

The Role of Sodium Driven Bicarbonate Transporters in Triple Negative Breast Cancer Metastasis



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1 Abstract

Triple negative breast cancer is the most difficult breast cancer subtype to treat due to; hormonal therapy resistance, regions of hypoxia, propensity to metastasize, and a lack of targeted therapy options. Metastasis is the cause of over 90% of breast tumour deaths. Hypoxic regions are areas of low oxygen and arise in tumours due to a high metabolic and proliferative rate, and aberrant vasculature. Hypoxia induces resistance to chemotherapy and radiotherapy. Hypoxic tumour regions are also generally more acidic, both hypoxia and acidosis induce metastasis. The sodium driven bicarbonate transporter (NDBT) family regulate intracellular pH. Preliminary data has shown that under hypoxic conditions (0.5% O₂) NDBT mRNAs are induced in triple negative breast cancers and knockdown of one of these (*SLC4A9*) or NDBT inhibition reduces migration and invasion *in-vitro*. The aim of this PhD was to investigate targeting NDBTs as a novel mechanism for inhibiting triple negative breast cancer metastasis.

To determine a functional role in breast cancer, generation of validated gene knockdowns of NDBTs was undertaken and these clones were compared to the effects of small molecule inhibitor. NDBT knockdown and inhibition was shown to reduce intracellular pH in normoxia and hypoxia. NDBT knockdown and inhibition reduced migration and invasion *in vitro* and metastasis *in vivo*. The mechanism of action for functional changes by NDBT knockdown were investigated with regards to intracellular signalling. In preliminary research this was primarily undertaken with regards to HIF-1 α . This study expanded on the preliminary research by determining activation status in an array of phospho-tyrosine signalling proteins. NDBT knockdown or inhibition reduced the activation of mTOR, LCK, LYN and many other tyrosine kinases. The reduced mTOR activation in turn resulted in an ablation of HIF-1/2 α stabilization and downstream signalling in hypoxia. Inhibitors against mTOR, LCK, and LYN were used to recapitulate the effects seen by NDBT knockdown but did not recapitulate the effects to the same extent.

Further to this, Epithelial to mesenchymal transition (EMT) status was assessed under hypoxic and acidic conditions to investigate intracellular pH control and EMT signalling. It was shown that knockdown of NDBT perturbed transcription of multiple EMT genes. This perturbation altered EMT protein expression culminating in an increased expression of E-cadherin and lower expression of vimentin by NDBT knockdown or inhibition.

NDBT knockdown or inhibition altered metabolism. Total ATP levels and extracellular lactate levels were reduced by NDBT knockdown or inhibition in normoxia and hypoxia. Furthermore, expression of PKM2 mRNA and protein expression was reduced by NDBT knockdown. Conversely, total mitochondrial mass was increased, but mitochondrial polarity was perturbed resulting in altered mitochondrial morphology by NDBT knockdown in normoxia and hypoxia.

This study presents evidence regarding the role of pH regulatory proteins in tumour metastasis. Accumulating evidence highlights the regulation of signal transduction, EMT, and cellular metabolism in response by acidosis and pH regulatory mechanisms.

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4 List of abbreviations

<u>Abbreviation</u>	<u>Definition</u>
ABCB1	ATP Binding Cassette Subfamily B Member 1
AKT	Protein kinase B
APC	Acid-Polyamine-Organocation
ARG1	Arginase 1
ARNT	Aryl hydrocarbon nuclear translocator
ATP	Adenosine triphosphate
bHLH	Basic helix loop helix
BNIP-3	BCL2/adenovirus E1B
BRCA1	Breast cancer type 1 gene
BRCA2	Breast cancer type 2 gene
BRD	Bromodomain
CAIX	Carbonic anhydrase 9
CD	Cluster of differentiation
CO ₂	Carbon dioxide
COX	Cyclooxygenase
CTAD	C terminus topologically associating domain
DIDs	4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
EPO	Erythropoietin
ER	Oestrogen receptor
ERK	Extracellular Signal-Regulated Kinase
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FIH1	Factor Inhibiting HIF-1

FRET	Fluorescence resonance energy transfer
GLUT-1	Glucose transporter 1
GSC	goosecoid
H ⁺	Hydrogen ion
HCO ₃ ⁻	Bicarbonate
HDAC	Histone deacetylase
HEK293	Human embryonic kidney cells 293
HER2	Erb2 receptor
Hg	Mercury
HIF	Hypoxia inducible factor
HRE	Hypoxic response element
HSP	Heat shock protein
IGF	Insulin like growth factor
IL2 β	Interleukin 2 beta
IL-6	Interleukin 6
LAMP2	Lysosomal-associated membrane protein 2
LAR	Luminal androgen receptor
LDH	actate dehydrogenase
LDHA	Lactate dehydrogenase A
LOX	Lectin-like oxidized low-density lipoprotein receptor-1
LPS	Lipopolysaccharides
MCP-1	Methyl-accepting chemotaxis protein
MCT1	Monocarboxylate transporter 1
MCT4	Monocarboxylate transporter 4
MDCK cells	Multicystic dysplastic kidney cells
MET	Mesenchymal to epithelial transition
MIP-1a	Macrophage Inflammatory Proteins 1a
miRNA	Micro ribonucleic acid
MMP	Matrix metalloprotease
mRNA	Messenger ribonucleic acid
MSL	Mesenchymal stem like
mTOR	Mammalian target of rapamycin

NA ⁺	Sodium
NAD ⁺	nicotinamide adenine dinucleotide
NBCe-1	<i>SLC4A4</i>
NBCe-2	<i>SLC4A5</i>
NBCn-1	<i>SLC4A7</i>
NDBT	Sodium driven bicarbonate transporter
NTAD	N terminus topologically associating domain
O ₂	Oxygen
ODDD	Oxygen dependant degradation domain
OXPHOS	Oxidative phosphorylation
PAI-1	Plasminogen activator inhibitor-1
PAS	Per and sim
PHD	Prolyl hydroxylase domain
PI3K	Phosphoinositide 3-kinase
PK	pharmacokinetics
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
Pyk2	Protein tyrosine kinase 2 beta
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Ser	Serine
SLC	Solute carrier
SLUG	Snail Family Transcriptional Repressor 2
SNAIL	Snail Family Transcriptional Repressor 1
SSAT	Spermidine/spermine N-acetyltransferase
TAD	Trans-activation domain
TCA	Tricarboxylic acid
TM	Transmembrane
TME	Tumour microenvironment
TNBC	Triple negative breast cancer

TNF	Tumour necrosis factor
TWIST	Twist Family BHLH Transcription Factor
VEGF	Vascular endothelial growth factor
VHL	Von hippel lindau protein
ZEB	Zinc Finger E-Box Binding Homeobox

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6 Chapter 1: Introduction

6.1 Cancer

Cancer is a large collection of related diseases characterised by uncontrolled growth and spread to distant tissues. Cancer can arise from a multitude of different cell types¹. Moreover, these can either be solid malignancies or blood borne depending on the origin of the cancer². It is a disease that arises from dynamic changes in the genome. Tumours are large masses of cells that have grown due to a loss of regulatory control of cell division. However, for a tumour to become cancer it must invade the surrounding tissue and then metastasize throughout the body. Cancer arises predominately due to mutations in two types of genes, oncogenes which are dominant gain of function genes; or tumour suppressors with recessive loss of function³.

Evidence has indicated that tumorigenesis is a multistep process and that these steps reflect genetic perturbations that drive transformation of healthy cells into a malignancy⁴. In concordance with this, most cancers are diagnosed with an age dependent incidence rate⁵. Pathological analysis of organ tissues reveal lesions with cells undergoing the immediate steps from healthy to malignancy⁶. Investigations into tumours at differing sites in the body have shown that genetic changes can be as subtle as point mutations or larger in scale; such as chromosome alterations⁷. The majority of deaths from cancer occur due to metastasis rather than tumour burden. Once cancer has entered distant sites, it grows and impairs normal bodily function resulting in death⁸.

6.2 Cancer statistics

Incidence and mortality of cancer is growing worldwide. The risk factors involved are highly complex and reflect not only a growing and aging population but an increase in risk factors associated with socioeconomic development. In 2018, lung was the most commonly diagnosed cancer (11.6%) across both sexes and the leading cause of cancer deaths (18.4%). This was closely followed by female breast cancer and colorectal cancer with an incidence at 11.6% and 10.2% respectively. Mortality was followed by colorectal at 9.2%

and stomach cancer at 8.2%. The most common type among males is lung cancer (14.5%) and among females it is breast (24.2%). Furthermore, breast cancer is also the leading cause of death in females worldwide in 2018 indicating an unmet clinical need (Fig. 6.1) ⁵.

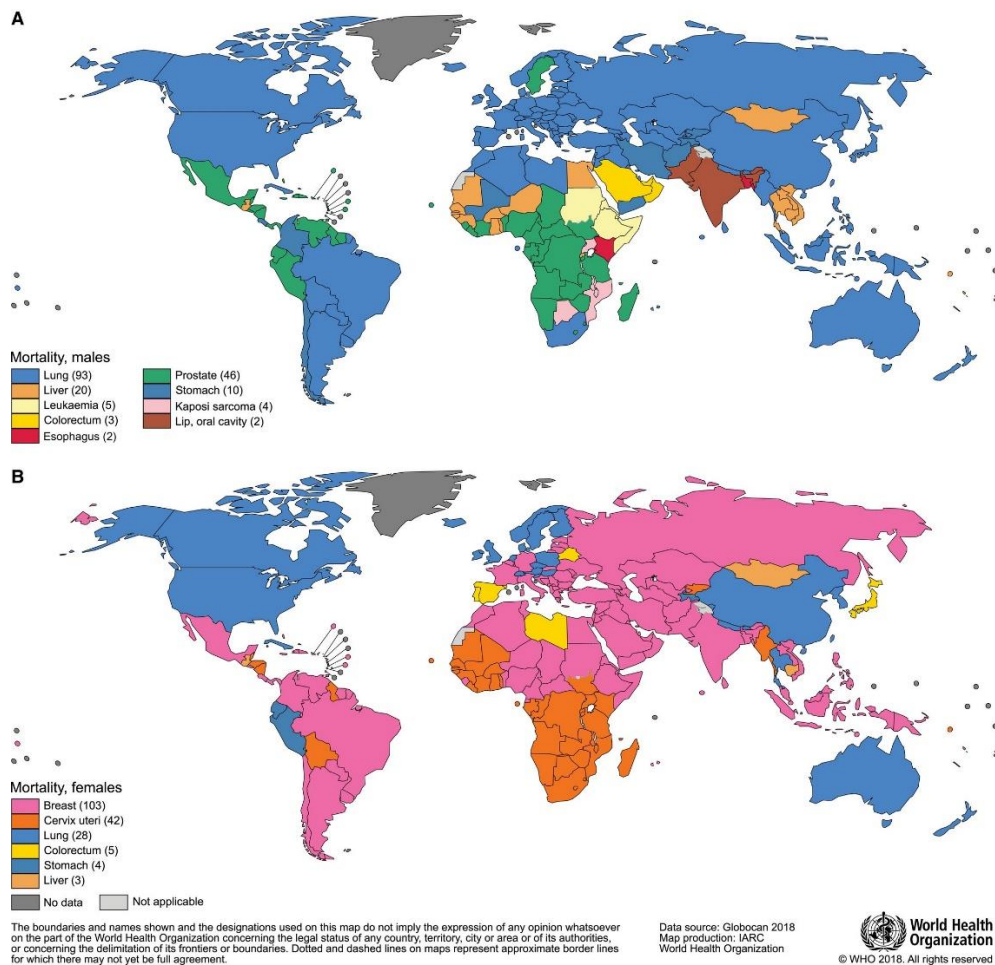


Figure 6.1 Cancer mortality for males (A) and females (B) worldwide in 2018.

Lung cancer has the mortality incidence for males worldwide whereas it is breast cancer for women by country. Cervical cancer is the second most common cancer among females followed by lung and colorectal.

Environmental risk factors

Whilst genetic factors play a role in determining risks for specific cancers many of these predispositions are unavoidable. Lifestyle and environmental factors however, can influence incidence of many types of cancer and are avoidable⁹. These risk factors can be attributed to nine potential modifiable risk factors.

Body weight, particularly excess body weight is a leading modifiable risk cancer worldwide. The risk of cancer increases when BMI (m/kg^2) is above 21. Obesity has been shown to be linked to a plethora of different cancers and risk increases as body weight increases¹⁰. Furthermore, a low fruit and vegetable intake in the diet is another risk factor for cancer. Consumption of less than 50g of fruit or vegetables each day increases the risk of: colorectal, stomach, lung, and oesophageal cancer¹¹. Additionally, physical activity levels are attributable to differing levels of cancer risk. Inactivity or insufficient activity (less than 2.5 h moderate activity a week) increased the risk factors of: breast cancer, prostate cancer, and colorectal cancer. These risk factors are interlinked i.e. someone with a high BMI is likely to be less physically active and thus further increase their risk factors of multiple cancer types¹².

Use of substances such as tobacco is another avoidable risk factor impacting cancer incidence and mortality. Smoking has been a known risk factor for lung cancer for many decades. Ultimately no smoking levels are safe and exposure to second hand smoke increases risk of cancer incidence. In addition to increasing the risk for lung cancer; smoking increases the risk for following cancers: (1) mouth, (2) oesophageal, (3) stomach, (4) liver, (5) and pancreatic cancer⁹. Similarly consumption of alcohol increases the risk of many cancer types. The safest consumption levels are expected to be zero although consumption has been shown to be beneficial in some diseases. This is due to the burden of alcohol use based on its neurophysiological diseases and injuries are greater than its potential benefits for specific diseases. Furthermore, no reduced risks have been seen with alcohol consumption for neoplastic disease. Moreover excessive consumption of alcohol results in increased incidence of: liver, mouth, breast and oesophageal cancers¹³.

Another avoidable risk factor for cancer risk is HPV infection from unsafe sex. Sexual transmission of HPV leads to persistent infection with oncogenic type of virus and as such results in increased risk for cervical cancer. Vaccinations for HPV at a young age are now

common practice in developed nations, however, HPV infection remains a major risk factor for women in the poorest regions¹⁴.

Environmental factors also contribute to increased incidence for many different cancer types. Urban air pollution, from particulate matter and car exhaust with a size less than 2.5 microns increase the incidence of lung cancer when exposure is higher than $7.5\mu\text{g}/\text{m}^3$ a year. Furthermore when particulate matter is less than 10 microns exposures of $15\mu\text{g}/\text{m}^3$ a year increases the risk of lung cancer¹⁵. Another environmental factor is indoor smoke exposure from the burning of solid fuels. Many lower economically developed countries use coal burners inside the home with poor ventilation resulting in increased exposure to particulate matter. Incidence of lung cancer is increased in homes with these burners¹⁶. The final avoidable risk factor is contaminated injections resulting in exposure to hepatitis B or hepatitis C. Exposure to any contaminated injection increases the risk of liver cancer due to the oncogenic effects of the hepatitis viruses¹⁷.

Implementation of many screening and preventative measures could significantly reduce incidence of cancer. However, many of these factors are socioeconomic in origin and thus implementation of policies regarding this will only be successful if they are accessible and used. These factors are reliant on cost and health system setup therefore limiting large scale intervention in less developed countries. Current preventative measures in developing countries such as improved HPV vaccination rates and use of healthy eating/exercise campaigns will likely reduce the incidence of some cancers in the future.

6.3 Genetic risk factors

With many types of cancer clustering within families, it was predicted that inherited alleles could result in a predisposition to develop cancers. Approximately 5 -10% of all cancers arise from this group of people¹⁸. The classic example of a heritable cancer susceptibility is the bilateral retinoblastoma resulting from mutations in RB1¹⁹. Currently, mutations for more than 50 hereditary cancer syndromes have been discovered²⁰. People carrying mutations for hereditary cancer syndromes are more likely to develop cancer at an early age and are additionally more likely to develop multiple tumours²¹.

In most hereditary syndromes the increased cancer risk is due to a single mutation in one gene²². These genes are commonly involved in cell cycle checkpoints or DNA damage

repair. As hereditary mutations are germline, it explains why those with these syndromes develop cancer earlier than the general public. Familial breast cancer arises from mutations in either BRCA1 or BRCA2 genes and affects approximately 1:1,000 women²³. Other examples of hereditary syndromes are: Hereditary nonpolyposis colorectal cancer, Neurofibromatosis type 1, and Von Hippel-Lindau disease²⁴.

6.4 Oncogenes

Proto-oncogenes are genes involved in normal cellular homeostasis that, when mutated, contribute to tumour progression and survival. In particular, proto-oncogenes regulate pathways involved in survival and proliferation³. However, cells have many intrinsic antineoplastic countermeasures to control expression and activation of these genes. Most commonly, overexpression of proto-oncogenes trigger apoptosis or senescence to prevent the cell from becoming cancerous²⁵. Alternatively some oncogenes don't have intrinsic mitogenic activity such as those that are anti-apoptotic. Thus, some oncogenes require other cooperating oncogenes to drive neoplastic transformation²⁶.

Whilst an oncogene has the capacity to regulate tumour progression in the context of aberrant expression or activation there are many diverse mechanisms of oncogene activation. These mechanisms include: overexpression, expression of mutant protein with increased stability or function, altered recruitment, altered subcellular localisation²⁷. Carcinomas often have aneuploidy and harbour many genetic mutations which means elucidating the original oncogenic mutations can be difficult²⁸. However others follow a clear stepwise progression and can be said to be attributed to the effects of a single oncogene. Thus some cancer therapeutics aim to target these oncogenes²⁹.

The classical example of an oncogene is MYC. Deregulation of MYC is one of the most frequently encountered mutations in carcinogenesis and is implicated in half of all malignancies³⁰. Mitogenic events induce signal transduction pathways that converge on MYC. The MYC gene promoter is bound by many transcription factors and the downstream targets of MYC are involved in many aspects of cellular physiology; however proliferation features most prominently³¹. With regards to proliferation MYC activates transcription of D-type cyclins and CDKs to promote cell cycle check point progression whilst also downregulating the expression of CIP/KIP family of cyclin/CDK inhibitors³². Uncontrolled

MYC activity is a potent oncogenic stimulus, requiring stringent regulation from transcriptional control to protein stability³³.

6.5 Tumour suppressors

Tumour suppressors were named so because many tumours develop in their absence. The most well-known tumour suppressor is P53 which is mutated in about 50% of all cancers and 20% in breast cancer³⁴. Tumour suppressors were shown experimentally to prevent neoplastic transformation. They are now known to have diverse functions in regulation, such as: growth, division, and genome integrity. Many tumour suppressor genes play crucial roles during development³⁵. Studies have shown in many cases that one copy of a tumour suppressor gene is sufficient to control growth and thus both alleles must be mutated or silenced to suppress function³⁶.

Tumour suppressor genes can be broadly classified into the following groups: (1) genes that control progression through cell cycle stages, (2) Inhibitors of cellular proliferation, (3) checkpoint inhibitors in response to DNA damage, (4) apoptosis inducers, and (5) DNA repair machinery.

As somatic inactivation of one allele is usually compatible with normal development, most hereditary cancer syndromes involve inactivation of tumour suppressor genes³⁷. Many tumour suppressors are inactivated by DNA methylation, however numerous alternative mechanisms have been described such as: proteasomal degradation, abnormal cellular location, and transcriptional regulation³⁸.

Due to the frequency of mutation in cancer, P53 is arguably the most crucial tumour suppressor and is known as the guardian of the genome. Upon cellular stress, P53 is activated by a phosphorylation cascade or other postranslational modifications resulting in the expression of target genes controlling cell cycle arrest, DNA repair, or apoptosis³⁹. P53 target genes have important roles in angiogenesis, senescence, and autophagy⁴⁰. The P53 signalling pathway is highly dynamic and connected to many others such as the mTOR pathway allowing for P53 to also be involved in the control of many cell phenotypes⁴¹. Expression of P53 is strictly controlled by the protein MDM2. MDM2 is an E3 ubiquitin ligase that signals for the degradation of P53. MDM2 and P53 form a negative-feedback loop, in which P53 induces the expression of MDM2, which in turn promotes the

degradation of P53 and quenches cellular P53 activity⁴². The most common P53 mutations occur in the DNA binding domain. These mutations can be subdivided into either contact mutations that remove essential DNA interaction sites or structural mutations that perturb the structure of the DBD, thereby reducing its kinetic and thermodynamic stability resulting in a loss of transcriptional activity⁴³.

6.6 Hallmarks of cancer

The hallmarks of cancer are a set of capabilities acquired by a cell during carcinogenesis. They rationalise the functions of many mutations in neoplastic disease (Fig. 6.2). Tumours are more than insular masses of proliferating cancer cells, they are composed of many cell types that interact. Many normal cells from the tumour associated stroma are known to contribute to these hallmarks. Thus, the role of cancer traits must be understood with the contribution of the tumour microenvironment in mind. The hallmarks of cancer are as follows:

6.6.1 Sustained proliferative signalling

The first and most fundamental trait of cancer cells is sustained proliferative signalling. Normal tissues stringently regulate the release of growth signals thereby controlling entry into the cell cycle and maintaining tissue architecture and homeostasis⁴⁴. Understanding the mechanisms by which mitogenic signals are released in normal tissues is poorly understood; but it is thought to be spatially and temporally regulated. Cancer cells by dysregulating these signals become detached from the normal growth inhibition⁴⁵. Cancer cells can acquire the ability to sustain proliferative signalling in numerous ways. They may produce the growth factors themselves, or they may stimulate normal cells to produce these mitogenic factors⁴⁶. Further methods by which the cancer cell can avoid this are by upregulating the levels of growth factor receptors rendering the cell more sensitive and responsive to growth factors. Alternatively, mutations in some receptors can result in ligand independent signalling⁴⁷. Many cases of downstream signalling pathways being mutated for constitutive activation result in bypassing the need for growth factor activation altogether. High throughput sequencing revealed that mutations can predict constitutive activation of certain signalling pathways. In melanoma 50% of cases contain B-Raf

mutations, resulting in constitutive activation of MAPK pathway signalling⁴⁸. In other tumour types, mutations in PI3k is frequently mutated into a hyperactive form. Further to this, its transducer AKT is also frequently mutated in addition to PI3k⁴⁹. The advantages of activation of upstream versus downstream signalling remain to be elucidated as does the impact of crosstalk between pathways emanating from the same growth factor receptors. However, alterations to the negative feedback loops in proliferative signalling are also common in cancer. This is commonly seen with RAS. The oncogenic effects of RAS do not occur due to hyper activation but due to a loss of the negative feedback messenger RAS GTPase, such that normally transient signalling is sustained⁵⁰.

6.6.2 Ability to evade growth suppressors

The second hallmark is the ability to evade growth suppressors. Cancer cells must circumvent the programs that negatively regulate proliferation; many of these pathways depend on the actions of tumour suppressor genes. Many of the tumour suppressor genes operate as larger networks wired to operate with functional redundancy. The cells which have mutations in tumour suppressor genes are able to ignore signals which stop entry into the cell cycle. These are most notably controlled by the tumour suppressor genes RB1 and TP53⁵¹. Within normal tissue, multiple anti proliferative signals function to maintain homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells⁵². Similarly to the pro-growth signals they bind and activate transmembrane proteins. Antigrowth signals can inhibit growth by forcing the cell into a G0 state or the cell could permanently be forced into a state of post mitotic stress and unable to replicate⁵³.

6.6.3 Ability to avoid apoptosis

The third hallmark is the ability to avoid apoptosis. Apoptosis is a barrier to carcinogenesis and can be triggered by many of the physiological stresses that a cancer cell may encounter. A common example would be the triggering of apoptosis by elevated oncogenic signalling or DNA damage⁵⁴. Many sensors which trigger apoptosis have been identified. The most common method by which cells evade apoptosis is loss of P53 function⁵⁵.

Alternatively, higher expression of anti-apoptotic proteins can enable the cell to halt apoptosis induction⁵⁶. Further to this cancer cells can alter cellular machinery involved in autophagy. Autophagy has pro-tumour and antitumor functions depending on cellular context and has crosstalk with apoptosis inducing pathways. By altering autophagy the cell can enhance survival when stressed⁵⁷.

6.6.4 Replicative immortality

Another hallmark is replicative immortality. Cancer cells have unlimited replicative potential whereas normal cells are constrained by a limited number of divisions. This limitation is normally associated with senescence⁵⁸. Evidence indicates that telomeres are involved in the potential for unlimited replication. Telomerase adds telomere repeat segments to the ends of DNA and is functionally absent in normal cells but is expressed highly in immortalised cells. By extending the length of the telomere the cancer cell is able to counter the loss of DNA that would otherwise occur⁵⁹.

6.6.5 Induction of angiogenesis

Induction of angiogenesis is the next hallmark. Similar to normal tissues, tumours require nutrients and oxygen for survival, as well as the ability to vent waste products. The tumour produces neovasculature to address this issue. Angiogenesis is controlled by a switch of opposing factors which either induce or halt angiogenesis⁶⁰. Some of these molecules are signalling proteins which bind to surface receptors displayed by endothelial cells. The most well-known molecules are the inducer VEGF-A and inhibitor thrombospondin 1. VEGF gene expression can be upregulated by both hypoxia and oncogenic signalling and the VEGF ligand acts upon three receptors VEGFR-1-3 indicating that regulation of angiogenesis is highly regulated at multiple levels and complex⁶¹. The blood vessels produced by tumours are commonly aberrant and prone to leakage and collapse. Angiogenesis is induced early on in tumour development⁶⁰. Many tumours display gradients of vascularisation with some areas being highly avascular whereas others are better vascularised⁶². It has been shown that many immune cells contribute to angiogenesis within the tumour⁶³.

6.6.6 Invasion and metastasis

The sixth hallmark of cancer is activation of invasion and metastasis. The multistep progress of metastasis has been rationalised as a sequence described as the metastatic cascade. EMT is a primary implicated mechanism by which transformed cells acquire the ability to invade and disseminate⁶⁴. By co-opting the process of embryogenic morphogenesis and wound healing cancer cells can acquire attributes enabling invasion. A set of pleotropic transcription factors orchestrate EMT such as Snail, Twist, and ZEB⁶⁵. Further to this, the loss of E-cadherin in epithelial cells and expression of vimentin have become markers of EMT⁶⁶. Cells that lose expression of E-cadherin can be free from being adherent to epithelial sheets and escape quiescence. Several of the EMT transcription factors actively repress the expression of E-cadherin. It is apparent that cross talk between the stroma and the cancerous cells is involved in the acquisition of such traits. Some mesenchymal stem cells can secrete factors which stimulate invasive behaviour⁶⁷. Macrophages can secrete matrix degrading enzymes. Indicating that the invasive phenotype does not occur in a cell autonomous manner. As the EMT process is inducible it implies it is also reversible once the cells have colonised the distant site. This plasticity may result in the formation of new tumour colonies⁶⁸. Furthermore, some tumour cells may only enter EMT partially thereby acquiring mesenchymal traits but still continuing to express epithelial markers⁶⁹. In addition, to the EMT program other distinct methods of invasiveness has been described. Collective invasion which involves the nodules of cancer cells migrating en-masse into adjacent tissues⁷⁰. Amoeboid invasion which involves morphological plasticity and the cells then slither through existing spaces in the extracellular matrix rather than making a passage for itself⁷¹. It is not conclusively determined if cells employing the previous types of invasion also employ components of the EMT programme.

6.6.7 Genomic instability and mutation

The next hallmark is genomic instability and mutation. The acquisition of traits above require the alterations in the genomes to produce these traits. Some mutated genotypes confer a survival advantage to neoplastic cells. Thus, tumorigenesis can be seen as multiple rounds of clonal expansion triggered by a cell gaining a new mutation and therefore a survival advantage⁷². Additionally, as some heritable phenotypes can be acquired by

epigenetic modifications some clonal expansions may be triggered by non-mutational changes⁷³. The high fidelity of DNA maintenance and repair systems normally ensures mutation rates are very low. During the course of carcinogenesis, tumour cells increase the frequency of mutation⁷⁴. This is achieved by increasing sensitivity to mutagens, or the inactivation of repair machinery. To amplify mutations further, many components of the DNA maintenance systems are compromised. Mutations in many of these proteins have been well documented. The loss of telomere DNA in many tumours can generate genomic instability and amplifications or deletions of chromosomes⁵⁹. Whereas the specifics of genome alterations vary dramatically between tumours; the large number of defects in DNA repair defects and thus destabilisation of gene copy number and nucleotide sequence, indicate that instability of the genome is inherent to many cancers⁷⁵.

6.6.8 Inflammation

Tumour inflammation is another hallmark of cancer. Pathological evidence has long shown that many tumours are infiltrated by immune cells. Both innate and adaptive arms of the immune system have been shown to be present. These infiltrations can range from subtle requiring highly specific/affinity antibodies, to gross inflammations, seen with basic histological techniques. Initially it was assumed that the immune response was only concerned with removing the lesion and in many cases evidence supports this idea⁷⁶. However, paradoxical evidence is emerging that inflammation can facilitate tumour progression, largely by the innate immune system. Inflammation can help support tumorigenesis by supplying molecules such as growth factors, angiogenic factors or MMPs⁷⁷. Furthermore, some immune cells can release chemicals such as ROS which are actively mutagenic for nearby cancer cells enhancing the speed at which traits may be acquired⁷⁸.

6.6.9 Deregulated metabolism

The penultimate hallmark is deregulated energy metabolism. Higher proliferative rates require high levels of cellular energy metabolism to sustain growth and division. Under normal conditions cells produce the majority of their energy by aerobic respiration via the mitochondria. However, in cancer cells there is a metabolic shift towards glycolysis termed the Warburg effect⁷⁹. While initially counterintuitive; this shift results in the production of

many by-products which can then be used for biosynthesis pathways⁸⁰. Glycolytic fuelling has been shown to be associated with many oncogenes⁸¹. This reliance on glycolysis can be further increased by hypoxic conditions which result in the upregulation of many glycolysis genes⁸².

6.6.10 Immune evasion

The final hallmark of cancer is evasion of immune destruction. The immune surveillance theory proposes that the immune system is ever vigilant and destroys incipient cancer cells. This theory is given weight by the increase in cancers of people with a compromised immune system⁸³. In genetically engineered mice, tumours grew faster in immune compromised mice than the controls⁸⁴. Many tumour cells have defective expression of MHC proteins as a method of evading immune control⁸⁵. Furthermore, many cancer secrete immunosuppressive factors to paralyse the adaptive immune response⁸⁶. Thus anti-tumour immunity is a significant barrier to carcinogenesis and by evading immune response cancer can propagate.



Figure 6.2 The hallmarks of cancer.

Multiple different hallmarks have been described to explain the common phenotypes exhibited by cancer cells to enable them to grow and survive. A huge number of different mutations can occur during carcinogenesis, however all these mutations can be explained by these hallmarks. Tumour promoting inflammation, avoiding immune destruction, genome instability, deregulating cellular energetics are the newest hallmarks to be added to the list. (Adapted from Hallmarks of cancer, 2011)

6.7 Breast cancer

Breast cancer is a complex and heterogeneous disease. Breast cancer is the most common female cancer in the UK accounting for 15.4% of all cancers; followed by lung 12.5%, and colorectal 11.6% in 2015⁸⁷. A total of 55,222 new cases were diagnosed in females in 2014 accounting for 31% of all female cancers. Likewise, worldwide breast cancer is the most common female cancer representing 12% of all new diagnoses. Similarly to other types of cancer, incidence correlates with age with the majority of breast cancers being diagnosed in patients over 70²³. Breast cancers can develop in different areas of the breast: such as the ducts, the lobules, or the tissue in between. Within the large group of breast carcinomas, there are various types of breast cancer denoted on their invasiveness relative to the primary tumour sites.

Age remains the largest risk factor associated with breast cancer. In addition, many other unchangeable factors can also contribute; however 27% of all breast cancer cases are due to preventable causes. Risk factors include: excessive alcohol consumption, Diethylstilbestrol exposure, contraceptive use, radiation exposure, obesity, and adult height. Hormone levels are a significant risk factor associated with breast cancer. It is thought many risk factors associated with breast cancer act via altering endogenous levels of hormones in the body. Those with the highest oestrogen levels are twice as likely to develop breast cancer than those with the lowest⁸⁸.

6.7.1 Subtypes of Breast cancer

Prior to the use of molecular profiling, taxonomy for breast cancer was based upon the use of morphological observations. Classification relied upon the usage of: histological type, tumour stage, lymph node status, and presence of receptors such as; oestrogen receptor (ER), progesterone receptor (PR), Erb2 receptor (HER2) to make treatment decisions. Receptor status still determines treatment regimens due to some subtypes being sensitive to target therapies. Oestrogen positive tumours can be treated with anti-hormonal therapy and carry the highest survival rates. Herceptin is a monoclonal antibody that targets the HER2 receptor. HER2 is amplified in 30% of all breast cancers. By binding to the HER2 receptor, it inhibits cells that express this receptor. The addition of herceptin to a

chemotherapy, as first-line therapy for metastatic breast cancer overexpressing the HER2 receptor, significantly increased time to disease progression, rate of objective response, duration of response and survival compared with chemotherapy alone⁸⁹.

DNA sequencing analysis has led to the creation of a molecular profile classification system with 5 distinct subtypes of breast cancer. These subtypes are; Luminal A, Luminal B, Basal, Claudin low, HER2⁹⁰. More recently, single cell RNA sequencing has revealed common signatures within the tumour as well as intertumoral heterogeneity regarding breast cancer subtype and crucial cancer-related pathways. This facilitated the creation of more complex molecular subtyping of breast cancers. Most of the non-tumour cells in the tumour were immune cells from three classes, T cells, B cells, and macrophages⁹¹. Data showed that aneuploid rearrangements occur early in tumour evolution and remained stable during clonal expansion. In contrast, point mutations evolved gradually, generating extensive diversity. Many of these diverse mutations were shown to occur at low frequencies in the tumour. It was shown that triple-negative tumour cells had an increased mutation rate (13.3X) while the ER+ tumour cells did not⁹². Owing to the high levels of heterogeneity within breast cancer further molecular analysis of breast cancers is likely to result in the creation of new molecular subtypes. Furthermore, breast cancer sequencing projects have been undertaken resulting in the generation of more subtypes of breast cancer.

6.7.2 Triple negative breast cancer subtype

The triple negative breast cancer subtype consists of tumours which fall into either basal or claudin low molecular subtypes^{93,94}. Likewise, TNBC display a wide range of mutations but lack the receptors for oestrogen, progesterone, or HER2. Due to a lack of these receptors there is a shortage of targeted therapies for this group. Thus treatment of triple negative breast cancers is more challenging. Currently, standard neoadjuvant chemotherapy (based on stage and grade), radiotherapy, and surgical resection are the only available treatment modality⁹⁵. Approximately 20% of breast cancers are triple negative⁹⁶. In addition, TNBC typically present late, are more hypoxic (low oxygen <2% O₂) and metastatic; therefore the prognosis for patients is poor⁹⁷. Typically, TNBC metastasise with 3 years of diagnosis or are diagnosed with metastasis. Whereas other breast cancers typically metastasize to the bone⁹⁸, TNBC typically metastasises to the brain and central nervous system⁹⁹.

Molecularly *TP53*, *Rb*, and *PTEN* are the most commonly mutated genes in TNBC with *TP53* occurring in 53-85% of cases. There is a well-established correlation between BRCA1 deletions and the risk of developing TNBC, with lifetime risks reaching as high as 50%. BRCA1 encodes an E3 ubiquitin ligase essential for homologous recombination mediated-repair of DNA double-strand breaks. However, unlike ER and HER2 breast cancers there is an overall lack of common set of mutations driving TNBC. Hence molecular targeting is difficult.

Further molecular subtyping has been undertaken on TNBCs to help determine therapeutic strategies¹⁰⁰. Survival rates for triple negative breast cancer are 77% compared with 93% for other cancers when detected at stage 3. Survival rates drop to 20% for those diagnosed with TNBC at stage 4. Recurrence rates are also higher for those with TNBC than other molecular subtypes⁹⁹. TNBC are more resistant to chemotherapy due to numerous different mechanisms. Copy number alterations such as MCL1, MYC and JAK2, as well as PTEN deletions or mutations have all been identified at a higher frequency in residual disease. Additionally, the high genomic instability and sub clonal nature of driver mutations in TNBC increase resistance¹⁰¹.

The first major TNBC genome array project identified 6 molecular subgroups⁹⁰. Basal like 1, which contain elevated levels of cell cycle and DNA damage response gene expression. Tumours of this type are more sensitive to genotoxic chemotherapy. Furthermore, they are the most proliferative molecular subtype¹⁰². Basal like 2 have an enrichment for growth factor signalling and myoepithelial markers. Additionally, they show alterations in glycolysis and gluconeogenesis. Inhibitors for many growth factor receptors would likely be the best molecularly targeted therapy for this subtype. BL2 subtype had the lowest pathological complete response to standard chemotherapy. The third subtype, Immunomodulatory contains tumour cells which have perturbed expression of antigens and cytokines as well as changes in immune signal transduction pathways. The mesenchymal phenotype contain increased expression of genes involved in EMT and growth factor pathways. The mesenchymal subtype is enriched for genes associated with related to cell motility and differentiation. Mesenchymal subtypes are sensitive to mTOR inhibition and has higher rates of PI3K mutations. Similarly to the mesenchymal phenotype the mesenchymal stem like phenotype has increased expression of EMT genes and growth factor pathways however, MSL type has decreased expression of proliferation genes¹⁰³. The subtype LAR is comprised of tumours which are driven by androgen receptor and express luminal genes.

LAR is the least proliferative and most chemo resistant subtype¹⁰². Basal like 1 and 2, immunomodulatory, and mesenchymal types fit within the basal like molecular classification of breast cancers whereas mesenchymal stem like was more closely related to the claudin low subtype¹⁰⁴.

The claudin low subtype has a worse overall survival when compared to patients with luminal A type subtype. Claudin low subtype was associated with young age of onset, higher tumour grade, larger tumour size, extensive lymphocytic infiltration⁹⁴.

6.8 Hypoxia

6.8.1 Oxygenation in normal and tumour tissue

Hypoxia is a low concentration of oxygen. The presence of hypoxia within tumours was first proposed by Thomlinson and Gray in 1955¹⁰⁵. They based this on observation of histological sections whereby the distribution of necrosis was reduced relative to the distance of blood vessels. Within tissues this is generally defined as levels less than $pO_2 < 9$ mmHg or $< 1\% O_2$ ¹⁰⁶. Oxygen levels within the body can vary widely depending upon tissue type and conditions¹⁰⁷. Within normal breast tissues, pO_2 measurements are around 65mm Hg with no area of the breast tissue dropping below 12.5 mm Hg. Within breast cancers the mean pO_2 was 10 mm Hg with areas within many of the tumours containing areas with between zero and 2.5 mm Hg¹⁰⁸. Within many types of solid tumours, hypoxia occurs frequently with greater than 50% of breast tumours displaying areas of hypoxia when measured using differing methods for detecting hypoxia such as pO_2 electrode or binding of pimonidazole¹⁰⁹. The development of tumour hypoxia is independent of clinical characteristics, e.g. clinical size, stage, histology, and grade at diagnosis¹⁰⁷. Multiple studies have shown a link between tumour hypoxia and lower disease-free survival, increased metastasis, and radioresistance¹¹⁰.

Within tumours, hypoxia arises due to a combination of multiple factors each compounding each other¹¹¹. Tumour cells have higher metabolic and proliferative rates resulting in a higher oxygen demand and increased distance from blood vessels¹¹². The vasculature structure within a tumour is aberrant, with many leaky and collapsed vessels. As oxygen delivery to cells is diffusion limited, many tumour cells in turn become hypoxic; with cells further away from the vasculature generally becoming the most hypoxic. As some vessels within the tumour are leaky and prone to collapse hypoxia is not only a chronic condition; it but acute as well, with the possibility of cells adjacent to a vessel becoming temporarily hypoxic¹¹³. Regions of necrosis within advanced tumours reflect the end results of cells which have been exposed to severe hypoxia for extended periods of time and failed to adapt¹¹⁴.

It has been demonstrated that hypoxia in tumours tends to select for a more malignant phenotype, and increases DNA mutation rates¹¹⁵. Tumour hypoxia also increases

chromosomal instability in addition to increasing DNA mutations¹¹⁶. Hypoxia results in oncogene amplification increasing signalling for cellular proliferation. This increase in oncogenic signalling in turn results in replication stresses, increasing replication errors and DNA damage¹¹⁷. Additionally, hypoxia can alter the expression levels of proteins involved in apoptosis allowing the cancer cells continue to proliferate, with what would otherwise be apoptosis inducing DNA damage⁵⁵. Hypoxia results in the downregulation of DNA repair genes particularly those involved in: homologous recombination, non-homologous end joining, and mismatch repair; resulting in any mutations more likely to go unrepaired¹¹⁸. Furthermore, if the cells are re-oxygenated a sudden burst of free radicals can also damage the DNA inducing more mutations¹¹⁹.

Hypoxia selects for a more malignant phenotype as hypoxic signalling results in: increases in expression of genes associated with angiogenesis, tumour invasion, and therapy resistance¹²⁰. Ultimately, this culminates in a more metastatic phenotype of human cancers. By enhancing metastasis, hypoxia can reduce the effectiveness of removing tumours by surgery as many cells have already disseminated. Therefore, hypoxia has a negative role in tumour prognosis, both because it causes resistance to chemotherapy and because it promotes a more aggressive phenotype. However, the very low levels of oxygen and the presence of necrosis are unique features of solid tumours¹²¹. Necrosis and hypoxia are not commonly seen in healthy tissue, with the exception of hypoxia being present in the bone marrow and colonic crypts, thus hypoxia presents a possible therapeutic avenue for exploitation¹²². These clear links between hypoxia and therapeutic resistance highlight the need for further investigation into this field.

6.8.2 Hypoxia Inducible Factors (HIFs)

Hypoxia results in large and rapid transcriptomic changes within the cell. These changes occur primarily via the stabilization and activation of Hypoxia Inducible Factor (HIF) transcription factors¹²³. HIFs are responsible for the regulation of over 100s of genes, both directly and indirectly¹²⁴. Many of these genes influence the hallmarks of cancer such as: metabolic changes, angiogenesis, cell growth, differentiation, survival, migration, and invasion. The HIFs target a specific promoter sequence called the hypoxic response element (HRE) found in many genes¹²⁵. Expression of HIF-1 α target genes are induced in

every cell type however, many genes are induced in a cell type specific manner. As hypoxia induces HIFs in many cell types it is likely HIF interaction with other transcription factors and proteins determining which subgroup of genes are activated¹²⁴. Activated HIF plays a crucial role in adaption, for example within a rapidly growing tumour HIF-1 activation facilitates the switch to aerobic glycolysis via glucose pathway activation to ensure energy production is maintained¹²³.

HIFs are heterodimeric transcription factors family consisting of alpha and beta subunits. It is composed of HIF-1 α /HIF-2 α /HIF-3 α ; and HIF-1 β also known as aryl hydrocarbon nuclear translocator (ARNT)¹²⁶. The HIF- α subunits are oxygen sensitive and only stabilized under hypoxic conditions whereas HIF-1 β is constitutively expressed and oxygen insensitive¹²⁷. All these subunits belong to the basic Helix Loop Helix-Per Sim (bHLH-PAS) transcription factor family based on their structure¹²⁸. The bHLH transcription factors are characterised by having regions capable of self-regulating expression as well as the expression of other transcription factors¹²⁹. The basic region was found to have the DNA binding properties required to bind to the HRE whereas the helix loop helix domain facilitates dimerization with ARNT¹³⁰.

The three HIF α analogues contain multiple functional domains¹²⁶. All HIF α subunits are distinct from ARNT via their inclusion of an oxygen dependant degradation domain (ODDD), this domain is the mediator of oxygen based protein stability. HIF-1 α and HIF-2 α contain two transactivation domains (TAD) required for transcriptional activity, and are structurally similar (Fig. 4, 5)¹³¹. The C terminal domain binds to coactivators such as p-300 in hypoxic conditions whereas the N terminal domain aids in protein stability in hypoxia¹³². Unlike HIF-3 α , HIF-1 α and HIF-2 α share a high degree (48%) of homology (Fig. 3)¹³³. This high level of similarity likely explains their similar mechanisms of stabilization and binding to HREs. However, they show different tissue distribution patterns whereas HIF-1 α is ubiquitously expressed throughout tissues HIF-2 α is tissue specific¹³⁴. In tissues where both are expressed under hypoxic conditions there it was predicted that competitive binding occurs; however HIF-1 α is biased towards promoter proximal binding whereas HIF-2 α is biased towards promoter-distant binding¹³⁵. All HIFs contain nuclear localisation signals and do not require dimerization with ARNT to enter the nucleus¹³⁶. HIF-1 α protein production was shown to be dependent on the PI3k-AKT-mTOR pathway. The 5' untranslated region of HIF-1 α mRNA contains a 5' -terminal oligopolypyrimidine tract which regulates the translational initiation of HIF-1 α in response to mTOR activation. Therefore, it has been shown that

activation of mTOR signalling stimulates the translation of HIF-1 α ¹³⁷. This translation and stabilization also occurs in normoxic conditions if the mTOR pathway is stimulated by various growth factors (EGF, FGF 2, IGF) ¹³⁸ and cytokines (IL2 β)¹³⁹.

HIF-3 α is unique among the HIF- α in that it is alternatively spliced into at least six isoforms due to the utilization of different promoters, different transcription initiation sites, and alternative splicing¹⁴⁰. HIF-3 α has been shown to be regulated by HIF-1 α and competitively bind to HIF-1 α gene targets¹⁴¹. Due to the lack of the C terminal TAD all but the full length isoform HIF-3 α is unlikely to act as an efficient transcription factor¹⁴². Instead of the C-TAD HIF3 has a leucine zipper domain. Furthermore, FIH regulation of HIFs binds at the CTAD and is therefore unlikely to be involved in HIF3a regulation. Some short HIF-3 α variants act as dominant-negative regulators of HIF-1/2 α actions, other HIF-3 α variants can inhibit HIF-1/2 α actions by competing for the common HIF-1 β .

HIF-1 β is the binding partner of the HIF α subunits. It is a member of the bHLH pas proteins. ARNT is the common binding partner for Aryl hydrocarbon receptor (AHR) as well as HIF-1 α . It is constitutively expressed and neither the mRNA or protein level is influenced by oxygen tension. ARNT is composed of a bHLH domain required for DNA binding, two PAS domains for dimerization and one transactivation domain. In addition, ARNT has a nuclear localization signal¹⁴³.

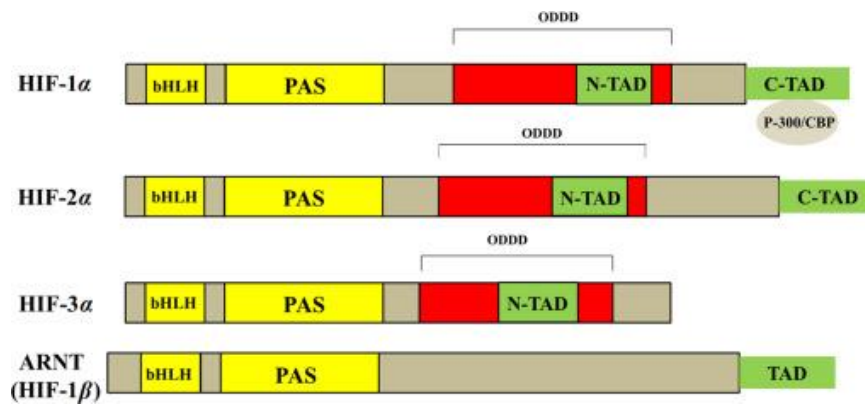


Figure 6.3 Structure and domains of the HIF transcription factor members.

There is a large amount of similarity between the members. All HIF members contain a: basic helix loop helix (bHLH) domain, Per-Arnt-Sim (PAS). All of the oxygen dependant domains contain a trans-activation domain. Only HIF3 doesn't contain a C-TAD. Hif-1 β does not contain an oxygen dependant degradation domain and is constitutively expressed in cells.

HIF α regulation is primarily controlled by the PHD proteins (Fig. 6.3). Under normal oxygen conditions HIF α subunits are hydroxylated by PHD proteins. Hydroxylation can occur at one or both highly conserved proline residues located near the N terminal transactivation domain¹⁴⁴. Hydroxylation of these residues provides a binding site for the Von Hippel Lindau (VHL) protein (Fig. 6.4). This ubiquitin ligase poly-ubiquitinates HIF α resulting in degradation via the proteasome complex¹⁴⁵. It is the PHD proteins which provide the oxygen sensitive aspect to HIF stabilisation. PHD proteins are members of Fe (II) and 2-oxoglutarate-dependent oxygenase superfamily whose enzymatic activity cannot function without oxygen as a cofactor¹²³. Furthermore the rate of HIF α hydroxylation is dependent on oxygen concentration, under hypoxia the PHD proteins cannot function and HIF α protein levels accumulate. Likewise HIF α protein levels can accumulate in normoxic conditions if the tumour is deficient in VHL protein, this is commonly seen in renal cancers which are highly HIF dependant¹⁴⁶.

