and complexes involved in cell proliferation, survival and apoptosis. As a biomarker, sortilin expression would have the potential use in predicting metastatic rates to some degree through its relationship with invasiveness. The findings of genetic sortilin miRNA control through exosomes is of great interest, as it implies that proteomic sortilin expression can be raised in cell types normally under-expressing sortilin, and from a medical point of view, exosomes in body fluid systems such as the circulatory and lymphatic systems can be screened for certain miRNAs known to be involved in sortilin post-transcriptional regulation as potential cancer biomarkers. Additionally, the increase in p50 expression as a result of exosome treatment from pSORT cells provides insight into the potential dual roles in cell survival and death, as sortilin is known for its involvement in the proNGF cell death complex, although the raised levels of p50 indicate a resistance to apoptosis. Further research would be required to identify the specific genetic involvement of exosomes influencing sortilin, and the subsequent protein pathways involved which influence the carcinogenic properties of cancer cells. The ever-expanding role of sortilin and its involvement in exosome-based communication clearly displays a great importance in understanding the mechanisms in which cancer and metastasis operates under.

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