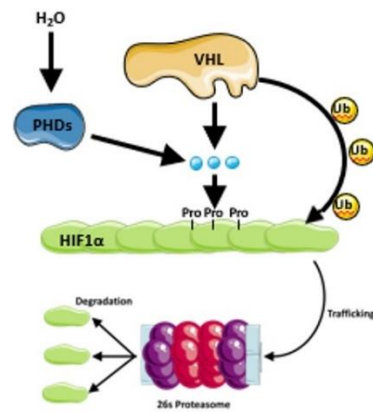
In addition to PHD, FIH1 also plays a role in the regulation of HIF α in an oxygen dependant manner (Fig. 6.4). FIH1 is also an Fe(II)⁺ and 2-oxoglutarate-dependent dioxygenase¹⁴⁷. Under oxygenated conditions it hydroxylates a conserved asparagine residue located near the C terminus transactivation domain. The FIH1 proteins are active at lower oxygen concentrations than the PHDs and thus may stop HIF activation at moderately hypoxic levels¹⁴⁸. Asparagine hydroxylation results in steric clash inhibiting the binding of P300 and CBP resulting in a halt in transcriptional activation¹³². HIF-2 α is relatively more resistant to FIH1 hydroxylation¹⁴⁹. Furthermore, hypoxic induction of HIF responsive genes may be more dependent on CTAD or NTAD requirements at the HRE and binding partners to determine if HIF-1 α or HIF-2 α transcribes the gene¹⁵⁰.

It is known that other proteins can contribute to the stabilization of HIF-1 α (Fig. 6.5). Heat shock protein 90 (HSP90) binds to HIF-1 α to stabilize it. Cells expressing the inactive unphosphorylated form of HSP90 β had a significantly delayed hypoxic response¹⁵¹. The PI3K/AKT pathway is also required for HSP90 to stabilize HIF-1 α ¹⁵². Treatment of cells with Geldanamycin, a HSP90 antagonist resulted in HIF-1 α destabilization in VHL negative cells. These data indicate HSP90 may be involved in blocking HIF-1 α from an oxygen insensitive degradation pathway¹⁵³. Rack1 another HIF-1 α binding protein competes with HSP90 for binding to HIF-1 α . RACK1 competes with HSP90 for binding to the PAS domain of HIF-1 α . HIF-1 α degradation induced by the HSP90 inhibitor 17-allylaminogeldanamycin is abolished by RACK1 loss-of-function. RACK1 binds to Elongin-C and promotes ubiquitination of HIF-

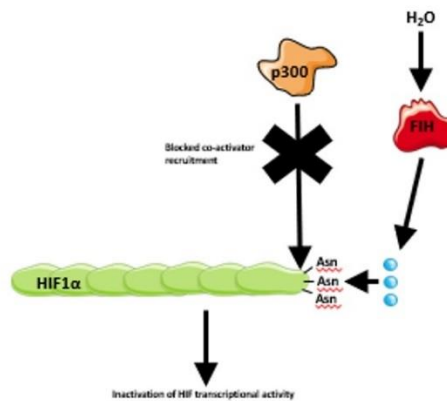
1 α . RACK1 promotes PHD/VHL-independent proteasomal degradation of HIF-1 α ¹⁵⁴. Other proteins also aid the degradation by binding to the elongin-C degradation complex. SSAT2 (spermidine/spermine N-acetyltransferase-2) binds to HIF-1 α and promotes its ubiquitination/degradation by stabilizing the interaction of VHL and Elongin C. Additionally SSAT1 also binds and works in a complementary role as SSAT2 in degrading HIF-1 α ¹⁵⁵.

1. Normoxic conditions

a. PHD mediated degradation



b. FIH mediated inhibition



2. Hypoxic conditions

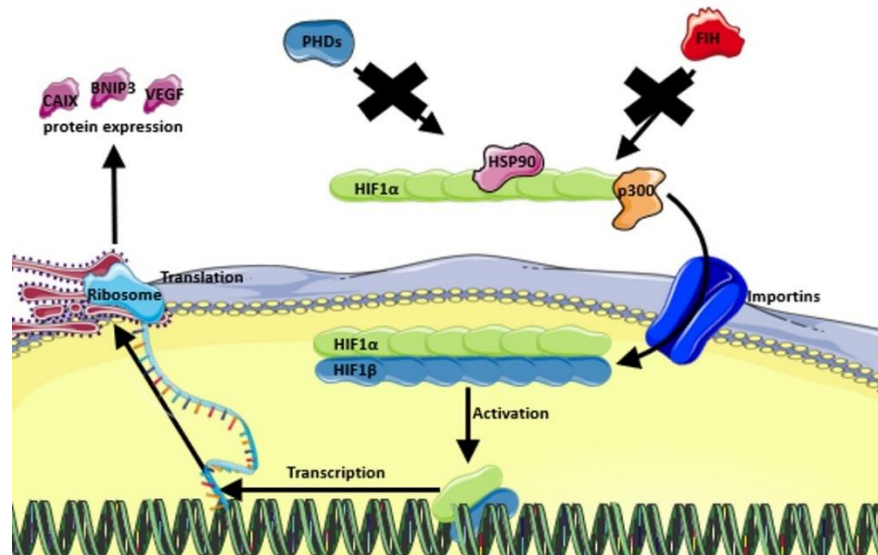
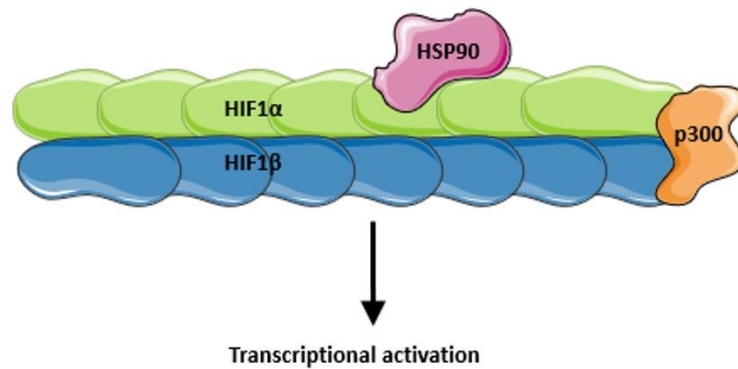


Figure 6.4 Stabilization of HIF transcription factor is dependent on oxygen.

Under oxygenated conditions PHD proteins use oxygen as a cofactor to hydroxylate proline residues on HIF oxygen dependant degradation domains. These hydroxyl moieties are then recognised by VHL which ubiquitinates HIF sending it for proteasome destruction. Alternatively FIH like PHD uses oxygen to add hydroxyl moieties to the c terminus of HIF. This blocks recruitment of p300 and decreases protein stability. Under hypoxic conditions both PHD and FIH are unable to function and thus coactivators bind to HIF resulting in nuclear translocation. Once inside the nucleus it dimerises with HIF-1 β and activates transcription.

O₂-independent Rack1 degradation of HIF1

A. Presence of HSP90



B. Inhibition of HSP90

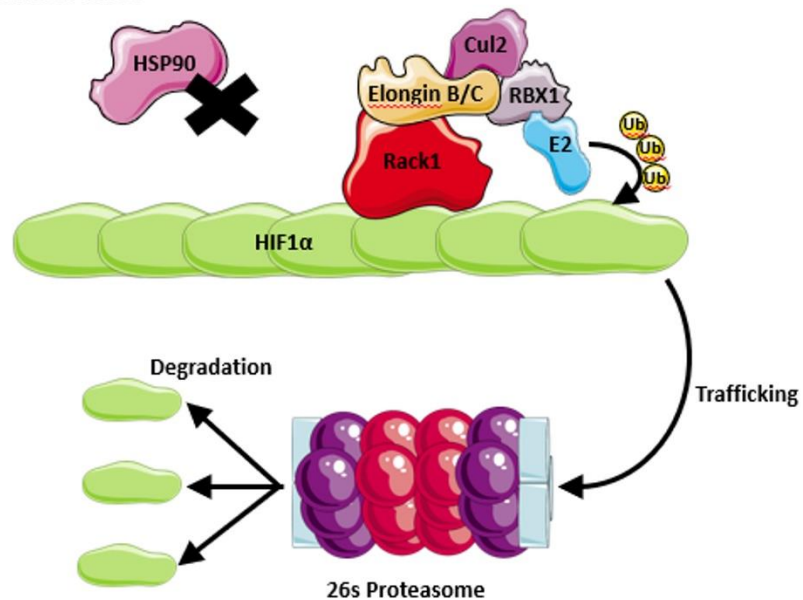


Figure 6.5 Degradation of HIF-1α by RACK1.

Degradation of HIF-1α can occur through other methods besides PHD proteins. Inhibition of HSP90 is permissive to the binding of Rack1 to HIF-1α. Once bound a number of other proteins form a complex that culminates in the ubiquitination of HIF-1α and it being sent to the 26S proteasome for degradation.

Under hypoxic conditions the stabilization of HIF α results in the translocation into the nucleus via importin α 1, α 3, α 5, and α 7¹⁵⁶. The NLS signal on the C terminal is more effective whereas the N terminal NLS was shown to be ineffective. Once inside the nucleus HIF α binds to its cofactors P300/CBP and dimerises with ARNT. The HIF transcription factor recognises the HREs in gene promoters' with the sequence RCGTG (R denotes either Adenosine or Guanidine)¹⁵⁷.

All chromosomes contain at least some hypoxia regulated genes with chromosomes 1, 2, and 17 containing the highest at 11%, 8%, and 6% respectively; indicating a strong evolutionary requirement for HIF signalling¹⁵⁸. Multiple studies into hypoxia have discovered a set of well characterised hypoxia regulated genes in many cancers¹⁵⁹. These include: CAIX¹⁶⁰, VEGF¹⁶¹, EPO¹⁶², PAI-1¹⁶³, LOX¹⁶⁴, GLUT1¹⁶⁵, BNIP3¹⁶⁶, and LDHA¹⁶⁷; with CAIX being one of the most commonly and highly upregulated in cancer. CAIX is commonly used as a positive hypoxia marker in studies of hypoxia induced effects, however no single marker can be universally used¹⁶⁸. For example, CAIX is only transcriptionally regulated by HIF-1 α not HIF-2 α ¹⁶⁹. Additionally, CAIX is also upregulated in acidic conditions independently of hypoxic conditions¹⁷⁰. Furthermore, based on chromatin accessibility some cells do not express CAIX in hypoxia even if HIF signalling is ongoing¹⁷¹.

Several studies have used gene expression arrays and RNA seq experiments to define a hypoxic signature. This approach investigated the signature in 2D using established cancer cell lines. A comparison of the 32 signatures generated so far revealed no single gene in common between all of them¹²¹. This could reflect the heterogeneity in the tumour samples used, as some were taken from different tumour types. Moreover, many signatures derived from cell lines when tested as normoxic vs hypoxic conditions might not be translatable as the transcriptional response of a cell line *in-vitro* culture might be partially an artefact and unrepresentative of the hypoxic phenotype observed in tumours. Many of these genes regulated by hypoxia can be classified by biological processes. The majority of hypoxia regulated genes are involved in cell metabolism, followed by gene transcription¹⁷². While the majority of these studies focused on the genes up-regulated in hypoxia via HIF-1 α ; there are also many genes which are repressed in hypoxia. Multiple signature arrays show as many genes are down regulated in hypoxia as are upregulated¹⁷³. Conversely, hypoxia downregulates genes primarily involved in cell metabolism and transcription. The mechanism controlling gene repression in hypoxia is not well understood. Many repressors in hypoxia don't contain any HIF enrichment sequences and

so are more likely to be regulated by other mechanisms¹⁷⁴. However one protein REST has been shown to bind to 20% of the promoters of hypoxia downregulated genes¹⁷².

Many hypoxia signatures have been exploited for therapeutic use¹⁷⁵. They have been used to generate a prognosis for a patient and also the prediction of therapeutic benefit from addition of hypoxia modifying agents to the use of radiotherapy¹⁷⁶. One study used four immortalised cell lines to produce an hypoxia signature from cells subjected to 0% and 2% oxygen. Patients with tumours characterised by expression of this signature had significantly decreased relapse-free and overall survival, which was maintained after adjustment for clinical covariates¹⁷⁷. Treatment stratification has been shown to be successful at improving patient outcome. They randomly assigned patients to nimorazole treatment or a placebo. They retrospectively found that those with their hypoxia gene signature benefitted from treatment with nimorazole¹⁷⁸.

6.8.3 Hypoxia and therapeutic approaches

Hypoxia is well known to be strongly correlated with treatment challenges in multiple cancer types. Resistance has been attributed to multiple mechanisms. Some of these challenges include: (1) reduction of oxygen radical formation resulting in less effective radiotherapy, (2) alterations in tumour microenvironment, (3) clonal selection for resistant phenotypes.

Hypoxic tumour cells have a decreased sensitivity for radiation¹⁷⁹. Oxygen plays a substantial role in radiotherapy, with oxygenated regions of tumour responding three fold better than hypoxic (<2% O₂) segments. This effect is known clinically as the Oxygen Enhancement Ratio, and oxygenated tumours prove much easier to treat than their hypoxic counterparts¹⁸⁰.

Radiotherapy functions via damaging the tumour cells DNA causing strand breaks. Double strand breaks have been shown to be the mechanism for lethality¹⁸¹. Radiation can also cause single strand breaks however, these are much more effectively repaired by the cell¹⁸². Higher doses of radiation cause more double strand breaks¹⁸³. DNA damage occurs either via direct ionization of the DNA causing a break or indirectly via the ionization of water. Once water is ionized it results in the formation of radicals, such as the hydroxyl radical which subsequently react with the DNA causing damage¹⁸⁴. As hypoxic tumours lack

oxygen and the mechanism of radiation induced cell killing is via reactive oxygen species it is therefore clear why hypoxic tumours are more resistant.

After the initial discovery that hypoxia reduced the effectiveness of the radiotherapy, hyperbaric oxygen treatment clinical trials were undertaken. The treatment modality was based on the hypothesis that if they increased the oxygen consumed it would result in higher levels in the blood which would then lead to an oxygenated tumour and increased cell kill. These trials resulted in little overall increase in efficacy as they failed to account for the tumour microenvironment¹⁸⁵. Tumour hypoxia can also be acute rather than solely a chronic phenomenon. The collapse of a vessel due to the leaky vasculature can result in a spike in hypoxia and a reduction in radiotherapy effectiveness. These trials were abandoned shortly afterwards and other angiogenic therapy attempted to produce a result similar which also failed. Treatments like bevacizumab attempted to inhibit angiogenesis to starve the tumour, however, in some instances resulted in a normalization of the tumour vasculature such that radiotherapy and chemotherapy was more effective. However, only 30% of patients responded and the therapy was effective for only a limited number of weeks due to tumours adapting quickly and becoming resistant to the treatment¹⁸⁶.

The oxygen fixation hypothesis has been proposed to explain how oxygen results in such an increased radiotherapy efficacy. Hydroxyl radicals are common and the cell does have the ability to repair damaged caused¹⁸⁷. However, if the hydroxyl radical reacts with oxygen it forms a peroxy radical. This radical permanently damages the DNA and fixes it in a damaged state. Therefore the theory states oxygen sensitises cells to therapy because the radicals formed in the presence of oxygen are far more difficult to repair¹⁸⁸.

An alternative hypothesis for oxygen's role in radiation induced DNA damage has been proposed. In this hypothesis oxygen acts in the level of a free radical. Ionizing radiation induces the double strand break, either directly or indirectly through radicals. Oxygen then oxidise the DNA radicals making the damage permanent. Alternatively in the absence of oxygen the DNA radicals are reduced by sulfhydryl group containing proteins which restore the DNA to its original form¹⁸⁹. Irradiation of tumors induces HIF-1 activity, resulting in the expression of vascular endothelial growth factor and basic fibroblast growth factor (bFGF), which act to prevent radiation-induced endothelial cell death¹⁹⁰.

To compound the issues of treating hypoxic cells with radiation evidence shows that hypoxia induces changes in DNA damage repair pathways and cell survival pathways

leading to an increasingly radioresistant phenotype¹⁹¹. HIF-1 α expression in tumour cells have been linked to poor prognosis for radiotherapy outcome. Knockdown studies on HIF-1 α resulted in reduced growth of tumour cells after radiation treatment when compared to HIF-1 α expressing counterparts¹⁹².

Radiotherapy is currently given as a fractionated treatment i.e. split into smaller daily treatments as opposed to a large single dose of radiation. This treatment modality was chosen primarily because it reduced the toxic effects seen on healthy cells. However the 5 R's theory can explain why treatment is given as fraction: (1) Repair, (2) Redistribution, (3) Reoxygenation, (4) Repopulation, (5) Radio sensitivity. Repair of sublethal damage is required for survival of the cells from radiotherapy and allow healthy cells to survive. Fractionation allows for the potentially lethal damage to accumulate until it is lethal. Depending on the phase of the cell cycle the cells can be more or less sensitive to treatment. During fractionations the cells can move to more sensitive stages of the cell cycle such as M and G2 phases. Hypoxic cells get reoxygenated during a fractionated treatment of radiotherapy making them more susceptible to treatment¹⁹⁰. Repopulation can occur if the fractionation time is longer than the cancer cell doubling time. Radiosensitivity is the final R and states that some cell types are intrinsically more sensitive to radiotherapy. Cancer cells have a greater reproductive capacity and therefore are more likely to be sensitive to the effects of radiation treatment. Thus by fractionating treatment cancer cell killing can be maximised such that the likelihood of resistance is reduced.

Hypoxic tumour cells are resistant to chemotherapy for numerous reasons¹⁹³. Firstly they are the most distant from the blood vessels. This limits drug delivery to the tumour cells and can also result in perfusion limited cell killing. Therefore many drugs become ineffective due to the fact the agent isn't delivered to the target¹⁹⁴. The high toxicity of many chemotherapeutics means that side effects are the limiting factor when determining dose and therefore failure to deliver the drug correctly results in treatment failures. Secondly, hypoxic cells are less proliferative; an effect at least in part caused by reduced ATP availability. Chemotherapy kills the most highly proliferative cells and therefore the reduction in proliferation results in a less effective treatment modality¹⁹⁵. Hypoxia in many cases can induce cellular senescence in cancer cells and many cancer therapeutics are rendered ineffective¹⁹⁶. Thirdly, hypoxia selects for cells which are no longer sensitive to TP53 mediated apoptosis, lessening the sensitivity to some anticancer agents. Many chemotherapeutic agents are DNA damaging agents and TP53 initiates an apoptotic

cascade when the DNA is rendered unrepairable. Cells without this protein are therefore less likely to undergo apoptosis¹⁹⁷. Additionally, the mechanism of some classes of anticancer agents resembles that of radiation in that oxygen increases the cytotoxicity of the DNA lesions they cause. Finally hypoxia is known to upregulate multiple genes known to increase drug resistance such as P-Glycoprotein¹⁹⁸ or ABCB1¹²⁰. Cells expressing high levels of these proteins effectively pump out the drug leaving them sequestered in the extracellular matrix or vented in a blood vessel such that they then target other cells in the body.

Hypoxia activated prodrugs have been designed as a potential solution to some of the issues discussed above (Fig. 6.6). Hypoxia activated prodrugs are inactive compounds that are reduced multiple times by endogenously expressed oxidoreductases, resulting in the generation of an anti-cancer therapeutic. The compounds are produced by the addition of a bio reductive group that ablates the anticancer effect but is released upon reduction and fragmentation of the compound (fig. 6.6). A range of chemical moieties have been identified as usable for the generation of hypoxia activated prodrugs¹⁹⁹. These prodrugs would provide a more targeted treatment to standard chemotherapy as adverse side effects are high. Hypoxic regions are not common in normal tissues and therefore the tumour microenvironment could be exploited for therapeutic gain. If these agents are combined with radiotherapy, the hypoxic prodrugs would target the hypoxic regions and the radiation would target the oxygenated regions resulted in full tumour cell killing.

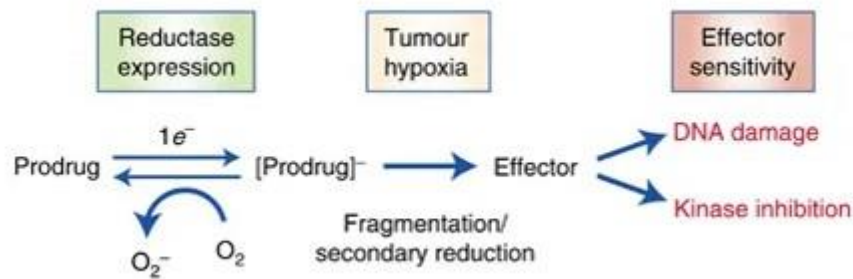


Figure 6.6 Activation of Hypoxia activated Prodrugs.

Reductase activity in hypoxic cells reduces the prodrug inside the cell producing an unstable prodrug fragmenting into a functionally active drug which subsequently inhibits a target or induces DNA damage. By requiring the expression of a reductase hypoxia activated prodrugs avoid activation in normoxic tissue and therefore the common side effects of drugs on normal tissue.

6.9 pH regulation

6.9.1 Introduction

It was assumed that as a direct result of the metabolic shift of the Warburg effect the tumour microenvironment would become more acidic and consequently tumour cells would be more acidic intracellularly (Fig. 6.7)²⁰⁰. Probes for pH indicated that the extracellular tumour environment was indeed acidic. However, many of these probes were larger than a single cell and unable to be used determine the intracellular pH²⁰¹. Recently research has identified alternative methods for measuring intracellular pH. Measurements by ³¹P-MRS indicate that intracellular tumour pH is slightly alkaline²⁰².

Whilst it is clear that the Warburg effect; increased glycolysis, increased glucose uptake, and lactate production even in normoxic conditions produces conditions likely to create an acidic tumour microenvironment²⁰³. This occurs via the conversion of pyruvate to lactic acid and produces a source of H⁺ ions in cancer cells²⁰⁴. There are multiple other metabolic pathways whereby other acidity can arise from and many of these pathways are upregulated in cancer. The pentose phosphate pathway for example converts 1 glucose-6-phosphate to 1 fructose 6-phosphate produces 3CO₂ and 6H⁺²⁰⁵. The pentose phosphate pathway is up-regulated in cancer, and stabilization of HIF-1α increases expression of genes involved in this pathway²⁰⁶. The Krebs cycle which while reduced still takes place in tumours also produces acidity as it produces 6CO₂ for each molecule of glucose generated, this CO₂ then is catalysed to H⁺ ions²⁰⁷. Fatty acid synthesis of palmitate results in the production of 7H⁺ per molecule of palmitate. Fatty acid synthesis is increased in cancer and closely linked to malignancy²⁰⁸. As tumour stage and grade increase hypoxia occurs, as the tumour reaches the more metastatic stages pH of the tumour microenvironment is reduced further (Fig. 7.7).

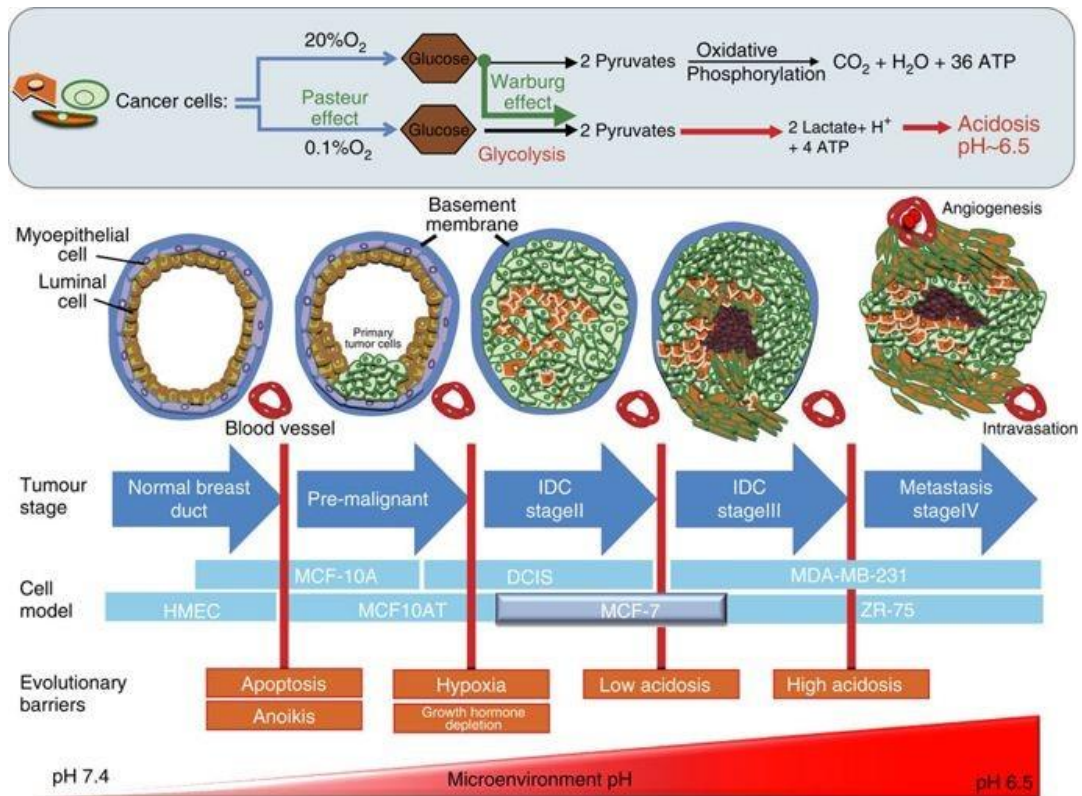


Figure 6.7 Cellular acidity and hypoxia increases with tumour stage and grade.

The generation of high levels of ATP by glycolysis results in the production of lactate and hydrogen ions as by-products and the acidification of the cell. As the tumour grows larger and its vasculature is insufficient for acid venting the tumour becomes acidic. The decrease in microenvironment pH is correlated to tumour stage and grade, and presence of hypoxia. The late stage tumours more frequently have areas of low pH which promotes the local invasion and dissemination of tumour cells which potentiating the formation of metastatic lesions at distant sites. (Adapted from Gillies., et al, 2015)

Despite this increased acidity production, tumour cells maintain a relatively alkaline intracellular pH (7.2-7.4)²⁰⁹. This indicated an increase in pH regulating and sensing proteins to maintain the pH. Many proteins are involved in pH sensing, some G-protein-coupled receptors are sensitive to cellular pH²¹⁰. For example, ORG1 is completely inactive at pH 7.4 but at pH 6.8 is functional. This pH sensing occurs via protonation of extracellular histidine residues. This activation in acidic conditions results in the formation of inositol phosphate, this increases phospholipase IP3 signalling, which can induce Ca^{2+} release from intracellular stores. Following this, ERK is activated by phosphorylation; ERK forms the major signalling route for acidosis induced CAIX activation²¹¹. Additionally, membrane potential changes induced by Ca^{2+} regulate many critical roles in the cell including cell cycle proliferation and pH regulation²¹². Additionally, there are acid sensing ion channels (ASIC). These channels open in response to an acidic extracellular pH. This opening allows the non-selective passage of ions (K^+ , Na^+ , Ca^{2+}) through the membrane. ASIC1 and ASIC3 knockdown has been shown to reduce tumour cell growth and reduced EMT induction in pancreatic cancer²¹³.

Multiple signalling pathways have been implicated in the response to acidosis, particularly signalling through p38²¹⁴. In another study p38 was rapidly phosphorylated in all cell lines tested and ERK1/2 phosphorylation was induced in half of cell lines. They surmised that acidosis induced ROS resulting in MAPK signalling activation and CREB activation via p38. Scavenging of ROS inhibited the activation of MAPK signalling²¹⁵. Furthermore, another study indicated that inhibition of ERK lead to a lack of induction of p-glycoprotein in acidosis and induced drug sensitivity. However, it is important to note that many of these experiments were undertaken at short time points and may be descriptive of the short term response to acidosis. Inhibition of ERK1/2 phosphorylation during acidosis led to necrotic cell death²¹⁶.

The HIFs are also stabilized by an acidic extracellular pH, even in normoxic conditions indicating a role for HIF in pH regulation. This stabilization occurs via sequestration of VHL ubiquitin ligase in the nucleus and therefore HIF is not hydroxylated²¹⁷. This leads to metabolic pathway rewiring induced by HIF transcriptional activity. CAIX is a main downstream target of HIF and regulates intracellular and extracellular pH. It was discovered that this upregulation of CAIX during acidosis can also occur independently of HIF but inhibition of the MAPK/PI3K pathway results in total suppression of expression¹⁷⁰.

6.9.2 Acidosis

Whilst all the aforementioned proteins work in effect to sense changes in acidity, multiple families of proteins are involved in the neutralisation of acid or its export outside the cell. There are five families involved in the mechanisms of pH regulation: monocarboxylate transporters²¹⁸, the sodium hydrogen ion exchangers²¹⁹, the vacuolar-type H⁺-adenosine triphosphatases²²⁰, the carbonic anhydrases²²¹, and the sodium driven bicarbonate transporters²²². Acidification of the tumour microenvironment shouldn't be viewed as a collateral event of tumour growth but a driver of tumour progression. The initial changes in tumour progression producing higher acidity cannot be tolerated by the intracellular machinery as this reduces activity and as such the tumour microenvironment becomes acidified. H⁺ ions directly ionise some amino acids in proteins resulting in function abnormalities and potentially death if not removed.

Monocarboxylate transporters (MCTs) facilitate the hydrogen ion linked transport across the plasma membrane along with L-lactate, ketone bodies, or pyruvate. They are part of a large super family of transporters. Both MCT1 and MCT4 have been shown to be increased in hypoxia in different cancers²²³. MCT4 is upregulated in a HIF-1 α manner²²⁴. Whereas MCT4 is P53 dependent MCT1 isn't²²⁵. MCTs facilitate the transport of their substrates in a unidirectional manner along a concentration gradient and can result in influx or efflux depending the concentration gradients of lactate or hydrogen ions²²⁶. The sodium hydrogen ion exchangers (NHEs) also regulate pH in cells, which NHE1 being the most extensively studied member of this family. NHE extrudes one proton for each sodium ion imported²²⁷. HIF-1 α regulates the induction of NHE1²²⁸. NHE1 has been implicated in migration, invasion, and proliferation of gastric cancer cells²²⁹. V-ATPase is a multi-subunit proton pump. It translocates protons into the extracellular space and also into intracellular compartments. Inhibition of v-ATPase results in an increase in intracellular pH and stabilization of HIF-1 α . Defective endo-lysosomal pH impairs receptor-mediated endocytosis of the transferrin receptor, which leads to cell-wide iron deprivation. Iron serves as co-factor for PHD and as such loss of iron inhibits activity and stabilises HIF²³⁰. The carbonic anhydrases; specifically carbonic anhydrase 9 & 12 are membrane bound enzyme that reversibly catalyse the hydration of CO₂ with water to produce HCO₃⁻ and H⁺. Both are upregulated in hypoxia²³¹. CA9 is HIF-1 α but not HIF-2 α regulated²³². CA9 has been shown to be important in many aspects of carcinogenesis. Knockdown or inhibition of CA9 reduces

migration²³³, invasion²³⁴, metastasis²³⁵, spheroid growth²³⁶, and survival²³⁷. In mammalian cells an increase of pH_i by 0.2 to 0.3 units promotes cell proliferation²³⁸. Whereas a decreased pH results in increased apoptosis, in part by the activation of pro-apoptotic caspases, cytochrome c and BAX.

6.9.3 Lactate acidosis

Multiple metabolic abnormalities commonly occur in cancer. With many cancer cells shifting towards a more aerobic glycolysis phenotype this results in large volumes of lactate being produced²⁰³. This shift towards lactate producing metabolic pathways has led to suggestions that this produces a survival advantage particularly to hypoxic conditions. In contrast to normal cells which normally metabolise glucose to pyruvate via glycolysis in the cytosol, and subsequently convert pyruvate to carbon dioxide in the mitochondria for oxidative phosphorylation; under hypoxic conditions; cancerous cells convert pyruvate to lactic acid. Cancerous cells convert pyruvate to lactic acid regardless of the oxygen state of the cell²³⁹. High levels of lactate are indicative of a worse prognosis²⁴⁰.

Lactate was originally thought of as only as a waste product of metabolism but recent research has elucidated a plethora of alternative functions. Lactate can have an immune suppressive function. Lowered extracellular matrix pH by secretion of lactic acid can impede T cell function resulting in an impotent T cell response. In mice studies T cell function was restored by buffering the pH to physiological levels²⁴¹. Acidosis by lactate results in ARG1 expression increases in macrophages, these macrophages then inhibit T cell function²⁴². Lactic acid also inhibits the innate immune through down-regulating the expression of Lipopolysaccharides (LPS) induced genes, delayed LPS-induced phosphorylation of AKT and the degradation of IKB. Lactic acid following LPS stimulation significantly reduced TNF, IL-6, MCP-1, MIP-1a secretion⁷⁹.

Lactate is a key player in angiogenesis. Lactate released from tumour cells stimulates VEGF expression in endothelial cells²⁴³. It can enter into tumour endothelial cells and lactate released from tumour cells via MCT4 is enough to stimulate growth²⁴⁴. Hyaluronan levels are increased by lactate and hyaluronan assists in angiogenesis^{245,246}. Treatment of cells with an LDHA inhibitor greatly reduces angiogenesis²⁴⁷. LDH knockout greatly reduces tumour cell proliferation²⁴⁸

Cell migration is affected by lactate signalling²⁴⁹. Lactate can increase endothelial cell migration²⁵⁰. In glioma cells lactate stimulates the release of transforming growth factor beta 2, which a key regulator of migration. Cell lines treated with lactate increased their migration potential in a dose dependant manner²⁵¹. High levels of lactate correlate with a higher incidence of distant metastasise²⁴⁰. Accumulating pyruvate inhibits the formation of 2-oxoglutarate. This molecule is part of the response for targeting HIF-1 α for degradation via the proteasome. When lactate and thus pyruvate levels increase, HIF-1 α drives angiogenesis via VEGF expression. This has been shown to also function independently of hypoxia. Import of lactate in cervical and colorectal cancer cell lines stabilized HIF-1 α ²⁵².

6.9.4 Carbonic acidosis

Whilst lactate acidosis is more studied, cellular acidity is also carbonic acid derived. Whereas lactate is produced in specific metabolic pathways, carbon dioxide and thus carbonic acid is produced as a by-product of many reactions²⁰⁷. Additionally, carbon dioxide is small enough to diffuse across the membrane should the gradient permit it. Carbonic acid itself only exists for fractions of a second after carbon dioxide dissolves in water²⁰³. Carbon dioxide rapidly dissociates into bicarbonate and hydrogen ion but has a pK_a of 3.9 is well below cytosolic or extracellular pH indicating most of compound exists as CO₂ and H⁺²⁵³.

Glycolysis results in the production of H⁺ ions, while the H⁺ ions generated during the conversion of glucose into pyruvate are consumed when pyruvate is reduced into lactate to regenerate NAD⁺, consumption of glycolysis-derived ATP by various ATPases and kinases leads to the production of H⁺ ions. Thus the pH is lowered. Whereas the glycolytic pathway occurs primarily during hypoxia, mitochondrial oxidation of pyruvate and more generally the metabolic use of the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) can also occur even under limited access to O₂²⁵⁴. Cytochrome C can undergo an isoform switch. This switch from isoform 1 to 4 increases efficiency²⁵⁵. The cells can remodel mitochondrial cristae to increase efficiency during low oxygen OXPHOS²⁵⁶. Acetyl-CoA is produced by oxidative metabolism. This is subsequently hydrated by intracellular carbonic anhydrases to produce bicarbonate and hydrogen ions²⁵⁷. Other metabolic

pathways produce CO₂ by the decarboxylation of metabolic intermediates such as the pentose phosphate pathway and Glutaminolysis²⁰⁶.

Whilst a key H⁺ transcriptional response element hasn't been found thus far, numerous studies have identified HIF-2α as having a prominent role in the adaption to metabolic acidosis²⁵⁸. HIF-1α however, has generally been found to be downregulated in response to acidosis. Acidosis has been found to affect histone deacetylases. Through NAD⁺ dependant increase in SIRT1 and SIRT6 it is possible that the differential HIF isoform expression is seen. In cervical, colon, and pharynx carcinomas HIF-2α activation in acidosis resulted in glutamine metabolism increases and the upregulation of SC-like Na⁺-dependent neutral amino acid transporter 2 and glutaminase 1 in juxtaposition to levels of glutamine synthesis seen at near neutral pH. Glut1, a well characterised HIF-1α target gene was downregulated in comparison²⁵⁸. However, this HIF expression changes may vary by tumour type. HIF-1A was upregulated in acidosis in glioma and HEK293 cells²⁵⁹. These arrays of gene changes are likely to arise from the field of pH sensors. Proteins that function as pH sensors function by protonation at histidine residues resulting in changes in conformation or activity²⁰³. Additionally, decreases in intracellular pH could also affect signalling proteins containing histidine residues. Many glycolytic enzymes are pH sensitive²⁰³. Additionally mutations in protein residues could affect activity. An arginine to histidine substitution in the tetra spanning domain of P53 resulted in a loss of activity at neutral pH²⁶⁰. An acidic environment has been shown to significantly enhance the radiation-induced expression of P53, partly by increasing the formation of P53 and also partly by slowing down the degradation of P53 through inhibiting P53-Mdm2 complex formation²⁶¹. Activation of P53 also aids in cellular adaption to oxidative stress in acidosis. Acidosis increased both glutaminolysis and fatty acid β-oxidation but decreases glutathione synthesis. The acidosis induced activation of P53 enhances pentose phosphate pathway activity by increasing the expression of G6PD and GSL2 genes. These changes all serve to increase NADPH production and counter the increased ROS induced under acidosis as novel glutathione synthesis is perturbed²⁶². Moreover hypoxia and acidosis when combined resulted in an increase in nuclear excision repair genes likely due to the increased damage caused by higher ROS levels²⁶³.

Many studies noticed an increase in invasiveness in tumours with a low pH²⁶⁴. These studies indicated many of the changes in cellular motility observed were due to changes in cytoskeletal dynamics and cell polarity²⁶⁵. Changes in the proteolytic activity of tumour associated macrophages and fibroblasts were also implicated²⁶⁶. In contrast to the tumour

microenvironment, intracellularly an increase in pH is shown to increase migration²¹⁹. Mild acidification has been shown to lead to a remodelling of the extracellular matrix and changes to cell to cell interactions²⁶⁷. Areas with the lowest TME pH are the most aggressive²⁶⁸. Metastatic dissemination was also shown to be driven by acidosis induced VEGF release. VEGF sequestered in the ECM was released during TME remodelling by proteases directly. Increasing VEGF transcription was also reported in acidic areas of human glioma cells implanted in mice²⁶⁹.

Lysosomes have been shown to have a role in the acidosis driven ECM remodelling²⁷⁰. Cathepsin B is one of the major proteases released by lysosomes²⁷¹. It has an acidic pH optimum and targets type IV collagen²⁷². In breast cancer cell lines lysosomal exocytosis was documented to occur through microtubule-dependent anterograde trafficking towards the cell surface²⁷³. LAMP2 was shown to be important in acidosis survival in breast cancer cell lines. The plasma membrane of acidosis-adapted cancer cells, where it was involved in the secretion of large amounts of H⁺ ions through exocytosis of acidic lysosomes²⁷⁴.

Autophagy has also been implicated to have a role in adaption to acidosis. Expression of the autophagy regulator autophagy-related 5 (ATG5) was increased in breast preinvasive cancer cells. MDA-MB-231 exposed to transient but also chronic low pH_e exhibited a similar increase in autophagy markers and was dependent on autophagy for prolonged survival in acidic environments²⁷⁵. ATG5 knockdown reduces melanoma cell survival under acidosis. Acidic pH leads to mTOR inhibition and an associated reduction in glucose and leucine uptake²⁷⁶. Another study using breast cancer cells identified a role for tuberous sclerosis 2, as a major regulator of mTOR complex 1 in sensing acute variations in pH_e through a mechanism involving ERK inhibition²⁷⁷. These studies suggest that autophagy could represent an adaptive mechanism to a restriction in nutrient availability upon cancer cell exposure to an acidic environment.

6.9.5 Protonation as a post translational modification

Posttranslational modification (PTM) by protons can change the charge of amino acid side chains resulting in alterations in conformation and function. Unlike other forms of PTMs protonation is rapid, reversible and does not require an enzyme²⁷⁸. This protonation state regulates the electrostatic energy of interactions on the protein altering binding affinities

of protein-protein, protein-phospholipid, and macromolecular assembly²⁷⁹. Protonation acts as a signalling mechanism inducing dynamic changes in protein localisation, including recruitment of cytosolic proteins to the plasma membrane or intracellular membranes by pH dependent binding to membrane specific phosphor-lipids. Intracellular compartments within the cell all have differing pH as such forcing the protonation state to match this. Whilst there are little structural studies analysis into pH dependant protein DNA binding, given the important of electrostatics in bindings generally the protonation state could also affect the ability of protein-DNA binding²⁷⁹.

The proton is a small molecule but its addition onto a residue increases the charge by one, resulting in the residue becoming a hydrogen bond acceptor to a hydrogen bond donator. Moreover these changes induces by protons are rapid and reversible. However the protonation state of any residue is not uniquely defined. The pH and pKa values together determine the probability or fraction of protein in such a protonation state. This could be view in the same vein as other PTMs; a fraction of the protein could be modified at any one time and this changes rapidly²⁸⁰.

Almost all proteins have titratable groups. Determining which sites are functionally relevant remains a challenge. Identification of a potential site by mass spectrometry does not guarantee any functional relevance. Similarly recent analysis of phosphorylation or acetylation suggested that only a fraction of identified modifiable site have any biologically relevant function. This problem is magnified for protonation as they are much more labile and harder to detect by mass spec or antibodies. Hence large scale proteomic approaches are not appropriate for protonation. To further compound the problems with attempting to study protonation, bioinformatic approaches based on the similarity to pH sensing proteins failed to detect anything meaningful²⁷⁸.

While any titratable site is a potential pH sensor, within physiological ranges there are only a minority that function as such²⁸¹. Histidine is the most appropriate choice as it has a PKa of 6.5 indicating the acid base dissociation is in equilibrium at this pH. Interestingly phosphate groups have a similar PKa value, but most of amino acid side chains have values far from 7²⁸¹. Those residues may still be important but in a cytoplasmic context when the protein environment shifts, desolvation, or local electrostatic potential dramatically shifts the PKa value²⁸¹.

Specificity of pH sensing is achieved by many of the methods mentioned previously. Notably only a small number of residues within a protein titrate within a physiological pH range. Comparing structurally related protein domains in pH sensing reveal many examples of pH specificity²⁸². The pleckstrin homology domain of several guanine nucleotide exchange factors contain several pH sensitive histidine switches for phosphoinositide binding. This specificity arises from the pH sensitive critical histidine residues at phosphoinositide binding sites that confer pH dependant electrostatic interactions with the negatively charged phospholipids. There are also examples of proteins from similar families having opposing responses to changes in physiological pH. The actin related proteins cofilin and twinfilin are activated by increased and decreased pH respectively. The focal adhesion kinase (FAK) and the related member proline rich tyrosine kinase 2 (Pyk2) are structurally similar pH sensory ability. However, increases in phosphorylation of FAK occurs at pH above 7.2 whereas decreases in pH below 7.2 increase the phosphorylation of Pyk2²⁸³. PTM by protons is not just related to activation but can also allosterically change proteins. The Bohr Effect is the classic example of pH driven allosteric protein changes. A less studied regulatory mechanism of protonation is coincidence detection. This mechanism requires multiple inputs or PTMs for a function to be executed²⁸³.

6.10 Metastasis

Metastasis is responsible for the vast majority of breast cancer patient deaths. Whilst breast cancer deaths have been in decline since the 1990s this is primarily due to increased screening resulting in early detection and adjuvant therapy. Nevertheless 30% of those who present with early stage disease will develop metastasis. Breast cancer starts as a localised disease but ultimately can spread to the lymph nodes and bone. At diagnosis the tumour is screened for prognostic markers to assess likelihood to progress or has progressed to a systemic disease. Thus metastasis is seen as a capacity only acquired at the later stages of tumorigenesis.

6.10.1 Stepwise progression

Metastasis is a multistep process encompassing: (1) the infiltration of tumour cells in local tissue, (2) intravasation of tumour cells into the circulatory system, (3) survival in the

circulatory system, (4) extravasation at a distant site, (5) colonisation of new niche. It is a very inefficient process due to the complexity and the number of events that could lead to failure of the cancer cell metastasising (Fig. 6.8).

Hypoxia and acidosis are potent tumour microenvironmental factors involved in metastasis. It has been implicated in nearly every step of the metastatic cascade. HIF protein expression is increased in metastatic tissue than the primary site and HIF-1 α expression enhances metastatic dissemination in pre-clinical models. Studies in animal models have shown that tumour cells can migrate either as a group or as single cells. Single cell migration can occur via mesenchymal migration. To migrate in this manner the cells must first undergo epithelial to mesenchymal transition (EMT).

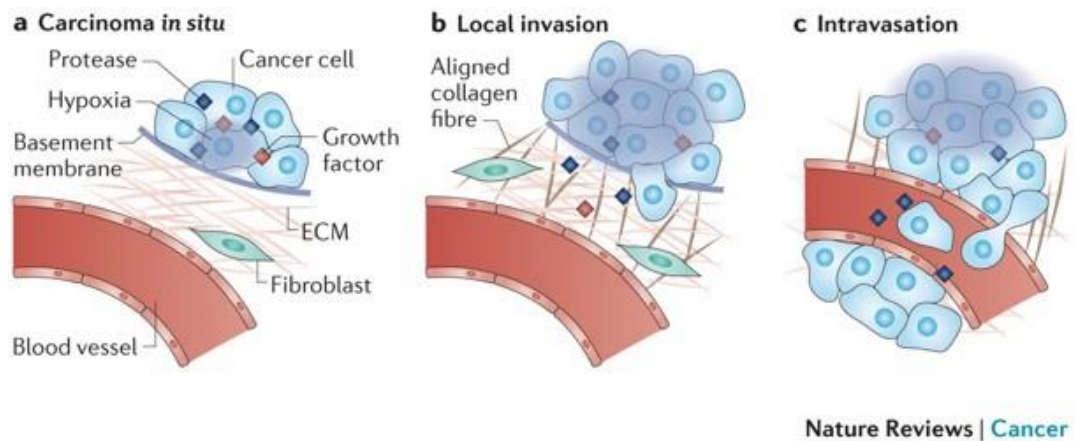


Figure 6.8 metastasis occurs in a stepwise progression.

Initially the carcinoma is in situ with proteases and growth factors localised within the tumours. As the tumour proliferates and outstrips its nutrient supply it becomes more acidic. This acidity activates proteases sequestered in the extracellular matrix facilitating ECM destruction and allowing the tumour to become locally invasive. As the tumour continues to invade it will begin to enter blood vessels and undertake intravasation beginning metastatic dissemination. (Adapted from Carmiet et al., 2011)

6.10.2 Hypoxia and metastasis

Hypoxia has been implicated as aiding metastasis at many points of the metastatic cascade (Fig. 6.9)²⁸⁴. Particularly hypoxia is heavily involved in remodelling the extracellular matrix. Tumour fibrosis is a common phenomenon of tumours²⁸⁵. Tumours are normally stiffer than the surrounding stroma²⁸⁶. It has recently been elucidated that there is a hypoxic specific fibrosis that occurs not just with fibroblasts but other cell types as well²⁸⁷. Furthermore HIF-1 α activation increases the expression of type I collagen²⁸⁸. However, there is evidence the greatest effects of hypoxia are on the post translation modification of collagen²⁸⁹. Reducing the expression of HIF-1 α reduced the expression of collagen modifying enzymes reducing the stiffness of the tumours when injected into mice but no change was seen with HIF-2 α ²⁹⁰. Collagen crosslinking is extracellularly initiated by the lysyl oxidase (LOX) family of secreted enzymes that oxidatively deaminate lysine or hydroxylysine collagen residues are also common downstream targets of HIF-1 α ²⁹¹.

Additionally hypoxia is implicated in the increased expression of MMP2 and MMP9²⁹². Hypoxic cancer cells also show increased proteolytic activity as a result of HIF-dependent increases in their expression of urokinase plasminogen activator surface receptor²⁹³. This protein promotes cell invasion by altering the interactions between integrin and the ECM; and when expression is ablated no intravasion is seen²⁹⁴. Thus, HIFs activate a transcriptional programme that results in the degradation of the basement membrane while simultaneously increasing synthesis of fibrillary collagens to function as a physical pathway for tumour invasion. Hypoxic signalling rapidly leads to the recruitment of macrophages and alters their transcription²⁹⁵. HIF-1 α knockdown mice have decreased myeloid cell infiltration in their tumours²⁹⁶. One mechanism for this attraction of macrophages is the secretion of semaphoring a. Once in the region of hypoxia, macrophages stimulate fibrosis by producing growth factors such as TGF α , TGF β 1, VEGF, FGF, PDGF, tumour necrosis factor- α (TNF α), interleukin-1 (IL-1)²⁹⁷. Furthermore they directly promote intravasion into blood vessels. Tumour associated macrophages can stimulate invasiveness of EGFR-expressing cancer cells by releasing EGF²⁹⁸.

Once in circulation cells must survive, however the attrition rate is high with less than 0.01% of cells predicted to survive²⁹⁹. Circulating tumour cells have been shown to express HIF-1 α , likely to help resist anoikis³⁰⁰. HIF signalling can also facilitate extravasation, VEGF secretion increase permeability of blood vessels of distant organs preparing them for

colonisation³⁰¹. Hypoxia signalling also upregulates the expression of adhesion molecules such as E-selectin, vascular cell adhesion molecule 1, intercellular adhesion molecule 1, or integrins, leading to enhanced adhesion to endothelial cells³⁰².

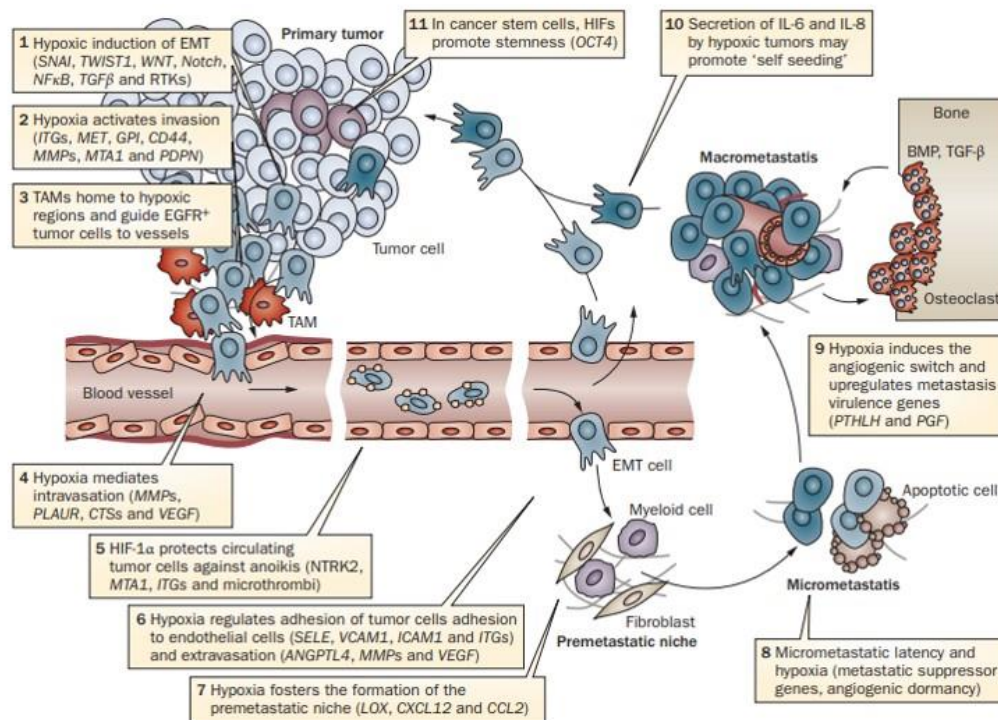


Figure 6.9 Hypoxia and the metastatic cascade

The metastatic cascade is modulated by HIF signalling at many points. Hypoxic signalling begins EMT, facilitating the acquisition of mesenchymal characteristics. Subsequently, invasion is increased by the upregulation and activation of MMPs and other proteins involved in the degradation of the tumour microenvironment. Intravasation is aided by HIF signalling upregulating VEGF and other vasculature remodelling signalling molecules. Furthermore, anoikis resistance is promoted by hypoxic signalling whilst the cells are circulating. Many genes upregulated in hypoxia signal for adhesion to endothelial cells to facilitate extravasation. Similarly LOX another secreted factor regulated by HIF fosters the formation of the premetastatic niche. (Adapted from Carmiet et al., 2011)

6.10.3 Acidosis and metastasis

Tumour acidosis has been witnessed in tumours with local invasion and distant metastasis²⁷⁰. An increasingly acidified tumour microenvironment altered cytoskeletal dynamics and the proteolytic activity of tumour associated macrophages and fibroblasts³⁰³. Acidosis leads to the activation of proteases such as cathepsin B that remodel cell matrix interactions and cell to cell adhesion²⁷¹. HCT116 cells were imaged over time and cell invasion and extracellular pH_e were correlated³⁰⁴. Increased angiogenesis occurs as the extracellular matrix is degraded facilitating metastasis. Direct increases in VEGF transcription occurred in acidic regions of glioma when imaged in real time³⁰⁵. Furthermore regardless of oxygen tension, acidosis increased the expression of stem like markers on cancer cells³⁰⁶. In melanoma cells treated with acidosis, the cells underwent an EMT induction and increased expression of TGF- β ³⁰⁷.

Lysosomes have a well-established role in degradation and remodelling of ECM which is driven by acidosis. Type 4 collagen is specifically degraded by cathepsin B which is activated at acidic pH³⁰⁸. Lysosomal exocytosis has been shown to follow pH gradients towards the cell surface in breast and prostate cancer cells³⁰⁹. Lysosome associated membrane protein 2 (LAMP2) an autophagy protein was shown to be critical for survival of breast cancer cells in acidosis²⁷⁴. LAMP2 has been shown to be upregulated in hypoxia³¹⁰. Exposure to acidosis for pre-invasive breast cancer cells induced the expression of autophagy related genes²⁷⁵. This was repeated in MDA-MB-231 cells and showed similar results²⁷⁵. Moreover they showed that knockdown of BNIP3 reduced survival under long term³¹¹. Knockdown of ATG5 in melanoma cells reduced acidosis survival, due to inhibition of mTOR and reduced glucose and leucine uptake²⁷⁶. ERK inhibition in acidosis has been proposed as one mechanism for sensing of extracellular pH which culminates on TSC2 regulation of mTOR²⁷⁷. This may be a mechanism by which the cells adapt to nutrient deprivation in acidic environments.

6.10.4 EMT

EMT was originally described in the context of normal cell differentiation during embryonic development facilitating the development of highly differentiated tissues³¹². Many of the molecular pathways commonly associated with EMT such as Snail, WNT, and Twist are

highly conserved across species³¹³. EMT is the process of cancer cells losing adherent properties and expressing a more invasive mesenchymal cell phenotype. Thus to migrate cancer cells undergoing EMT must, become resistant to anoikis, down-regulate cell-to-cell attachment, up-regulate the cell molecules in movement, degrade cell-to-cell junctions, and activate proteases³¹⁴. EMT is a heterogenous process, not all tumour cells will undergo EMT and not all that do so will successfully metastasise³¹⁵. EMT is a multi-step process that involves numerous signalling pathways and alterations in gene expression³¹⁶. Multiple environmental factors influence the cell to undergo EMT: the original cells characteristics, the tumour microenvironment, intracellular and extracellular signalling, and epigenetic changes³¹⁷. If migrating collectively, EMT cells migrate at the forefront and excavate matrix fibers, thereby generating tracks for follower cells to move through³¹⁷. However, EMT is not always a requirement for metastasis as those which follow behind the leading cells can still successfully metastasise³¹⁷.

Many transcription factors are upregulated during EMT. The pattern of transcription factors varies greatly, however some such as Snail, Twist, and Zeb are commonly upregulated in many types of tumours³¹⁵. Cytokines are known to induce EMT by activating transcription factors for example TGF- β functions through activating Snail³¹⁸. Many of the EMT transcription factors function as parts of interconnected networks (Fig. 6.10)³¹⁹. Hypoxia is known to influence these first steps in the metastatic cascade³²⁰. Hypoxic signalling promotes this switch by upregulating the expression of EMT promoting transcription factors³²¹. Snail, Twist, Zeb, and gooseoid transcription family members, which are members of the classical model of EMT initiators have been shown to be upregulated in hypoxia³²². Additionally other transcription factors such as WNT, which is intertwined with the HIF signalling network is upregulated in hypoxia³²³.

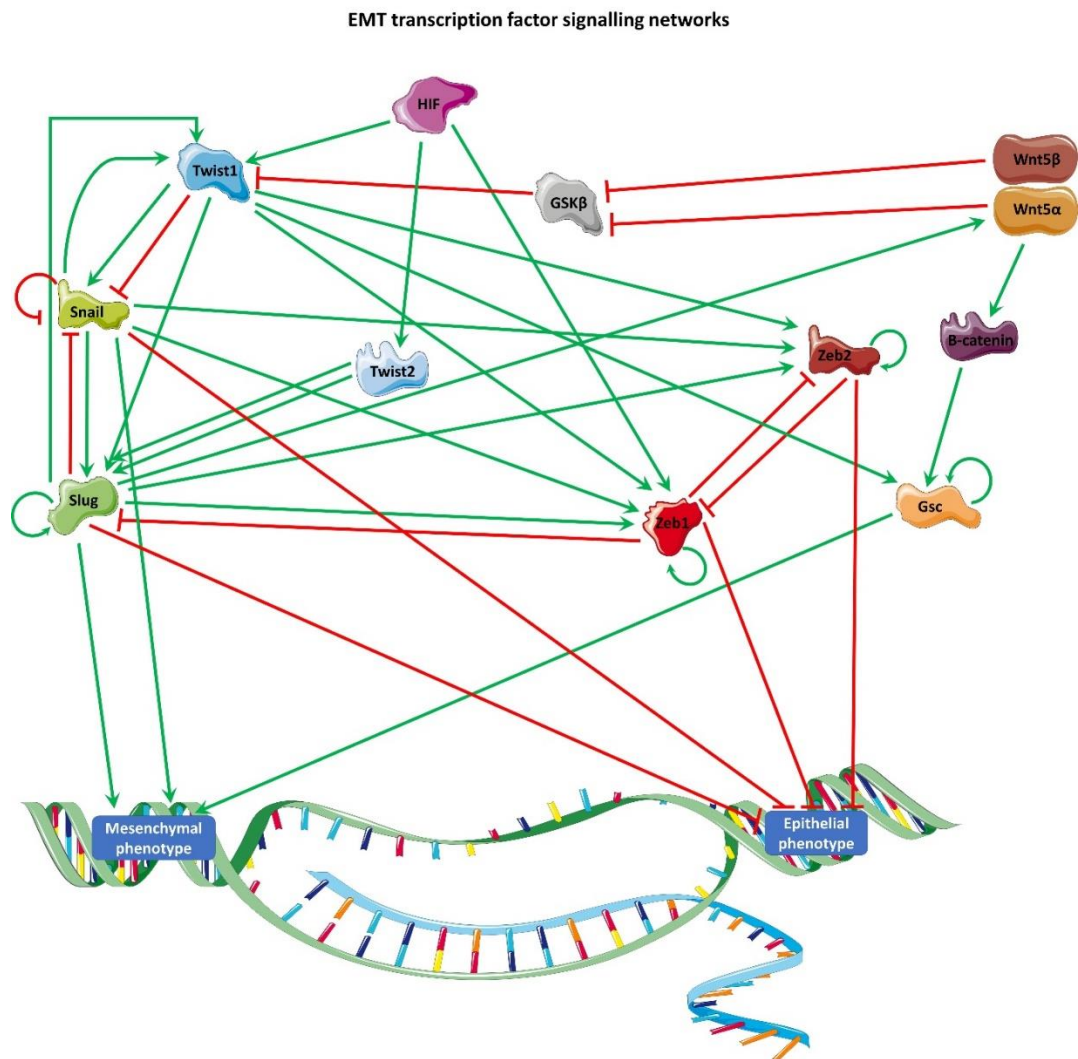


Figure 6.10 EMT transcription factor signalling networks in breast cancer.

Many EMT transcription factors are intertwined and actively enhanced or repress the activity of each other. All these signalling networks converge onto two points, the increase in a mesenchymal phenotype by enhanced transcription of mesenchymal genes; and a decrease in an epithelial phenotype by repression of transcription at these genes. Some EMT members such as Twist1/2 induce their effects by changing the activity of other EMT transcriptional proteins.

6.10.4.1 *Snail*

The Snail family of transcription family contains three members, Snail, Slug, and Smuc. Both Snail and Slug repress the expression of E-cadherin³²⁴. However, Smuc has been shown to be a poor inducer of EMT³²⁵. All the family members encode transcriptional repressors and share a similar organization with a conserved C-terminal domain, which contains four to six zinc fingers and bind to the E-box motif (5'-CANNTG-3') in target gene promoters³²⁶. Snail members contains the evolutionarily conserved SNAG (Snail/Gfi) domain essential for the binding of several transcriptional co-repressor complexes. In the central region of Snail, a serine-rich domain (SRD) and a nuclear export sequence (NES) are found in the regulation of protein stability and subcellular localization of Snail, respectively. Snail interacts with many co-repressor complexes. The SNAG domain, located at the N-terminus, is required for Snail to interact with several co-repressor complexes, including Sin3A and histone deacetylase 1/2 (HDAC1/2) complex³²⁷.

Snail is closely regulated by other transcription factors such as NF κ B³²⁸. TGF- β and EGF can induce Snail activation³²⁹. In hepatocytes and MDCK cells Snail expression resulted in resistance to TGF- β induced apoptosis³³⁰. Once induced by TGF- β Snail activation results in the upregulation of pro-inflammatory interleukin and MMPs³³¹. Thus activation of Snail not only alters the profile of the cell itself but shifts the tumour microenvironment to a more invasion profile. During TGF- β -induced EMT, it has also been shown that Snail forms a transcriptional repressor complex with SMAD3/4. This complex targets the adjacent E-boxes and Smad-binding elements in genes encoding junction proteins such as E-cadherin, CAR and occludin resulting in genes repression³³². It has been demonstrated that Snail can interact with chromatin remodelling factors through the SNAG domain to exert its function of transcriptional repression. For instance, Snail interacts with the Sin3A-HDAC1/2 complex through the SNAG domain to deacetylate histone H3 and H4, leading to the repression of E-cadherin expression³³³. Inactive Snail is localised to the cytoplasm and activation results in accumulation in the nucleus³³⁴. Once activated it functions as a transcriptional repressor at target promoters. The protein p21 activates snail by phosphorylation on Ser246³³⁵. Additionally gsk2b also phosphorylates Snail at two consensus motifs in the Snag domain. This phosphorylation mediates Snail ubiquitination by β -TRCP resulting in cytoplasmic localisation and proteasomal degradation³³⁶. PDK1 can also phosphorylate Snail resulting in the nuclear export and thus EMT inhibition³³⁷.

Snail induced EMT expression results in physical cellular changes due to altered cytoskeletal gene expression³³⁸. Some of the genes under the control of Snail include E-cadherin, Vimentin, cytokeratin 18³³⁹. Snail promotes EMT in breast cancer via the activation of ERK1/2. Snail induced nuclear translocation of p-ERK, and the activation of its subcellular downstream effector, Elk-1³⁴⁰. Overexpression of snail in MCF-7 resulted in increased migration and tumorigenicity and reduced cell adhesion. Blocking the activity of Slug resulted in reduced metastasis³⁴¹. Snail is required for lymph node metastasis of MDA-MB-231 cells³⁴². Slug is required for the metastasis of the transformed melanoma cells. Slug is an essential mediator of Twist-induced EMT and metastasis. Twist needs to induce Slug and other intermediate factors to suppress the epithelial branch of the EMT, then Twist and Slug act together to promote EMT and metastasis. Expression of Snail has been associated with tumour recurrence and resistance to chemotherapy and radiotherapy. Aberrant expression of Snail and/or Slug promotes resistance to doxorubicin³⁴³. Expression of Snail also represses the expression of oestrogen receptor making cells resistant to tamoxifen³⁴⁴. Snail also represses the aromatase gene³⁴⁵. High levels of Snail predict decreased relapse-free survival in women with breast cancer³⁴⁶.

Snail also alters the metabolic phenotype of the cell³⁴⁷. Snail represses fructose-1,6-biphosphatase and other enzymes in gluconeogenesis³⁴⁸. This gives the cells a more stem like phenotype and tumorigenesis by increasing glycolysis, reducing ROS and oxygen consumption³⁴⁹. Snail signalling can also inhibit mitochondrial respiration and COX activity³⁵⁰.

6.10.4.2 *Twist*

Twist transcription factors are members of the basic loop helix-loop-helix transcription family³⁵¹. Similarly to the Snail transcription factor family, twist transcription factors are important during embryonic development³⁵². Twist1 significantly contributes to tumour metastasis, initiation, and growth. The twist transcription family consists of Twist1 and Twist2³⁵¹. The twist family act as dimers either homodimers or heterodimers with E proteins in the BHLH family³⁵³. The homodimers are functionally active whereas the heterodimers are functionally silenced as the other member competes with twist for binding to the hexanucleotide sequence E-boxes (CANNTG) so that they can function as

transcription factors³⁵⁴. An E box independent mechanism of Twist1 function has been demonstrated when Twist1 binds to P53³⁵⁵.

Twist1 is tightly regulated at many functional levels. Phosphorylation by PKA, PKB, CK2, MAPK, etc., act to regulate the function of Twist proteins³⁵⁶. Regulation at additional levels, such as micro-RNA regulation, transcriptional regulation including chromatin conformation also occur³⁵⁷. Recently methylation of promoter region of TWIST1 was described as another regulatory mechanism in breast cancer³⁵⁸. Overexpression of twist1 has been linked to metastasis³⁵³. Knockdown of twist1 has been shown to inhibit this³⁵⁹. The promotion of EMT by Twist accounted for this increase in metastatic capacity³⁶⁰. In breast cancer models, cooperation of TWIST1 with RAS pathway promoted tumour initiation and development³⁶¹. Twist1 can induce gene expression by interactions with bromodomain proteins. Twist1 interacts with BRD4 and a BRD4-BRD1 complex binds with acetylated H4-K5ac/K8ac. Twist1 then recruits this complex to target gene promoters which activates pause release factor P-TEFb to phosphorylate and release RNA-Pol II. Once such target of this complex is WNT5a, increased expression of WNT5a subsequently promotes tumourigenicity³⁶².

Twist1 can bind directly to the E-cadherin promoter, down-regulate promoter activity, and repress E-cadherin gene expression. Twist1 promotes invadopodia formation via up-regulation of platelet-derived growth factor receptor expression³⁶³. Invadopodia-mediated matrix degradation is also induced indirectly by TWIST1³⁶⁴. AKT2 can phosphorylate Twist1 at Ser42 to inhibit P53 activity in response to DNA damage³⁶⁵. *In-vivo*, Twist1 is positively associated with angiogenesis³⁶⁶. Over-expression of Twist1 in breast cancer increases vascular endothelial growth factor (VEGF) expression and induces angiogenesis *in vivo*. Twist1 mediates the thrombin induced up-regulation of angiogenesis growth factors and receptor proteins such as VEGF, GRO- α , KDR, Ang-2, MMP-1, and CD31 in breast cancer lines³⁶⁷.

TWIST1 knockdown completely blocked EMT and partially reversed multidrug resistance *in-vitro*; in addition it improved the efficacy of Adriamycin for breast cancers *in-vivo*³⁶⁸. Co-delivery of TWIST1 shRNA and paclitaxel inhibits breast growth and restricts the metastasis of tumours³⁶⁹. TWIST1 is phosphorylated in 90 % of invasive human breast tumours and the phosphorylation levels positively correlate with TWIST1 protein levels³⁷⁰. When phosphorylated on Ser42 by Akt, Twist1 promotes EMT by modulating TGF- β ³⁷¹. TGF- β induced by phosphorylated TWIST1 maintains hyperactivation of PI3K/Akt³⁷². The Ser68 site can also be phosphorylated by MAPKs including p38, JNK, and ERK1/2. Activation of MAPK

by active Ras protein or TGF- β significantly increases Ser68 phosphorylation and TWIST1 protein levels. It has been shown that TNF- α promotes EMT and cancer stemness through induction of TWIST1 with a direct binding of NF- κ B with TWIST1 promoter³⁷³. Overexpression of p65 up-regulates TWIST1 and EMT in breast epithelial cells. IL-6 level elevation correlates with advanced tumour grade and poor patient survival. IL-6 stimulates TWIST1 expression in breast cancer cells, which in turn activates STAT3. Additionally, TWIST1 induces IL-8 expression, through interactions with NF- κ B and inhibition of IL-8 by a neutralizing antibody reduces the invasiveness of breast cancer cells³⁷³. Generation of cancer stem cells is improved when breast cancer cells are over expressing twist1. The resultant CD24^{low}-CD44^{high} cells have an increased capacity to form mammo-spheres and exhibit increased tumorigenic potential. TWIST1 down-regulates CD24 through direct binding to its E-box sequence³⁷⁴.

6.10.4.3 Zeb

Zeb is a zinc finger E-box binding transcription factor family. This family is characterized by the presence of 2 zinc finger clusters, which are responsible for DNA binding, and a centrally located homeodomain³⁷⁵. There are two members of the Zeb transcription factor family ZEB1 and ZEB2 which are highly homologous. In contrast to Snail and Twist, Zeb is far larger with the molecular weights of these proteins being 29, 20, and 134 KDa respectively³⁷⁶. Zeb was initially discovered as a transcriptional repressor in chicken embryos in the 1990s³⁷⁷. Null mice die perinatally exhibiting respiratory failure and skeletal deformities and sever T cell deficiencies in the thymus³⁷⁸. In adult tissues the expression is heterogenous with barely detectable levels in the liver and pancreas and high levels in bladder and uterus³⁷⁹. Expression of ZEB1/2 is correlated with a poor prognosis³⁸⁰. ZEB1 is responsible for downregulation of basal membrane constituents at the invasive front of colorectal carcinomas³⁸¹. ZEB2 has been implicated in the progression of malignant carcinoma. It was demonstrated that ZEB2 can induce EMT and enhance invasion in vitro³⁸². Further investigations identified several junctional proteins as ZEB2 targets, and reports have linked ZEB2 expression with advanced carcinoma stages³⁸³.

ZEB1 is tightly regulated by multiple signalling pathways. These pathways include: WNT,³⁸⁴ TGF- β , HIF-1 α , and microRNAs³⁸⁴⁻³⁸⁶. HIF-1 α directly regulated ZEB1 expression through the

hypoxia response element which is located in the ZEB1 proximal promoter³⁸⁷. This binding was through HRE-3. HIF-1 α expression increased lung metastasis of a bladder cancer model via ZEB1 modulation³²². HIF-1 α also directly increased ZEB1 expression in colorectal cancer cells³⁸⁸. Both ZEB1 and ZEB2 are involved in a regulatory loop with the miR200 microRNA family. Members of the miR200 family are involved in stabilizing the epithelial phenotype³⁸⁹. ZEBs can transcriptionally inhibit the expression of miR200 to allow EMT to take place³⁹⁰. Reciprocally, members of the miR200 family inhibit protein expression of both ZEB factors. Depending on the stimulus, the existence of this loop permits rapid switching to an epithelial or a mesenchymal phenotype. Other EMT transcription factors, Slug and Twist have been shown to bind to the E box sequences upstream of Zeb and upregulate its expression³⁹¹. TGF- β also controls Zeb expression indirectly through ETs1 induction³⁹². Induction of ZEB1 with TGF- β in glioblastoma leads to invasion³⁹³. Furthermore, TGF- β also induces splicing changes via ZEB1/2 activation resulting in downregulation of epithelial splicing regulatory proteins³⁸⁶.

Aberrant expression of ZEB1 has been characterised in lung, gastric, colorectal, and breast³⁹⁴⁻³⁹⁷. ZEB1 is able to downregulate the expression of E-cadherin by binding to its promoter site and repressing expression³⁹⁸. In addition, ZEB1 is able to regulate other epithelial genes such as Mucl and Pkp3 by binding to their promoters resulting less adhesion and increased invasiveness³⁹⁹. Smad3 has shown to be an interacting partner of Zeb before they interact with DNA⁴⁰⁰. Cellular context must also be taken into account when evaluating the role of the Zeb proteins. ZEB1 transfected in MCF-7 repressed the E-cadherin promoter by 50%, but in SW13 cells the reduction was only 25%. The absence of the ATPase subunit BRG1 the chromatin remodelling protein SWI/SNF in the latter lines has been shown to be responsible for this difference in repression⁶⁵. Triple-negative human breast cancers express high *ZEB1* mRNA levels and exhibit features of EMT. Genome wide binding screens of ZEB1 in Hs578T triple negative breast cancer cells have shown that ZEB1 interacts with circa 2,000 genes⁴⁰¹. Another transcriptomic analysis of control and ZEB1 knockout cells, revealed 1,372 differentially expressed genes⁴⁰¹.

Additionally, ZEB1 has been shown to increase radiotherapy resistance regardless of EMT status⁴⁰². Knockdown of ZEB1 lead to increase radio-sensitivity in breast cancer cells. Mechanistically, ZEB1 regulates radio-sensitivity by promoting homologous recombination mediated DNA damage repair CHK1 upregulation mediated this effect. Interestingly, ZEB1 also interacted with USP7 and stabilized it which deubiquitinates CHK1 leading to

stabilization of Chk1⁴⁰². Upon ionising radiation ZEB1 was upregulated but not Twist or Snail⁴⁰³. Radioresistant subpopulations of Sum159 cells derived from ionizing radiation exhibit hyperactivation of the kinase ATM and upregulation of ZEB1⁴⁰². This activation of ATM promotes tumour cell radio-resistance in vitro and in vivo. Mechanistically, ATM phosphorylates and stabilizes ZEB1 in response to DNA damage⁴⁰².

6.10.4.4 *Goosecoid*

Goosecoid (GSC) is a transcription factor highly expressed during embryonic development in Spemann organizer, an area of the embryo where the establishment of body plan is initiated in gastrulation⁴⁰⁴. GSC is far less studied than other classical EMT transcription factors. GSC expression is high in breast carcinomas. Goosecoid was shown to modulate EMT in hepatocellular carcinoma and increase metastasis⁴⁰⁵. TGF- β induced goosecoid expression in breast cancer cells. Additionally, this increased expression resulted in increased metastasis in in-vivo⁴⁰⁶. In ovarian cancer overexpression is associated with therapy resistance and poor prognosis⁴⁰⁷. Goosecoid induced the expression of FOXc2 and enhanced TGF- β induced EMT⁴⁰⁸. In *Xenopus* gain of function experiments using GSC resulted in impaired cell polarity⁴⁰⁹. GSC is a target gene of FAST2. FAST2 forms a complex with Smad2 and Smad4 to bind to the DNA promoter. Smad3 if it binds to the FAST2-SMAD2 complex results in repression of GSC expression⁴¹⁰.

6.10.4.5 *WNT*

WNT signalling is another EMT regulating pathway⁴¹¹. The WNT pathway is usually divided into two distinct cascades; canonical (β -catenin dependent) and non-canonical (β -catenin independent). In canonical WNT signalling an absence of WNT ligands results in a signalling inactive state. This results in the phosphorylation of β -catenin by the destruction complex. The destruction complex contains the proteins: Axin, APC, CK1 α , and GSK-3 β . Upon phosphorylation by GSK-3 β , β -catenin is ubiquitinated by TrCP β resulting in proteasomal degradation⁴¹¹. Without β -catenin in the nucleus, HDACs are recruited by TCF/LEF to repress target genes. In the presence of WNT secreted ligands such as WNT1 the canonical pathway is activated. WNT is bound by Fzd and co receptors LRP. LRP receptors are subsequently phosphorylated by CK1 α and GSK-3 β which recruits Dvl to the cell membrane

whereby they polymerise and are activated⁴¹². Activated Dvl polymers inactivate the destruction complex resulting in translocation and stabilisation of β -catenin to the nucleus. Once localised into the nucleus β -catenin competitively binds LEF and TCF to form an active complex; displacing TLE complexes⁴¹². This activates complex recruits histone modifying co-activators such as p300 and ultimately results in a transcriptional switch. In contrast to canonical signalling the non-canonical signalling is independent of β -catenin⁴¹². These WNT ligands bind and activate the frizzled-ROR receptor which recruits and activates Dvl. Dvl subsequently binds to Rho, Rho then interacts with Rac1 to activate ROCK and JNK⁴¹². JNK then translocate to the nucleus resulting in transcriptional responses via activation of ATF for example⁴¹¹.

6.11 Sodium Driven Bicarbonate Transporter (NDBT) Proteins

The Bicarbonate transporter gene family is a sub family of the solute carrier family and part of the Amino Acid-Polyamine-Organocation (APC) Family. Proteins encoded by these genes are involved in cellular pH regulation. The bicarbonate family control the passage of bicarbonate across the plasma membrane providing transport for the buffering component of pH regulation. The bicarbonate transporters can be split into three distinct groups based upon mechanistic action: (1) Electroneutral chloride bicarbonate exchangers (AE1-3), (2) electrogenic sodium coupled bicarbonate transporters (NBCe1 and NBCe2), and (3) electroneutral sodium coupled bicarbonate transporters (NBCn1, NBCn2, NDCBE respectively). Bicarbonate control is an integral component of pH regulation but is also vital in other physiological processes including the transport of solutes and fluids across the basolateral membranes. Mutations in this gene family result in multiple pathologies with many mutations resulting in fetuses incompatible with life^{128,413}.

The Bicarbonate transporter gene family all encode transmembrane proteins which transport bicarbonate (or carbonate) and a monomeric ion (Na^+ or Cl^-) across the plasma membrane⁴¹⁴⁻⁴¹⁶. Members 1-3 transport chloride ions whereas the remaining members excluding *SLC4A11* transport sodium ions, *SLC4A11* is unique among the members as it transports borate⁴¹⁷. Two members, NBCe1 and NBCe2 are electrogenic, one cycle of transport results in the movement of one or two net negative charges across the membrane carrying a current and causing a shift in membrane potential. The change in potential is dependent on stoichiometry of the two members which shifts from 1:2 to 1:3 depending on conditions (Fig. 6.12)⁴¹⁸. The remaining members are electroneutral and as such no change in potential occurs during each cycle of transport. NBCn-1 transports a stoichiometry of 1:1⁴¹⁹. NDCBE has multiple possible stoichiometry but all are electroneutral⁴²⁰.

The topology of the sodium dependant bicarbonate transporters contain structural similarities to AE1-3 and data concerning this is based on hydropathy plots of NBCe-1 (Fig. 6.11)⁴²¹. The proteins contain a long N terminus which is intracellular and also a shorter C terminus which is also intracellular⁴²². Dimerization of bicarbonate transporters is stabilized by either the transmembrane domain interactions⁴²³, or N terminus interactions⁴²⁴, or interactions with cytoskeletal proteins such as ankarin¹²⁸. Oligomerization is expected to be required for functional expression⁴²⁵. 10-14

transmembrane domains separate the N and C terminus. The first 6 assignments of transmembrane loops have been validated. However, there are differences among the remaining loops indicating experimental methods differences or that these TM loops are more flexible.⁴²² Another similarity is that most bicarbonate transporters are inhibited by 4, 4'-Diisothiocyano-2, 2'-stilbenedisulfonic acid (DIDS). For AE1 and NBCe-1 DIDS interacts on residues of TM5 extracellular domain and most likely elsewhere. DIDS however, interacts with most transporters within a cell. Glycosylation is another similarity among bicarbonate transporters. Currently four of the bicarbonate transporters are known to be *N*-glycosylated: AE1 is *N*-glycosylated on only its fourth extracellular loop, whereas AE2, AE3, and NBCe1 are all *N*-glycosylated on only their third extracellular loops. The rest of the bicarbonate transporter members have at least two consensus *N*-glycosylation sites on the third loop¹²⁸. Glycosylation is not vital for the function of NBCe-1⁴²⁶ however a mutation in NBCn-1 which the three glycosylate asparagine groups are replaced with glutamine shows reduced expression and localisation. Physiologically the NDBTs control pH_i regulation in every cell they are expressed. They ensure the cell can recover from acute levels of high acid loads returning the pH_i to homeostatic levels⁴²⁵.

6.11.1 Inhibition of NDBTs

The bicarbonate transporters are amenable to inhibition with small molecule inhibitors however no inhibitor specific for a single member has been discovered¹²⁸. Still benzene derivatives are capable of inhibiting NDBTs⁴²⁷. DIDS is a still benzene that is capable of inhibiting the NDBTs but also the NHEs⁴²⁸. Whereas most members of NDBTs will be inhibited at a concentration of 200μM, NBCn1 is poorly inhibited and a concentration of 500μM is required⁴²⁸. DIDS is also a protein crosslinker that alkylates either amino or thiol groups and thus has many off target effect such as inhibiting caspase activity⁴²⁹.

S0859 is a small molecule inhibitor of sodium dependant bicarbonate transporters⁴³⁰. This compound inhibits the majority of acid extrusion by the NDBTs but has non-specific effects⁴³¹. It is more specific than stilbene derivatives as they inhibit chloride-bicarbonate exchanger activity in addition to NDBTs¹²⁸. Initially tested on ventricular myocytes with 30μM resulting in complete inhibition. Further validation of the compound indicated it did not have off target effects which inhibited CAIX activity⁴³⁰. Additionally, it has been shown

inhibition blocks NDBT activity in mammary tumour cells⁴³². Furthermore S0859 has been shown to be ineffective at blocking NHE activity⁴³⁰.

S3705 is another small molecule inhibitor of NDBTs¹²⁸. It has been shown to inhibit NDBTs in breast cancer at a concentration of 40 μ M⁴³³. This was repeated in cholangiocarcinoma lines as well; furthermore inhibition slowed tumour growth and induced apoptosis.

Other methods for inhibiting the bicarbonate transporters have been described as well. Antibodies raised against NBCe1 third extracellular loop inhibited activity (Figure 12). However the antibody interacts with a small protein of 56-KDa⁴³⁴. Antisense probes have been shown to reduced expression of NDBTs with no lethality in 2D⁴³⁵. Phosphatase inhibition can also reduce NDBT activity indicating phosphorylation state is important for NDBT function⁴³⁶. Agents such as levetiracetam and hydrochlorothiazide have been reported to inhibit NDBTs in isolated tissues^{437,438}.

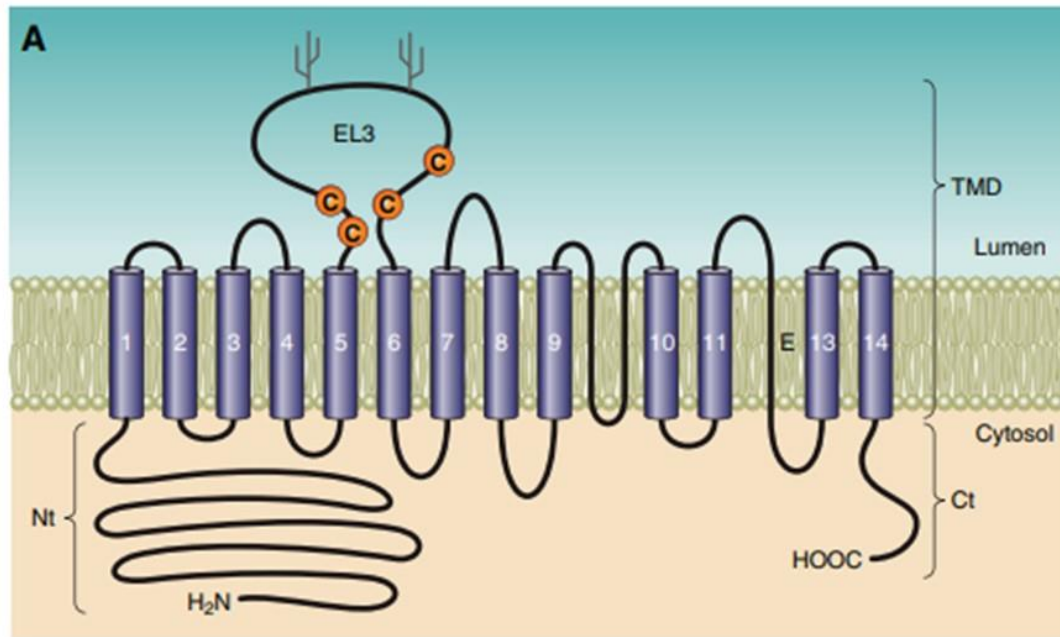


Figure 6.11 Generalised topology of Sodium Driven Bicarbonate Transporters.

Schematic diagram of NBCe-1 used as the basis for all NDBTs. The diagram highlights the core regions of the proteins: N-terminus, trans-membrane domain, extracellular loop, and the C-terminus. The relative size of the C and N terminus depend on the splicing state of the protein.

6.11.2 SLC4A4

SLC4A4 (NBCe-1) is the first of the electrogenic (each reaction results in a change in the electrical potential of the cell) NDBTs. It is the second most widely studied transporter in the NDBT gene family after *SLC4A1* (AE1). It was first discovered in 1983 in salamander renal proximal tubule. NBCe-1 catalyses the transport of Na^+ with 2 or 3 HCO_3^- into the cell, resulting in a net negative charge in the direction of transport (Fig. 6.12). Similarly to other transporters *SLC4A4* proteins forms oligomers⁴³⁹. The *SLC4A4* gene is located on chromosome 4. There are two promoters for *SLC4A4* and 5 splice sites resulting in the production of multiple splice variants. In addition, expression of *SLC4A4* varies between splice variants⁴⁴⁰. NBCe-1a is constitutively active whereas others (variant B) are induced. Acidic conditions have been shown to induce the expression of NBCe-1 as has hypercapnia. This is due to the conditions increasing the concentration of HCO_3^- in the blood¹²⁸. In cancer, expression of NBCe-1 is induced in hypoxia⁴³¹.

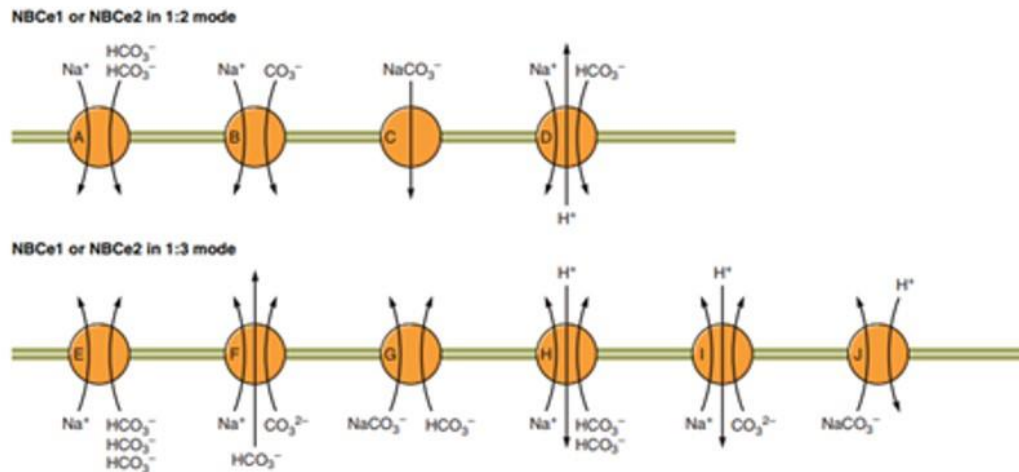


Figure 6.12 Stoichiometry configurations of NBCe-1/2.

Different mechanisms have been proposed for the passage of bicarbonate and sodium across the membrane based on experimental results. NDBCe-1/2 have been shown to function in either a 1:2 or 1:3 mode with both resulting a net change in electrical charge on the membrane (electrogenic).

Promoter 1 lies upstream of non-coding exon 1 and encodes for the transcription of variants NBCe-1B,C, E. The transcription of NBCe-1b has been shown to be pH dependant and the human p1 contains a 284 bp pH responsive sequence. This pH sensitive sequence ends 8bp upstream of the transcription start site. This responsive sequence resides within on an area of the genome containing consensus binding sequences for the nuclear factor of NFkB1, JUN, and TP53 binding sites ⁴⁴¹.

The second promoter lies upstream of exon 4 and is responsible for the transcription of NBCe-1a and presumably NBCe-1d⁴⁴⁰. This promoter is very active in renal proximal tubule cells leading to a higher abundance of NBCe-1a in this tissue¹²⁸. There are three main and two minor splice variants of *SLC4A4*. Splicing results in three main variations among NDBT-e1; (1) N-terminal appendage, (2) homologous cassette excision, and (3) C terminus appendage. There are two possible N-terminus appendages. The first is expressed in variants A, and D; it is 41 amino acids long and contains an auto stimulatory domain and is encoded by exon 4, the second is an 85 amino acid terminus. It is encoded in exon 2 and contains an auto stimulatory domain and consensus binding site for IRBIT. In the minor splice variants D/E omission of a 27 nucleotide nt terminal region is seen. This arises from a splice site in exon 6. The omission of this region removes a casein II phosphorylation site which may have a regulatory role. Excluding splice variant C all splice variants contain the same C terminus. In splice variant C exon 24 is omitted resulting in a frameshift mutation⁴⁴⁰.

NBCe1-A was the first to be discovered and is most highly expressed in the renal proximal tubules (Fig. 6.13). This isoform arises from second promoter in exon 3 and has a non-glycosylated molecular mass of 116KDa. As NBCe-1A contains the auto-stimulatory domain it has the highest per molecule activity of the splice variants. NBCe1-B is the most widely expressed isoform of *SLC4A4* it arises from the dominant promoter in exon 1. It was first cloned in the pancreas and differs from isoform A at the N-Terminus. Due to this alternative N-terminus it has a lower per molecule activity than A and a similar level of activity to C. NBCe1-C is predominantly expressed in the brain. It contains the same N-terminus as variant B containing the auto inhibitory domain. Variant C is most distinguished by its C terminus which has arisen due to frameshift mutation. The consequences of this alternative terminus is unclear. The remaining splice variants D and E are expressed at a much lower level. D is identical to A except for the excision of the 27bp from cassette 1. E is similar to B except for the excision of the 27bp in cassette 1⁴⁴⁰.

Recently the structure of NBCe-1 was solved by cryoEM and it showed that NBCe-1 is a dimer (Fig. 6.15)⁴⁴². This was in concordance with previous FRET and spIDA data indicating it was dimeric^{439,443}. NBCe-1 is similar to other members of the APC family as it contains structures with internal pseudosymmetry of either 5 + 5 Transmembrane or 7 + 7 Transmembrane inverted repeats⁴⁴⁴. The 7 + 7 TM inverted repeat fold and domain organization of NBCe1 resemble the structure of AE1. Additionally, NBCe-1 shares global structural features with other transporters such as: a gate domain that is smaller than the core domain, two highly tilted antiparallel TM helices (TMs 5 and 12) in the gate domain, and residues in the core domain mediating substrate/ion specificity⁴⁴⁵. Interestingly the mutagenesis experiments of Huynh et al indicate that the structurally flexible NBCe1 ion coordination region near TM3, TM8, and TM10 helices and the antiparallel β -strands preceding TM3 and TM10, showed that NBCe1 could be converted from an electrogenic Na⁺-coupled symporter to an electroneutral anion exchanger⁴⁴⁶. The region corresponding to the middle of the transporter within each monomer formed by short TM3 and TM10 and the antiparallel β -strands preceding these TMs (has been proposed to contribute to substrate coordination in other ion transporters. This region of NBCe1 is accessible from the extracellular side representing an outward-open conformation. The pathway contains an electropositive opening from the extracellular side with an external diameter <12 Å and the most constricted region formed by TM3, TM10, and the antiparallel β -strands in the middle of each NBCe1 monomer. Furthermore, residues Phe443, Leu446, Asp555, and Lys559, whose substitution has been shown to significantly alter NBCe1 function, lie within the ion accessibility pathway. It has been proposed that DIDs via its interaction with Lys559/27 mediates the blocking of the ion accessibility pathway and/or the conformational changes during the transport cycle⁴⁴⁶.

Mutations in NBCe-1 have shown to give rise to disease (Fig. 6.14). Due to the high abundance and importance of NDBTs in renal cells mutations in NBCe-1 commonly give rise to renal tubular acidosis. Additionally many children who have defects in this gene present with mental retardation indicating that the acidosis may be whole body not just renal in cases with the Q29X mutation. The build-up of aqueous humour in some mutations in NBCe-1 result in Glaucoma, band keratopathy, cataracts, and corneal edema. These effects may be secondary to the renal failure experienced. Growth retardation is common in some mutations of NBCe-1. Individuals possessing mutations show reduced height and weight as well as bone dysplasia and reduced muscle mass.

Due to its physiological role as a protein able to transport bicarbonate across the membrane it can play a role in many cellular processes. In cell lines overexpressing *SLC4A4* processes sensitive to pH changes are influenced. Whereas the electroneutral isoforms are unresponsive to changes in electrical potential *SLC4A4* is electrogenic and potential affects function. Under normal conditions the chemical gradients of Na^+ and HCO_3^- dictate transport resulting in the net inwards transport of bicarbonate into the cell (acid extrusion). When the electrical potential overcomes the chemical gradients net bicarbonate efflux occurs resulting in cytosolic acidification. In hypoxic cells it has been proposed that *SLC4A4* localise with CA9 and form a metabolon, the removal of bicarbonate from the cell surface working in conjunction with CA9²⁰³. It has also been proposed that NDBT have a minor role in cell migration and invasion by producing localised pH gradients⁴⁴⁷.

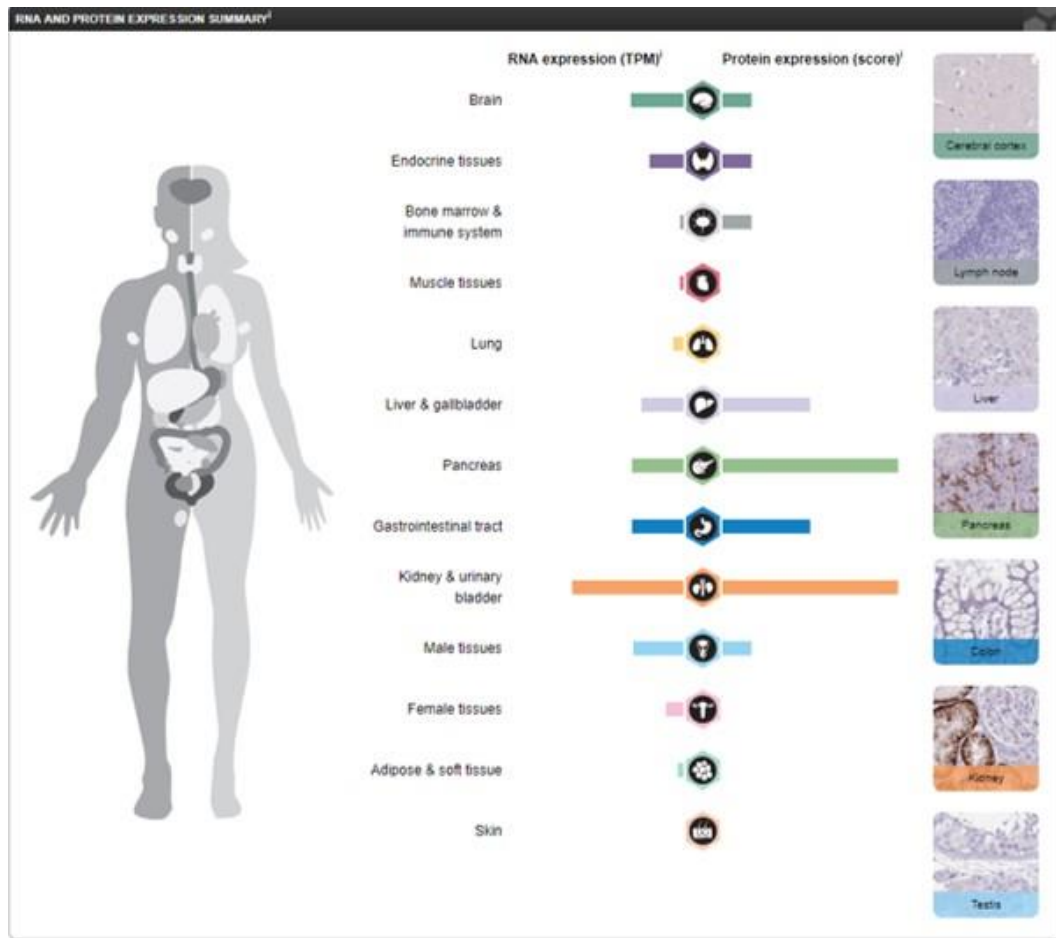


Figure 6.13 Expression of SLC4A4/NBCE-1 is expressed in many normal tissues.

Expression of SLC4A4/NBCE-1 is localised to distinct tissues in the body. High protein expression was found in the pancreas, renal system, and gastrointestinal tract. Expression of NBCE-1 in normal breast tissue is low. Expression is highest in the pancreas and kidney. However, notable levels of expression of NBCE-1 is also seen in the gastrointestinal tract, liver, bone marrow, endocrine tissues and the brain. Similarly RNA expression across tissues is similar to protein levels. (Adapted from Human Protein Atlas)

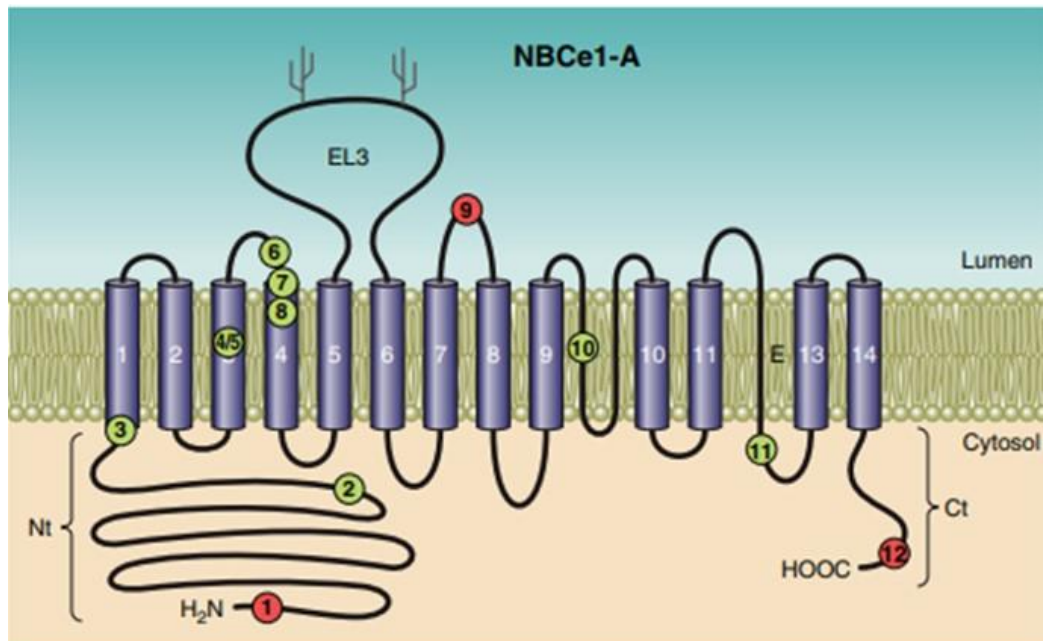


Figure 6.14 Mutation sites in NBCe-1.

Diagram of mutation sites and location in the protein for mutations in NBCe-1. Multiple sites of mutation are seen on NBCe-1 resulting a range of disease states. Red are non-sense mutations. Green are missense mutations. Many mutations have been seen in NBCe-1 each resulting in differing phenotypes. Mutations do not have to occur in the portion of the protein responsible for ion transport to reduce activity. Most mutations commonly result in renal system issues.

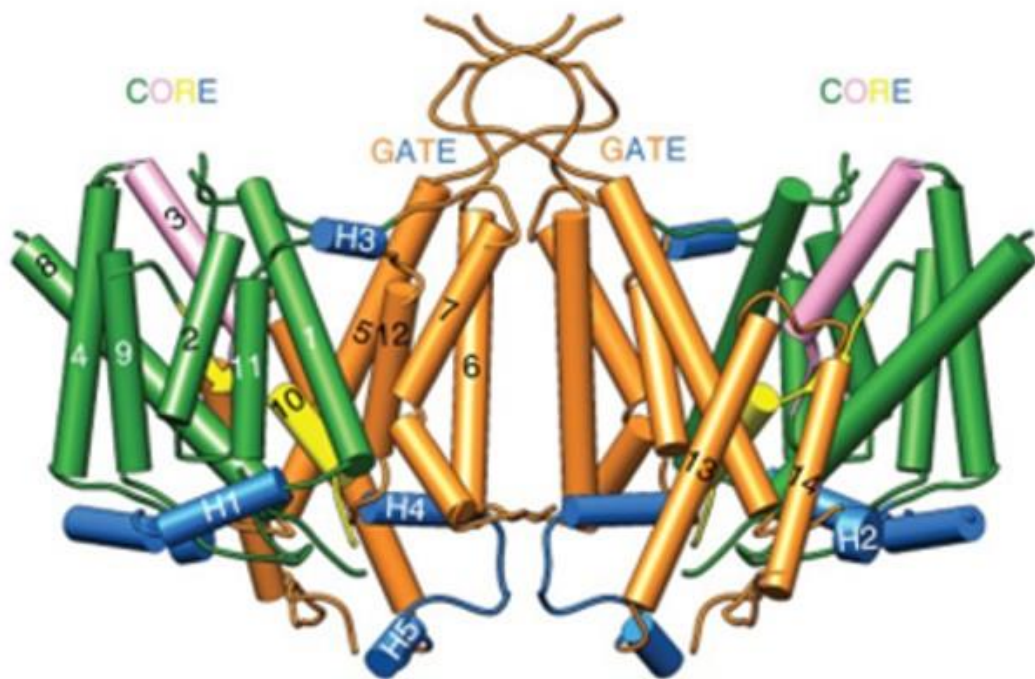


Figure 6.15 Three dimensional model of NBCe-1 dimer.

3D dimeric structure of NBCe-1. The Gate and core domains are clearly defined. The model shows clear points in the protein where interactions occur to generate a dimer. Interactions occur near the gate domain and near the core transmembrane sections. The 3D model indicates the transmembrane regions of NDBTs loop round into a cylindrical formation and that NDBTs function as a dimer to facilitate the passage of ions.

6.11.3 SLC4A5

SLC4A5 is the second electrogenic sodium driven bicarbonate transporter. It is expressed widely throughout the body but is abundant in the brain and liver. In the brain it contributes via cerebral spinal fluid secretion into brain vesicles. In the liver high expression likely contributes to pH homeostasis. Only one variant of *SLC4A5* is known to be functional. It is also the only NDBT not to be stimulated by IP(3)R binding protein (IRBIT). *SLC4A5* proteins form higher oligomers similar to other bicarbonate transporters¹²⁸.

SLC4A5 expression is often associated with hepatocytes however, *SLC4A5* mRNA is expressed in many organs (Fig 6.16). It is expressed in the brain and IHC studies have shown localisation to the apical membrane of the choroid plexus epithelium in rat and mouse studies. Human brain samples were analysed by RT-PCR showed the presence of *SLC4A5* especially in the hippocampus, cerebrum, cerebellum, and choroid plexus epithelium. Despite the presence of transcripts in human CPE it was not possible to detect the presence of NBCe-2 with existing antibodies⁴⁴⁸. Mutations in the *SLC4A5* genes leads to individuals with elevated blood pressure⁴⁴⁹.

The *SLC4A5* gene is located on chromosome 2 and contains at least 31 exons. As the fourth member of the bicarbonate transporter family to be discovered it was originally named NBC4. However, the protein has been renamed to express its characterization better. It is now known as NBCe-2. Six splice variants have been identified¹²⁸.

Only two splice variants are likely to produce a functional protein. Currently only NBCe2-c has been shown to be functional. Splice variant C is also the most structurally similar to other NDBTs in its transmembrane domain structure. However splice variant A contains a 16 amino acid cassette between transmembrane regions 11-13 believed to encode a PKA phosphorylation site. Splice variant A is the largest variant. It is expected this splice variant is functional as currently no evidence has shown this insertion affects function. The other splice variants contain frameshift mutations and poorly match the human genome and therefore predicted to be physiologically inactive⁴⁴⁰.

Despite being located frequently throughout the body evidence describing its functional roles are scarce. Similarly to other NDBT it is expected to contribute to pH_i regulation in the cell types it is *expressed*. The co-transport of *SLC4A5* functions indistinguishably to

SLC4A4. It functions as a Na^+ and HCO_3^- cotransporter thereby controlling intracellular pH. SLC4A5 protein functions with a 1:3 or 1:2 stoichiometry^{450,451}.

Some evidence of its role is based upon the signs exhibited in knockout mice. Currently only *SLC4A5* upregulation has only been shown in response to HDAC and methyl transferase inhibitors and in response to hypoxia in cancer cells. Similarly to NBCe-1 it is electrogenic and therefore each passage of sodium and bicarbonate results in a net negative charge.

Knockout mice have been produced and despite the expected importance of NBCe-2 in the liver the mice exhibit no hepatic phenotype. Mice with a disrupted *SLC4A5* gene exhibit defective CSF secretion. In addition, some *SLC4A5* deficient mice display retinal abnormalities presumably due to decreased intracranial pressure. Null mice also display increased blood pressure and metabolic acidosis which fits with *SLC4A5* role in Na^+ and HCO_3^- reabsorption⁴⁵².

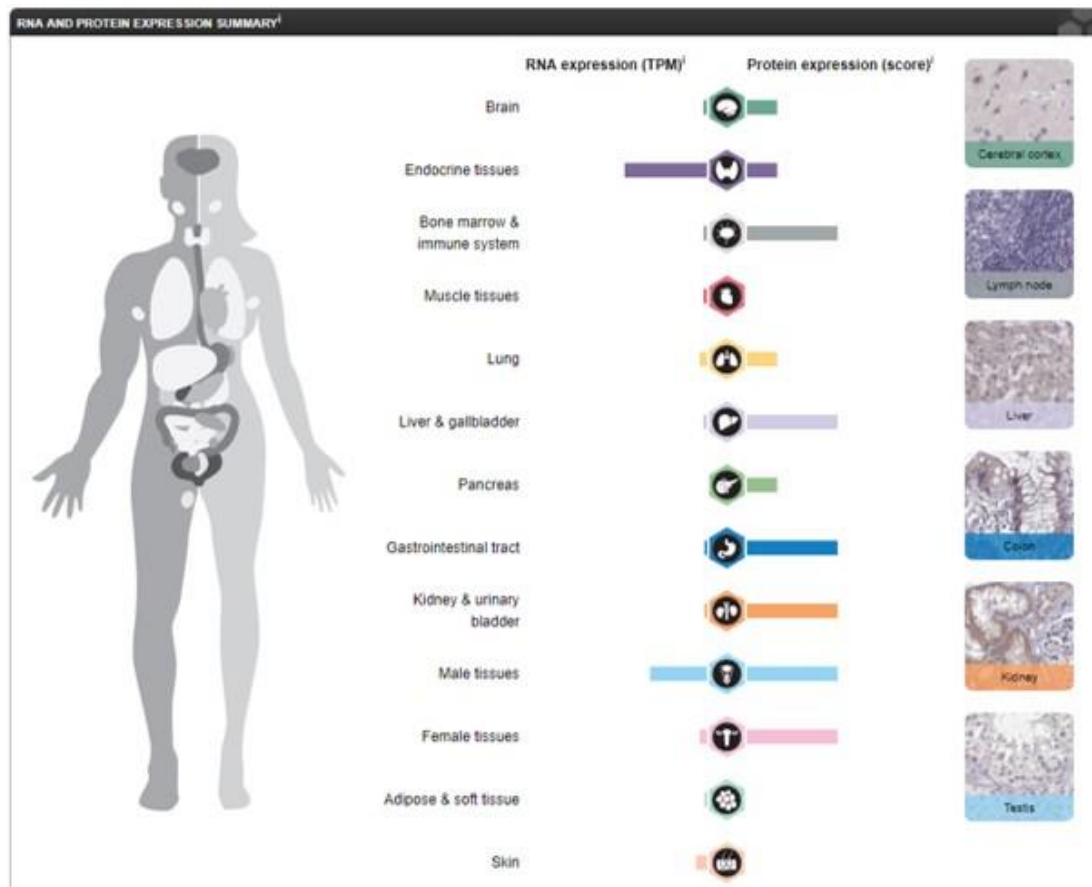


Figure 6.16 Expression of NBCe-2 is localised to multiple tissues in the body.

Most tissues in the body expressed NBCe-2 highly with the exception of adipose, skin, and muscle tissues. Furthermore, protein expression levels were commonly higher than RNA expression in the tissue. (Adapted from Human Protein Atlas)

6.12 Preliminary data

It was observed that expression of NDBTs was increased in response to anti-angiogenic treatment in xenografts¹⁷¹. It was hypothesized that NDBTs are part of a pH regulatory metabolon. A panel of ten cancer cell lines (3 of which were breast) were investigated for the expression of NDBT and other anion transporters mRNA in response to hypoxia. Increased expression of many of these members were identified in response to hypoxia (8/10). The most frequently upregulated in hypoxia were SLC4A9 in 5/10 cell lines including MDA-MB-231 (7.5 fold) and MCF-7 (4 fold); and SLC4A5 in 5/10 cell lines including MDA-MB-231 (4 fold)⁴³¹. Subsequently hypoxic induction of AE4 (SLC4A9) was confirmed in MDA-MB-231⁴³¹. To tie the role of NDBTs and the anti-angiogenic treatments, hypoxic regulation of NDBTs was investigated. These proteins were shown to be regulated by HIF-1 α and HIF2 α ⁴³¹. Knockdown or inhibition of SLC4A9 in MDA-MB-231 resulted in increased intracellular acidification in spheroids. The growth of MDA-MB-231 was unaffected in 2D, however in spheroids the growth rate was reduced by 48%⁴³¹. Similarly knockdown of SLC4A9 reduced the growth rate of xenografts by 79%⁴³¹. This effects was seen *in-vitro* with the inhibitor S0859. MDA-MB-231, HCC-1806, and MCF-7 all showed reduced wound healing in hypoxia. These effects were comparable to the effects of shRNA knockdown of AE4 in MDA-MB-231. To further investigate the in-vitro effects trans-well invasion assays were performed. Both SLC4A9 knockdown and inhibition with S0859 reduced invasion in normoxia and hypoxia⁴³¹. Other groups have also investigated the roles of NDBTs in regards to migration and invasion. In 2012 Pastorekova et al showed that CAIX and NBCe-1 co-localise in migrating cells⁴⁵³. This localisation was at the leading edge of the cells indicating a role of NDBTs in the migratory apparatus⁴⁵³. Building upon this Pouyssegur and Parks showed that NBCe-1 plays a role in the migration of breast and colon cancer cells⁴⁵⁴. They showed that spheroids grown with NBCe-1 knockdown had increased cell death⁴⁵⁵. Additionally, it was shown that NBCe-1 knockdown increased cells sequestered in S phase⁴⁵⁴. Furthermore NBCe-1 KD in MDA-MB-231 cells reduced migration and invasion in normoxia and hypoxia in a small number of *in-vitro* assays⁴³¹. However, not all members of the NDBT family have been shown to reduce migration. Investigations into the effect of NBCn-1 have shown that it aided cellular motility and also co-localised with ERM proteins but knockdown did not reduce overall motility⁴³⁵. However, NBCn-1 was shown to have an important role in arterial smooth muscle migration and response to pH changes⁴³⁵.

Based upon its function as a NDBT it is expected it may play a role in the pH metabolon of migrating tumour cells. SLC4A4 and SLC4A5 would localise to the leading edge of the migrating tumour cell. Functioning to remove bicarbonate from the local extracellular environment would result in acidification of the leading edge of the migrating tumour cell. This in turn would degrade the ECM, increase MMP activity and also release growth factors resulting in increased tumour migration and invasion. Intracellularly this would lead to the maintenance of a slightly alkaline pH conducive for cellular growth and proliferation. Many of those signalling pathways are controlled by the Ser/Thr kinase mTORC1⁴⁵⁶. mTORC1 is a master regulator of cell metabolism, survival and growth⁴⁵⁷. mTORC1 expression amplifies the expression of HIF which is essential for survival in hypoxia conditions⁴⁵⁸. Acidosis rapidly and reversibly inhibits mTORC1 via the TSC1-TSC2 pathway²⁷⁷. Maintenance of alkaline pH via the function of NDBTs would therefore result in higher levels of mTORC1 and HIF expression presenting the cells with a distinct survival advantage.

6.13 Aims of this study

This study aims to investigate if NDBTs are required for migration and invasion *in vitro* and metastasis *in vivo*. I hypothesise that NDBT localise to the leading edge of migrating tumour cells and bind to CAIX as part of a pH regulation metabolon driving migration and invasion on the leading edge. In addition, I hypothesise that, NDBT knockdown will reduce intracellular kinase signalling; inhibiting the induction of epithelial-to-mesenchymal-transition (EMT) and reducing metabolic adaption lowering ATP levels. This study was split into hypotheses that were broken down into 5 key aims which are as follows:

- 1) Establish which NDBTs are hypoxia regulated and determine if they colocalise to the leading edge of migrating cells with CAIX.
- 2) Produce knockdown cell lines of the most frequently upregulated NDBTs in multiple cell lines. Subsequently the impact of NDBT knockdown will be investigated with regards to migration, invasion, pH regulation, and spheroid formation *in vitro*.
- 3) Investigate the impact of NDBT knockdown on reducing tumour metastasis *in vivo*.
- 4) Establish whether NDBT reduces pH sensitive tyrosine kinase signalling and in turn produce large transcriptomic changes. Moreover the impact of these signalling changes will be investigated downstream with regards to the induction of EMT.
- 5) Ascertain whether NDBT knockdown alter ATP production by perturbing glycolysis.

7 Chapter 2: Materials and Methods

7.1 Cell culture

7.1.1 Maintenance of cell lines

All cell culture was carried out in sterile conditions in a class II biological safety cabinet (Walker, Glossop, UK). All cell lines: Cal-51, HCC-1806, Sum-159, MDA-MB-231, MCF-7, JIMT-1, and SKBR3 were maintained in Dulbeccos modified eagle medium, high glucose (DMEM) supplemented with 10% foetal calf serum (Sigma), 1% L-glutamine (Sigma), 1% Pen/strep (Sigma) and incubated in a CO₂ incubator at 37°C (MCO-5AC-UV-PE, Panasonic, UK). T225cm² culture flasks (Corning) containing 25ml of DMEM complete media were used to culture the cells. Cell lines were routinely tested for mycoplasma.

7.1.2 Passaging cells

Prior to routine experimental preparation cells were passaged three times a week. Dissociation of cells from T225cm² flask was performed by aspirating media from flask, followed by two washes with 5ml of dPBS. Next, 5ml of 1X trypsin/EDTA was added to the cells and flasks placed in the incubator for 3 minutes. The trypsin was neutralised using 10ml of complete media. The cells were concentrated by spinning at 800 x g for 10 mins. Supernatant was removed and cell pellet re-suspended in 10ml of media. Cells were counted using a haemocytometer using the method described below and 1.5×10^6 cells were seeded into a fresh flask.

7.1.3 Cell counting

Cells were counted using a haemocytometer (Neubauer chamber, Celeromics). Glass coverslips were attached to the chamber by breathing onto the coverslips and pressing

them against the chamber. Cells were harvested from culture flasks according to the method described above. Pellets were re-suspended in 5ml of fresh media. A 1 in 10 dilution was performed to make 20µl of counting suspension. 12µl of this suspension was used to fill the haemocytometer chamber. All 4 sets of 16 squares were counted and an average taken. The total number of cells and cells/ml were calculated using the following formulas.

Equation 1 Cell concentration

$$\text{cells per ml} = \frac{\text{average counter per mm}^2 \times \text{dilution factor}}{0.0001}$$

$$\text{Total number of cells} = \text{cells per ml} \times \text{total volume}$$

7.1.4 Cryopreservation of cell lines

For long term storage and use in future experiments, cells were cryopreserved at the lowest passage possible in freeze media. Freeze media consists of 10% DMSO (Sigma) and 90% foetal calf serum (Sigma). Cell lines were harvested and counted as per previous protocols. Cells were pelleted and re-suspended at a concentration of 1×10^6 cells per ml in freeze media. 1ml of cell suspension was transferred into separate 1.8ml cryo vials (Starlabs). Vials were placed into a Mr Frosty freezing container and placed into a freezer at -80°C overnight. Following this the vials were transferred to -150°C for long term storage.

7.1.5 Lentiviral shRNA knockdown

Stable NDBT knockdown cell lines were generated using a pKLO-puro lentiviral vector (Sigma). Sigma MISSION validated shRNA was used to generate the knockdowns in this study. The service included shRNA cloning, sequence verification, DNA quantification and titre verification. Multiple shRNA sequences were tested for the most efficient knockdown (codes and sequences in appendix). To generate a control knockdown shRNA cell line, cells were transduced with MISSION shRNA non-targeting control which induces the RISC/RNAi pathway. These lines are referred to as shCTL.

Cells were pelleted, re-suspended and counted using the methods previously described. 5×10^4 cells were incubated with the appropriate lentiviral particles with a MOI of 5. Polybrene was added to the cells prior to incubation with the lentiviral particles at a concentration of $6 \mu\text{g}/\text{ml}$ to overcome cell/lentiviral membrane charge differences. One well for each shRNA was transduced on a 48 well plate and a negative (puromycin) control included. Cells were incubated with lentiviral particles for 48h and washed 3 times with PBS. Cells were then split into 24 well plate and cultured under the presence of antibiotic selection to generate transduced pools. Prior to selection a puromycin kill curve had been produced and antibiotic selection validated with the negative control.

7.1.6 Clonal selection

Single cell clonal selection was achieved by harvesting and counting as previously described. Cells were serially diluted until a final concentration of 1 cell per $\sim 200 \mu\text{l}$. The cell suspension was plated out into a 96 well plate (Griener) and 4 hours later checked for the presence of a single colony. Wells containing more than 1 cell were discarded. Single clones were expanded until viable for banking and RNA extraction.

7.1.7 Spheroids

Cells were cultured in T175cm² flasks until 70% confluent. Cells were trypsinised and counted as described previously. Cells were resuspended 1×10^5 cells/ml. 100ul of cell suspension was aliquoted into each well of a low adhesion plate (96 well, Round bottom plate, Corning). A buffer jacket of PBS was used on the outside wells. The plate was spun at 2000rpm for 10 minutes and placed into an incubator. Spheroid form approximately 3 days afterwards. Cell lines requiring Matrigel (growth factor reduced, Corning) were prepared in the same method however, 5ul of Matrigel was added per well when aliquoting into the low adhesion plate and placed on ice. The plate was also spun at 4°C . The Matrigel was thawed on ice. The day after plating the 100ul of fresh media was added to the wells. Media was changed every two days by tilting the plate and slowly aspirating half the media.

7.2 RNA

7.2.1 RNA extraction

TriZol extraction method was used to extract RNA. All steps involving TriZol were undertaken in a fume hood. Plates were removed from the incubator and placed on ice. Media was aspirated using a serological pipette. 1ml of TriZol was added per dish and incubated at room temperature for 5 minutes. 1ml of TriZol was removed from each dish and placed into an Eppendorf tube. 200µl of chloroform (Fisher Scientific) was added and the samples were centrifuged for 15 minutes at 12,000 RPM. Following centrifugation 400µl of the aqueous phase was removed and placed into an Eppendorf tube containing 0.5ml isopropanol. The RNA was pelleted by centrifugation for 15 minutes at 12,000 RPM. Subsequently the supernatant was removed and 800µl of 70% ethanol was added. The tubes were centrifuged for 5 minutes at 12,000 RPM and the supernatant discarded. The pellet was dissolved in 20µl ultrapure water for quantification and cDNA synthesis.

7.2.2 RNA quantification

RNA was quantified using a Nanodrop (ThermoScientific, Nelson, UK). The wavelength was set to 260/280nm. 1µl of ultrapure water was dispensed onto the pedestal and used as a blank. Pedestal was cleaned with tissue and sample was added to pedestal and quantified.

7.2.3 cDNA synthesis

cDNA synthesis was carried out using a 2µg RNA reaction using a high capacity reverse transcriptase kit (Applied Biosystems). A reverse transcription master mix was prepared using the table below.

Table 1 cDNA synthesis reaction

Component	Volume
RNA	2µg
10x RT buffer	2µl
dNTP (100mM)	0.8µl
RT random primers	2µl
PCR grade water	4.2µl
Multiscribe reverse transcriptase	1µl
Total volume per reaction	20µl

The cDNA programme was selected and ran for 2.5H. Once finished samples were removed and placed on ice. The samples were diluted 1 in 10 prior to future experimentation and stored at -20°C.

7.2.4 qRT-PCR

QRT-PCR was performed using a lightcycler 480 II thermal cycler (Roche diagnostics LTD, Burgess hill, UK). Reactions were prepared using the Lightcycler 480 SYBR green I master mix. All samples were run in duplicate on a 96 well plate (lightcycler 480, white, Roche). A master mix was prepared according to the table below.

Table 2 qRT PCR reaction

Component	Volume
Forward primer (10uM)	1µl
Reverse primer (10uM)	1µl
Molecular biology grade water	6µl
Master mix (sybr I green)	10µl
cDNA	2µl
Total volume per reaction	20µl

All samples were probed for actin as an internal control and PCR grade water used as a negative control. Upon completion of the programme data was transferred to a .txt file and analysed using the delta CT method.

Table 3 qRT-PCR primer sequences

Gene	Forward Primer Sequence	Reverse Primer Sequence
<i>CAI</i>	CCAAACATGACACCTCTCTGAA	GCCCCACATTGATAATTTCTTT
<i>CAII</i>	CACCCCTCCTCTCTGGAAAT	GTTAAGTTTACGGAATTTCAACACC
<i>CAIII</i>	CTTCGGATGATCATGGCTCT	CCAGTGAACCAATGAAGCTC
<i>CAIV</i>	TGTCCAAAATAACGGGCACT	CAGTCCTCCTCCAGAAATGC
<i>CAVα</i>	GACATCTTGCCGGAATAAAA	CCCGCGTAGGTCCAGTAAT
<i>CAVβ</i>	CATGATGGCCACTATCAGAAGA	ACAGTGCACAAGGGTTTCAA
<i>CAVI</i>	GTCAACAATGGCCACACAGT	ATCTCCGAGGACGCACCT
<i>CAVII</i>	AGAGAATTTCCCGCAGAAG	CCCCTTCCCTCACGTACC
<i>CAIX</i>	CTTGGAAGAAATCGTGAGG	TGGAAGTAGCGGCTGAAGTC
<i>CAXII</i>	CCCTCGAGTTCCAAGGCTA	GGGCAGGTTCACTTCACT
<i>CAXIII</i>	TTTTTGCCTGGTGTTTGG	TTGACAGAACAATGACCCACA
<i>ACTB</i>	ATTGGCAATGAGCGGTTT	GGATGCCACAGGACTCCAT
<i>SLC4A4</i>	CCATTGCTCACATCGACAGT	CACAAGGGTTCCAGTGACTCT
<i>SLC4A5</i>	CAGGGAGTGATGGAGAGCTT	GAGGCTGTCCGAGAAGA
<i>SLC4A7</i>	AAAGTGGAGAAATTAAGGTAATGGA	CAACCTTGCTGAAGTCAACAGT
<i>SLC4A8</i>	AGAATTGAGCCACCCAAAAA	TTGGAACTCCAGGCATTTTC
<i>SLC4A9</i>	GGGGTCCCTTACTGAGGTGT	TAGCCCTTTCCAGCATACA
<i>SLC4A10</i>	AGCCTCCAAAAATGTTCTT	GAGCTGCTGTTCCATTTGGT
<i>Snai1</i>	CTTCCAGCAGCCCTACGAC	CGGTGGGGTTGAGGATCT
<i>Snai2</i>	TGGTTGCTTCAAGGACACAT	GCAAATGCTCTGTTGCAGTG
<i>Twist1</i>	AGGGCCGGAGACCTAGATT	TCATCTGGACATGTGCTTCTTC

<i>Twist2</i>	CATGTCCGCCTCCCACTA	GAGGGGACACTGGAGGTAGTC
<i>ZEB1</i>	CGAAACGCGAGGTTTTGTA	TCTAGACAGGAAATCCACACA
<i>ZEB2</i>	GTTTACCGTTTATCTGAACTAGAAGG	TTTTTGTTAGCATTTGCTTTTAGC
<i>WNT5a</i>	CGTCTGGAAGCAGACGTTTC	TCACGCCTCCTGATCTCC
<i>WNT5b</i>	TGCCTTTCCAGCGAGAAT	CCCCCTTCTCTTCAGGTA
<i>GSC</i>	CCTCCGCGAGGAGAAAGT	CGTTCTCCGACTCCTCTGAT
<i>Vimentin</i>	TACAGGAAGCTGCTGGAAGG	ACCAGAGGGAGTGAATCCAG
<i>LOX</i>	TTTCCCGCTCTCTGATTCTC	TGGAAAAGCTTGGACATGAA
<i>E-cadherin</i>	TGCTCTTCCAGGAACCTCTG	GCGGCATTGTAGGTGTTCA
<i>LDHA</i>	AACATGGCAGCCCTTTATG	ATTGGAGTTTGAGTGACCA
<i>Glut1</i>	GCCCATGTATGTGGGTGAA	AGTCCAGGCCGAACACCT
<i>tRNA-Leu(UUR)</i>	CACCCAAGAACAGGGTTTGT	TGGCCATGGGTATGTTGTTA
<i>B2-microglobulin</i>	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
<i>SLC4A4 nested</i>	ACTGGAGGAGCGACGGAA	CGTATTGATTCTGAAAAGTAATGCGTACG
<i>SLC4A5 nested</i>	CTCAACCCTGGACATGGTTCA	CTGTCAGTTTCTCTCGGTATT
<i>SLC4A7 nested</i>	TCGCTCAGTTCTAGCTTCAGGTTT	TTAAAAACCCGGTAGTCACACAT
<i>SLC4A8 nested</i>	GATCTGCTCAGACCCGACCA	TTTGAGATGACCAAGGACAGGG
<i>SLC4A9 nested</i>	CTGTACTGGTTCTGAGATTCT	CCTTTTAATGAGTAGAGAGTGAATG
<i>SLC4A4 eGFP</i>	AAGCTTAGCATAATGGAGGATGAAGCTGCTCG	CTCGAGACGTTAGTACAATGTAGTTTCTGTTCCC
<i>SLC4A5 eGFP</i>	ATGAAGGTGAAGGAGGAGAAGGC	CAGAGTGAGTAACTCCAAGTGGAA
<i>SLC4A7 eGFP</i>	AAGCTTACGTACATGGAAAGATTTCTGCTGGAGAAG	CTCGAGACAGTCGTATAATGAAGTTTCAGCATCCACG
<i>SLC4A8 eGFP</i>	GGATCCACAGTAATGCCGGCCGCCG	CTCGAGACAGCCGTAGTTGAAGAGACTCTTTCTTTGCG
<i>SLC4A9 eGFP</i>	AAGCTTGCAATCAATGGAAATGAAGCTGCCAGGCCAG	CTCGAGGACATAATAATTACAGAAATGTTGATTT
<i>Hexokinase1</i>	GACCAAGTTTCTCTCAGATCG	CCTAGCTGCTGGAGGATAGC
<i>Phospho-Fuctokinase 1</i>	CCCAGTGGCACATTGACTC	GCAGGTGTGAGGTGAGGTG
<i>Pyruvate kinase M2</i>	GGTGGAGATGCTGAAGGAGA	TTGATGGTCTCCAAGTACTCATA
<i>Aldolase A</i>	CCTATTAAGCGGAGGTGTTCC	GTCACGAGGGAAGAAAGAGC
<i>Phospho-glyerate kinase 1</i>	GAGCCAGTTGCTGTAGAATCA	CTTCTGGGCTACACAGTCC
<i>Enolase 1</i>	TCCAACATCCTGGAGAATAA	ATGCCGATGACCACCTTATC

7.3 Protein

7.3.1 Protein extraction

Cells were grown to a density of 3×10^6 cells or 70% confluency in a dish. Dishes were removed from the incubator and placed on ice. Cells were washed with ice cold PBS. Dishes were scraped using a cell scraper and the solution pelleted by centrifuging at 12,000 RPM in a labelled Eppendorf. Supernatant removed and 200 μ l of RIPA + protease inhibitors (Sigma, P8340) added to the pellet. The pellet was re-suspended in the lysis buffer by pipetting. Samples were syringed to remove unwanted DNA and proteins attached. Samples were spun at 12,000g to pellet remaining DNA and histones. Protein samples were kept on ice at all times and stored at -80°C .

7.3.2 Bradford Assay

Total protein concentration was calculated using Bradford assay. A standard curve was produced using BSA at a starting concentration of 4mg/ml and serially diluted 1 in 2 to a final concentration of 15.625 μ g/ml. An Eppendorf containing PBS was used as the negative control for the assay. Samples were diluted 1 in 10 for use in the assay. The Coomassie brilliant blue dye (BioRad) was diluted 1 in 5 in H_2O . 10 μ l of samples were placed into each well and samples were run in triplicate. 200 μ l of dye was added onto to each well. The plate was read on a spectrophotometer at 620nm (Infinite F50, Tecan, UK). Protein concentration was determined via comparison against standard curve using Microsoft Excel.

7.3.3 Gel electrophoresis

Volumes required for 100 μ g of protein per sample were calculated via Bradford assay. The correct volume of 4x Lamelli loading buffer (In-House) was added to each sample and total volume of each sample normalized to 40 μ l with dH_2O . Samples were boiled for 5 minutes

at 95°C. 3 µl of protein standard (BioRad, Cat# 1610399) was loaded into well 1 of a precast mini PROTEAN 4-20% polyacrylamide gel (Biorad, Cat# 4561033). Protein samples were separated according to size via the mini-PROTEAN tetra-cell 1-D vertical gel electrophoresis set up. Electrophoresis was performed at 80V for 120 minutes.

7.3.4 Immunoblotting

Protein samples were transferred to nitrocellulose via the transblot turbo transfer system on high molecular weight protein settings (Biorad). Membranes were blocked in 5% BSA-TBST solution for 1H. Immediately following the blocking step primary antibody was added and left overnight at 4°C. The following morning membranes were washed 3 times in TBS-T 0.5% for 10 minutes. Each secondary antibody (Licor) was added for 1h at 1:15,000 dilution away from light. Samples were washed three times for 5 minutes in TBS-T 0.5% and placed in TBS for storage. Blots were imaged using LICOR odyssey. Actin was probed for as a loading control.

Table 4 Antibodies

<u>Target</u>	<u>Company</u>	<u>Catalogue #</u>	<u>Dilution</u>
NBCe-1	Cell signalling Technology	11867S	1:500
NBCe-2	SigmaAldrich	HPA036621	1:250
NBCn-1	Abcam	ab82335	1:1000
AE4	Abcam	ab151133	1:500
CAIX	Absolute antibodies	Ab00414-1.1	1:1000
HIF-1α	BD biosciences	610959	1:250
HIF-2α	Novus	NB100-122	1:250
HIF-1β (ARNT)	R&D systems	AF5630	1:500
pMTOR	Cell signalling Technology	2971S	1:500
MTOR	Novus biologicals	NBP2-43658	1:500
β-actin	Santa Cruz Biotechnology	sc-1616	1:5000
Glut-1	Santa Cruz Biotechnology	sc-377228	1:1000
Pdgfr-α	Abcam	Ab5460	1:1000
Fgfr2	Abcam	Ab10648	1:1000

pALK	Abcam	Ab73996	1:1000
ALK	Abcam	Ab16770	1:1000
pcMET	Abcam	Ab5662	1:1000
cMET	Abcam	Ab71472	1:1000
Hgf	Abcam	Ab83760	1:1000
VEGFr2	Abcam	Ab39256	1:1000
EGFr	Abcam	Ab52894	1:1000
EGF	Abcam	Ab9695	1:1000
ZEB1	Abcam	Ab124512	1:1000
ZEB2	Santa Cruz Biotechnology	sc-271984	1:500
E-cadherin	Santa Cruz Biotechnology	sc-71009	1:500
Vimentin	Dako Omnis	GA63061-2	1:200
Snail	ThermoFisher	MA5-14801	1:2000
Slug	Santa Cruz Biotechnology	sc-166476	1:500
Twist1	ThermoFisher	MA5-17195	1:2000
Twist2	ThermoFisher	CF805832	1:2000
GSC	ThermoFisher	PA5-40495	1:250
WNT5a	ThermoFisher	MA5-15511	1:500
WNT5b	ThermoFisher	PA5-72844	1:500
MAPK	Cell signalling Technology	9012S	1:1000
FAK	Cell signalling Technology	3258S	1:1000
AKT	Cell signalling Technology	9272S	1:1000
CREB	Cell signalling Technology	9197S	1:1000
Integrin- β	Abcam	ab183666	1:1000
SHMT2	Abcam	ab224428	1:1000
P53	Cell signalling Technology	2524T	1:1000
Luciferase	Abcam	ab21176	1:1000
MDM2	Santa Cruz Biotechnology	sc-5304	1:500
LOX	Cell signalling Technology	58135S	1:1000
LDHA	Cell signalling Technology	3582S	1:1000
ETS1	Santa Cruz Biotechnology	sc-55581	1:500
cMYC	Santa Cruz Biotechnology	sc-56634	1:500
FOSL1	R&D systems	AF4935	1:5000

FOSL2	Cell signalling Technology	11967	1:5000
FOSB	Cell signalling Technology	2251	1:1000
cFOS	Biolegend	61402	1:1000
ATF4	Cell signalling Technology	3753	1:1000
JUNB	Cell signalling Technology	3753	1:1000
cJUN	Cell signalling Technology	9165	1:1000
JUND	Cell signalling Technology	5000	1:1000
FDPS	Abcam	ab38854	1:1000
ATG3	Abcam	ab108251	1:1000
TIA1	Abcam	ab140595	1:1000
pLYC	R&D systems	MAB7500	1:500
LYC	Cell signalling Technology	2787T	1:1000
pLYN	Biorbyt	orb315590	1:1000
LYN	Cell signalling Technology	4576S	1:1000
PKM2	Cell signalling Technology	4053S	1:1000

7.3.5 Co-immunoprecipitation

Co-IP was performed using Ser-Mag speed beads protein A/G magnetic particles (GE lifesciences). 25 µl of bead slurry was placed into each Eppendorf tube and 175µl of wash buffer added. Samples were vortexed. Beads were engaged on magnet and buffer removed. A second wash was repeated using 1ml of wash buffer.

The lysates were pre cleared by adding the appropriate volume for 1mg of protein per tube containing cleaned beads. The volume was made up to 500µl using NP40 + protease inhibitors. Samples were vortexed for 10 seconds. Samples were incubated for 30 minutes at room temperature. Beads were engaged on magnet and samples moved to a fresh Eppendorf tube.

Lysates and antibody were incubated overnight at 4°C with gentle rotation. The following morning samples were incubated with 25µl of cleaned beads for 1H at room temperature. Beads were engaged onto magnet and washed with 500ul of wash buffer each time and 10s of vortexing. A final wash was undertaken with 500µl of ultrapure water.

Protein was eluted from beads using 65 μ l of elution buffer incubated at room temperature for 10 minutes. Following this, 10 μ l of neutralization buffer was added. Beads were pelleted by pulse centrifugation. Beads were engaged on magnet and samples removed and placed into fresh Eppendorf tube. 25 μ l of 4X lamelli was added to the samples. Samples were stored at -80°C.

7.4 Immunocytochemistry and immunohistochemistry

7.4.1 Immunostaining with fluorescent stains

Cells were grown to roughly 70% confluence on coverslips in a 6 well dish. When assay was complete media was aspirated and cells washed once with PBS and then all wash aspirated. The cells were subsequently fixed in 4% formaldehyde/PBS for 30 minutes at room temperature. 0.1% Triton X-100 in PBS was added the fixed cells for 5 minutes to increase permeability. The permeabilized cells were washed 3 times in PBS. 100x Phalloidin working solution was added to each well and cells were incubated at room temperature for 90 minutes. Cells were rinsed with PBS 3 times and mounted onto a slide using DAPI containing mounting media. Cells were observed using a confocal microscope.

7.4.2 Immunostaining with fluorescent antibodies

Cells were grown on a cover slip in a 6 well dish to desired confluence. Media was aspirated and cells washed once with PBS. Cells were fixed with 100% Ice cold methanol at -20 for 5 minutes. Cells were washed 3 times with ice cold PBS. Antigen retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) was preheated 95°C. Coverslips were placed into antigen retrieval buffer and heated to 95°C for 10 minutes. The coverslips were removed and immersed in PBS. The cells were washed in PBS 3 times for 5 minutes each time. Cells were incubated with 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 minutes. Primary antibody was added to the cells in 1% BSA in PBST and incubated for 1 hour at room temperature. The solution was then removed and the cells washed 3 times with PBS for 5 minutes a time. The cells were incubated with secondary antibody in 1% BSA for 1H at room temperature in the dark. Remove the solution and wash 3 times with PBS for 5 minutes each time. DAPI stain was added to the cells for 5 minutes and washed with PBS. Cells were mounted onto coverslips using mounting media.

7.4.3 Fixation, embedding, and sectioning of spheroids and tissue

10ml of neutral buffered formalin was prepared by diluting stock solution in distilled water. Spheroids or tissue was left in formalin overnight.

Spheroids or tissue were removed from the formalin and placed into a mould. In the case of spheroid the mould was made from the cut off bottom half of a cryo vial. All remaining fixative was removed from the spheroids. Liquid 2% agarose was prepared by heating agarose solution in the microwave until molten. 1ml of agarose was pipetted into each mould to cover the spheroids. Once the agarose had set the agarose dome was removed from the mould. The domes were submerged in 50% methional until ready for use in the tissue processor.

The domes were transferred into a sealed histology container for use in the tissue processor (TP1020 semi-enclosed benchtop tissue processor Leica biosystems, Milton Keynes, UK). Name tags were written on using pencil so that the xylene wouldn't remove the markings. The containers were then placed into a metal cassette ready for use with the processor. The metal cassette was then clipped into the rotating carrousel. The processing cycle was started and run for 14h. The following day the samples were removed from the processor and placed onto the hot wax section of the embedder (EG1160 embedding station). The agarose containing spheroids from the cassettes and placed face down into metal mould so samples are in the correct orientation. Fill the metal mould with hot wax and place tissue cassette on top. Moved samples to the cold block and chill for 1h so the wax is solid. Once set pop samples out of the cassette and trim excess wax using the Para trimmer. Samples are now ready for storage and further experimentation.

Microtome blade was placed into holder and the clearance angle was set to 4. The paraffin block was then inserted into the holder. The block was then trimmed until a surface level section of the sample could be cut from the block using a thickness of 10uM. Sections were cut with a thickness of 4uM into a ribbon. The ribbon was subsequently picked up using teasers and placed on the surface of a water bath and left until sections flattened out. Microscope slides were used to pick up the sections and left to dry overnight.

7.5 Kits

7.5.1 ATP production

ATP assay kit was used to determine ATP levels (ab113849, Abcam, Cambridge, UK). Cells were seeded into 96 well white luminescence plate at a concentration of 5,000 cells per well in 100µl media. Cells were grown for 24h. 50µl detergent was added and incubated for 5 minutes at room temperature on an orbital shaker (700rpm). 50µl substrate solution was added to each well for 5 minutes at room temperature and shaken as previously. Plate was then covered in foil for 10 minutes to dark adapt luminescence. The plate was then read on plate reader (flourostar).

7.5.2 Lactate production

Extracellular lactate levels were determined using Lactate-Glo kit (Promega). 10,000 cells were plated and grown for 24h in dialysed serum (one-shot dialysed serum, Gibco). Medium was collected after 24h and diluted 1 in 50 in PBS. 50µl of diluted media was added to a plate. 50µl of prepared detection reagent was added to each well. Plate was shaken gently for 60 seconds and incubated for 1h. Luminescence was recorded using plate reader (flourostar).

7.5.3 Mitochondria staining

10,000 cells were seeded onto a black 96 well plate and left to grow for 24h in either normoxic or hypoxic conditions. Prior to staining cells were washed with warm PBS (37°C). Warm serum free media containing the staining solution and placed in the incubator for 30 minutes (Active Motif, #15005, Chromeo live cell mitochondrial staining kit). Subsequently cells were washed with warm PBS twice. Cells were placed in PBS and imaged by confocal microscopy. Hypoxic condition was undertaken in the hypoxic chamber using degassed PBS. Plates were sealed before imaging with parafilm.

7.6 Functional Assays

7.6.1 Migration

Cells were seeded into a 24 well plate at a concentration of 50,000 cells per well. Cells were left overnight at 37°C until confluent. Afterwards cells were scratched with a P200 pipette to form a cross. An equal amount of pressure was added each time to ensure each scratch was the same size. Following the scratch wells were washed 3 times with media. Drug was added to the media at a concentration of 10 μ M. Wells were imaged using fixed camera and XYZ coordinates of each image saved for imaging consistently for each wound. Cells were placed in incubator or hypoxia for 24H and then imaged again using the same coordinates. Changes in wound size were calculated using Image J and compared to control.

7.6.2 Invasion

Matrigel (Corning) was thawed overnight at 4°C. Matrigel was added to boyden chamber to a final volume of 100 μ l. Concentrations of matrigel were optimized for the cell lines used. Cal-51 were optimized for a 1 in 6 dilution. Matrigel was diluted in serum free media. The matrigel was left to set overnight in the incubator. Following this 500 μ l of 10% serum was added to the bottom of the well. Cells were extracted and counted as mentioned above. 100,000 cells were added to the boyden chamber in 1% FBS media. Cells were incubated in normoxia and hypoxia for 48H. Media was aspirated from top of the chamber. The inserts were placed in ice-cold methanol for 30 minutes to fix cells. Afterwards the Matrigel was removed using a cotton bud. The cells were stained by placing the inserts into 0.1% crystal violet 10 minutes. Afterwards the inserts were washed 3 times by submersion into PBS. Afterwards the cells were counted at 40X magnification.

7.6.3 Spheroid invasion

Spheroids were grown as described previously. Once formed the cell culture media was removed and replaced with 100ul 3mg/ml growth factor free geltrex (ThermoFisher). Cells were placed in the incubator for 1h to allow the geltrex to solidify. Subsequently 100ul of media was added on top. Spheroids were imaged using widefield microscope for 0h timepoint and then again at 24h.

7.6.4 pH_i quantification

10,000 cells were seeded onto a black 96 well plate and left to grow for 24h in either normoxic or hypoxic conditions. Media was removed and warmed PBS with staining solution was added (SNARF™-1 Carboxylic Acid, Acetate, Succinimidyl Ester, Invitrogen, and S22801). Cells were incubated with staining solution for 15m and then placed in fresh PBS. Cells were imaged using confocal microscopy.

8 Chapter 3: NDBT expression and binding partners

8.1 Introduction:

The NDBT family are involved in the process of pH regulation, as such, expression of these proteins are widespread throughout the body⁴⁵⁹. However, in normal tissue, expression of these transporters is particularly high in renal cells⁴⁶⁰ and proximal tubules⁴⁶¹. This is to control absorption of salt in urine. RNA Sequencing analysis identified high levels of transcripts in the testes and endocrine tissues⁴¹³. Data from the Human Protein Atlas also indicated high expression in: bone marrow, liver, gastrointestinal tract, and reproductive tissues⁴⁶². SLC4A4 expression in particular has been shown to be high in the plasma membrane of neurone and glia to control neuron activation; secretory epithelial cells where it facilitates luminal secretion, and renal proximal tubules to control ion reabsorption¹²⁸. Similarly to SLC4A4, SLC4A5 expression is widespread throughout the body. Expression of SLC4A5 is particularly high in choroid plexus where bicarbonate absorption helps facilitate cerebrospinal fluid secretion in vesicles, and hepatocytes where it controls liver pH⁴⁶³.

Loss of expression of NDBTs have been shown to result in disease¹²⁸. Many individuals with SLC4A4 gene defects display mental retardation and whole body acidosis¹²⁸. Mutations in the C-terminus of SLC4A4 have been shown to be linked to epilepsy⁴⁶³. Furthermore many individuals with SLC4A4 gene defects present with poor eyesight or blindness⁴⁶⁴. In SLC4A4 null mice, death occurs at week 4, before possible ocular defects could manifest. Individuals with SLC4A4 gene defects also display lowered growth and weight, similarly SLC4A4 null mice have bone dysplasia and muscle mass⁴⁶⁵. Loss of SLC4A4 results in proximal renal tubule acidosis indicating the requirement for these proteins in pH regulation⁴⁶⁶. Although expression of SLC4A5 is high in hepatocytes no known defects in the liver have been seen in those presenting with SLC4A5 mutations¹²⁸. Mice with SLC4A5 defects display decreased cerebrospinal fluid secretion and thus lowered brain ventricle volume⁴⁶⁷. Furthermore SLC4A5 null mice display ocular abnormalities such as detached retina, likely due to the changes induced by brain ventricle pressure¹²⁸. Single nucleotide polymorphisms have been linked to hypertension⁴⁶⁸. SLC4A5 null mice also display

compensated metabolic acidosis, with normal pH but increased levels of bicarbonate in the urine⁴⁵².

Multiple cancers express NDBTs¹²⁸. Protein expression studies of NCBe1 displayed high expression levels in: thyroid, prostate and liver cancers¹²⁸. Expression of NBCe2 was highest in breast, thyroid and prostate cancers⁴⁶⁹. Thyroid tumour tissue had three times higher expression of SLC4A4 than matched normal tissue⁴⁷⁰. Previous work indicated a heterogeneous expression of NDBTs in: breast, colorectal, and brain cancers¹²⁸. Hypoxia leads to an induction of many of these transporters at the RNA level⁴³¹. Investigations into the role of bicarbonate transporters in HER2 positive models showed an important role in pH regulation⁴⁷¹. In addition, mutations in NDBT *SLC4A7* show an increased breast cancer risk⁴⁷². While many studies have looked at the role of bicarbonate transporters in ER+ve breast cancers, few studies look into triple negative breast cancer⁴⁷³. MDA-MB-231 showed induction of multiple NDBTs in hypoxia indicating a role of NDBTs and pH regulation in triple negative breast cancer⁴³¹. Expression of SLC4A4 has been shown to have been exceptionally high in the colorectal cell line HT29⁴⁷⁴. Knockdown of SLC4A4 in HT29 has been shown to sensitise the cells to methotrexate⁴⁷⁴.

Tumour cell migration requires the tightly controlled coordination of: cell adhesion contacts, cytoskeletal remodelling, the digestion and reorganization of the extracellular matrix, and local ion and water transport across the plasma membrane. These processes are dependent on changes in intracellular and extracellular pH. The NDBTs play an important role in controlling intracellular pH and function as part of a cellular pH metabolon in the lamellipodia (Fig. 8.1). NHE1, a protein similar to the NDBTs, has been shown to transport protons to the cytosol⁴⁷⁵ and acidify the extracellular space⁴⁷⁶. NHE1 enhances the invasiveness of melanoma cells⁴⁷⁷, modifies migration⁴⁷⁸, morphology⁴⁷⁹, and adhesion⁴⁸⁰. SLC4A7 has also been shown to localise to the leading edge of lamellipodia⁴⁸¹. The NDBTs function to extrude acid but function indirectly. The bicarbonate is transported into the cell and reacts with hydrogen ions to form CO₂ and H₂O. The CO₂ then diffuses outside the cell and is used by CAIX to generate protons and maintain the CO₂ gradient (Fig. 8.1). Additionally, the enzymatic activity of CAIX is higher in the presence of NDBTs⁴⁵³.

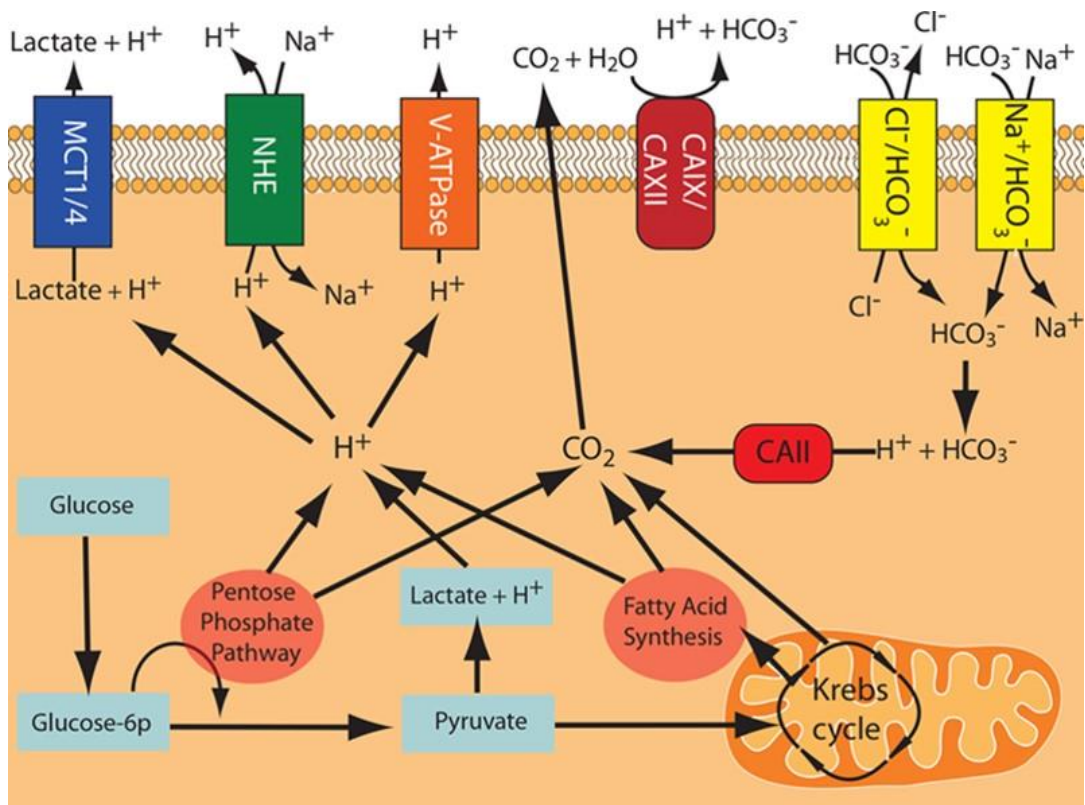


Figure 8.1 Methods of H⁺ generation and extrusion from cell into extracellular environment⁴³¹.

Cellular metabolism results in the production of hydrogen and carbon dioxide. To adequately control intracellular pH the cells have multiple different proteins involved export of hydrogen ions. However, the most effective methods for regulating intracellular ions is for bicarbonate to titrate the H⁺ ions intracellularly to form CO₂ which can freely diffuse across the plasma membrane. To maintain a concentration gradient CO₂ is then hydrated to produce H⁺ and bicarbonate extracellularly.

8.2 Hypothesis:

I hypothesise that expression of NDBTs are upregulated in hypoxia and localise to the leading edge of migrating tumour cells. To investigate this hypothesis I will undertake the following aims:

1. *Determine the mRNA expression levels of NDBTs at different time points in normoxia and hypoxia (0.5% O₂).*
2. *Evaluate the protein expression levels of NDBTs at different time points in normoxic and hypoxic conditions.*
3. *Investigate if NDBTs and CAIX are binding partners in hypoxia*
4. *Investigate if NDBTs co-localise to the leading edge of migrating tumour cells in hypoxia*
5. *Produce and validate shRNA knockdown cell lines*
6. *Determine the pH in cell lines with NDBT knockdown or inhibition*

8.3 Results:

The expression of the sodium driven bicarbonate transporter family members in normoxia and hypoxia (0.5% O₂ 72h time course) was investigated by qRT-PCR. The expression of these genes was investigated in a panel of four triple negative cancer cell lines (HCC-1806, MDA-MB-231, Cal-51, and Sum-159pt; (Fig. 8.2). This revealed 28 cases where expression was significantly increased and one case where expression was significantly reduced. All cell lines showed significant increases in at least 3 of the sodium driven bicarbonate transporters. The pattern of increased expression in hypoxia was unique in each of the four cell lines and did not correlate to the overall expression pattern in normoxia. The bicarbonate transporters SLC4A4 (4/4), SLC4A5 (3/4), and SLC4A8 (4/4) were the most frequently upregulated by hypoxia. SLC4A4 was upregulated 50-fold in HCC-1806 ($P < 0.05$ $n = 3$), 9-fold in MDA-MB-231 ($P < 0.01$ $n = 3$), 6-fold in Sum-159pt ($P < 0.001$ $n = 3$), and 5-fold in Cal-51 ($P < 0.001$ $n = 3$). SLC4A5 upregulated 5-fold in HCC-1806 ($P < 0.001$ $n = 3$), 4-fold in MDA-MB-231 ($P < 0.01$ $n = 3$), 5-fold in sum-159pt ($P < 0.001$ $n = 3$), 5-fold in Cal-51 ($P < 0.05$ $n = 3$) (Fig. 8-2).

To determine if increases in NDBT mRNA expression results in increased protein expression, western blot time courses were undertaken in normoxia and hypoxia (Fig. 8-3). In HCC-1806, expression of NBCe-1 was upregulated at 24h by 50%, reduced at 48h by 30%, upregulated 5% at 72h, and reduced 60% at 96h. Expression of NBCe-2 was upregulated significantly at all four time points tested compared to normoxia. Furthermore, expression of SLC4A7 and SLC4A9 were not modulated by hypoxia and both maintained a consistent expression level at all time points ($n = 3$).

Further to this a hypoxic RNA time course was undertaken for the alpha-carbonic anhydrase family in hypoxia to determine if other points in the proposed pH metabolon are also hypoxia regulated (Fig. 8-4). Multiple members were shown to be either upregulated or downregulated in hypoxia. *CAII* was upregulated at both 24h and 72h hypoxia ($P < 0.05$, $n = 3$), *CAIII* ($P = 0.05$, $n = 3$), *CAXII* ($P < Ns$, $n = 3$), and *CAXIII* ($P < Ns$, $n = 3$) were upregulated at all time points in hypoxia. Conversely *CAI* ($P = Ns$, $n = 3$) was downregulated in hypoxia at 24h and 48h. This indicated that some members may be regulated by hypoxia.

To determine if expression of NDBTs was localising to the edge of tumour cells the cells were grown on cover slips and scratched (Fig. 8-5). The cells were placed in hypoxia to

induce the expression of CAIX. As can be seen from the images expression of SLC4A4 and SLC4A5 (green) was high and their expression is ubiquitous in non-migrating cells. NDBTs co-localise with CAIX in hypoxia in HCC-1806. Expression of NDBTs was localised at the leading edge of the tumour cells (green). CAIX expression localised to the edge of cells (red). Co-localisation displayed as yellow when signals overlapped. No expression of CAIX was detected in normoxic conditions (n = 3).

Determination of binding partners for NBCe-1, NBCe-2, and NBCn-1 was undertaken using Co-immunoprecipitation, pulling down with anti-CAIX or anti-CAXII antibodies. Both NBCe-1 and NBCn-1 showed binding to CAIX in hypoxic conditions (1% or 0.5% O₂) in HCC-1806 (Fig. 8.6-8.7), and MDA-MB-231 (Fig. supplementary, n = 3). It was not possible to determine interactions between NBCe-2 and CAIX. NBCe-2 was shown to interact with CAXII in HCC-1806 (Fig. 8.8, n = 3).

Prior to the generation of lentiviral transduction cell line resistance to the selection reagent had to be determined. Cells were treated at differing concentrations of puromycin to determine lowest effective dose determined by 100% cell death (Fig. 8.9). The effective concentration of puromycin needed for both HCC-1806 and MDA-MB-231 was 3µg/ml. For Cal-51 the effective concentration was 40µg/ml.

Cell lines were transduced with lentiviral shRNA to produce NDBT knockdown cell lines. Initial transduction produced cell pools with insufficient knockdown thus single cell clonal selection was undertaken. Single cell clones were grown and tested for expression of NDBTs and produced high knockdown efficiency clones. Knockdown was quantified by qRT-PCR. (A) *SLC4A4* expression in HCC-1806 was reduced: 90%, 95%, and 55% in constructs: 1, 2, and 3 respectively in normoxic conditions. (B) *SLC4A4* expression in Cal-51 was reduced by: 85% by construct 1, 90% by construct 2, and 75% by construct 3. (C) *SLC4A5* expression in HCC-1806 was reduced: 75%, 80%, and 78% in constructs: 1, 2, and 3 respectively in normoxic conditions. (D) *SLC4A5* expression in MDA-MB-231 was reduced: 80%, and 85% in constructs: 1, and 2 respectively in normoxic conditions (Fig. 8.10).

Expression of NBCe-1 was reduced with lentiviral transduction in HCC-1806 clones (Fig. 8.11). The expression levels of NBCe-1 were reduced 10% in normoxia and 20% in hypoxia (0.5% O₂) in construct 1. Expression levels of NBCe-1 were reduced 80% in normoxia and 95% in hypoxia (0.5% O₂) in construct 2. Expression levels of NBCe-1 were reduced 70% in normoxia and 75% in hypoxia (0.5% O₂) in construct 3. ***, P < 0.001; **, P < 0.01; *, P <

0.05; n = 3. Expression of NBCe-2 was reduced with lentiviral transduction in HCC-1806 clones. The expression levels of NBCe-1 were reduced 40% in normoxia and 70% in hypoxia (0.5% O₂) in construct 1. Expression levels of NBCe-1 were reduced 90% in normoxia and 75% in hypoxia (0.5% O₂) in construct 2. Expression levels of NBCe-1 were reduced 90% in normoxia and 95% in hypoxia (0.5% O₂) in construct 3. ***, P < 0.001; **, P < 0.01; *, P < 0.05; n = 3. Expression of NBCe-1 was reduced with lentiviral transduction in Cal-51 clones. The expression levels of NBCe-1 were reduced 20% in normoxia and 50% in hypoxia (0.5% O₂) in construct 1 (Fig. 8.11). Expression levels of NBCe-2 were reduced 50% in normoxia and 50% in hypoxia (0.5% O₂) in construct 2. Expression levels of NBCe-1 were reduced 70% in normoxia and 85% in hypoxia (0.5% O₂) in construct 3. ***, P < 0.001; **, P < 0.01; *, P < 0.05; n = 3. Expression of NBCe-2 was reduced with lentiviral transduction in MDA-MB-231 clones. The expression levels of NBCe-1 were unchanged by S0859 treatment (10uM) in normoxia and hypoxia (0.5% O₂). Expression levels of NBCe-1 were reduced 90% in normoxia and 75% in hypoxia (0.5% O₂) in construct 1. Expression levels of NBCe-1 were reduced 95% in normoxia and 95% in hypoxia (0.5% O₂) in construct 2. ***, P < 0.001; **, P < 0.01; *, P < 0.05; n = 3.

It was predicted that loss of NDBT activity would lower intracellular pH. To determine if inhibition or knockdown of NDBTs directly impacts intracellular pH cells were grown and stained with a pH sensitive ratiometric fluorescent dye carboxy-snarf (Fig. 8.12). Inhibition of NDBT reduced intracellular pH by 0.3 (P < 0.05, n = 3) and 0.1 (P < 0.05, n = 3) units in normoxia and hypoxia respectively in HCC-1806; 0.15 (P < 0.05, n = 3) and 0.2 (P < 0.05, n = 3) units in normoxia and hypoxia respectively in MDA-MB-231; 0.18 (P < 0.05, n = 3) and 0.23 (P < 0.01, n = 3) units in normoxia and hypoxia in CAL-51; and 0.24 (P < 0.05, n = 3) and 0.22 (P < 0.05, n = 3) units in normoxia and hypoxia in SUM-159PT (Fig. 10-4). Knockdown of SL4A4 reduced intracellular pH by 0.3 (P < 0.05, n = 3) and 0.2 units (P < 0.05, n = 3) in normoxia and hypoxia in HCC-1806 and 0.3 units (P < 0.05, n = 3) in normoxia and hypoxia in CAL-51 (Fig. 10-4). Similarly SLC4A5 knockdown reduced pH by 0.2 (P < 0.05, n = 3) and 0.17 (P < 0.05, n = 3) units in normoxia and hypoxia in HCC-1806 and 0.23 and 0.22 units in normoxia and hypoxia respectively in MDA-MB-231 (fig. 8.12).

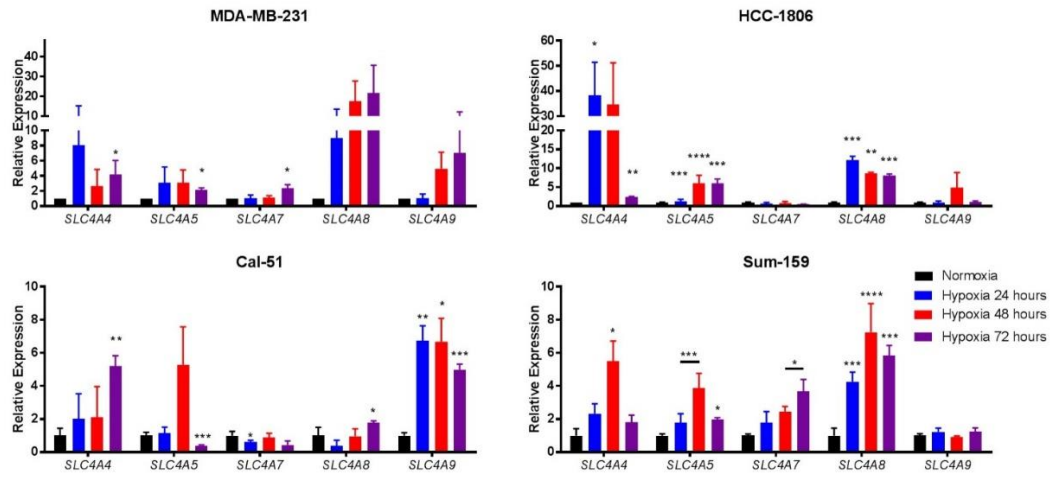


Figure 8.2 Expression of NDBT mRNA is upregulated in multiple Triple Negative Breast cancer cell lines in hypoxia.

Expression of sodium driven bicarbonate transporters is varied among different cell lines. The most frequently upregulated NDBT was SLC4A4, followed by SLC4A5 and SLC4A8. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

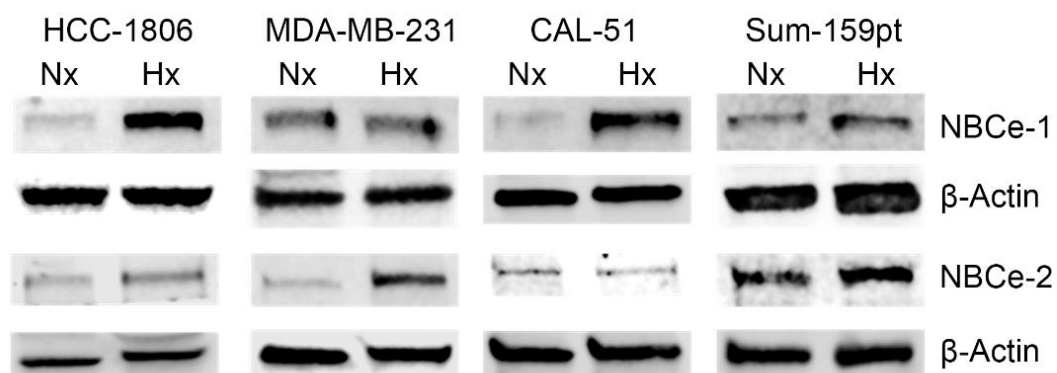


Figure 8.3 Expression of NDBT proteins are upregulated in hypoxia in HCC-1806.

Hypoxia induced the expression of NBCe-1 was upregulated in hypoxia in HCC-1806, CAL-51, and Sum 159pt at 24h at 0.5% O₂. Whereas NBCe-2 was upregulated in HCC-1806, MDA-MB-231, and Sum-159pt in hypoxia. (n = 3).

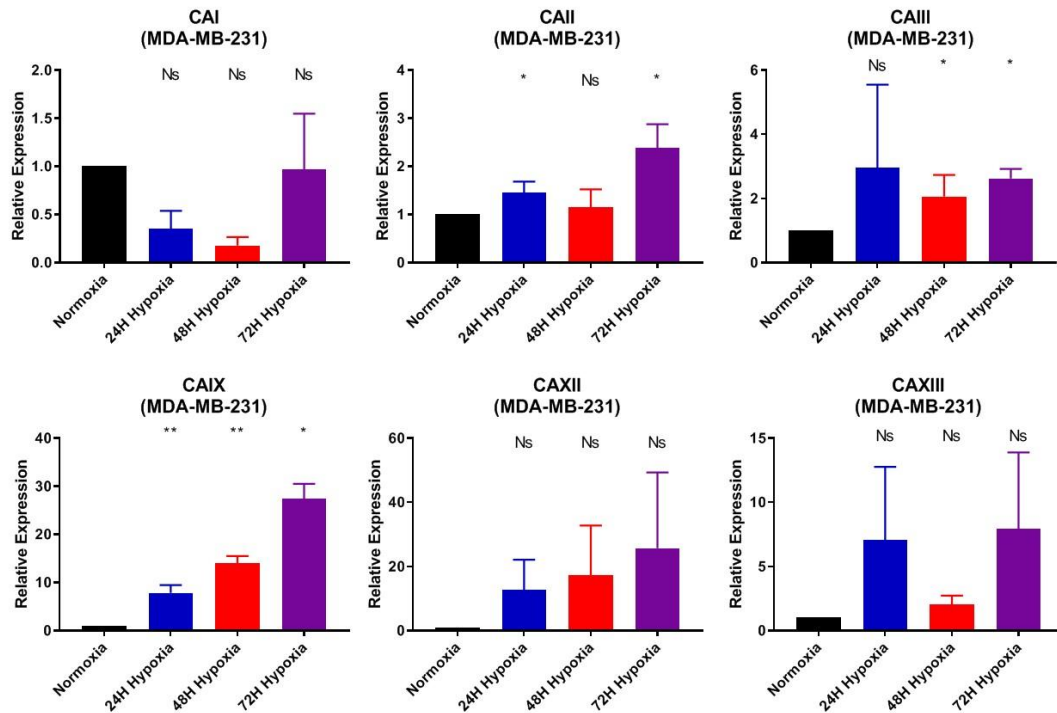


Figure 8.4 Carbonic anhydrase family mRNA expression is increased in hypoxia in MDA-MB-231.

RNA expression of carbonic anhydrase family members is modulated by hypoxia (0.5% O₂) in MDA-MB-231. (A) Expression of CAI is reduced by 60%, 75%, and 5% at 24h, 48h, and 72h hypoxia respectively. (B) Expression of CAII is increased 1.5, 1.2, 2.2 fold at 24h, 48h, 72h hypoxia respectively. (C) Expression of CAIII is increased 2.5 fold at 24h, 2 fold at 48h, and 5 fold at 72h hypoxia. (D) Expression of CAIX is increased 10 fold at 24h, 15 fold at 48h, and 30 fold at 72h hypoxia. (E) Expression of CAXII was increased 17 fold, 19 fold, and 22 fold at 24h, 48h, and 72h hypoxia respectively. (F) Expression of CAXIII is increased 1.5 fold, 2 fold, and 1.9 fold at 24h, 48h, and 72h hypoxia respectively. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n = 3.

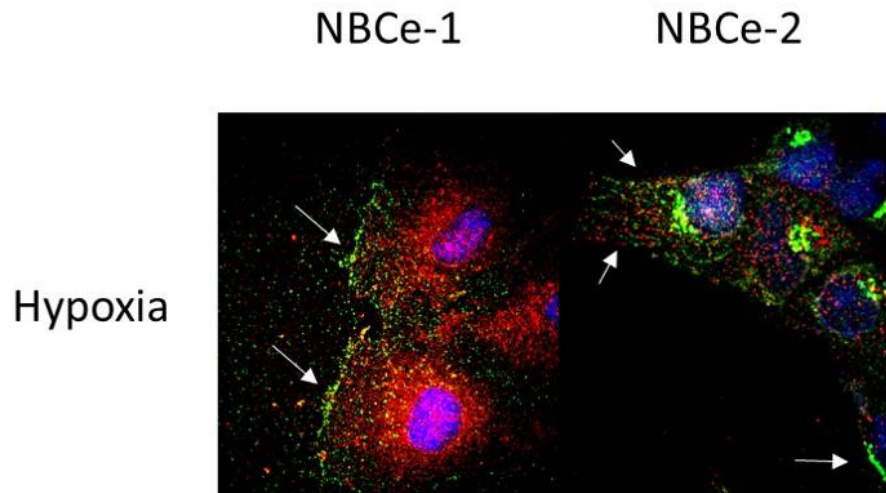


Figure 8.5 Co-localisation of NDBT and CAIX in migrating HCC-1806 cells.

Expression of NDBTs localise to the leading edge of migrating tumour cells. NDBT = green, CAIX = red, DAPI = blue. Cells were scratched on the left and migrated for 24h. Expression of NBCe-1 localised to the leading edge of the tumour cell, whereas expression of NBCe-2 also localised to the leading edge to a lesser extent. CAIX expression was ubiquitous through the cell but yellow staining indicated co-localisation of CAIX and NDBTs at the leading edge. n = 3).

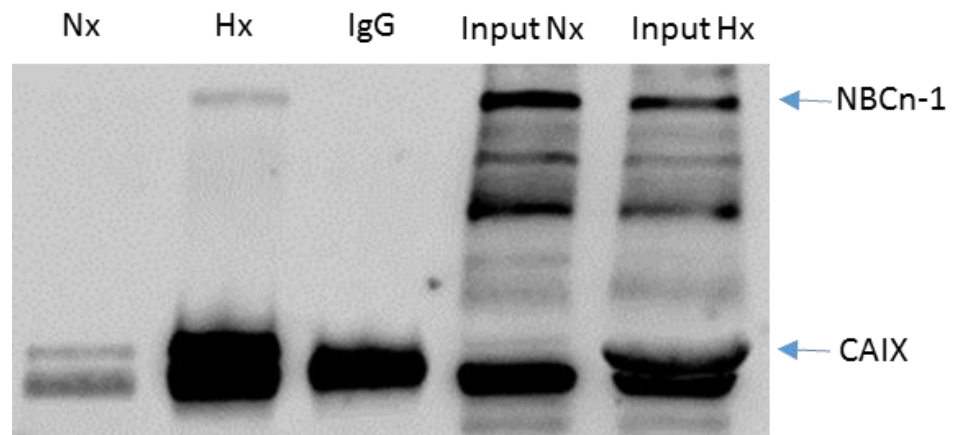


Figure 8.6 NBCn-1 Co-IP using CAIX pulldown shows binding in hypoxia.

An interaction between NBCn-1 and CAIX was detected in hypoxia but not in normoxia in HCC-1806 (0.5% O₂). Expression of CAIX is too low in normoxia so an interaction could not be detected (n = 3).

HCC-1806

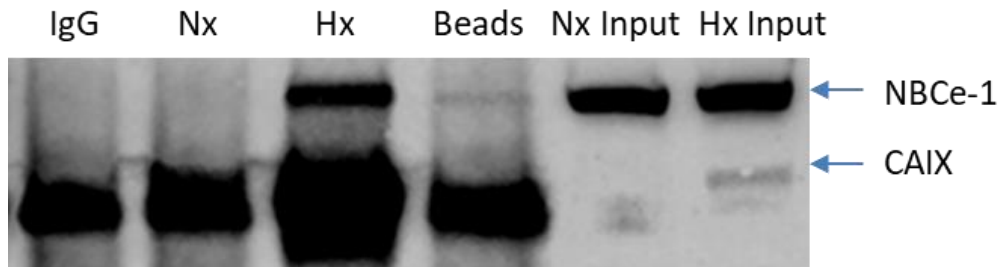


Figure 8.7 NBCe-1 Co-IP using CAIX pulldown shows binding in hypoxia.

NBCe-1 interacts with CAIX in hypoxic conditions. High levels of NBCe-1 were seen with the pulldown and no interaction was seen during normoxia (n = 3)

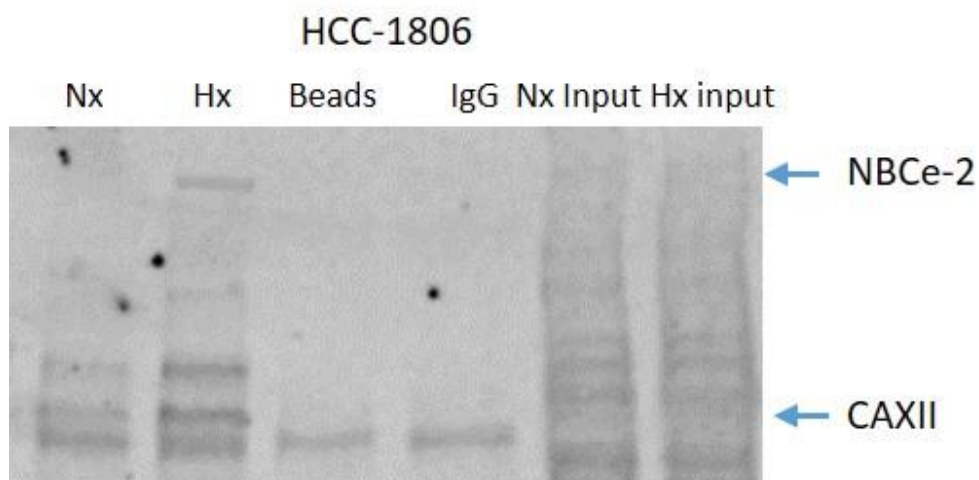


Figure 8.8 Co-IP of NBCe-2 using CAXII pulldown shows binding in hypoxia.

NBCe-2 interacts with CAXII in hypoxic conditions but not normoxic conditions. (n = 3).

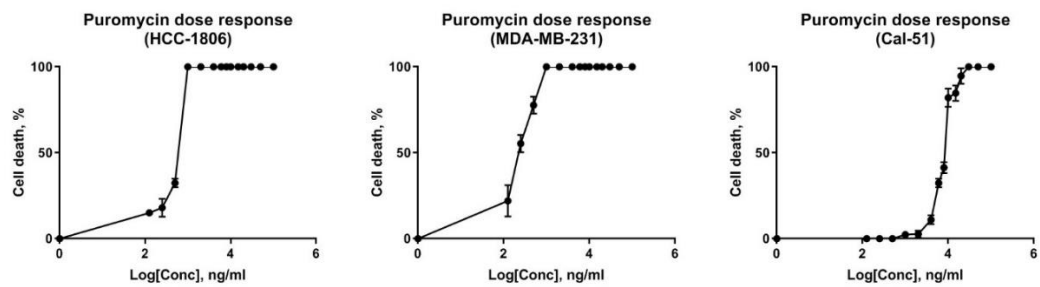


Figure 8.9 Puromycin kill curves for Lentiviral selection in multiple cell lines.

Cell survival was reduced upon treatment of puromycin. Viability was quantified 72h post treatment. Trypan blue was used to differentiate between live and dead cells. The cells lines were tested (A) HCC-1806, (B) MDA-MB-231, (C) Cal-51. The lowest concentration that resulted in <95% cell death was used for selection. n = 3

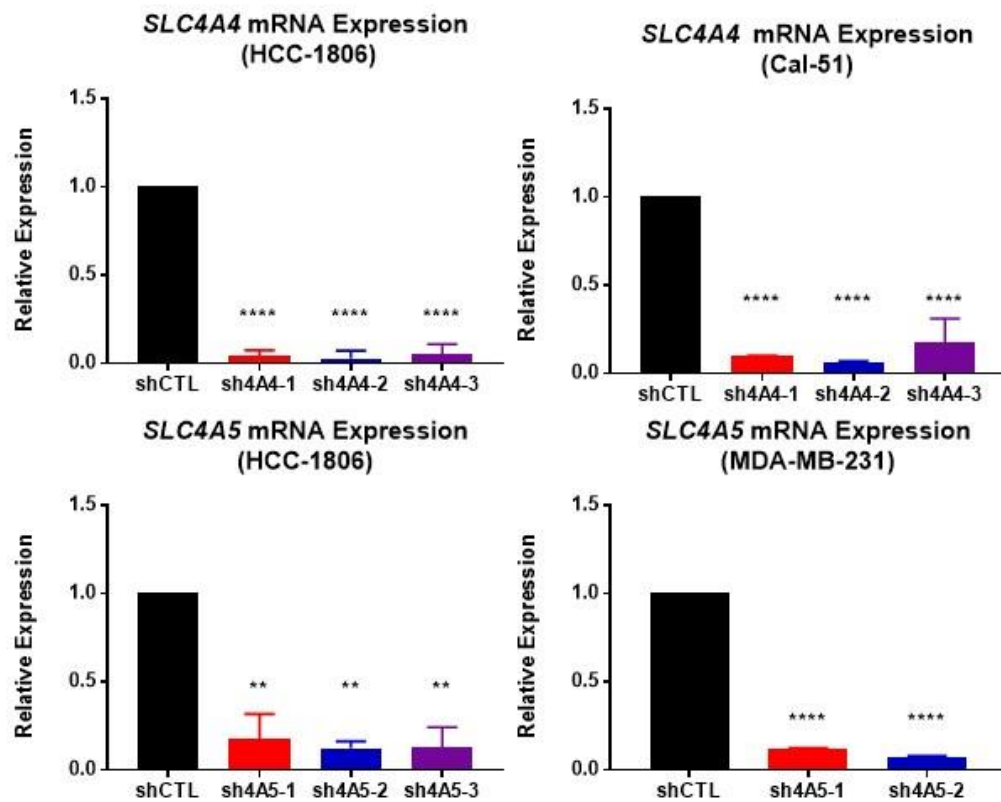


Figure 8.10 Lentiviral transduction and clonal selection reduced expression of NDBTs in multiple cell lines.

HCC-1806 transduction produced three clones with SLC4A4 knockdown all greater than 90%. Transduction produced three clones with at least 75% knockdown of SLC4A5 in HCC-1806. Three clones were produced with greater than 75% knockdown of SLC4A4 in CAL-51. Transduction of MDA-MB-231 produced two clones with greater than 75% knockdown. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

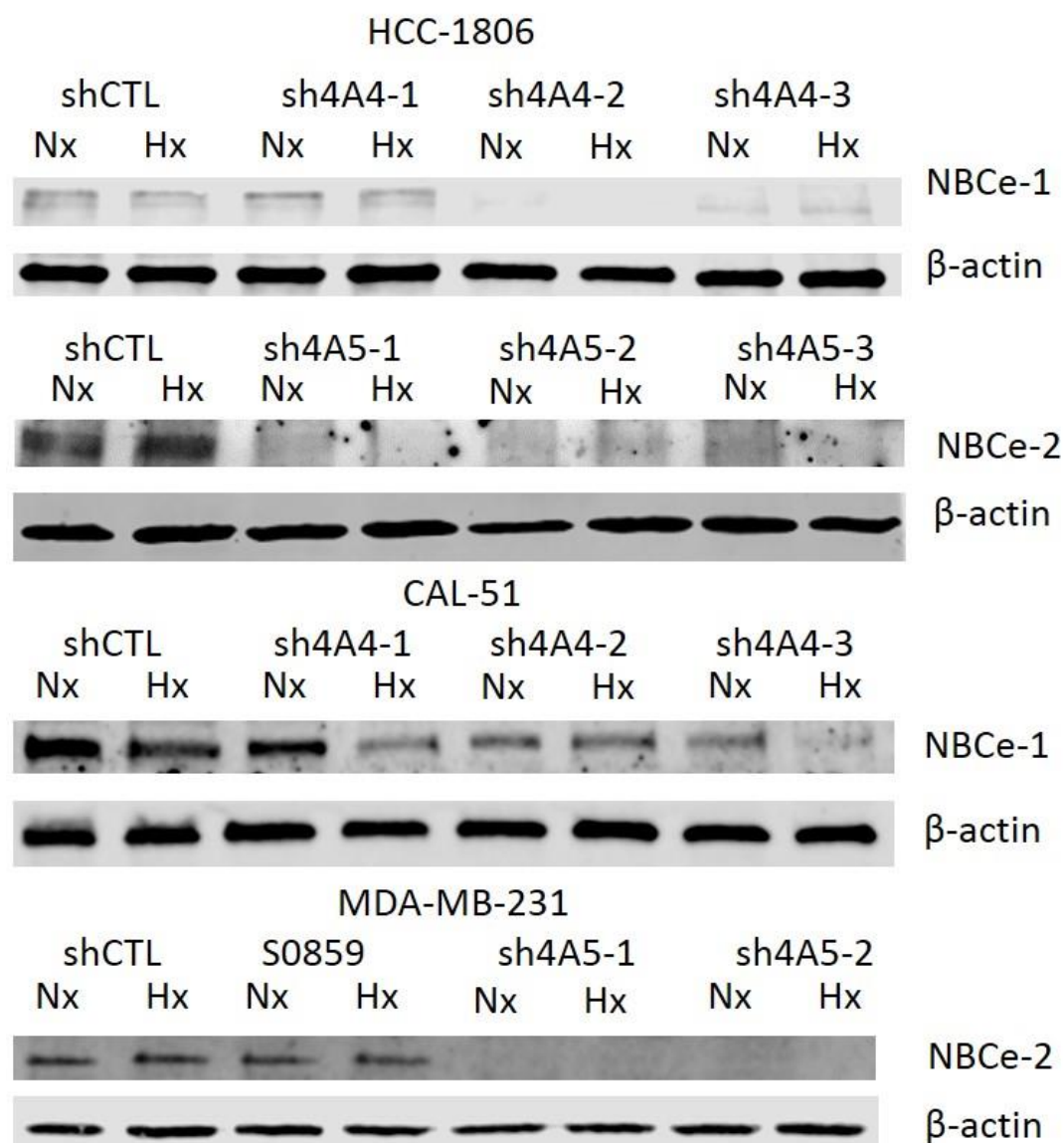


Figure 8.11 Transduction with lentiviral constructs reduced expression of NDBTs in multiple cell lines.

Three clones were produced for SLC4A4 knockdown in HCC-1806 and CAL-51. Three clones were produced for SLC4A5 knockdown for HCC-1806 and two clones for MDA-MB-231. Expression was reduced in both normoxic and hypoxic conditions (0.5% O₂) for all conditions tested. Additionally, the effect of S0859 treatment was tested to determine if inhibition affected protein stability (n = 3).

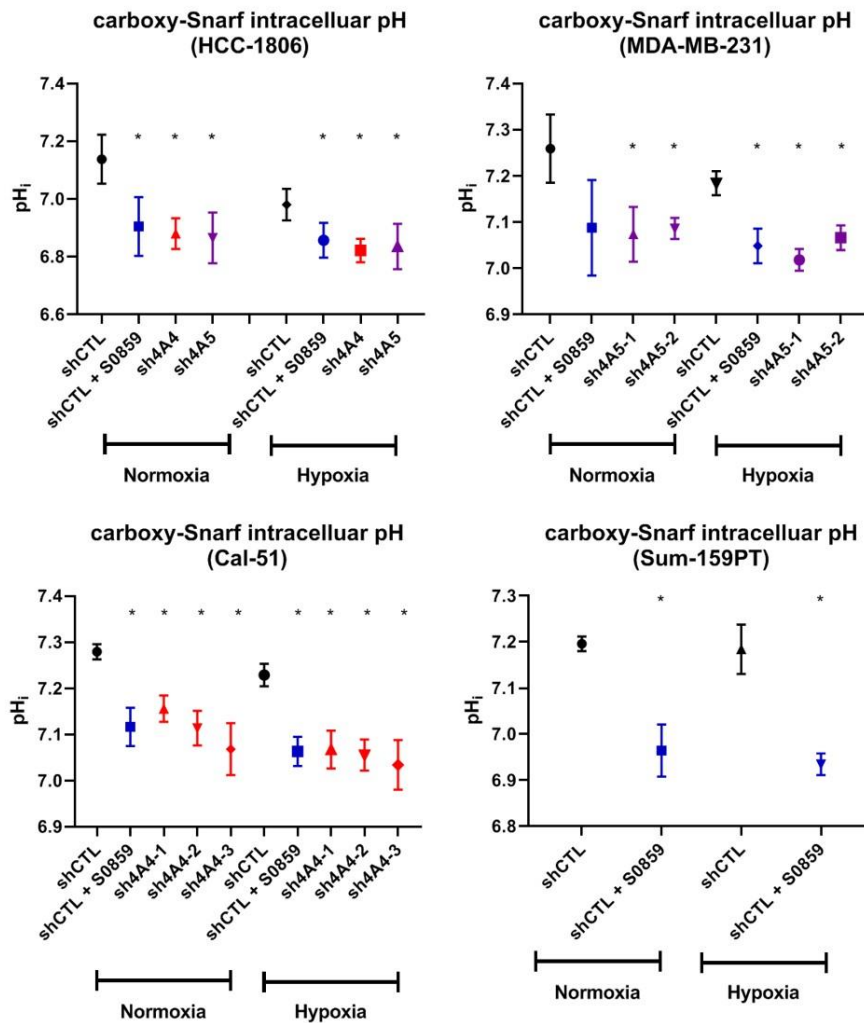


Figure 8.12 Intracellular pH was lowered by knockdown and inhibition of NDBTs.

Inhibition of NDBT reduced intracellular pH in multiple cell lines in both normoxia and hypoxia. Inhibition with S0859 (10uM) for 24h reduced pH by 0.2 units in both normoxia and hypoxia in all cell lines tested.

8.4 Discussion:

The results presented in this chapter answers the hypothesis “Is NDBT expression upregulated in hypoxia and are they binding partners of CAIX *in vitro*” in triple negative breast cancer cells. Expression of NDBT mRNA’s were upregulated in 4 breast cancer cell lines at 0.5% O₂ with a heterogeneous expression within cell lines and between cell lines. Protein expression of NDBTs were either upregulated or maintained in many cell lines in hypoxia. Similarly, expression of other members of the pH regulatory metabolon were upregulated in hypoxic conditions, particularly membrane bound and cytoplasmic carbonic anhydrases. Thus, many members of the metabolon are upregulated to ensure the enzymatic reactions are not rate limited by a single member. NDBTs colocalised to the leading edge of migrating tumour cells and colocalised with CAIX in hypoxia. CAIX and CAXII were shown to be binding partners of NDBTs in hypoxia.

0.5% O₂ hypoxia was chosen as the physiologically relevant partial pressure for breast tumours¹⁰⁷ and previous work⁴³¹. Hypoxia (0.5% O₂) resulted in an upregulation of NDBT mRNA in multiple cell lines (Fig. 8.2). The expression was heterogenous (Fig. 8.1). Expression of NDBT mRNA has been investigated in other studies in different cell lines and oxygen tensions⁴⁵⁴. It was shown that at 1% O₂ only LS174T cells had induction of an NDBT at 24h⁴⁵⁴. Only *SLC4A4* was upregulated in these conditions⁴⁵⁴. The other cell lines tested (MDA-MB-231, PC3, A549, A375, and CAL51) failed to demonstrate an induction of NDBT at 24h or 48h hypoxia⁴⁵⁴. However, in this study induction was defined as higher than 20 fold induction, and lower levels of induction were seen⁴⁵⁴. In 0.5% hypoxia *SLC4A4*, *SLC4A5*, *SLC4A8*, and *SLC4A9* were induced. Additionally, under the same conditions, *SLC4A4* expression was induced in HCT116, and SCC25; whereas *SLC4A5* was induced in SW480, MDA-MB-231, and U87 cells. *SLC4A7* was only induced in one cell line, HCT116, in hypoxia⁴³¹. *SLC4A9* was the most frequently upregulated across all the cell lines⁴³¹. When the same cell lines were tested at 1% oxygen, expression of the NDBTs was not significantly induced except in LS174T⁴³¹. It was shown that chronic hypoxia reduced the expression of *SLC4A7*⁴⁸². HIF stabilization results in the upregulation of many pH regulatory proteins²²⁴. These data indicate that expression of NDBTs is only induced under more severe hypoxia. Whereas, other pH regulatory proteins such as CAIX are more readily induced at higher oxygen tension.

HIF-1 α has been shown to upregulate the expression of *SLC4A4* in 1% hypoxia⁴⁵⁴. This was also shown in 0.5% hypoxia⁴³¹. Furthermore, HIF-1 α , but to a greater extent HIF-2 α , was shown to be involved in regulation of *SLC4A9* in 0.5% hypoxia⁴³¹. Knockdown of CAIX has been shown to induce the expression of *SLC4A4* in hypoxia; this induction was increased further when CAIX knockdown was combined with CAXII knockdown⁴⁵⁴. The loss of pH regulatory control and subsequent intracellular acidification likely increased *SLC4A4* expression. HIF-1 α stabilization has been shown to vary across cell lines in hypoxia⁴⁸³. This stabilization also varies at differing timepoints in hypoxia⁴⁸³. HIF-2 α , similarly to HIF-1 α , has varying levels of stabilization and at differing oxygen tensions and durations of hypoxia¹⁴⁹. Further to this, tissue distribution of the HIF transcription factors varies, HIF-1 α is ubiquitous expressed whereas HIF-2 α is more restricted in tissue distribution⁴⁸⁴. The heterogeneity in NDBT induction in hypoxia is likely due to varying levels of HIF transcription factor distribution and stabilization in hypoxia as well as the time point undertaken.

Protein expression of *SLC4A4* and *SLC4A5* was induced in 0.5% hypoxia whereas *SLC4A7* and *SLC4A9* were maintained. Overall protein translation is reduced in hypoxia due to limited ATP availability, highlighting the importance of these proteins in adaption to hypoxia⁴⁸⁵ whereas expression of *SLC4A4* was shown to be increased in 1% hypoxia in MDA-MB-231 at 24h⁴⁵⁴. Expression of *SLC4A7* was shown to be reduced in hypoxia. Furthermore expression levels of *SLC4A7* were lower in breast cancer samples than in matched tissue⁴⁸⁶. This data conflicts with later results in the same study which showed in a MCF10aT breast cancer progression model the expression of *SLC4A7* was increased in the low and intermediate grade lesions compared to normal tissue⁴⁸⁶. Thus, this data indicates *SLC4A7* may have a role in early breast cancer progression.

Expression of multiple members of the cytoplasmic and transmembrane carbonic anhydrase family has been shown to be upregulated in 0.5% O₂ (Fig. 8.4). Induction of CAIX mRNA indicated hypoxic induction. Induction was highest in CAXII, and CAII. Induction of HIF-1 α by dimethyl oxalylglycine failed to induce CAII protein levels in multiple cell lines⁴⁸⁷. However, in MCF7 cells, expression of CAII was increased in hypoxia. CAII has been shown to co-localise with MCT1, another pH regulatory protein⁴⁸⁸. Moreover, CAII has been shown to bind to NHE1 and enhance NHE1 activity⁴⁸⁹. Similarly CAII also has been shown to bind to NHE3 and increase NHE3 activity⁴⁹⁰. This increased expression and localisation likely occurs to facilitate efficient pH control within the cell⁴⁸⁸. Knockdown of CAII has been shown to

reduce colony survival and size⁴⁹¹. Furthermore, CAII xenografts had reduced growth and higher levels of apoptosis⁴⁹¹. The role of CAXII in hypoxia is well documented²³¹. Expression of CAXII has been shown to be increased under hypoxic conditions in cell lines and tumour xenografts⁴⁹².

NDBT expression was shown to be localised to the leading edge of migrating tumour cells⁴³¹. Similarly expression of CAIX was shown to be leading edge of the migrating cells and co-localising with NDBTs (fig. 8.5). It has previously been shown CAIX localises to the leading edge of migrating tumour cells. Knockdown of CAIX has been shown to reduce migration in hypoxia⁴⁹³. SLC4A4 has been shown to co-localise with CAIX in migrating MCDK cells, however this is not a cancer cell line⁴⁵³. Additionally, localisation was shown for NHE1²²⁷. Transfection of cells with a mutated NHE1 isoform with perturbed localisation resulted in lower directed migration but not overall migration²²⁷. Thus the localisation of pH regulatory proteins induces cell polarity and migratory direction of cells.

CAIX was shown to be a binding partner of SLC4A4 and SLC4A7 whereas CAXII was shown to be the binding partner of SLC4A5 (Fig. 8.6-8.8). No binding was seen in normoxic conditions as expression of CAX and CAXII is not induced under these conditions²³¹. While data has shown that some of these proteins co-localise in the leading edge of migrating cells, data showing that these proteins are binding partners is novel. However, CoIP doesn't distinguish if the interaction is direct or indirect. Furthermore, based upon this data the pH metabolon schematic was redrawn to factor in the binding of CAIX/CAXII and the NDBTs (Fig. 8.13).

Knockdown or inhibition of NDBTs lowered intracellular pH in multiple cell lines (Fig. 8.12). Knockdown produced a slightly bigger reduction (0.1 unit) in intracellular pH than inhibition. Hypoxia has been shown to reduce intracellular pH by 0.25 units⁴⁹⁴, the lower the oxygen tension the further the reduction in intracellular pH, with anoxia producing the largest change in pH (0.5 units), however, this study used 3% O₂ and pH was measured after 5 minutes⁴⁹⁵. Moreover, as time in hypoxia increases intracellular pH decreases further; until 1h where intracellular pH changes plateau, in HEPES buffered media or bicarbonate media this pH change was 0.3 units⁴⁹⁶. In pancreatic cell line PK-8, hypoxia reduced intracellular pH by 0.25 units after 72h hypoxia. Knockdown of KRAS in addition to hypoxia reduced intracellular pH by 0.3 units. Furthermore, knockdown of CAIX in this cell line reduces intracellular pH by an additional 0.2 units in hypoxia²³⁷. In addition to this, primary cells isolated from pancreatic cancers in mice showed a reduction of 0.4 units in

hypoxia. Knockdown or inhibition of CAIX in this cell line reduced intracellular pH by 0.5 units²³⁷. Recently investigations into pH regulation has shown that hypoxia reduces recovery speed from an intracellular acid load prepulse⁴⁹⁷. In colon cell line HCT116 recovery was reduced by 47% when incubated with DMOG and 54% slower in 2% hypoxia⁴⁹⁷. Furthermore, resting pH_i was reduced by 0.03, 0.04, 0.0375, and 0.035 units in RT112, MCF10a, HCT116, HT29 cell lines upon treatment with DMOG⁴⁹⁷. The reduction in intracellular pH indicates that the NDBTs are important for regulating intracellular pH. Hypoxia results in large transcriptional alterations, with a switch towards glycolysis. Lactate and pyruvate are produced as a by-product of glycolysis hence a decreased pH_i in hypoxia⁷⁹. The additive reduction seen in hypoxia indicates the importance of the NDBTs in hypoxia. Knockdown of other pH regulatory proteins have been shown to reduce intracellular pH. Knockdown of NHE1 reduces intracellular pH and resulted in reduced activation of MAPK signalling⁴⁹⁸. Inhibition of CAIX lowers intracellular pH in colon cancer²³⁶.

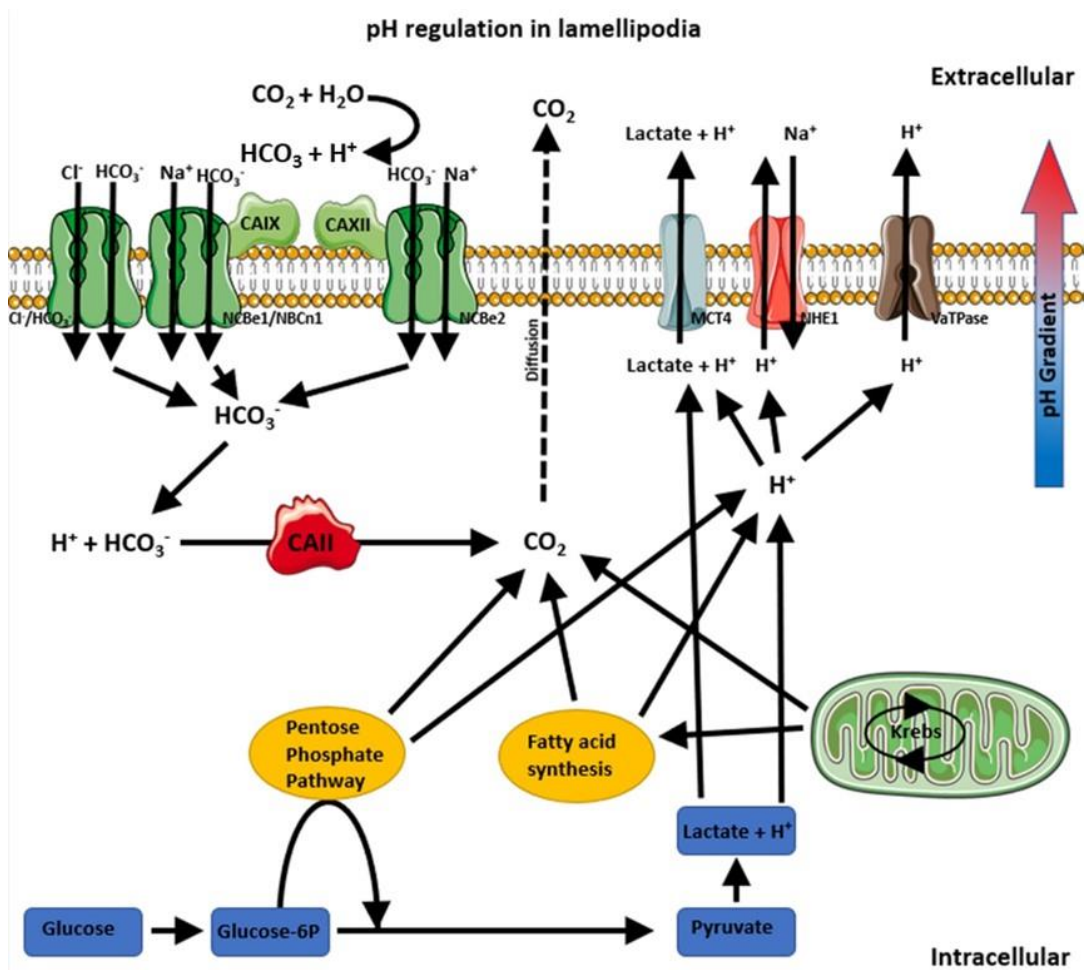


Figure 8.13 Updated pH regulatory metabolon schematic based on generated data.

Levels of H^+ ions are produced as by-products of metabolic pathways. Intracellularly these are titrated by CAII to form CO_2 which can diffuse across the lipid bilayer assuming a positive gradient. To maintain this gradient CAIX and CAXII hydrate CO_2 to produce bicarbonate and H^+ . Bicarbonate is then retaken up by the cells through NDBTs to facilitate further titration.

8.5 Conclusion:

In summary, I have shown that NDBTs expression is increased in hypoxia at both the RNA and protein level. In hypoxic conditions NDBTs co-localised to the leading edge of migrating HCC-1806 cells with CAIX. Furthermore, CAIX was shown to be a binding partner of SLC4A4 and SLC4A7, whereas SLC4A5 was a binding partner of CAXII but not CAIX in HCC-1806. The data in this chapter provides a strong rationale for targeting the NDBTs as an approach to inhibiting pH regulation in hypoxia to determine a role in migration and invasion *in-vitro*.

9 Chapter 4: Functional roles of NDBT genes in migration and invasion

9.1 Introduction

Further to the work discussed in the previous chapter *in vitro* assays with the lentiviral shRNA knockdown cell lines to determine a role for NDBTs in migration and invasion. Two NDBTs (*SLC4A4* and *SLC4A5*) were selected as targets for future experiments. *SLC4A4* was chosen based on the high induction in HCC-1806 and CAL-51 at the mRNA level and maintained protein levels in hypoxia. Similarly *SLC4A5* was selected due to the high levels of induction at the mRNA level in HCC-1806 and MDA-MB-231 and the large increases in protein expression in hypoxic conditions. Additionally, the NDBT inhibitor S0859 was selected for use to determine if inhibition of NDBTs could reproduce similar effects to knockdown. This chapter investigates the functional consequence of NDBT inhibition or knockdown on migration and invasion *in vitro*.

An acidic tumour microenvironment has been correlated with increased invasion and distant metastasis²⁷⁰. The extracellular pH of normal tissues is between 7.2-7.5, whereas the extracellular pH in tumours is typically between 6.5 and 6.9⁴⁹⁹. The mechanism by which increased acidity increases invasion is multifaceted⁵⁰⁰. Acidosis degrades the extracellular matrix (ECM) and functions to facilitate a physical path to invasion⁵⁰⁰. This degradation of the ECM occurs via the activation of matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases collectively capable of degrading essentially all components of the ECM. They function to remodel the ECM in diverse biological roles such as: tissue morphogenesis, growth, uterine cycling and postpartum involution, tissue repair, and angiogenesis⁵⁰¹. This family of enzymes are tightly regulated by many factors from the transcriptional to activation level⁵⁰². Activation of many MMPs relies on pH and many are functionally active at more acidic pH. MMP-9 for example is active at a pH_e of 6.8⁵⁰³. MMPs are secreted as zymogens with a pro-peptide region containing cysteine that interacts with the zinc region in the active site. Under acidic conditions peptidases undertake proteolysis on the pro-peptide region of removing part of the peptide. Complete removal of the peptide is subsequently by the MMP intermediate or another active MMP⁵⁰⁴. Many peptidases have more acidic pH optima hence the MMP

activation in more acidic conditions⁵⁰⁴. Studies have identified MMP expression as a poor prognostic marker in breast cancer⁵⁰⁵. Studies investigating inhibitions of MMP9 have shown that migration and invasion is reduced when it is inhibited⁵⁰⁶. Reducing the expression of an inhibitor of the MMPs resulted in less invadopodia formations and lower migration and invasion⁵⁰⁷. MMP2 knockdown reduced the formation of lung metastasis⁵⁰⁸. Downregulation of MMP7 decreased metastasis⁵⁰⁹. Knockdown of MMP12 reduced inhibited migration, invasion, and proliferation^{510,511}. Overexpression of MMP13 increased migration and metastasis¹²⁷, as did overexpression of MMP26⁵¹². In addition to degradation of the ECM, MMPs can also facilitate invasion by alternative mechanisms. MMP activity can promote tumour growth by degrading the ECM and enhancing angiogenesis⁵¹³. Additionally, MMP activity can release active growth factors from the cell surface and ECM⁵¹⁴. This release of growth factors occurs by active MMPs cleaving growth factor binding proteins inducing chemo-attraction and migration of the tumour cells⁵¹⁵. Ultimately clinical trials targeting MMPs were largely unsuccessful⁵¹⁶. These trials were unsuccessful due to a lack of efficacy for the inhibitors and toxicity issues⁵¹⁷. Attempts to modulate tumour pH_e by oral dosing with bicarbonate in mouse models⁵¹⁸. The treatment was shown to increase tumour pH_e but did not alter tumour pH_i. Formation of intrahepatic metastases were reduced following intrasplenic injection⁵¹⁸. In tail vein injections, formation of metastases were reduced in prostate cancer cells but not melanoma cancer cells⁵¹⁸. Low pH was shown to reduce the release of the matrix remodelling protease cathepsin B, indicating a role of reduced for increasing pH_e in reducing matrix remodelling and reducing invasion⁵¹⁸. Whilst the MMPs were an attractive target it was not possible to translate that into clinical efficacy thus new molecular targets are needed to help treat pH control, ECM degradation and metastasis.

CAIX functions catalyse the extracellular hydration of CO₂ to produce bicarbonate and a hydrogen ion. In normal tissues expression of CAIX is limited to gastrointestinal mucosa, pancreatic ducts, biliary tract and the gallbladder, ovaries, testes, hair follicles, mesothelium, and choroid plexus⁵¹⁹. CAIX activity facilitates the acidification of the tumour microenvironment²³⁶. Expression of CAIX is induced by either hypoxia or acidosis and CAIX has been implicated in the control of cancer proliferation and cellular transformation⁵²⁰. CAIX is overexpressed in a variety of tumour types and is strongly associated with increased metastasis and poor prognosis⁵²¹.

Many studies have shown a role for CAIX in migration⁵²¹. Overexpression of CAIX induces migration and activation of MMPs⁴⁹³. Knockdown of CAIX reduced invasion in a series of triple negative breast cancer models ranging from: cell lines in normoxia and hypoxia, 3D spheroids and in ex-vivo explants⁵²². These results were not limited to breast cancer as similar results were seen in renal carcinomas⁵²³. Moreover inhibition of CAIX reduced proliferation and migration *in vitro*⁵²⁴. In-vivo models have shown that CAIX is a critical regulator of breast tumour growth and metastasis⁵²⁵. Inhibiting CAIX attenuated the growth of tumours in mouse mammary models⁵²⁶. Anti-angiogenic therapy inhibits the development of new vasculatures in tumours. Antiangiogenic therapy induces hypoxia and as a consequence CAIX expression is induced. Knockdown of CAIX increased the efficacy of antiangiogenic treatment reducing tumour growth in-vivo¹⁷¹. The catalytic activity of CAIX has been shown to be greatly reduced in the absence of NDBTs⁴⁵³. I previously showed that NDBTs and CAIX are binding partners and co-localise to the leading edge of migrating tumour cells. Thus the knockdown of NDBTs are likely to reduce migration and invasion.

Other pH regulatory proteins have been implicated in migration and invasion *in-vitro* and metastasis. Downregulation of MCT1 reduced migration and invasion and in turn expression of MMP9 by suppressing the AKT signalling pathway *in-vitro*⁵²⁷. MCT4 knockdown reduced migration and invasion in pancreatic carcinoma cells⁵²⁸. Knockdown of MCT1 *in-vivo* reduced migration and invasion in breast cancer cell lines⁵²⁹. MCT4 knockout mice developed fewer lesions and those lesions were less invasive⁵³⁰.

V-ATPase has been implicated in migration and invasion. In breast cancer cells v-ATPase localises to the leading edge of migrating cells⁵³¹. Inhibition of v-ATPase reduced MDA-MB-231 cell migration and invasion *in-vitro*⁵³². Knockdown of subunits of v-ATPase reduced growth of hepatocellular carcinoma xenographs.⁵³³

NHE proteins have been shown to regulate cell migration⁵³⁴. Inhibition of NHE1 reduces directed but not random motility⁵³⁴. NHE localises to the leading edge of migrating cells²²⁷. Cells expressing a translocation defective NHE1 are unable to migrate in a standard wound healing assay⁵³⁴. NHE1 affects cell migration by: altering cell volume, regulating the intracellular pH, and thereby the assembly and activity of cytoskeletal elements⁵³⁵. This migration by NHE1 has been shown to be activated by AKT signalling induced by cleaved FASL signalling⁵³⁶. Invasion is also modulated by NHE1 expression, inhibition reduced invasion of gastric cancer cells²²⁹. NHE1 facilitates invasion by extruding hydrogen ions and facilitating MMP activity⁵³⁷. The role of NHE1 in invasion has also been shown to vary

between cell types. In MDA-MB-231 expression of NHE1 depends on expression of CD44 and subsequent binding of hyaluron⁵³⁸. However, in MCF7 overexpression of NHE1 alone was sufficient to increase expression⁵³⁹. Inhibition of NHE1 suppresses the tumour cell growth in-vivo⁵⁴⁰.

Investigations into the functional roles of NDBTs in cancer cell: growth, migration, invasion, and metastasis have been limited to a four studies^{431,453,454,541}. It was shown that CAIX improves cell migration in both the presence and absence of hepatocyte growth factor (HGF), and that HGF upregulates the expression of CAIX⁴⁸¹. Subsequently, CAIX localises to the leading edge of lamellipodia in lung cancer (A549) cells⁴⁵³. It was shown in lamellipodia CAIX co-localizes with NDBce-1 and anion exchanger 2 (AE2) forming a migration apparatus, increasing the catalytic activity of CAIX⁴⁵³.

Disrupting activity by inhibition with S0859 reduced 3D tumour spheroid growth in breast, colon, and brain cancer cell lines⁴³¹. Furthermore inhibition increased the pH at the core of tumour spheroids, this in turn lead to an increase in apoptosis at the core of the spheroids⁴³¹. Knockdown of SLC4A9 reduced spheroid growth rate and also increased the pH in the core of tumour spheroids⁴³¹. Similarly to NDBT inhibition, SLC4A9 knockdown also increased apoptosis in the core of spheroids. Tumour growth rate was reduced by knockdown of SLC4A9 in-vivo with breast and brain cancer cell lines. SLC4A4 and SLC4A9 were shown to be regulated by Hypoxia. SLC4A4 was more heavily controlled by HIF-1A whereas SLC4A9 was controlled by HIF-1 α and HIF-2 α ¹⁷¹.

It has been also been shown that knockdown of HIF-1 α reduced SLC4A4 mRNA expression in colon cancer cells⁴⁵⁴. Conversely, of SLC4A4 reduced the expression of CAIX and p21 in hypoxia⁴⁵⁴. Furthermore, knockdown of SLC4A4 in combination with CAIX knockdown reduced cell survival in hypoxia and 3D spheroid growth⁴⁵⁴. Knockdown of SLC4A4 reduced migration in normoxia and hypoxia in MDA-MB-231 cells⁴⁵⁴. However, these functional results were only undertaken in a single cell line and didn't expand the study into invasion or metastasis.

In colon cancer cells, SLC4A4 mRNA was induced in hypoxia; and knockdown of CAIX reduced SLC4A4 mRNA expression in hypoxia⁴⁵⁴. Furthermore, in combination with CAIX knockdown, SLC4A4 knockdown reduced LS741T cell survival in normoxia and hypoxia⁴⁵⁴. Knockdown of SLC4A4 in MDA-MB-231 cells reduced migration and invasion in normoxia but the effect in hypoxia was not investigated⁴⁵⁴.

SLC4A7 has been shown not to increase migration in MCF7 cells⁵⁴². Expression of SLC4A7 was also not upregulated at 1% O₂ in LS741T cells⁴⁵⁴. However, it was shown SLC4A7 is required for the migration of smooth muscle cells⁴³⁵. In contrast to the data for SLC4A4 and SLC4A9, SLC4A7 was shown not to reduce tumour spheroid growth²³⁸. These differences indicate an alternative role for SLC4A7 compared to that of the other transporters. It was shown that SLC4A7 regulation is required for MCF7 cell cycle progression and that SLC4A7 functions to induce phagosome acidification^{543,544}.

Multiple experimental metastasis models have been designed, however, due to the complexity of cancer metastasis no one model alone is sufficient. The data I will show demonstrates a role of NDBTs in invasion. Orthotopic transplantation of cell lines into the mammary fat pad and evaluation of metastasis would therefore be one possible model. However, due to differences in 3D growth rates and necrosis in spheroids results from this model would be compounded (Fig 9.3). The use of an inducible knockdown system would be a method to overcome this however stable knockdowns were already available. Therefore, using a metastatic colonisation model would be an appropriate alternative. There are two types of this model, intra-cardiac and tail vein injection. The route of administration results in different patterns of metastatic colonisation. In breast cancer cell lines tail vein injection normally results in colonisation in the lung as the primary site and less frequently in the liver. Intra-cardiac injection commonly results in colonisation in the bones and less frequently the brain.

Hypothesis

I hypothesise that inhibition or knockdown of NDBTs will reduce migration and invasion *in-vitro* and metastasis *in-vivo* by disrupting the pH metabolon in lamellipodia. Furthermore, I hypothesise that knockdown of NDBTs will reduce metastatic colonisation *in-vivo*. To investigate this hypothesis I will undertake the following aims:

1. Evaluate the impact of NDBT inhibition or knockdown on 2D cell growth
2. Investigate the effect of NDBT knockdown or inhibition on 3D spheroid growth
3. Determine if NDBT knockdown or inhibition reduces 3D spheroid invasion
4. Undertake a pilot study to determine the most appropriate cell lines for use *in-vivo*.
5. Undertake an full *in-vivo* study to determine that effect of NDBT knockdown on metastasis.

9.2 Results

A time course was undertaken to determine the impact of NDBT knockdown or inhibition on spheroid growth. In contrast to 2D where growth was unaffected spheroid size was affected (fig. 9.1). In the control spheroid sized increased; NDBT knockdown lines became necrotic and shrunk in size. Conversely growth rates in spheroid was altered. In HCC-1806 growth was inhibited by 20% by S0859 ($P < 0.05$ $n = 3$). Knockdown of NBCe-1 resulted in spheroids that couldn't grow and began to undergo necrosis after day 2 ($P < 0.001$ $n = 3$). This was also seen with NBCe-2 knockdown whereby the cells necrosis after day 2 ($P < 0.001$ $n = 3$). For spheroids with NBCe-1 or NBCe-2 knockdown at the day 12 endpoint the spheroid was almost entirely disintegrated (Fig. 9.2). Total PARP was increased in treated and knockdown cells compared to the controls. However, none expressed the cleaved form (Fig. 9.3).

NBCe-1 knockdown reduced wound healing in two cell lines. In HCC-1806 NBCe-1 knockdown reduced wound healing 7%, 20%, and 45% in the three constructs respectively (NS, $P < 0.05$, $P < 0.001$, $n = 3$) in normoxic conditions, under hypoxic conditions (1% O_2) wound healing was reduced 20, 30, and 30% in the three constructs respectively ($P < 0.01$, $P < 0.001$, $P < 0.001$, $n = 3$). Knockdown of NBCe-1 reduced wound healing 30% in normoxia in all three constructs ($P < 0.05$, $n = 3$) and 20% in hypoxia (1% O_2) in all three constructs ($P < 0.05$, $n = 3$) in Cal-51 (Fig. 9.4). NBCe-2 knockdown reduced wound healing in two cell lines. In HCC-1806 NBCe-2 knockdown reduced wound healing 70%, 60%, and 50% in the three constructs respectively ($P < 0.001$, $n = 3$) in normoxic conditions, under hypoxic conditions (1% O_2) wound healing was reduced 50, 45, and 30% in the three constructs respectively ($P < 0.001$, $n = 3$). Knockdown of NBCe-2 reduced wound healing 65% and 35% in normoxia in the two constructs ($P < 0.001$, $P < 0.01$, $n = 3$) and 50% and 55% ($P < 0.001$, $P < 0.01$, $n = 3$) in hypoxia (1% O_2) in all three constructs ($P < 0.05$, $n = 3$) in MDA-MB-231 (Fig. 9.4).

Knockdown of NBCe-1 reduced invasion in two cell lines; HCC-1806 NBCe-1 knockdown resulted in a 50% reduction ($P < 0.01$, $n = 3$) in normoxia and a 50%, 80%, and 50% reduction ($P < 0.01$, $P < 0.001$, $P < 0.01$, $n = 3$) in each construct respectively in hypoxia (1% O_2) in invasion in all constructs. In Cal-51, NBCe-1 knockdown reduced invasion 70, 80, and 80% ($P < 0.001$, $n = 3$) respectively in each construct. In hypoxia knockdown reduced

invasion 50, 60, and 70% ($P < 0.01$, $P < 0.001$, $n = 3$) in hypoxia (1% O_2) (Fig. 9.5).NBCe-2 knockdown reduced invasion in both HCC-1806 and MDA-MB-231. In HCC-1806 invasion was reduced 80%, 80%, and 90% in normoxia ($P < 0.001$, $n = 3$) in each construct respectively. In hypoxia (1% O_2) the reduction was 75%, 65%, and 75% ($P < 0.001$, $n = 3$) for each construct respectively. In MDA-MB-231 invasion was reduced 75% ($P < 0.001$, $n = 3$) in both normoxic and hypoxic (1% O_2) conditions for both constructs (Fig. 9.5).

Spheroid invasion assays were undertaken to determine if these migration and invasion results were comparable to a 3D context (Fig. 9.6). Spheroids were formed after 3 days and encased in Matrigel. After 24h the invasion was measured using a widefield microscope. Inhibition of NDBTs and knockdown of NDBTs reduced 3D spheroid invasion (figure 9.10). S0859 treatment reduced invasion by 35% ($P < 0.05$, $n = 3$), SLC4A4 knockdown by 70% ($P < 0.01$, $n = 3$), and SLC4A5 knockdown by 60% ($P < 0.01$, $n = 3$).

Validation of the cell lines for use *in vivo* required that the cells were mycoplasma negative, luciferase positive, and compatible with the strain of mice being used. The cells were tested for mycoplasma 1 week prior to implantation. Expression of luciferase was well above the detection limit for all cell lines tested (Fig. 9.7). Cells were serially diluted to determine the number at which luminescence was well below the detection limit. For all cell lines the number of cells required were low. To explore this effect further luminescence per 1000 cells was calculated to determine if transfection efficiency and expression was similar across the cell lines. This would enable easier interpretation of the data when the cells are implanted. Accordingly the expression was similar across all cell lines when controlled for cell number. Expression across the HCC-1806 and MDA-MB-231 was also similar with HCC-1806 expressing at a slightly higher level (Fig. 9.8).

The final requirement for the validation of future *in vivo* studies was a pilot study using the two cell lines to determine compatibility. Pilot study data indicated that both cell lines are compatible with SCID mice with HCC-1806 growing very aggressively. Initial imaging on implantation day indicated that HCC-1806 cells had been successfully injected into the left ventricle and began to spread around the body. MDA-MB-231 cells showed no expression of luminescence in the mice on implantation day (Fig. 9.9). Expression of luminescence in the tubes containing the cells were also tested to determine a possible cause. No luminescence was detected in the tubes either indicating the cells were not expressing luminescence after hypoxic treatment (Fig 9.10). Subsequent imaging at day 3 indicated

that HCC-1806 had metastasised around the body. In addition, MDA-MB-231 had also spread and was expressing luminescence after reoxygenation. Upon tumour burden becoming too great the mice were sacrificed and organs stained. Micro-metastases were detected in: brain, spinal cord, and bones with HCC-1806. MDA-MB-231 micro-metastases were detected in: spinal cords, wrists, and tail (Fig. 9.11).

A full intracardiac study was undertaken using HCC-1806 with 8 mice per group. Mice were imaged weekly until termination at 5 weeks. Whole mice images displayed evidence of metastasis in 7/8 control group mice, 8/8 SLC4A4 mice, and 4/8 SLC4A5 group mice (Fig. 9.12). Overall signal was highest in SLC4A5 knockdown group followed by SLC4A4 knockdown and then the control group. Whole mice images indicated that large amounts of signal from the mice were localised to the chest cavity suggesting cells hadn't been pumped round the body once injected. Upon imaging of total mice, organs and bones were separated to determine exact locations of metastatic colonisation. In the control group 6/8 and 7/8 mice had cells in the heart or lungs indicating that all mice initiation failed in this group. Moreover, in the SLC4A4 knockdown group 5/8 and 6/8 mice cells in the heart and lungs respectively indicating most initiations had failed. However, SLC4A5 group had 3/8 and 2/8 in the heart and lung indicating half of the initiations were successful (Fig. 9.13). Bones metastasis was investigated as it was the primary site of HCC-1806 metastasis in the pilot study (Fig. 9.11). As the control and SLC4A4 group had failed initiations it was expected metastasis would lower. As predicted SLC4A5 group had the highest levels of metastatic colonisation. However, the number of mice with colonisation was lower in this group (Fig. 9.14). Overall, the data indicates that the intra-cardiac *in-vivo* experimentation was not initiated correctly and therefore the data cannot be used.

To overcome the issues of initiation observed in the previous *in vivo* study, tail vein injections were undertaken. SLC4A4 knockdown reduced growth by 20% ($P < 0.05$), and SLC4A5 knockdown reduced growth by 26% ($P < 0.05$) (Fig 9.17). NDBT knockdown reduced lung metastasis (Fig 9.18). Control group has lung metastasis in (6/8), SLC4A4 in (4/6), and SLC4A5 in (1/6) (Fig. 9.17). Lung metastasis was reduced by 94% ($P < 0.001$) by SLC4A4 knockdown and 98% ($P < 0.001$) by SLC4A5 knockdown (Fig. 9.18).

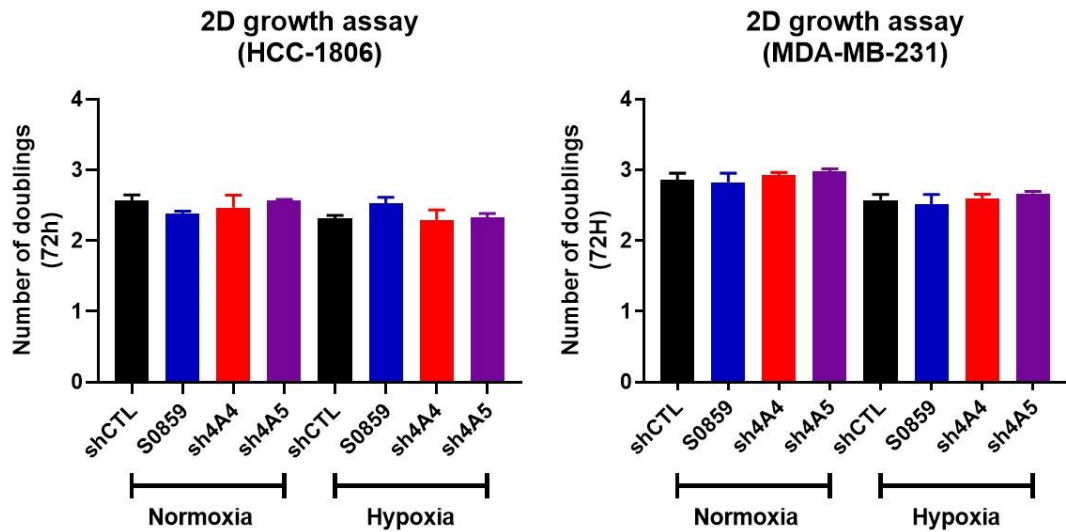


Figure 9.1 Growth is unaffected by NDBT knockdown in HCC-1806 and MDA-MB-231.

Cellular proliferation was unaffected by NDBT knockdown or inhibition in in normoxia or hypoxia. Doubling number was unchanged by knockdown of NDBTs in normoxia or hypoxia. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

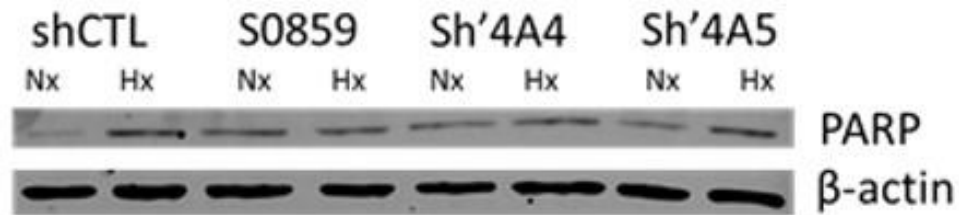


Figure 9.2 Parp expression in HCC-1806. Expression of full length PARP was altered by NDBT knockdown or inhibition in a normoxic and hypoxic manner.

Under normoxic conditions expression of full length PARP in increased 3 fold with NDBT inhibition, 2 fold with SLC4A4 knockdown, and 1.5 fold with SLC4A5 knockdown. Under hypoxic conditions expression of PARP is reduced 60% by NDBT inhibition, 50% by SLC4A4 knockdown, and 70% by SLC4A5 knockdown. It was not possible to detect either long or short cleaved PARP fragments in any condition indicating apoptosis was not occurring.

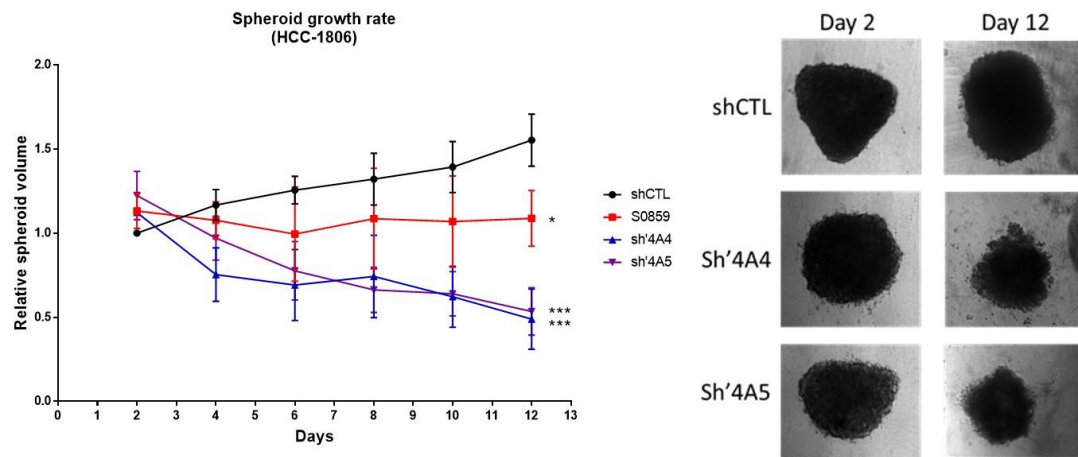


Figure 9.3 Knockdown or inhibition of NDBT reduces spheroid growth.

Knockdown and Inhibition of NDBTs reduces spheroid growth and size. Spheroid size was measured every two days after formation on day 2. (A) Representative time course of spheroid size over 12 timeframe. (B) Representative images of spheroids at start and end-point. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

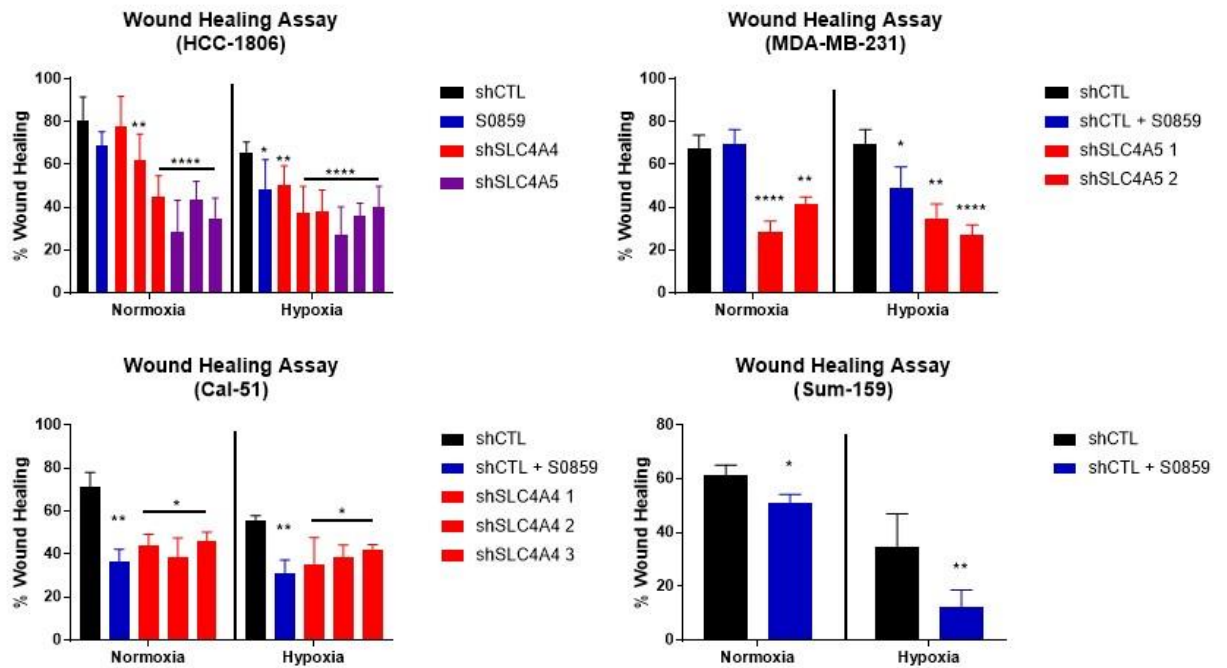


Figure 9.4 Knockdown or Inhibition of NDBTs reduce migration in normoxia and hypoxia.

The effect on migration by NBDT knockdown or inhibition was investigated with wound healing assays. Inhibition of NBDT with S0859 resulted in a reduction in migration in normoxia in HCC-1806, Cal-51, Sum-159pt, however in hypoxia (1% O₂) S0859 reduced migration in all cell lines. Similarly knockdown of *SLC4A4* reduced migration in HCC-1806 and Cal-51 in normoxia and hypoxia. Knockdown of *SLC4A5* reduced migration in HCC-1806 and MDA-MB-231 in normoxia and hypoxia.

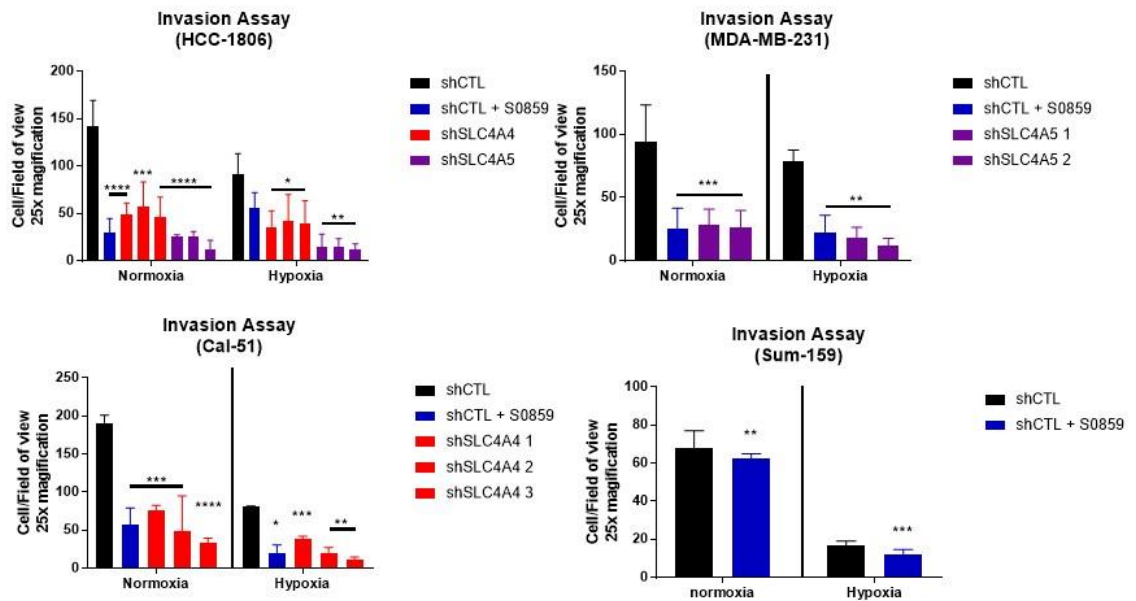


Figure 9.5 NDBT inhibition or knockdown reduces migration in normoxia and hypoxia.

The role of NDBTs in cell invasion was investigated with NDBT knockdown and inhibition using modified boyden chamber assays coated with low growth factor matrigel. Application of S0859 resulted in a reduction in invasion of 60% ($P < 0.05$, $n = 3$) in normoxia and 50% reduction ($P < 0.05$, $n = 3$) in hypoxia (1% O_2) in HCC-1806. In Cal-51 S0859 treatment reduced migration 75% ($P < 0.001$, $n = 3$) in normoxia and 60% ($P < 0.05$, $n = 3$) in hypoxia (1% O_2). Invasion was reduced 70% ($P < 0.001$, $P < 0.05$, $n = 3$) in both normoxia and hypoxia (1% O_2) in MDA-MB-231. Normoxic and hypoxic (1% O_2) invasion was reduced 10% ($P < 0.05$, $P < 0.01$, $n = 3$) in Sum-159PT.

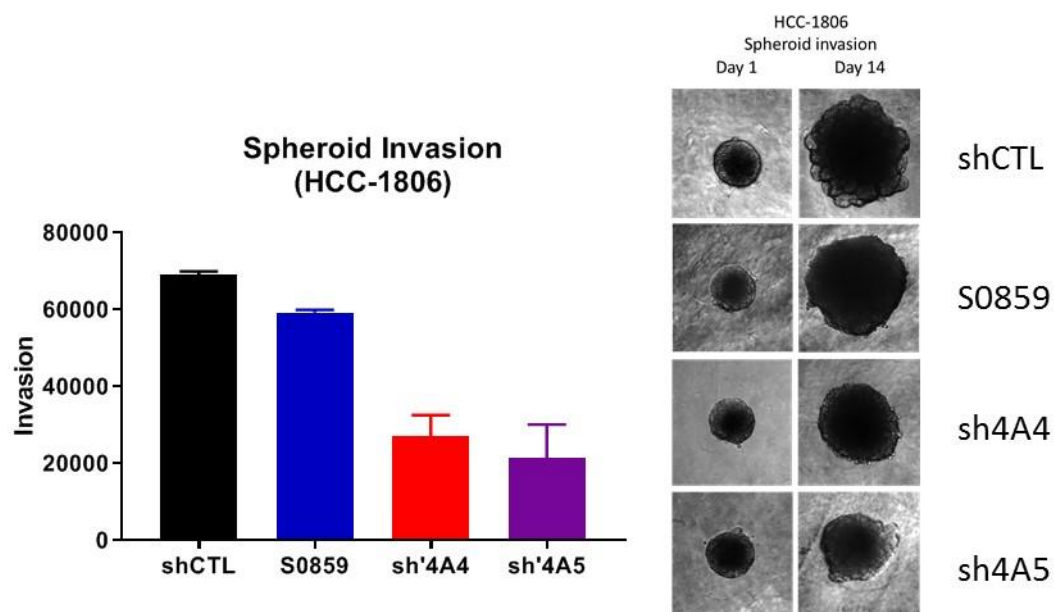


Figure 9.6 Spheroid invasion is reduced by NDBT knockdown and inhibition.

Knockdown of NDBT and inhibition reduced spheroid invasion. S0859 treatment reduced invasion by 351 pixels ($P < 0.05$), SLC4A4 knockdown by 50% ($P < 0.01$) and SLC4A5 knockdown by 60% ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

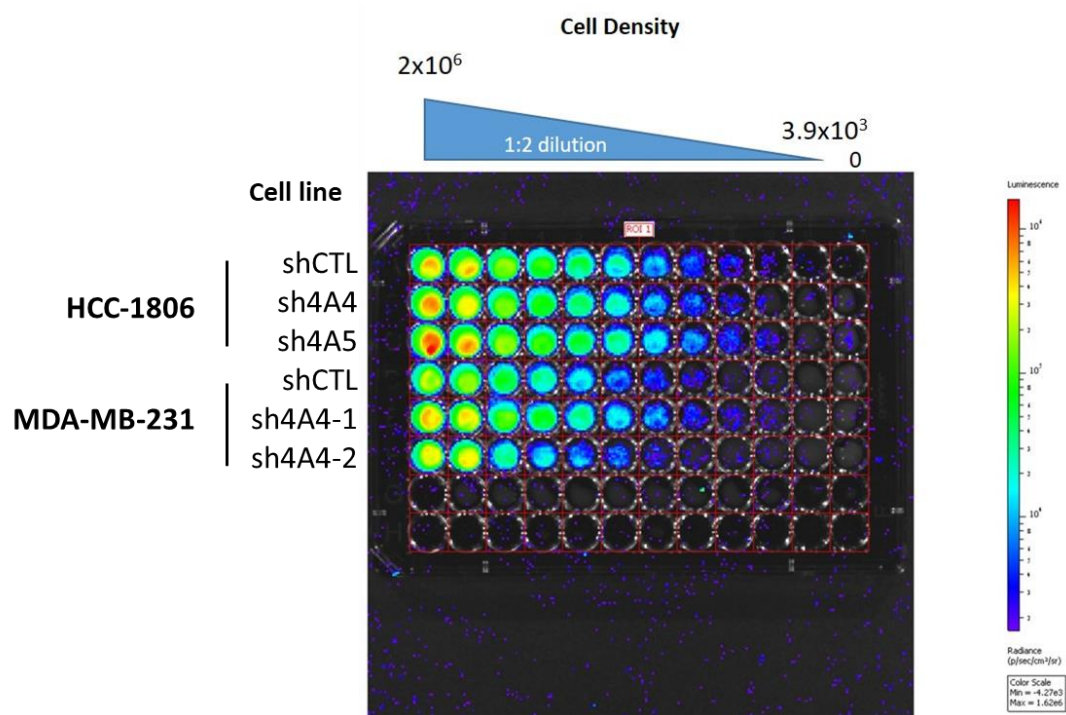


Figure 9.7 Luminescent expression of fLUC transduced cell lines.

Expression of photons produced in each well by luminescence transduced cell lines showed expression of luciferase was high in transduced lines. A dilution series was undertaken with the cells diluted 1 in 2 across the plate. Luminescence expression was linear with cell number. All cell lines produced a signal stronger than the detection limit even at low numbers.

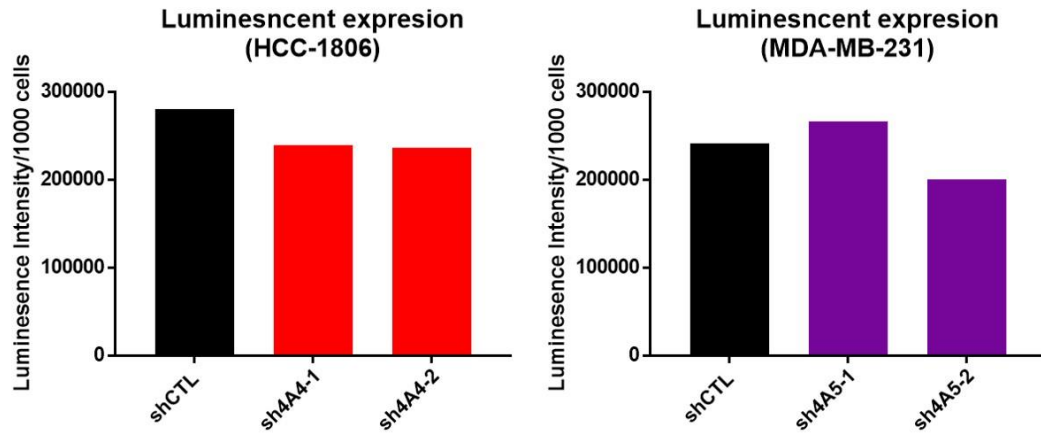


Figure 9.8 Relative luminescence of each cell line tested on IVIS imaging system.

Quantification of luminescence expression from IVIS imaging system relative to cell number showed that cell lines transduced with fLUC expressed luminescence post treatment with luciferin. Expression in HCC-1806 was consistent across all constructs tested. The knockdown cell lines had marginally lower luminescence intensity. Similarly to the HCC-1806 cell line MDA-MB-231 luminescence expression was consistent across the constructs. One knockdown line had a slightly higher expression and the other a slightly lower expression.

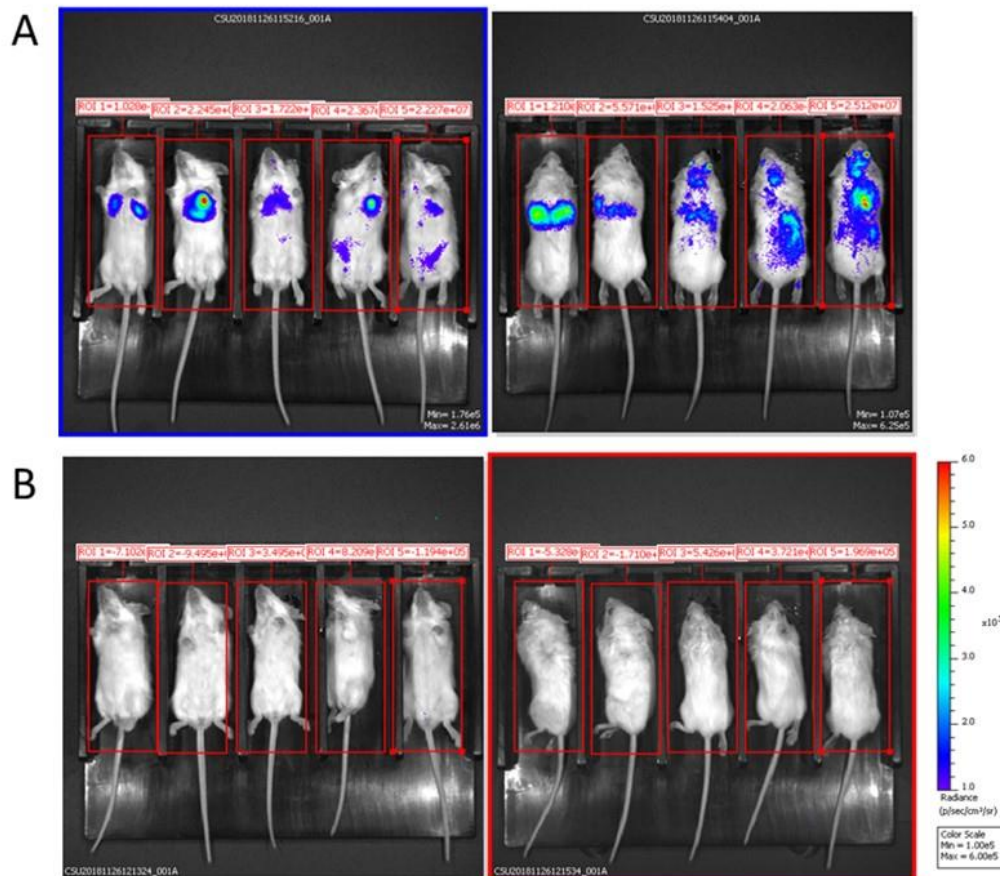


Figure 9.9 HCC-1806 but not MDA-MB-231 cell lines expressed luminescence in the mice on Implantation day in pilot study.

Luminescence was detected in mice injected with HCC-1806 cells but not those injected with MDA-MB-231 cells. Ventral and dorsal imaging of the mouse post injection on implantation day. Imaging showed that the injections were successful for mice 2-5 however, mouse one displayed expression in the lungs indicating the experiment may not work. The HCC-1806 cells had already begun to be spread around the body as in some mice the cells were shown to be in other sections of the body. As no luminescence was seen for MDA-MB-231 it is not possible to determine if the cells were successfully injected into the left ventricle of the heart.

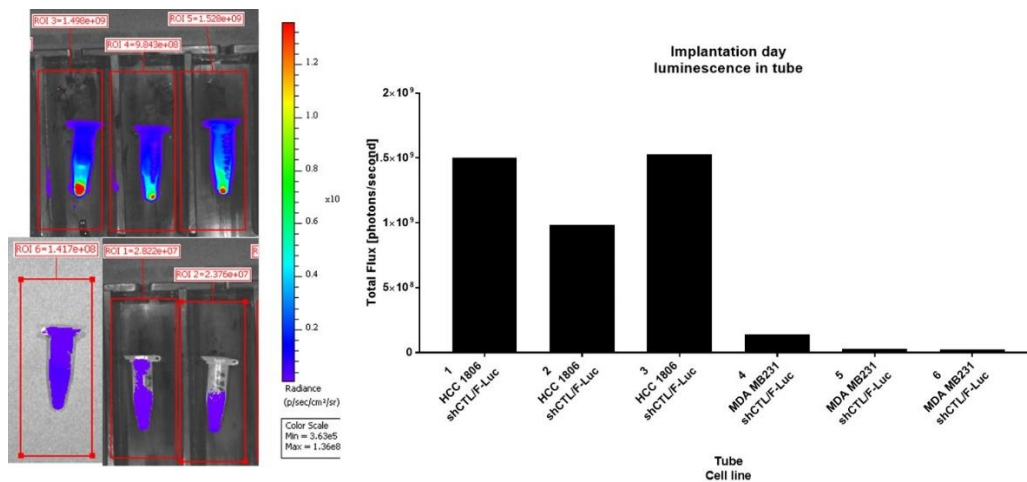


Figure 9.10 Expression of cell luminescence in tube post injection shows expression in HCC-1806 but not MDA-MB-231.

Post injection test of luminescence expression in cell suspensions post injection into the mice indicated that HCC-1806 but not MDA-MB-231 cells were expressing luminescence. The lack of expression in the mice necessitated the testing of the cells to determine a possible cause of the absence of signal. HCC-1806 expressed the highest levels of luminescence. Expression of luminescence was not seen in MDA-MB-231 in any of the tubes due to oxygen levels being too low intracellularly in these cells to drive the luciferase reaction.

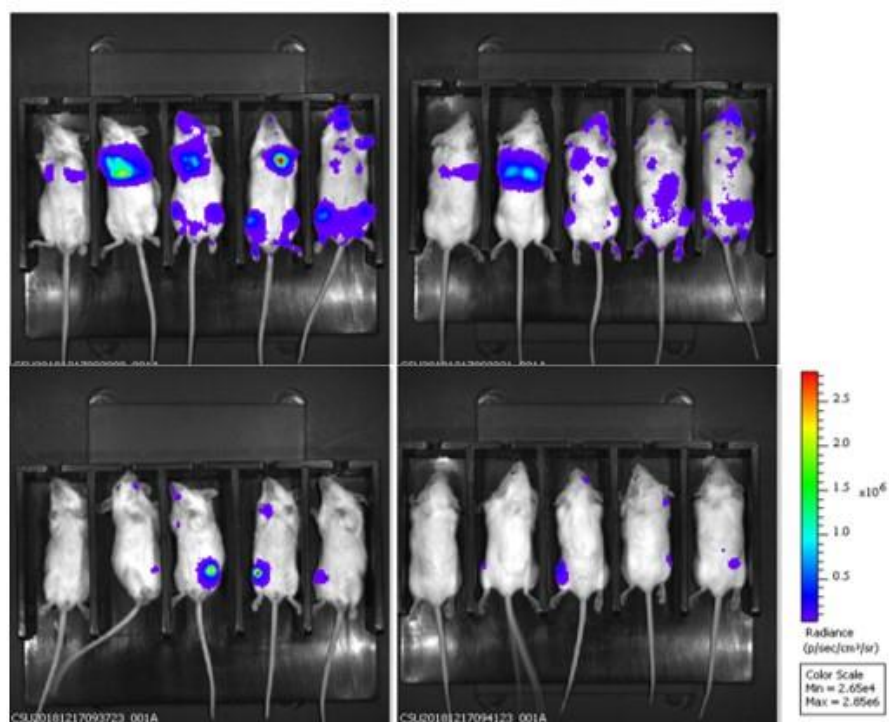


Figure 9.11 Luminescence expression Pre-termination in pilot study.

Luminescence was detected in all but one mice on termination day. The first five mice with HCC-1806 implanted had far higher levels of luminescence and a greater degree of metastasis. The second set of five were MDA-MB-231 expressed some metastasis with one mouse not expressing any luminescence.

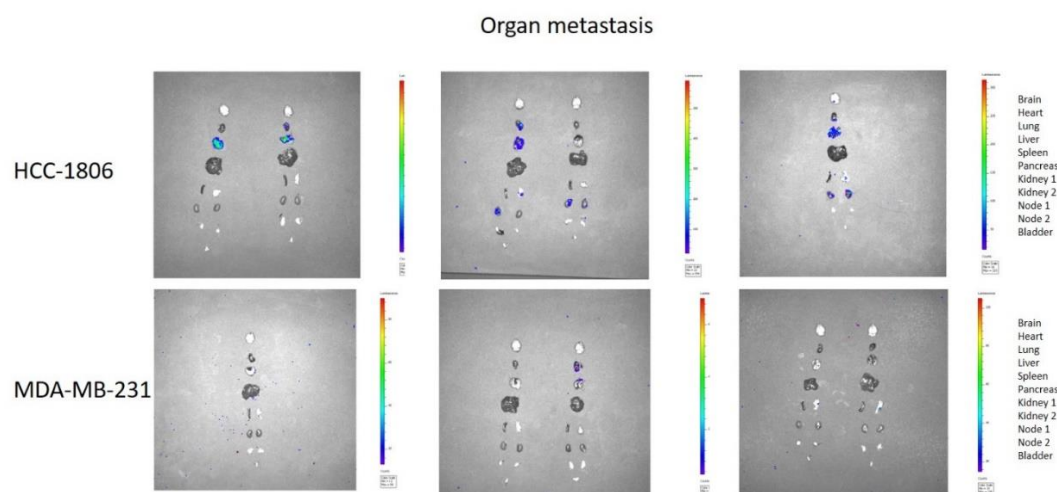


Figure 9.12 Luminescence expression in organs in pilot study.

Luminescence was detected in organs of mice. Organs were dissected from the mouse and imaged separately to provide better resolution and information regarding metastatic sites. HCC-1806 expression was present in the heart and lungs indicating cells had been lodged there. Expression was also detected in the pancreas and kidneys and possibly in the brain to a low extent. In MDA-MB-231 expression was detected in the heart and spleen.

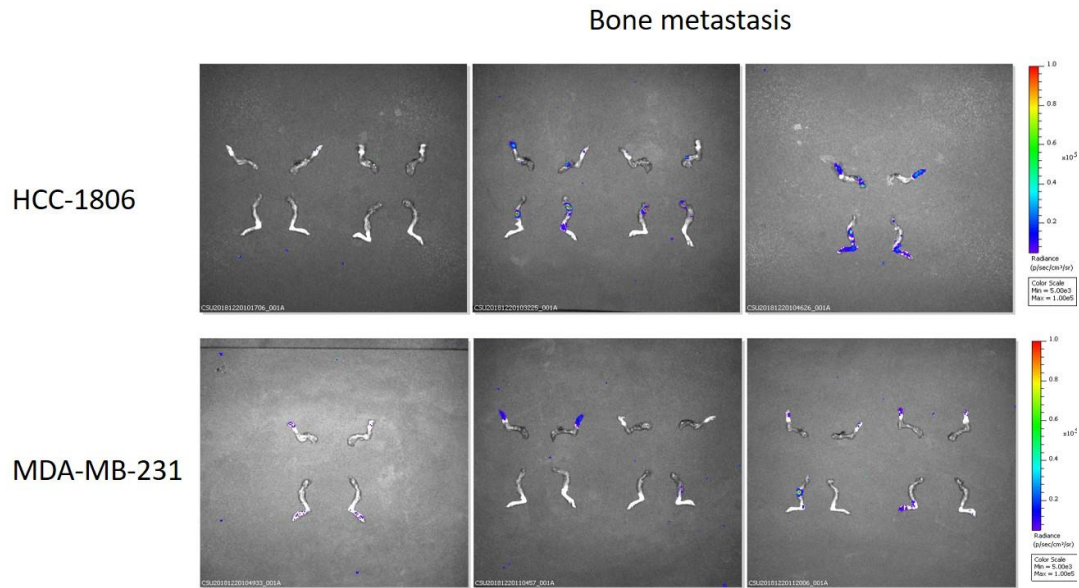


Figure 9.13 Luminescence expression in limbs.

Expression of luminescence was detected in the front and hind limbs of the mice. Front and hind limbs were extracted from the mice post termination and imaged separately for each mouse. Bone metastasis to the knuckles and ankles were seen in both HCC-1806 and MDA-MB-231. The highest levels of luminescence was detected in mouse injected with HCC-1806.



Figure 9.14 Whole mouse luminescence imaging in intracardiac study.

Whole mice were imaged for each group upon termination. Expression of luminescence was detected in 7/8 control group mice, 8/8 SLC4A4 mice, and 4/8 SLC4A5 group mice. Although the number of mice with successful cocolonization was highest in SLC4A5, total luminescence expression was highest in SLC4A5 group.

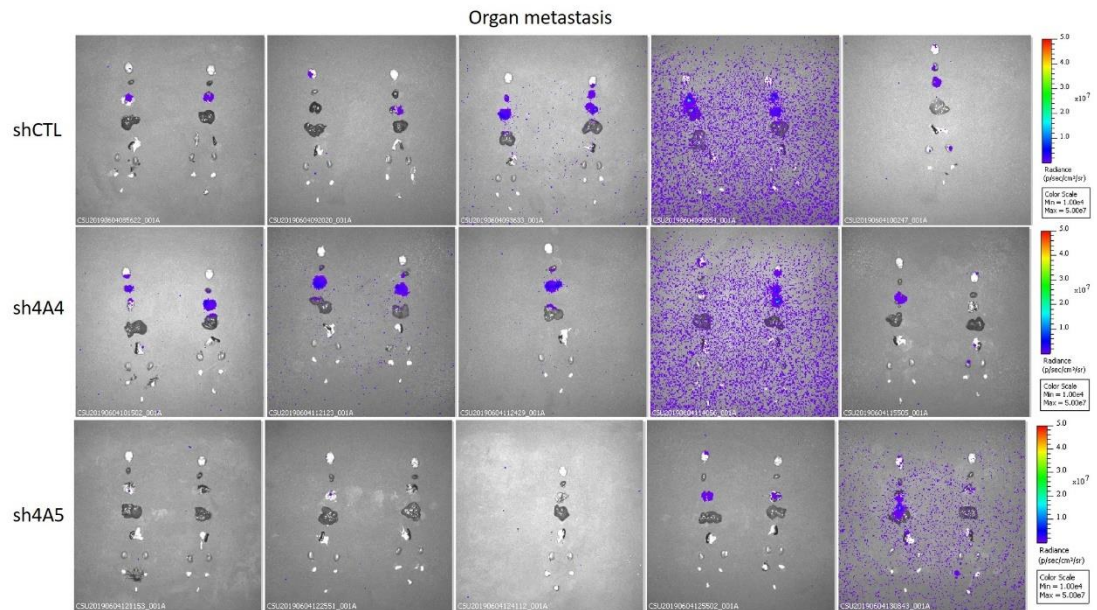


Figure 9.15 Luminescence expression in organs in intra-cardiac study

Expression of luminescence was detected in many organs in the mice. Expression of luminescence in lungs was seen in 6/8 mice in the control, 5/8 in SLC4A4 group and 3/8 in SLC4A5 group. Similarly expression was seen in heart in 7/8 in the control, 6/8 in SLC4A4 group and 2/8 in SLC4A5 group. Therefore indicating that initiation of the intracardiac models had failed in these mice.

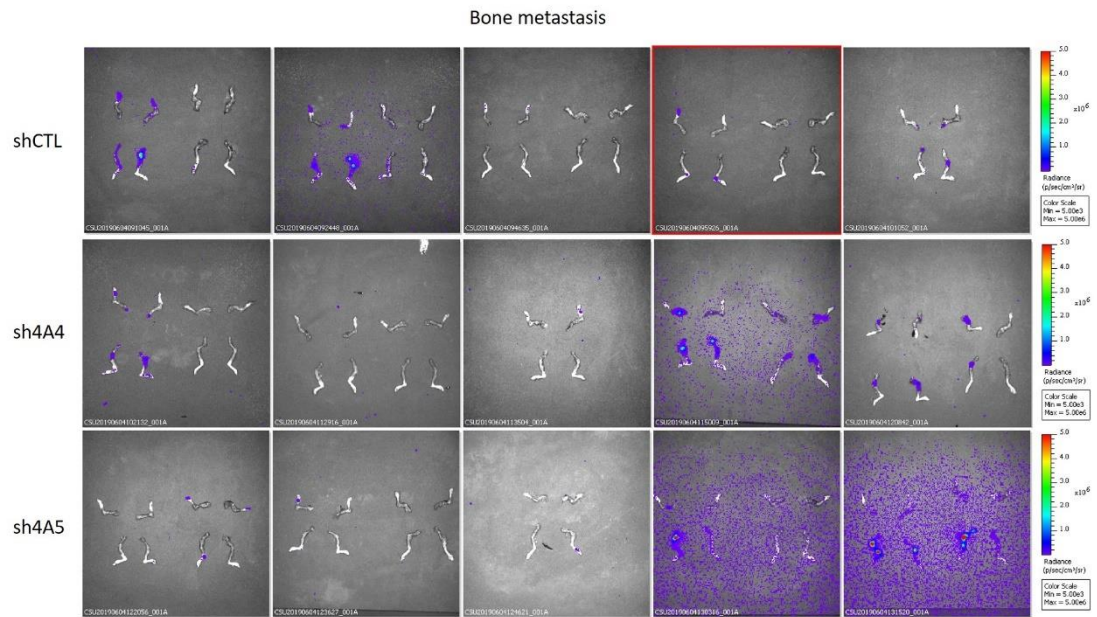


Figure 9.16 Luminescence expression in bones in intra-cardiac study.

Expression of luminescence was seen in 4/8 in the control group, 5/8 in the SLC4A4 group and 5/8 in SLC4A5 group. Expression was highest in SLC4A5 group. Overall, metastasis was highest in limbs compared to organs.

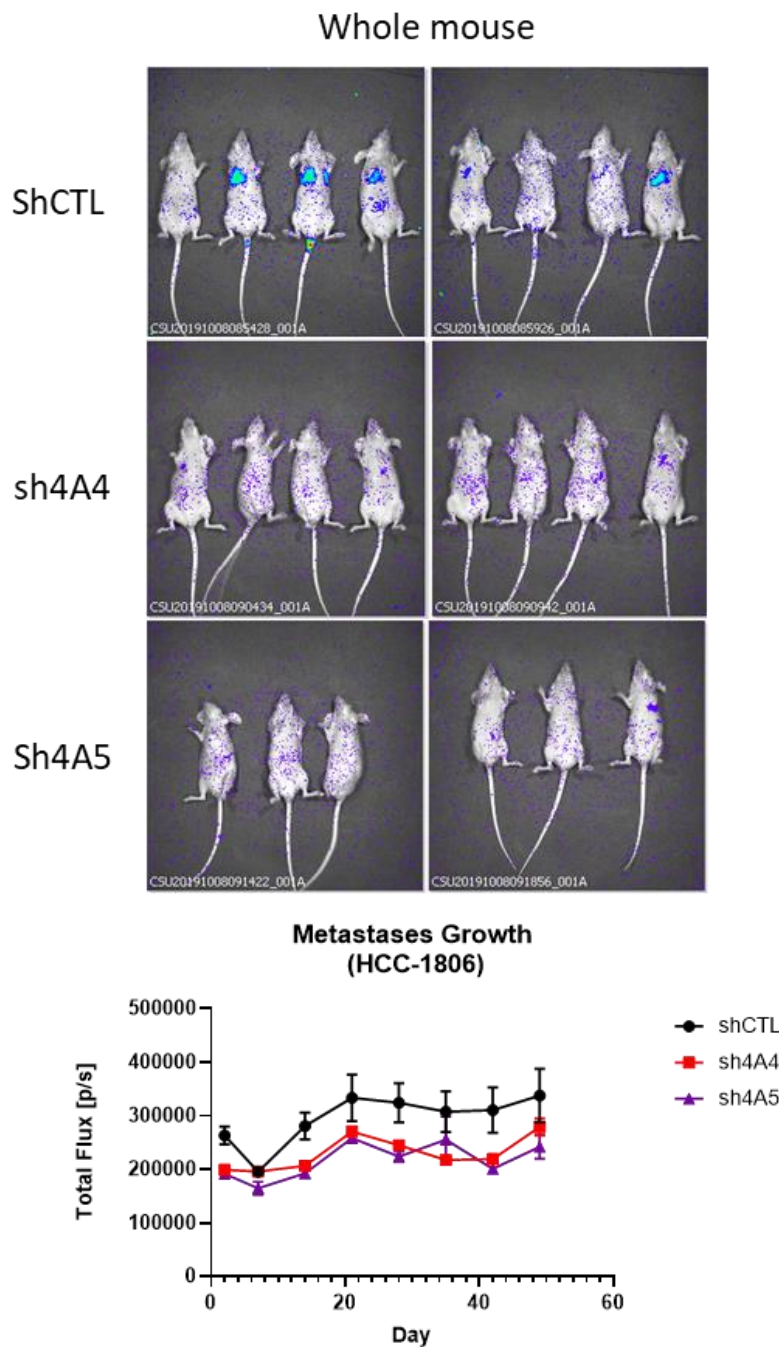


Figure 9.17 NDBT knockdown reduces tumour growth *in-vivo*

Knockdown of NDBTs reduced metastatic growth *in-vivo*. Both SLC4A4 knockdown and SLC4A5 knockdown reduced metastatic growth 30%. Total number of mice with identifiable tumours was reduced by NDBT knockdown. Tumours were detected in 7/8 in control group, 5/8 in SLC4A4 knockdown, and 3/6 in SLC4A5 knockdown.

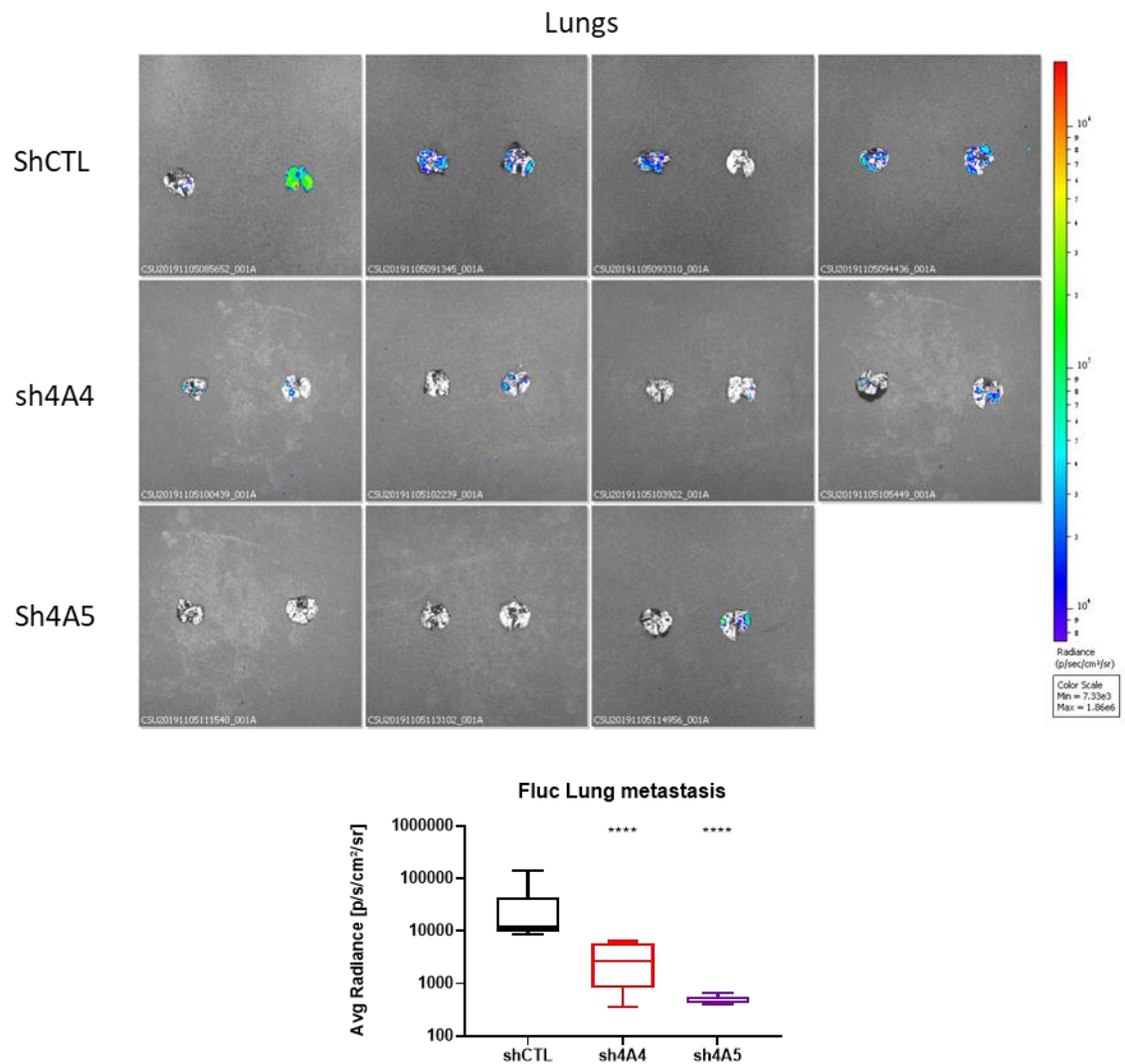


Figure 9.18 NDBT knockdown reduces lung metastasis

The number of mice with lung metastasis was reduced by NDBT knockdown. Tumours were detected in 7/8 in control group, 5/8 in SLC4A4 knockdown, and 3/6 in SLC4A5 knockdown.

9.3 Discussion

The results in this chapter answered the hypothesis “NDBT knockdown or inhibition will reduce migration and invasion in-vitro and metastasis in vivo” in triple negative breast cancer cells. However, further studies are required to fully elucidate the role of NDBTs in migration and invasion. Knockdown or inhibition of NDBTs was shown to reduce migration in multiple cell lines, however, this reduction was not specific to hypoxic conditions. Similarly to migration, knockdown or inhibition of NDBTs reduced invasion but was not specific to hypoxia. However, the effect of knockdown or inhibition was greater with invasion than migration indicating the 3D microenvironment is required for full elucidation of the functional role of NDBTs. The role of the pH metabolon; that the NDBTs are members of is to maintain pH_i and as a consequence acidify the pH_e . This acidification of the tumour microenvironment results in the activation of MMPs and degradation of the ECM facilitating a path to invasion. In a 2D migration assay there is no requirement for the ECM to be degraded for migration to occur, however, NDBTs and other pH regulatory proteins have been shown to be involved in directed migration via a proton gradient^{447,545}. Thus a reduction in migration was expected but the invasion assay recapitulates the micro environment mechanism better. This was evident as growth in 2D was unaltered by NDBT knockdown or inhibition but spheroid growth was reduced. In a 2D proliferation assay the media is buffered and metabolic by-products can be vented from the cells reducing the impact of high proliferative rates until the cells become near confluent. In a 3D spheroid, there is no vasculature or space for metabolic by-products to be removed and as such build up in the core of the spheroid. Therefore, as NDBT knockdown or inhibition reduced intracellular pH regulation the impact of this would be less severe in 2D than 3D. Furthermore, this inhibition or knockdown of NDBTs resulted in a lack of 3D spheroid invasion. To mitigate the compounding factor of spheroid growth being reduced and inducing necrosis if the spheroid became too large, spheroids in the migration were formed much smaller and encased in low growth factor Matrigel. The loss of NDBT function either by NDBT knockdown or inhibition not only resulted in necrosis in the spheroids but also that the ECM surrounded was degraded slower reducing invasion.

2D proliferation is not reduced by NDBT knockdown or inhibition in HCC-1806 or MDA-MB-231 (fig. 9.5). This was in both normoxic and hypoxic conditions (fig 9.5). It has previously been shown that SLC4A4 knockdown reduced proliferation in MDA-MB-231 cells in

normoxia and hypoxia⁴⁵⁴. This discrepancy between my results and the literature may be due to the timescale of my experiments. In the literature the proliferation assay was undertaken for 7 days whereas mine was undertaken for 72h⁴⁵⁴. When comparing for similar time points minor differences were seen by SLC4A4 knockdown at 72h⁴⁵⁴. Additionally in another study knockdown of SLC4A9 did not reduce 2D proliferation of MDA-MB-231 cells⁴³¹. It has previously been shown that knockdown of SLC4A4 reduces 3D proliferation in LS174T and MDA-MB-231 cells. Knockdown of SLC4A9 did not reduce survival in normoxia or hypoxia in neutral or acidic conditions in MDA-MB-231 and U87 cells. Similarly, in LS174T colon cell lines knockdown of SLC4A4 or SLC4A9 didn't reduce survival. When combined with CAIX knockdown SLC4A4 knockdown reduced cell survival in normoxia and hypoxia⁴⁵⁴. As PARP expression is increased by NDBT expression normoxia and maintained at similar levels in hypoxia. It is likely that for SLC4A4 knockdown to impact proliferation and survival in 2D, other members of the pH regulatory apparatus must also be inhibited or removed.

In contrast to 2D, knockdown or inhibition of NDBTs reduced spheroid growth rate in HCC-1806 (Fig. 9.2). It has been shown that knockdown of SLC4A9 reduces spheroid growth and increases intracellular pH in the tumour core⁴³¹. Similarly knockdown of SLC4A4 reduces spheroid growth⁴⁵⁴. Similar results; reduced spheroid proliferation and lowered pH in the spheroid core have been observed with CAIX knockdown²³⁶. Additionally knockdown of NHE1 also reduces spheroid growth rates²²⁷. In addition, the ability to form spheroids and proliferation was reduced with v-ATPase subunit knockdown⁵⁴⁶. It has been shown that the core of spheroids increased apoptosis and caspase activity in MDA-MB-231 cells⁴³¹.

It has previously been shown that knockdown of SLC4A4 in MDA-MB-231 cells reduces migration⁴⁵⁴. It was shown that this effect occurred in normoxia⁴⁵⁴. I have shown that knockdown of SLC4A5 reduces migration in both normoxic and hypoxic conditions In HCC-1806 and MDA-MB-231 (Fig. 9.4). Furthermore, knockdown of SLC4A4 in Cal-51 and HCC-1806 also reduced migration in normoxia and hypoxia (Fig. 9.4). The reduction in migration was not enhanced in hypoxia in any of the cell lines tested. Other pH regulatory proteins, when knocked down or inhibited, have also been shown to reduce migration; NHE1 knockdown reduced directed but not random motility⁵³⁴. This action occurred via alteration of the cytoskeleton⁵³⁵. Similarly inhibition of CAIX also reduces migration⁵²². CAIX enzymatic activity is enhanced by NDBT function⁵⁴⁷. However, as the effect of NDBT knockdown or inhibition were not hypoxia specific it is unlikely to occur via this mechanism (Fig. 9.4).

Knockdown of SLC4A4 has been shown to reduce invasion in MDA-MB-231 in normoxia. This effect was not investigated in hypoxia. I have shown that inhibition of NDBTs reduced invasion of breast cancer cells in normoxia and hypoxia (fig. 9.5). Invasion has been shown to be reduced by knockdown or inhibition of other pH regulatory proteins⁴⁵³. I have shown that knockdown of NDBTs has a greater effect on invasion than migration (Fig 9.4 and 9.5). However, knockdown of SLC4A4 in MDA-MB-231 cells had been shown to have a similar effect on migration and invasion⁴⁵⁴. Each of the NDBTs have different expression levels across cell lines (Fig 9.1). Differences in the expression between cell lines may be due to: the cell type the cell line was derived from⁵⁴⁸, mutational landscape⁵⁴⁹, and epigenetic landscape⁵⁵⁰. Thus to increase the efficacy of targeting NDBTs, targeting the most highly expressed NDBT may be the most effective method to reduce migration and invasion. Alternatively S0859 which targets the NDBT family may be used to induce similar effects.

3D spheroid invasion was reduced with NDBT knockdown and inhibition. However, the effect with S0859 was much less pronounced (fig. 9.6). Spheroids were placed in matrigel at a much smaller volume to reduce the effects of necrosis seen with NDBT knockdown on larger spheroids (Fig. 9.3), thus it is unlikely to be necrosis reducing invasion. Necrosis was likely occur due to the build-up of metabolites and acidity due to high proliferation⁴³¹. Furthermore, cell lines were placed in low growth factor matrigel to reduce proliferation. It has been previously shown that S0859 penetration into spheroids in some cell lines is poor⁴³¹. Inhibition of CAIX produced similar reductions in spheroid invasion. Thus inhibition of pH regulation by NDBT knockdown or inhibition reduces invasion.

As triple negative breast cancer commonly occurs with metastatic lesions in the bone and brain in patients; intra-cardiac metastatic model was taken forward. Pilot study data indicated that HCC-1806 was the more metastatic cell line compared to MDA-MB-231. Luciferase expression was higher in HCC-1806 overall and more metastatic lesions were formed. Furthermore, the attrition rate of this experiment was determined to be about 30% due to the needle puncturing the lung or leaving cells in the heart. HCC-1806 spread to the bones as expected.

Full study data had shown that the experiment hadn't worked for technical reasons. Almost all mice in each group had cells in the lung or chest cavity. Only three mice did not have cells in the lung or chest, which were all in the 4A5 knockdown group. These mice had the highest levels of metastasis. However, this level was lower than that seen in the control from the pilot data. The control cell lines had the highest number of lesions compared to

other groups although the luciferase expression was lower indicating that metastatic potential was reduced by NDBT knockdown.

Tail vein injections displayed a higher success rate than the intra-cardiac study. NDBT knockdown completely ablated metastasis in the SLC4A4 and SLC4A5 groups. It has previously been shown that knockdown of SLC4A9 reduces xenograft growth⁴³¹. I have shown that NDBT knockdown reduces spheroid growth rate, therefore metastatic colonisation was investigated via injection to reduce complications from a reduced growth rate.

9.4 Conclusion

In conclusion I have shown that knockdown or inhibition of NDBT reduces migration and invasion *in-vitro* in both normoxia and hypoxia. This knockdown and inhibition did not alter the growth of cells in 2D but resulted in slower growing and overall smaller 3D spheroids. Moreover, this reduction in intracellular pH resulted in a reduction in migration and invasion *in-vitro*. The data in this chapter provided strong supporting data to take NDBT knockdown cells into a xenograft model to determine if the reduction in migration and invasion *in-vitro*, translates into a reduction in metastasis in an *in-vivo* setting. Initial *in-vivo* data required the change from an intra-cardiac to tail vein model due to difficulties with initiation. NDBT knockdown ablated metastasis *in-vivo*.

10 Chapter 5: NDBT impact upon signal transduction and Epithelial to Mesenchymal transition

10.1 Introduction:

10.1.1 1: intracellular signalling

Signal transduction is pivotal for many if not all cellular functions⁵⁵¹. Deregulation of cellular function can result in many types of cancers and other diseases. All cells in a multicellular organism are exposed to a variety of extracellular signals that they need to interpret and respond to appropriately⁵⁵¹. These signalling molecules can be generated local to the cell, such as in the cases as synaptic transmission or autocrine signalling's⁵⁵² or produced distantly in the body such as hormones and growth factors⁵⁵³; additionally ligands on cell surfaces can interact, as can factors sequestered in the extracellular matrix and subsequently released⁵⁵⁴. To respond to these stimuli the cells express a plethora of receptors capable of binding these stimuli. These receptors are classified into families based on structure and function. These signalling pathways do not function in isolation and continually undergo cross talk to enable a greater variety of responses to the stimuli. The major classes of receptor are: G protein coupled receptors, receptor tyrosine kinases, ligand gated ion channels, integrins, and cytokine receptors⁵⁵⁵.

Intracellularly these signalling pathways function by a receptor upstream binding its cognate ligand and becoming activated⁵⁵¹. This activated receptor then signals on its intracellular domain to other signalling molecules resulting in a signalling cascade⁵⁵¹. However, many factors are involved resulting in extensive levels of modulation of the cascade⁵⁵¹. The large number of proteins involved in a signalling cascade and the plethora of transcription factors available for phosphorylation results in the cells being able to acutely respond to many stimuli⁵⁵¹. Many signalling molecules are involved in multiple pathways and converge on a single downstream target⁵⁵¹. Thus some pathways may work synergistically or antagonistically⁵⁵⁶. To further apply more complexity to intracellular signalling there are many master regulators which are genes which code mainly for transcription factors⁵⁵⁷. These are at the top of the hierarchy and disruption to these result

in large cellular changes⁵⁵⁷. Not all signal transduction results in an activation, repression of a protein function can induce large effects for example phosphorylation of GSK3- β at serine 9 results in steric hinderance⁵⁵⁸.

Signals are passed by post translational modifications of a protein⁵⁵⁹. There are multiple PTMs which alter protein function such as: phosphorylation, ubiquitination, hydroxylation, glycosylation, protonation, lipidation, acylation, or sumoylation among many others⁵⁶⁰. Many of these modifications serve differing functions; ubiquitination results in degradation, glycosylation in protein stability and regulation⁵⁶¹. The most extensively studied is phosphorylation and this is how many signalling cascades occur⁵⁶². Many proteins contain multiple types of these modifications allowing the activity of these proteins to be very tightly regulated⁵⁶². Depending on the site of the protein some moieties can either be activating or repressing to the proteins function⁵⁶².

10.1.2 2: Phospho-tyrosine-kinase signalling

Protein kinase activity regulate key cellular processes such as proliferation and migration and such if deregulated contribute to processes such as cancer growth and metastasis⁵⁶². The first proto-oncogene to be discovered was SRC a kinase. Large scale genome sequencing projects have greatly enhanced the knowledge of somatic mutations in common cancer types (Fig 10.1). The most commonly encoded gene region encoded by a cancer gene was the kinase domain. Some mutations are relatively common depending on the cancer type, EGFR was mutated in 27% of glioblastoma samples⁵⁶³. Particular mutations can also be associated with certain molecular subtypes. In luminal A breast cancer loss of function mutations in MAP3K1 and MAP2K4 occur in 20% of cases⁵⁶⁴. However only relatively few of these kinases have such frequent mutations in a specific cancer type with most being mutated at an intermediate rate of 1-10% of cases.

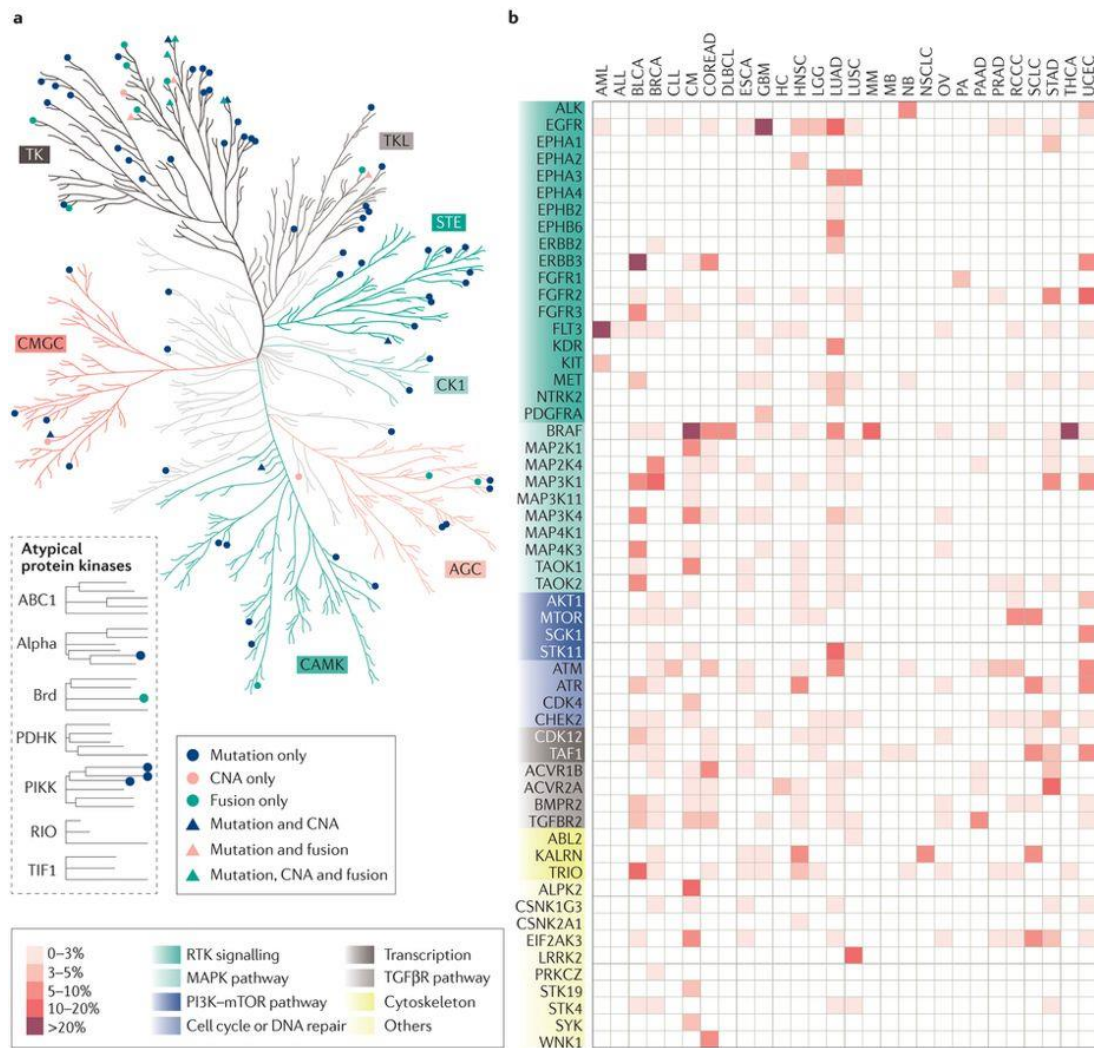


Figure 10.1 Driver protein mutations identified in large scale kinome genomic studies.

Mutations in RTK signalling have been shown to be involved in cancer progression in many cancer cell types. The most commonly mutated RTK is EGFR. Expression of RTK mutation is heterogenous across cancer types. (Adapted from Daly et al., 2016)

Hypoxic signalling occurs primarily via the stabilization of the HIF transcription factors¹⁴⁴. This stabilization results in large signalling and transcriptional changes within the cell¹⁴⁴. For example, hypoxic signalling results in the reduction of cell cycle progression and increased invasiveness⁵⁶⁵. Expression of cell cycle inhibitors p21 and p27 are induced in hypoxia leading to G1 arrest. p21 and p27 bind directly to cyclin dependent kinases (CDKs) and inhibit the interaction of CDK and its activating cyclin⁵⁶⁵. Thus, the CDKs cannot be phosphorylated on the threonine residue required for activation halting cell cycle progression⁵⁶⁵. Moreover, HIF signalling has been implicated in the regulation of RTK signalling. Expression of many RTK genes are upregulated in response to hypoxia e.g. VEGF-A both *in-vivo* and *in-vitro*⁵⁶⁶. This upregulation and secretion of VEGF-A in tumours functions in a paracrine fashion to stimulate angiogenesis and VEGFR intracellular signalling⁵⁶⁷. Hypoxia has been shown to increase the activity of SRC in tumour cells resulting in increased VEGF-A expression⁵⁶⁸. Met, a protein strongly correlated with metastasis is upregulated by HIF-1 α . Induction of MET is via HIF activated transcription, the MET promoter contains two HRE binding sites and one AP1 binding site⁵⁶⁹. Increased expression of met increases oligomerisation and ligand independent activation but also increased sensitivity to HGF ligand⁵⁶⁹. Thus, the increased migration and invasion of tumour cells in hypoxic conditions is in part due to HIF regulation of MET⁵⁶⁹. Increased ROS expression in hypoxia induced ERK activation promoting invasiveness by suppressing dual specificity phosphatase 2 RNA and protein levels⁵⁷⁰. ERK activation was shown to be independent of MET signalling⁵⁷⁰. Another signalling protein that mediates invasion and metastasis, AXL is upregulated in hypoxia⁵⁷¹. Both HIF-1 and HIF-2 bind to the AXL promoter and increase expression⁵⁷¹. AXL forms a complex with SRC, increasing kinase activity and activating MET signalling via phosphorylation of MET intracellular domain⁵⁷¹. Furthermore, HIF-1 α interacts with nRTK RON⁵⁷². The HIF-1 α /RON complex binds to the c-JUN promoter and increased the expression of c-JUN culminating in AP-1 signalling for survival and metastasis⁵⁷². HIF-1 α and c-JUN are functionally cooperative in hypoxia, c-JUN is phosphorylated on ser63 in hypoxic conditions resulting in activation⁵⁷³. Activated c-JUN interacts with HIF-1 α on its ODDD masking the ubiquitination site and therefore propagation of hypoxic signalling⁵⁷⁴. However, mutations in Ser63 and Ser73 on c-JUN result in a dominant negative binding to HIF-1 α reducing hypoxic signalling⁵⁷³.

Epithelial to mesenchymal transition (EMT) is a process by which cells lose polarity and cell-cell adhesion and acquire a migratory phenotype³¹². EMT facilitates metastatic dissemination of breast cancer cells. EMT is induced by multiple signalling pathways,

particularly hypoxic signalling. HIF signalling targets include the EMT factors ZEB, SNAIL, Twist⁵⁷⁵. Transcription of SNAIL by HIF-1 α resulted in the repression of E-cadherin³²⁷. Furthermore, hypoxia induced SLUG expression plays a role in the cadherin switch of EMT³⁴⁵. HIF-1 α signalling increases the expression of ZEB1³²². This in turn increased the expression of Vimentin and N-cadherin whilst reducing the expression of cytokeratin 18³²². The TWIST1 promoter contains a HRE site which HIF-1 α has been shown to bind⁵⁷⁶. Knockout mice of HIF-1 α or TWIST1 show a similar phenotype and reduced metastasis⁵⁷⁶. Similarly to HIF-1 α , HIF-2 α also regulates EMT transcription factor expression⁵⁷⁷. Expression of SNAIL, ZEB1, Vimentin, and TWIST1 were all shown to be regulated by HIF-2 α ⁵⁷⁷. HIF-2 α binds to β -catenin forming a transcriptional complex and driving EMT⁵⁷⁸.

Aside from the direct regulation of some EMT transcription factors, downstream targets of HIF also induce EMT⁵⁷⁹. LOX is a downstream target of HIF-1 α ⁵⁷⁹. LOX activates the transcription of EMT factor snail, which subsequently binds and represses the transcription of E-cadherin⁵⁷⁹. Expression of TGF- β is induced by hypoxic signalling⁵⁸⁰. Hypoxia activates autocrine TGF- β signalling; activation of TGF- β downregulates PHD2 via smad dependant signalling resulting in further HIF stabilization and EMT⁵⁸⁰. Notch signalling is increased by hypoxia, SNAIL and SLUG are downstream targets of notch and are subsequently upregulated, driving EMT⁵⁸¹.

Similarly to hypoxia, extracellular acidosis results in downstream transcriptional and signalling changes⁴⁹⁹. Acidosis is initially sensed by the pH sensitive ion channels⁵⁸². Under acidic conditions protons bind to the extracellular domain resulting in a conformational change in the ASICs and opening the transmembrane pore in the protein⁵⁸². Once opened the transmembrane pore facilitates the passage of sodium ions across the membrane⁵⁸². ROS signalling is increased by extracellular acidosis²¹⁵. Rapid phosphorylation of p38 and ERK1/2 occurs in acidosis and inhibition of ERK1/2 in acidosis lead to necrosis²¹⁵. Scavenging of ROS in acidosis inhibited the activation of p38²¹⁵. CREB was subsequently activated by p38 activation and remained active 24h after cells had been placed back into neutral media²¹⁵. *In-vivo* acidosis studies have linked acidosis to inflammation. expression of TNF- α , COX-2 and iNOS in parallel to an activation of p38 and ERK1/2 kinases seen *in-vitro*⁵⁸³. Expression of COX-2 and iNOS depended on p38 kinase, but not on ERK1/2⁵⁸³. Furthermore, acidosis induced p38 and ERK1/2 signalling increases drug resistance⁵⁸⁴. P-glycoprotein activity is increased in acidic tumours *in-vivo*⁵⁸⁴. Caspase 3 activity was reduced 30% in acidosis conditions but was restored with p38 inhibitors⁵⁸⁴. Further to this,

acidosis sensing protein GPR65 prevents apoptosis when activated⁵⁸⁵. GPR65, via MEK.ERK signalling increases expression of BCL-2 and BCL-XL⁵⁸⁵. This attenuates apoptosis via PUMA and BIM under cellular stress such as glucose starvation⁵⁸⁵. Colorectal cancer cells suppress the expression of vitamin D receptor in acidic conditions, overexpression of vitamin D receptor in these conditions reduced migration and invasion⁵⁸⁶. Moreover, knockdown of the vitamin D receptor increased the expression of stem like markers such as SOX2⁵⁸⁶. In glioma cells, extracellular acidosis mediated reduction in vitamin D resulted in an increase in CTP24A1 and increased mitochondrial respiration⁵⁸⁷. Acidosis signalling results in increased migration, invasion and an EMT phenotype²⁶⁷. Tumour cells placed in acidic media displayed increased migration and invasion²¹³. However, when combined with ASIC1 or ASIC3 migration and invasion were remained at levels comparable to cells in neutral media²¹³. Furthermore, tumour cells placed in acidic media had increased expression of EMT markers vimentin, zeb1, and snail1 via activation of the RhoA pathway²¹³. Knockdown of ASIC1 reduced metastasis *in-vivo*²¹³. Increased intracellular acidosis also leads to dynamic intracellular signalling changes²⁷⁷. Activation of mTORC1 is rapidly and reversibly reduced by intracellular acidification resulting in reduced phosphorylation of downstream target S6K²⁷⁷. Knockdown of Tuberous Sclerosis Complex 2 (TSC2) resulted in an insensitivity to intracellular acidosis²⁷⁷. The upstream AKT pathway was unaffected by lowered pH whereas ERK pathway was inhibited. Treatment with MEK inhibitors reduced phosphorylation of MTORC1 modestly indicating multiple pathways also play major roles.

SRC family kinases play important roles as signalling intermediaries⁵⁸⁸. They relay and modulate various upstream signals to regulate downstream effects such as migration or proliferation⁵⁸⁸. LYN kinase is a member of the SRC family and has been implicated in breast cancer⁵⁸⁸. Drug resistant cancer cells have increased activation of LYN⁵⁸⁹. In colorectal cancer cells LYN kinase signalling forms part of the AKT anti-apoptotic cascade⁵⁸⁹.

Directional migration of cancer cells requires LYN signalling⁵⁹⁰. Inhibiting LYN signalling in prostate cancer cells reduced proliferation *in-vitro* and *in-vivo*⁵⁹¹. Targeting LYN with dastatinib reduced lymph node metastasis in breast cancer⁵⁹². LYN kinases expression in breast cancer is associated with the basal subtype and reduced overall patient survival⁵⁹³. Knockdown of LYN in breast cancer reduced migration and invasion but not proliferation *in-vitro*⁵⁹⁴. LCK kinase is another SRC family kinase and is commonly mutated in triple negative breast cancer malignancies⁵⁹⁵. LCK expression is required for T cell mediated signal transduction⁵⁹⁶. Binding of IL-2 to cognate receptor activates LCK signalling⁵⁹⁷. LCK is localised to the plasma membrane and binds to the cytoplasmic domains of many cell

surface receptors such as AXL, CD2, CD4, and CD5⁵⁹⁸. However, LCK has also been shown to bind other kinases such as CDK1, RAF1, and SYK⁵⁹⁹. Downstream phosphorylation targets of LCK are: ZAP-70, ITK, protein kinase C, and PI3k⁶⁰⁰. LCK signalling controls cell cycle, cell adhesion, and cell migration⁶⁰¹. LCK phosphorylates the microtubule associated protein MAPT⁶⁰¹. Activated LCK results in the activation and DNA binding of STATb⁶⁰². RUNX3 is another transcription factor that is a target of LCK signalling⁶⁰³. LCK signalling protects cancer cells from apoptosis and induces proliferation⁶⁰⁴. Expression of LCK is high in metastatic colon cancers⁶⁰⁵.

10.2 Hypothesis:

I hypothesise that inhibition or knockdown of NDBTs will reduce intracellular phospho-signalling because of increased intracellular acidification. To investigate this hypothesis I will undertake the following aims:

1. *Undertake a phospho-signalling array to determine the effect of hypoxia on intracellular phospho-protein activation.*
2. *Determine the effect of acidosis on intracellular phospho-signalling*
3. *Determine the effect of NDBT knockdown or inhibition on intracellular phospho-signalling in hypoxic, or hypoxic and acidic conditions.*
4. *Evaluate the impact of NDBT knockdown or inhibition on receptor tyrosine kinase expression.*
5. *Investigate the activation and expression of mTOR, HIF-1 α , HIF-2 α , and downstream targets.*
6. *Determine the expression of p53 and the P53 negative regulator MDM2.*
7. *Investigate the expression of top hits LYC and LYN under hypoxic and acidic conditions.*
8. *Evaluate the functional outcome of LYC or LYN inhibition on: migration, invasion, survival, and proliferation in normoxia and hypoxia.*
9. *Determine if Inhibition of LYC or LYN reduces HIF stabilisation in hypoxia.*
10. *Investigate the role of NDBT knockdown or inhibition on EMT transcription factor mRNA and protein expression in normoxia or hypoxia*

- 11. Determine the impact of NDBT inhibition or knockdown on expression of EMT markers E-cadherin and Vimentin in normoxia and hypoxia*
- 12. Examine the impact of NDBT knockdown on expression of AP1 transcription factor subunits in normoxia and hypoxia*
- 13. Determine the impact of NDBT knockdown or inhibition on c-MYC and ETS1 expression in normoxia and hypoxia*

10.3 Results:

Evidence regarding the effects of extracellular acidosis on phospho-signalling was few and focused primarily on the ERK signalling pathway. To evaluate these perturbations an intracellular phosphor-RTK signalling array was used such that multiple pathways could be evaluated simultaneously.

Initially the effects of acidosis and hypoxia independently were evaluated using the array. To identify the role of hypoxia on signalling HCC-1806 shCTL cells were placed in either normoxia or hypoxia for 48h (Table 3). Hypoxia resulted in a near global induction of phosphor-signalling. A total of 38 phospho sites were increased in hypoxia. JNK1/2/3 (T183/Y185, T221/Y223), P53 (S392), STAT5a/b (Y694/Y699), PRAS40 (T246), AMPK α 1 (T186), STAT2 (Y689), ERK1/2 (T202/Y204, T185/Y187) were the most significantly increased in terms of total expression. The largest increases in fold expression were for Lyn (Y397) 6.5 fold, Lck (Y394) 4.3 fold, HSP27 (S78/S82) 4 fold, p38 α (T180/Y182) 4 fold. Three proteins: WNK1 (T60), RSK1/2/3 (S380/S386/S377), and Fgr (Y412) had reduced phosphorylation reduced in hypoxia 13%, 18%, 59% respectively. Subsequently, HCC-1806 shCTL cells were placed in either neutral (pH 7.4) or acidic media (pH 6.4) for 48h. Acidosis resulted in an almost overall reduction in phosphor-signalling state (Table 4). A significant reduction in activation was seen in 30 out of 43 phospho sites tested. The largest reductions were seen in GSK-3 α/β (S21/S9), ERK1/2 (T202/Y204, T185/Y187), FAK (Y397) and AKT (S473). An upregulation in signalling of three proteins; p27 (T198), eNOS (S1177), P53 (S392) was identified. The highest upregulation was P53 (S392) with 10%. Little to no change was identified in AKT (T308), HSP60, P70 S6k (T421/S424), P53 (S15), STAT3 (Y705), PYK2 (Y402), C-Jun (S63).

To further expand upon these results, an investigation into the effects hypoxia and acidosis in combination was undertaken (Table 5). The cells were placed in hypoxia and either neutral or acidic media for 48h. The changes in expression were much more variable than either condition in isolation. The majority of the proteins tested had reduced activation in comparison to the neutral condition. However, the reduction in signalling was still higher than the normoxic levels of expression. Four proteins had a higher expression with the combination: FGR (Y412), HSP27 (S78/S82), p38 α (T180/Y182), MSK1/2 (S376/S360). 28 proteins had a major reduction in activation levels with GSK3-3 α/β (S21/S9), HSP60 (x),

ERK1/2 (T202/Y204, T185/Y187) P53 (S392). The remaining 11 proteins were either unchanged in activation or only minor reductions in expression.

Subsequently we wished to identify changes in intracellular RTK phospho-signalling due to the knockdown or inhibition of NDBTs (Fig. 10.2). ShCTL, shCTL + S0859, sh4A4, and sh4A5 were subject to either hypoxia and neutral conditions or hypoxia and acidosis conditions for 48h. In hypoxia and neutral conditions NDBT knockdown or inhibition resulted in upregulation of five proteins. The remaining phospho-proteins were all severely reduced in activation. ERK1/2 (T202/Y204, T185/Y187) was upregulated by NDBT inhibition by 50%, WNK1 (T160) was increased by 20% by NDBT inhibition, HSP60 (X) by 5% by *SLC4A4* knockdown, CREB (S133) by 50% and 30% by *SLC4A4* and *SLC4A5* respectively, and β -catenin (X) by 20% and 40% by *SLC4A4* and *SLC4A5*. The greatest reduction were seen in GSK-3 α/β (S21/9), JNK 1/2/3 (T183/Y185, T221/Y223), FAK (Y397), AMPK α 2 (T172), STAT5a/b (Y694/Y699) SRC (Y419), AMPK α 1 (T183), TOR (S2448) for *SLC4A4* and *SLC4A5*. Interestingly the general pattern for *SLC4A4* and *SLC4A5* were generally quite similar in both terms of targets but also level of changes to expression levels. NDBT inhibition was generally similar although slightly less reduced but divergent results were seen for GSK-3 α/β (S21/S9), PRAS40 (T246), JNK1/2/3 (T183/Y185, T221/Y223), STAT5a/b (Y694/Y699) and STAT6 (Y641) and AKT1/2/3 (T308) whereby activation levels of these proteins were much higher than the NDBT knockdowns.

When hypoxic and acidosis (pH 6.4) conditions were combined, in all cell lines there was less total phospho-signalling (Table 5). Most signalling proteins had reduction in activation of 50%. Five proteins had higher expression than the control: ERK1/2 (T202/Y204, T185/Y187) in *SLC4A4*, HSP60 (X) in *SLC4A4* and *SLC4A5*, CREB (S133) in *SLC4A4*, P70 s6 kinase (T421/ S424) in NDBT inhibition, and RSK1/2/3 (S380/S386/S377) in NDBT inhibition. The loss of pH control in the knockdown cell lines resulted in the loss of activation of pH sensitive protein. Interestingly some proteins which were reduced by NDBT knockdown were only slightly reduced in activation with NDBT inhibition. Indicating either the inhibitor is resulting in lower rates of intracellular acidification or a knock on structural effect is cause of other results seen. The proteins less affected by NDBT inhibition in hypoxia and acidosis were: p27 (T198), STAT3 (Y705), WNK1 (T60), p70 s6K (T421/S424), and PDF rb (Y751).

The expression of RTKs were investigated in hypoxia and hypoxia + acidosis after 24h (Fig. 10.2). The investigation primarily focussed on cMET as it is the most potent known inducer

of metastasis. Inhibition of NDBT with S0859 increased expression of Pdgfr- α by 20% and 30% in normoxia and hypoxia. FGFR2, pALK, ALK were unchanged by NDBT inhibition in both normoxia and hypoxia. PcMET was increased by 20% by NDBT inhibition in normoxia and 30% in hypoxia. Total cMET was unchanged by NDBT inhibition. Additionally HGF, VEGFr2, and EGFR were unchanged by NDBT inhibition. Expression of EGF was reduced by 30% by NDBT inhibition.

Signalling by mTOR impacts many pathways such as proliferation and migration. As mTOR signalling is required for HIF stabilization we investigated mTOR activation by blotting for S2448 as mTOR activation was reduced on the signalling array⁴⁵⁸. The S2448 phosphorylation site on mTOR is phosphorylated by its downstream target S6k and reflects the cells nutrient and amino acid status⁶⁰⁶. Thus as the NDBTs are expected to increase intracellular acidity and reduce metabolic activity this site was chosen. Other phosphorylation sites are present on mTOR however some of them are autophosphorylation sites and not appropriate. We hypothesized that mTOR activation was reduced by NDBT knockdown in hypoxia thus leading to a loss in HIF stabilization. mTOR induction was validated by western blot (Fig. 10.3). Hypoxia resulted in a 2 fold increase in expression of Phospho 2448 mTOR in HCC-1806 shCTL with no significant increase in overall protein expression levels. Inhibition with S0859 resulted in no change to activated mTOR levels whereas NDBT knockdown reduced mTOR activation by half.

HIF signalling is the major cellular response to hypoxia. This occurs via the stabilization of Hif-1 α or Hif-2 α due to the PHD proteins lacking oxygen as a cofactor. HIF-1 α signalling was reduced in response to NDBT knockdown. In HCC-1806, knockdown of NDBT ablated HIF-1 α signalling; no stabilization of HIF-1 α was seen (Fig. 10.4). This led to an absence of expression of downstream target CAIX (Fig. 10.6). These findings were replicated in MDA-MB-231, no stabilization of HIF-1 was seen and no induction of CAIX was present in hypoxia. Inhibition of NDBT with S0859 resulted in no change to HIF signalling and CAIX was expressed in similar levels to the controls. Similarly to HIF-1 α , NDBT knockdown ablated HIF-2 α stabilization in hypoxia (Fig 10.5). This lack of HIF-2 α stabilization was seen with shRNA constructs for *SLC4A4* and *SLC4A5*. However, S0859 had no effect on HIF-2 α stabilization.

Hypoxia has been shown to induce EMT in tumours. Many EMT related transcription factors contain a hypoxic response element (HRE). Furthermore an acidic extracellular tumour microenvironment has been shown to induce EMT. As NDBT knockdown ablated

HIF signalling, reduced migration, and stopped invasion, we decided to investigate if this was caused by a reduction in the ability to induce EMT via perturbed HIF signalling.

Due to the size and number of conditions available it was more pertinent to interrogate the data with specific functional outcomes in mind. Thus it was asked what effect did NDBT knockdown or inhibition have on the activation of EMT related phospho-proteins. The EMT transcription factors we investigated were: Snail1, Twist1, WNT5a, and ZEB1. Of the signalling proteins involved in Snail1 regulation, only ERK1/2 (T202/Y204, T185/Y187) was unperturbed by NDBT knockdown or inhibition in hypoxia. The remaining molecules were all reduced. As with many of the other results from the array the results for inhibition was less pronounced than knockdown. When the cells were placed in acidosis the expression of these proteins was greatly reduced in the control but more so in the NDBT knockdowns. When comparing expression levels placing the shCTL cells into hypoxia and acidosis brought phosphorylation levels of these proteins to the same levels as just NDBT knockdown in hypoxia. Similarly to Snail1; WNT5a related phospho-proteins were also reduced in response to NDBT knockdown in hypoxia with the exception of β -catenin. Expression of β -catenin (X) was increased with hypoxia in *SLC4A4* and *SLC4A5* knockdown but not NDBT inhibition. When placed into hypoxia and acidosis shCTL levels of β -catenin (X) were reduced but levels in the knockdowns were also reduced to similar levels as the controls. Interestingly JNK1/2/3 (T183/Y185, T221/Y223), *ampka2* (T172) showed no change by the control when placed in hypoxia and acidosis compared to just acidosis. However, NDBT knockdown reduced GSK-3 α/β (S21/S9) levels in hypoxia alone but when in hypoxia and acidosis were combined levels were comparable with the control and just hypoxia. Twist1 similarly had reduced activation of phospho-proteins related to its activation with NDBT knockdown and hypoxia. All proteins involved were reduced and further reduced in activation with NDBT knockdown in hypoxia and hypoxia plus acidosis. ZEB1 phospho-proteins expression were all reduced except for ERK1/2 (T202/Y204, T185/Y187) which was higher in hypoxia and acidosis with NDBT knockdown. One protein was unchanged in the control in hypoxia vs hypoxia plus acidosis which was p38 α (T180/Y182). These data together indicate that it is likely that NDBT knockdown and inhibition is likely to lead to inhibition of downstream EMT activation.

Transcriptional regulation of key EMT genes were altered by NDBT knockdown or inhibition, and a heat map was produced (Fig.10.7-10). In HCC-1806 with either NBCe-1 or NBCe-2 knockdown: *twist1* (2 fold), *twist2* (4.5 fold), and *snai2* (3 fold) were unregulated in

hypoxia. In contrast *snai1* (3 fold), ZEB1 (2 fold), ZEB2 (4 fold), WNT5b (2 fold), GSC (5 fold) were significantly downregulated in hypoxia whereas in the control they were upregulated. WNT5a was not significantly changed by knockdown or inhibition of NDBT. The patterns were similar in normoxia.

In MDA-MB-231, a different pattern was seen. WNT5b was the only transcription factor for which expression in hypoxia was significantly increased (2.6 fold) by NBCe-2 knockdown. The remaining EMT genes were decreased: *Snai1* (1.5 fold), *Snai2* (1.5 fold), *Twist1* (2 fold), *Twist2* (1.5 fold), ZEB2 (3.7 fold), WNT5a (5 fold), GSC (3 fold). Treatment with s0859 showed similar effects however the changes were more subtle compared to knockdowns. Knockdown and inhibition of NDBT showed a similar pattern in normoxia.

Cal-51 showed an overall increase in EMT gene expression in hypoxia with *Snai2* as the only exception (2 fold decrease). NBCe-1 knockdown reduced the induction of *twist1* (50%), *twist 2* (90%), WNT5b (30%), and GSC (20%). Induction of ZEB1 was increased by 2 fold. Under normoxic conditions knockdown had an overall reduction in EMT gene expression.

EMT gene transcript levels were also quantified in normoxia and hypoxia with NDBT inhibition. S0859 treatment resulted in a significant decrease in *twist2*, WNT5a, and WNT5b levels and a significant increase in ZEB1 in hypoxia. In normoxia treatment with S0859 resulted in a decrease in all EMT genes tested except for *twist1*, *snai1*, and ZEB1 where there was no significant change.

Western blot of EMT proteins were undertaken to see how these results translated to the protein level (Fig. 10.11). NBCe-1 knockdown in HCC-1806 showed an upregulation of E-cadherin in normoxia and hypoxia. Vimentin, WNT5b, *snai2*, and ZEB1 expression was reduced in normoxia and hypoxia. *Twist1* and ZEB2 expression was unchanged. NBCe-2 knockdown showed a reduction in ZEB1, ZEB2, Vimentin, GSC, WNT5b, *Snai2*, *Snai1*, and *twist1* in both normoxia and hypoxia. E-cadherin expression was increased by NBCe-2 knockdown in both normoxia and hypoxia.

Further to the describing the EMT signalling changes that occurs during NDBT knockdown we wished to elucidate a possible mechanism. Thus investigations were undertaken into three separate areas: Firstly the expression of RTKs and their activation was blotted for, secondly mTOR was inhibited but as individual isoforms and pan inhibition to determine if this resulted in a different EMT expression pattern both at the RNA and protein level, and

functional outcome investigated. Thirdly alternative intracellular proteins were inhibited to see if similar effects were produced.

The function consequences of mTOR inhibition were investigated in HCC-1806 and MDA-MB-231 (Fig. 10.12-13). Individual isoform and a pan mTOR inhibitor was used.

Interestingly a differential effect was seen with between the two cell lines. In MDA shCTL and S0859 treated cells inhibition in both normoxia and hypoxia resulted in no significant change in wound healing. However, there was an additive effect with sh4A5 knockdown cells. Inhibition of mTORc1 resulted in a 20% reduction in construct 1 and a 15% reduction in construct 2 in normoxia. In hypoxia mTORc1 inhibition reduced wound healing by 0% in construct 1 and 10 in construct 2. Pan inhibition reduced wound healing by 10 in construct 1 and 5% in construct 2 in hypoxia. In hypoxia it was reduced by 5% for both constructs. Inhibition of mTORc2 inhibition reduced wound healing by 15% and 20% in constructs 1 and 2 respectively in normoxic conditions. Similarly, in hypoxia wound healing was reduced 10 for both constructs. These data indicate that mTORc2 is not the pathway by which migration is perturbed by NDBT knockdown in MDA-MB-231. However, the additive effects seen in the NDBT knockdown cell lines point towards an increased dependence on mTORc2 in MDA-MB-231.

Conversely the effects in HCC-1806 were markedly differently. Inhibition of mTORc1 resulted in a 20% reduction in wound healing in both normoxia and hypoxia. Similarly mTORc2 inhibition resulted in a 20% reduction in both normoxic and hypoxic conditions. Furthermore pan mTOR inhibition resulted in 18% reduction in normoxia and a 22% reduction in hypoxia. Similar results were seen in S0859 treated cells. In normoxia wound healing was reduced 10%, 8%, and 5% for mTORc1, pan mTOR, and mTORc2 respectively. In hypoxia wound healing was reduced 10%, 15%, and 10% for mTORc1, pan mTOR, and mTORc2 respectively. However, in SLC4A4 knockdown cells wound healing was reduced by 10% by mTORc1 inhibition, 10% by pan mTOR inhibition, and 12% by mTORc2 inhibition in normoxia. In hypoxia mTORc1 inhibition reduced wound healing by 0%, pan mTOR inhibition by 5%, and mTORc2 inhibition by 3%. Similarly with the SLC4A5 knockdown cells treatment with mTORc1 inhibitor reduced wound healing by 10%, pan mTOR by 6%, and mTORc2 by 10%. Furthermore these results were mirrored in hypoxia with mTORc1 inhibition reducing wound healing by 5%, pan inhibition by 4%, and mTORc2 by 8%. These data indicate that mTOR signalling plays a significant role in wound healing and are a possible functional pathway for the intracellular acidosis induced by NDBT knockdown.

Additionally, LYC and LYN were identified as alternative potential functional pathways by which intracellular acidosis produces the functional effects seen. Lyk inhibition reduced wound healing by 10% in normoxia and 15% in hypoxia. Lyn did not reduce wound healing in normoxia but reduced wound healing by 20% in hypoxia. When Lyk and Lyn were combined wound healing was reduced by 15% in normoxia and 20% in hypoxia. This data indicates that Lyk reduces wound healing in both normoxia and hypoxia whereas Lyn is more hypoxia specific. Combining inhibitors of both proteins resulted in no additive inhibition in wound healing (Fig. 10.17).

Protein expression was investigated to determine a mechanism by which inhibiting mTOR, Lyk, or Lyn resulted in wound healing reductions. EMT markers and signalling targets were investigated based upon previous array results. ZEB1 expression was unchanged by mTOR inhibition in normoxia, however in hypoxia expression was increased by C2 inhibition but reduced by a pan inhibition. WNT5b expression was reduced by all mTOR inhibitors in normoxia, whereas in hypoxia MTORc1 and MTORc2 increased expression. Goosecoid expression was reduced in normoxia by all inhibitors and no effect was seen in hypoxia. Vimentin expression was unchanged by all mTOR inhibitors in both normoxic or hypoxic conditions (Fig. 10.14). Further to this MAPK expression and activation was reduced by all mTOR inhibitors in normoxia and increased in expression in hypoxia. FAK expression was reduced by all mTOR inhibitors in normoxia and hypoxia. Expression of AKT was unperturbed by mTOR inhibition likely as AKT is upstream of MTOR. Interestingly CREB expression is increased by mTOR inhibition in normoxia. In hypoxia no increase in expression was seen.

Inhibiting LYK increased ZEB1 expression in normoxia but reduced expression in hypoxia. ZEB2 expression was increased by LYK inhibition in both normoxia and hypoxia. Vimentin and Slug expression was unchanged in normoxia and hypoxia by LYK inhibition. Similarly GSC was unchanged by LYK inhibition. LYN increased expression of ZEB1 in normoxia but decreased expression in ZEB2. In hypoxia the reverse occurred, ZEB1 expression was decreased but ZEB2 expression increased. No effect on Vimentin, slug, and goosecoid was seen in both normoxia and hypoxia. Expression of TIA1, Integretin beta, SHMT2, P53, were investigated with LYK and LYN inhibition. However, no changes were seen in any targets in both normoxic and normoxic conditions. Thus no clear mechanism could be determined such that the effect of NDBT knockdown could be reproduced.

Further experiments were undertaken to determine the role of LYC and LYN in hypoxia. Survival was evaluated using a clonogenic assay in normoxia and hypoxia (Fig 10.19). In normoxia no change in survival was seen with either LYC or LYN inhibition alone. However, when inhibition of the targets were combined a minor reduction (4%) in survival was seen. In hypoxia Inhibition of LYN resulted in a 20% reduction in colony survival was seen. LYN inhibition resulted in a 25% reduction in survival. Inhibition of LYN and LYK had an additive effect in reducing colony survival. These data indicate the LYC and LYN have a hypoxia specific effect on survival.

In the previous chapters it was previously shown that NDBT inhibition and knockdown resulted; in reduced migration and invasion, necrosis in spheroids, altered intracellular signalling. Thus it was decided to investigate some potential mechanistic causes. Firstly apoptosis was investigated and P53 expression quantified. This was investigated by possible pathways i.e. the intrinsic and extrinsic pathways were blotted for as well as P53 due to its role in apoptosis. Additionally, cMYC expression was quantified due to its role in invasion and migration. Further to this LOX and LDHA expression was quantified as they are closely linked to hypoxia and also invasion (Fig. 10.21). It is possible that some of the perturbations seen were due to a switch in translation efficiency. EIF4A1/2 were blotted for as they are known to have altered translation preferences in hypoxia due to binding affinity changes to long 5' UTRs (Sup Fig). Subsequently to this an alternative explanation was the formation of different heterodimers in large transcription complexes. It is known that members of the AP1 transcription factor are upregulated to differing extents in hypoxia, with FOSL2 being particularly implicated with hypoxic survival. This may induce a change in binding affinity for one of its DNA binding sequences.

Expression of P53 total was completely ablated with NDBT knockdown and reduced by NDBT inhibition in hypoxia by 70%. No effect on P53 by NDBT inhibition was observed in normoxia. Additionally the P53 inhibitory protein MDM2 was reduced by 50% in normoxia and hypoxia by NDBT knockdown. NDBT inhibition increased expression of MDM2 by 30% in normoxia and hypoxia. LOX and LDHA expression were quantified by western blot in normoxia and hypoxia. LOX and LDHA expression were reduced by 50% and 70% respectively compared to the control by SLC4A4 knockdown. However, SLC4A5 knockdown had no effect on LOX and LDHA expression. ETS1 is a transcription factor with some regulation under P53, additionally it control transcription of MMPS and angiogenesis. Furthermore it is under the regulatory control of VEGF. Thus it may be a possible

mechanism by which the knockdown of NDBTs is reducing migration and invasion. ETS1 expression is reduced by 30% in hypoxia in the control. Inhibition of NDBTs reduces expression of ETS1 by 20% in both normoxia and hypoxia respectively. Knockdown of both SLC4A4 and SLC4A5 ablated expression of ETS1. MYC expression is markedly increased compared to other breast cancer subtypes. High expression of MYC is associated with a poor prognosis and higher levels of invasion and metastasis. Expression of MYC results in the transcription of a large number of downstream targets including those involved in metabolism, invasion, and drug resistance. In the control cMYC expression was unchanged. NDBT inhibition increased the expression of cMYC 2 fold in normoxia however it was reduced by 10% in hypoxia. Knockdown of SLC4A4 increased cMYC expression 50% in normoxia but ablated expression in hypoxia. Knockdown of SLC4A5 did no change cMYC expression in normoxia however in hypoxia expression was ablated.

The effect on large multimeric transcription factors by NDBT inhibition and knockdown were investigated by western blotting. AP1 has two DNA binding consensus sequences, and AP1 sequence and a CRE sequence. Depending on the arrangement of subunits affinity to these sequences will be altered (Fig. 10.22). Fos1 expression was unchanged by treatment with S0859 in normoxia and hypoxia. Knockdown of SLC4A4 or SLC4A5 increased expression of FOSL1 in normoxia by 60% and 40% and in hypoxia by 50% and 40% respectively. Fos12 expression was reduced by treatment with S0859 by 70% in normoxia and 10% in hypoxia. Knockdown of SLC4A4 reduced normoxic FOSL2 expression by 75% and hypoxic expression by 80%. Fos12 expression was reduced by 60% in normoxia and 65% in hypoxia by SLC4A5 knockdown. Fosb expression was increased by treatment with S0859 in normoxia and hypoxia by 40%. Fosb expression was reduced by SLC4A4 knockdown, 40% in normoxia and 30% in hypoxia. Fosb expression was reduced by SLC4A5 knockdown, 40% in normoxia and 15% in hypoxia. JunB was not expressed in normoxia or hypoxia in the control or S0859 treated cells however in SLC4A4 and SLC4A5 knockdown cells it was highly expressed. The expression of cJun was reduced upon treatment with S0859 in normoxia by 60% and hypoxia by 30%. Expression of cJun was almost completely ablated in normoxia and by 75% in hypoxia by SLC4A4 knockdown. Expression of cJUN was reduced by 75% in normoxia and hypoxia by SLC4A5 knockdown. Both pJUND and total JUND expression was altered by NDBT knockdown but not inhibition. Inhibition of NDBTs with S0859 had no effect on either total or phospho JunD. Total junD was reduced 25% in normoxia and hypoxia by SLC4A4 and 40% by SLC4A5 knockdown. Expression of phospho JunD was reduced by 50% by SLC4A4 knockdown and 45% by SLC4A5 knockdown. ATF4 expression

was unchanged by treatment with S0859 in normoxia or hypoxia. Knockdown of SLC4A4 reduced ATF4 expression by 80% in normoxia and hypoxia. Knockdown of SLC4A5 reduced ATF4 expression by 70% in normoxia and hypoxia. Expression of cFOS was unchanged upon treatment with S0859 in either normoxia or hypoxia. SLC4A4 knockdown reduced the expression of cFOS by 50% in normoxia but no change expression was seen in hypoxia. SLC4A5 knockdown reduced normoxic cFOS expression by 70% and by 20% in hypoxia.

Table 3 Hypoxia increases phospho-signalling in HCC-1806.

Target	Effect	Normoxia	Hypoxia
HSP60	N/A	0.0094	0.0109
WNK1 T60	Contentious	0.0086	0.0075
GSK-3a/b S21/S9	Inhibition	0.0041	0.0054
JNK 1/2/3 T183/Y185, T221/Y223	Activation	0.0038	0.0076
p53 S392	Stabilization	0.0035	0.0078
RSK1/2/3 S380/S386/S377	Activation	0.0030	0.0024
c-Jun S63	Activation	0.0028	0.0062
AKT1/2/3 T308	Activation	0.0027	0.0036
FAK Y397	Activation	0.0027	0.0062
p53 S46	Activation	0.0026	0.0031
PYK2 Y402	Activation	0.0022	0.0026
p53 S15	Activation	0.0022	0.0028
PRAS40 T246	Inhibition	0.0021	0.0044
STAT5a/b Y694/Y699	Activation	0.0019	0.0037
STAT3 Y705	Activation	0.0019	0.0022
STAT2 Y689	Dimerization	0.0018	0.0043
AMPKa2 T172	Activation	0.0017	0.0036
ERK1/2 T202/Y204 T185/Y187	Activation	0.0017	0.0038
p70 s6 kinase T389	Activation	0.0017	0.0023
MSK1/2 S376/S360	Activation	0.0016	0.0035
eNOS S1177	Activation	0.0015	0.0017
Src Y419	Activation	0.0014	0.0033
p70 s6 kinase T421/S424	Activation	0.0014	0.0021
PLC-γ1 Y783	Activation	0.0014	0.0018
STAT6 Y641	Activation	0.0012	0.0028
STAT3 S727	Activation	0.0012	0.0016
STAT5b Y699	Activation	0.0011	0.0024
Chk-2 T68	Activation	0.0010	0.0029
AMPKa1 T183	Activation	0.0010	0.0017
AKT1/2/3 S473	Activation	0.0010	0.0021
Yes1 Y426	Activation	0.0008	0.0022
b-catenin	N/A	0.0007	0.0014
TOR S2448	Activation	0.0007	0.0020
CREB S133	Activation	0.0006	0.0015
p27 T198	Activation	0.0006	0.0006
PDGF-Rb Y751	Activation	0.0005	0.0016
STAT5a Y694	Activation	0.0005	0.0009
Hck Y411	Activation	0.0005	0.0019
p38a T180/Y182	Activation	0.0004	0.0018
HSP27 S78/S82	Activation	0.0004	0.0015
EGF R Y1086	Contentios	0.0003	0.0011
Fgr Y412	Activation	0.0003	0.0001
Fyn Y420	Activation	0.0002	0.0008
Lyn Y397	Activation	0.0002	0.0015
Lck Y394	Activation	0.0001	0.0003

Currently the relationship between NDBT regulated pH control and signal transduction is not well-known. Thus, the phospho-kinase array assay was performed. Initially, the signalling changes induced by 48H hypoxia (0.5% O₂) were tested in HCC-1806. An overall trend of increased phosphorylation was seen, 38 out of 43 (88%) proteins were increased by at least 20%. (Two technical repeats)

Table 4 Acidosis causes an overall reduction in phosphorylation in HCC-1806.

Target	phosphorylation	Effect	shCTL NX	shCTL acid
GSK-3a/b	S21/S9	Inhibition	0.015	0.004
PRAS40	T246	Inhibition	0.012	0.005
JNK 1/2/3	T183/Y185, T221/Y223	Activation	0.012	0.004
STAT2	Y689	Dimerization	0.010	0.003
FAK	Y397	Activation	0.008	0.002
AMPKa2	T172	Activation	0.008	0.003
ERK1/2	T202/Y204, T185/Y187	Activation	0.008	0.001
STAT6	Y641	Activation	0.008	0.003
STAT5a/b	Y694/Y699	Activation	0.008	0.002
MSK1/2	S376/S360	Activation	0.008	0.003
src	Y419	Activation	0.006	0.002
WNK1	T60	Contentious	0.006	0.003
HCK	Y411	Activation	0.006	0.002
CHK-2	T68	Activation	0.006	0.002
yes	Y426	Activation	0.005	0.002
TOR	S2448	Activation	0.005	0.002
STAT5b	Y699	Activation	0.005	0.002
HSP60	X	N/A	0.004	0.003
AKT1/2/3	S473	Activation	0.004	0.001
lyn	Y397	Activation	0.004	0.001
AMPKa1	T183	Activation	0.004	0.001
CREB	S133	Activation	0.004	0.001
p53	S392	Stabilization	0.004	0.004
p38a	T180/Y182	Activation	0.003	0.001
HSP27	S78/S82	Activation	0.003	0.001
p53	S46	Activation	0.003	0.003
fyn	Y420	Activation	0.003	0.001
PDF Rb	Y751	Activation	0.003	0.001
B-catenin	X	N/A	0.003	0.001
STAT5a	Y694	Activation	0.003	0.001
EGF R	Y1086	Contentious	0.002	0.001
AKT1/2/3	T308	Activation	0.002	0.002
c-jun	S63	Activation	0.002	0.002
p70 s6 kinase	T421/S424	Activation	0.002	0.001
RSK1/2/3	S380/S386/S377	Activation	0.001	0.001
p53	S15	Activation	0.001	0.001
p70 s6 kinase	T389	Activation	0.001	0.001
lck	Y394	Activation	0.001	0.000
STAT3	Y705	Activation	0.001	0.001
PLC-γ1	Y783	Activation	0.001	0.001
PYK2	Y402	Activation	0.001	0.001
fgr	Y412	Activation	0.001	0.000
eNOS	S1177	Activation	0.001	0.001
STAT3	S727	Activation	0.001	0.001
p27	T198	Activation	0.000	0.000

In addition to the effect of acidosis alone was investigated related to phospho-signalling. HCC-1806 was exposed to either acidic (pH 6.4) or neutral (pH 7.4) in normoxia for 48H. In contrast to hypoxia, acidosis induced an overall reduction in phosphorylation. 32/43 proteins had at least 20% reduction in phosphorylation. The largest reductions were seen in: ERK1/2 (T202/Y204, T185/Y187) 83% reduction, fgr (Y412) 80%, AKT1/2/3 (S473) 19%, EGF-R (Y1086) 18%. One protein showed a significant increase was seen in phosphorylation in P27 (T198) 28%.

Table 5 Phosphorylation state is altered by NDBT knockdown and acidic conditions.

Target	phosphorylation	Effect	NEUTRAL				ACIDIC			
			shCTL	S0859	sh'4A4	sh'4A5	shCTL	S0859	sh'4A4	sh'4A5
GSK-3a/b	S21/S9	Inhibition	0.032	0.028	0.013	0.015	0.017	0.010	0.011	0.013
FAK	Y397	Activation	0.027	0.015	0.010	0.007	0.020	0.007	0.004	0.006
PRAS40	T246	Inhibition	0.025	0.021	0.014	0.016	0.022	0.011	0.010	0.010
HSP60	X	N/A	0.022	0.015	0.022	0.017	0.008	0.004	0.011	0.009
JNK 1/2/3	T183/Y185, T221/Y223	Activation	0.021	0.012	0.006	0.005	0.020	0.009	0.007	0.006
ERK1/2	T202/Y204, T185/Y187	Activation	0.020	0.029	0.018	0.020	0.005	0.004	0.010	0.004
p53	S392	Stabilization	0.017	0.007	0.001	0.001	0.004	0.002	0.002	0.002
STAT5a/b	Y694/Y699	Activation	0.017	0.011	0.005	0.005	0.015	0.007	0.004	0.004
src	Y419	Activation	0.017	0.007	0.004	0.004	0.011	0.006	0.005	0.005
WNK1	T60	Contentious	0.015	0.018	0.013	0.012	0.006	0.005	0.004	0.003
STAT6	Y641	Activation	0.012	0.007	0.003	0.004	0.010	0.001	0.003	0.004
AMPKa2	T172	Activation	0.012	0.007	0.004	0.003	0.010	0.004	0.003	0.003
TOR	S2448	Activation	0.012	0.006	0.005	0.005	0.009	0.004	0.004	0.005
HCK	Y411	Activation	0.011	0.005	0.004	0.005	0.008	0.004	0.003	0.004
STAT2	Y689	Dimerization	0.011	0.008	0.006	0.006	0.009	0.005	0.004	0.004
AKT1/2/3	S473	Activation	0.010	0.009	0.005	0.008	0.005	0.003	0.002	0.005
fyn	Y420	Activation	0.010	0.003	0.005	0.003	0.007	0.004	0.004	0.003
MSK1/2	S376/S360	Activation	0.010	0.006	0.005	0.004	0.010	0.005	0.005	0.005
yes	Y426	Activation	0.010	0.004	0.004	0.002	0.007	0.003	0.003	0.003
CHK-2	T68	Activation	0.010	0.005	0.005	0.004	0.006	0.003	0.003	0.004
p53	S15	Stabilization	0.009	0.004	0.002	0.002	0.004	0.001	0.001	0.001
p53	S46	Activation	0.009	0.004	0.002	0.003	0.004	0.002	0.002	0.002
AMPKa1	T183	Activation	0.009	0.003	0.001	0.001	0.004	0.002	0.001	0.001
STAT5b	Y699	Activation	0.009	0.005	0.003	0.004	0.007	0.002	0.003	0.003
c-jun	S63	Activation	0.009	0.005	0.004	0.003	0.003	0.002	0.002	0.001
lyn	Y397	Activation	0.009	0.004	0.003	0.002	0.006	0.003	0.001	0.003
B-catenin	X	N/A	0.008	0.004	0.009	0.013	0.006	0.003	0.002	0.005
p70 s6 kinase	T421/S424	Activation	0.007	0.003	0.003	0.001	0.003	0.005	0.001	0.001
STAT5a	Y694	Activation	0.007	0.004	0.004	0.004	0.005	0.003	0.002	0.004
AKT1/2/3	T308	Activation	0.007	0.003	0.002	0.002	0.003	0.001	0.002	0.001
p70 s6 kinase	T389	Activation	0.007	0.003	0.003	0.002	0.003	0.002	0.000	0.001
RSK1/2/3	S380/S386/S377	Activation	0.007	0.003	0.003	0.002	0.002	0.003	0.002	0.001
p38a	T180/Y182	Activation	0.007	0.002	0.003	0.002	0.008	0.004	0.004	0.003
CREB	S133	Activation	0.007	0.004	0.014	0.009	0.007	0.005	0.011	0.007
STAT3	Y705	Activation	0.007	0.003	0.002	0.002	0.003	0.001	0.001	0.001
HSP27	S78/S82	Activation	0.007	0.004	0.002	0.002	0.007	0.003	0.004	0.003
PLC-γ1	Y783	Activation	0.006	0.003	0.002	0.002	0.005	0.001	0.001	0.002
Ick	Y394	Activation	0.006	0.003	0.002	0.002	0.006	0.002	0.002	0.001
STAT3	S727	Activation	0.006	0.001	0.003	0.002	0.002	0.001	0.000	0.001
PYK2	Y402	Activation	0.005	0.002	0.002	0.002	0.002	0.001	0.001	0.001
EGF R	Y1086	contentious	0.005	0.002	0.002	0.001	0.003	0.002	0.001	0.002
eNOS	S1177	Activation	0.005	0.002	0.001	0.001	0.002	0.001	0.000	0.001
fgr	Y412	Activation	0.005	0.003	0.002	0.001	0.006	0.001	0.001	0.001
PDF Rb	Y751	Activation	0.004	0.002	0.001	0.001	0.003	0.002	0.001	0.001
p27	T198	Activation	0.003	0.001	0.000	0.001	0.001	0.001	0.000	0.001

To determine the perturbations in phospho-signalling by NDBT knockdown, the phospho signalling array was performed with the following conditions: shCTL, NDBT inhibition, NBCE-1 knockdown, NBCE-2 knockdown; in both neutral (pH 7.4) hypoxia (0.5% O₂, 0% CO₂) and acidic (pH 6.4) hypoxia in HCC-1806. Knockdown or inhibition of NDBT in hypoxia resulted in a near global reduction in phospho-signalling, resulting in levels of phosphorylation in the normoxic control. In hypoxic acidosis there was also a reduction in signalling due to NDBT knockdown or inhibition. This reduction was cumulative with acidosis resulting in much lower levels in phosphorylation than acidosis alone. The following phospho-signalling proteins were identified as hypoxic and pH sensitive to NDBT knockdown and are involved in migration and invasion: FAK (Y397) reduced 66%, Lyn

(Y397) reduced 75%, Lck (Y394) reduced 75%, JNK1/2/3 (T183/Y185) reduced 50%, Stat6 (Y641) reduced 70%, mTOR (S2448) reduced 50%.

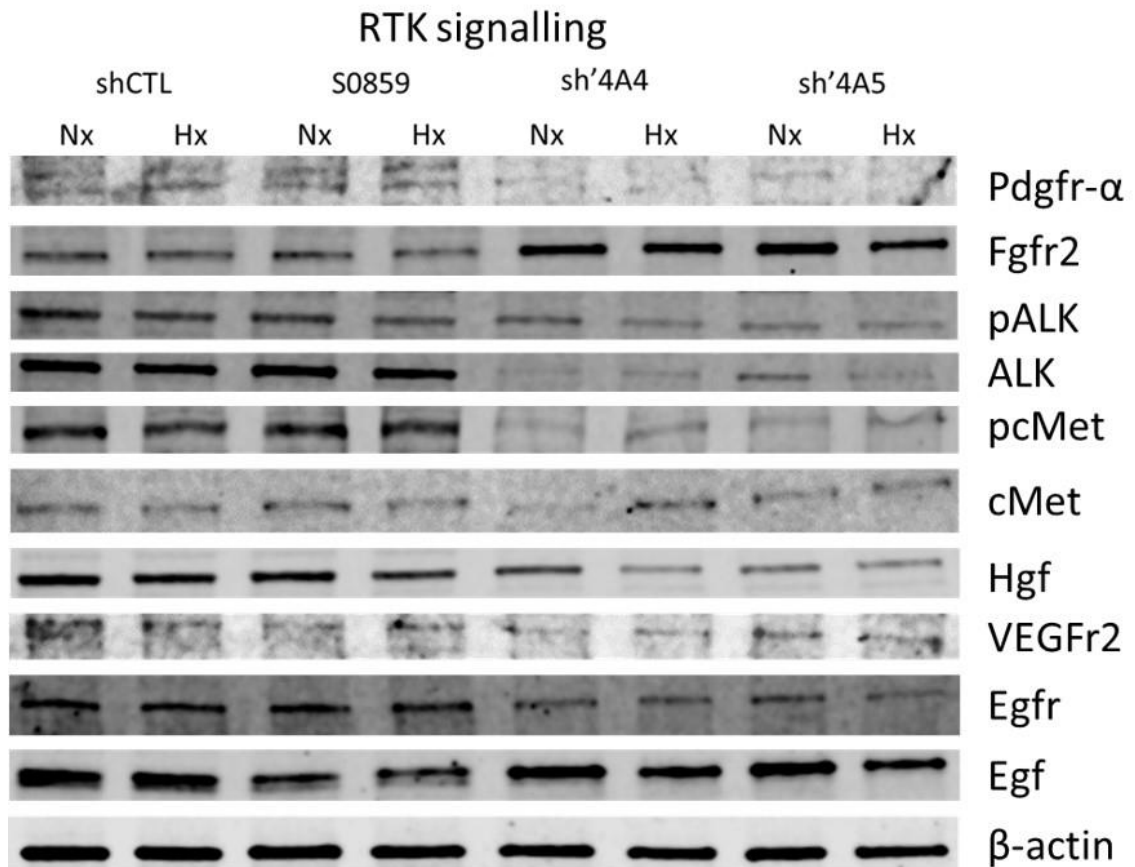


Figure 10.2 RTK expression is modulated by NDBT knockdown and inhibition.

Expression of Pdgfr-α, ALK, and pcMET were ablated by NDBT knockdown but not inhibition in both normoxia and hypoxia. Expression of pALK was unchanged by NDBT inhibition in normoxia and hypoxia, however knockdown of SLC4A4 reduced pALK expression by 80% in normoxic and hypoxic conditions whereas SLC4A5 knockdown reduced expression by 75%, and 85% in normoxia and hypoxia respectively. Knockdown of SLC4A4 reduced total cMet expression by 95% and 10% whereas SLC4A5 knockdown reduced expression by 60% and 40% in normoxia and hypoxia respectively. HGF expression was unchanged by NDBT inhibition. Knockdown of SLC4A4 reduced HGF expression by 30% and 70% in normoxic and hypoxic conditions; similarly SLC4A5 knockdown reduced HGF expression by 50% and 75%. EGFr expression was unchanged by NDBT inhibition. However, SLC4A4 and SLC4A5 knockdown reduced expression by 65% and 85% in normoxia and hypoxia respectively. Expression of EGF was unchanged in all conditions tests. Expression of FGFr2 was increased with NDBT knockdown. SLC4A4 knockdown increased expression 2 and 2.5 fold in normoxia and hypoxia. Whereas SLC4A5 knockdown increased expression 3 fold and 1.7 fold in normoxia and hypoxia. n = 3

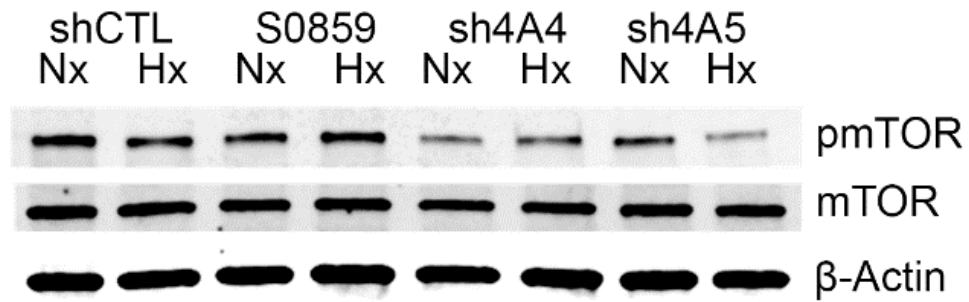


Figure 10.3 NDBT knockdown reduces mTOR activation in hypoxia, whereas total levels remained constant.

S0859 treatment increased activation by 30% in normoxia whereas activation was reduced 40% in hypoxia. Knockdown of SLC4A4 reduced activation by 35% in normoxia and 65% in hypoxia. Similarly SLC4A5 knockdown reduced mTOR activation by 40% in normoxia and 80% in hypoxia. SLC4A5 knockdown also reduced total mTOR expression in hypoxia by 30%. n = 3

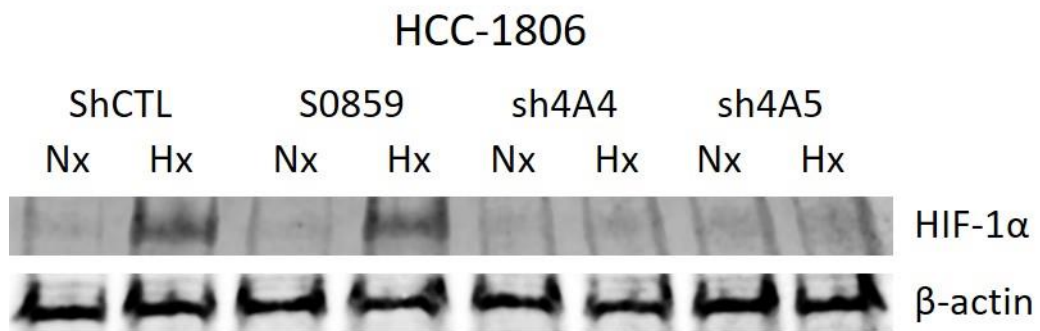


Figure 10.4 HIF-1α stabilization is ablated by NDBT knockdown.

Knockdown but not inhibition of NDBT reduced Hif-1α stabilization in hypoxia. No expression of HIF-1α was detected in normoxic conditions. No change in stabilization in hypoxia was seen with S0859 treatment. Knockdown of SLC4A4 or SLC4A5 resulted in no hypoxic Hif-1α stabilization. n = 3

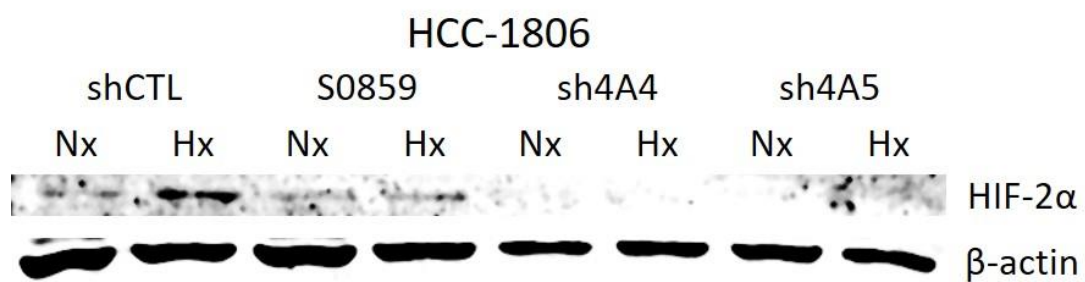


Figure 10.5 HIF2α is stabilization is ablated by NDBT knockdown.

Stabilization of Hif-2α occurs in hypoxia. In the control and drug treated samples HIF-2α is stabilized and expressed in hypoxia. However, NDBT knockdown ablated stabilization. n = 3

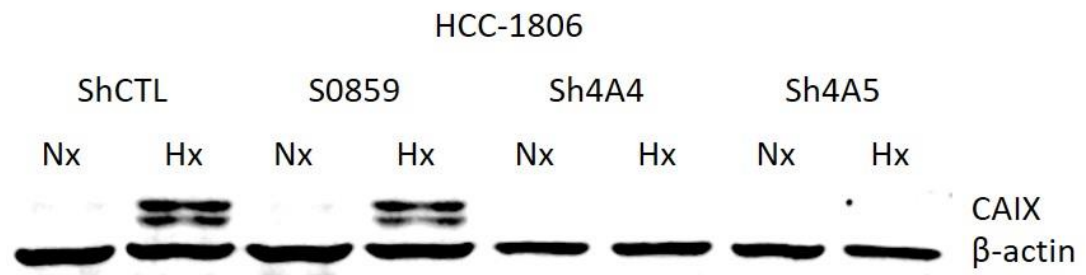


Figure 10.6 CAIX induction was reduced by NDBT inhibition and completely ablated by SLC4A4 or SLC4A5 knockdown. n = 3

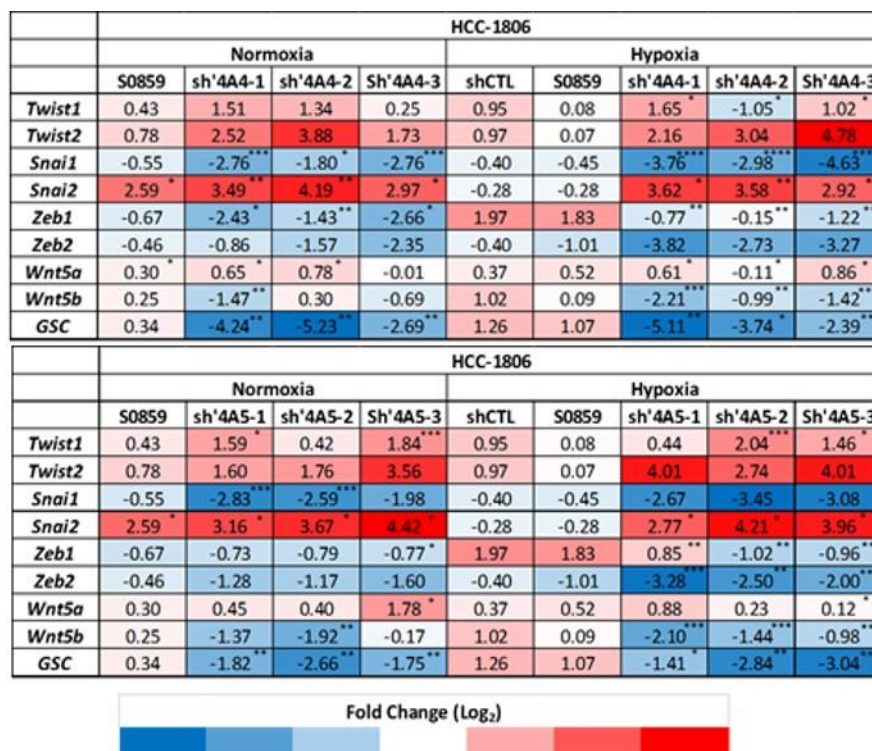


Figure 10.7 Knockdown and inhibition of NDBT altered the mRNA expression of many EMT transcription factors in HCC-1806.

Expression of *Twist1* was increased by SLC4A4 knockdown or inhibition in normoxia. However, in hypoxia expression of *Twist1* was reduced by S0859 and only increased in one SLC4A4 knockdown construct. *Twist2* expression was increased by SLC4A4 knockdown or NDBT inhibition in normoxia. *Twist2* mRNA in hypoxia was reduced by S0859 treatment but increased with SLC4A4 knockdown with all constructs. *Snai* expression was reduced by NDBT inhibition or SLC4A4 knockdown in both normoxia and hypoxia. *Snai2* expression was increased by NDBT inhibition and SLC4A4 knockdown in normoxia whereas in hypoxia NDBT inhibition or SLC4A4 knockdown reduced and increased expression respectively. *Zeb1* and *Zeb2* expression was reduced by NDBT inhibition or SLC4A4 knockdown in both normoxia and hypoxia. Expression of *WNT5a* was unchanged by NDBT inhibition or SLC4A4 knockdown in normoxia and hypoxia. Expression of *WNT5b* was unchanged by treatment with S0859 however, SLC4A4 reduced expression in normoxia and hypoxia. *GSC* expression was reduced in normoxia and hypoxia by SLC4A4 knockdown. However, S0859 treatment increased expression in normoxia but reduced expression in hypoxia. SLC4A5 knockdown increased the expression of *Twist1* and *Twist2* in normoxia and hypoxia. Expression of *Snai1*, *Zeb1*, and *Zeb2* were reduced by SLC4A5 knockdown in normoxia and hypoxia. Expression of *Slug* was increased by SLC4A5 knockdown in normoxia

and hypoxia. Wnt5a expression was increased by SLC4A5 knockdown in normoxia and hypoxia, whereas WNT5b expression was increased in normoxia and hypoxia. GSC expression was reduced in normoxia and hypoxia by SLC4A5 knockdown. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$

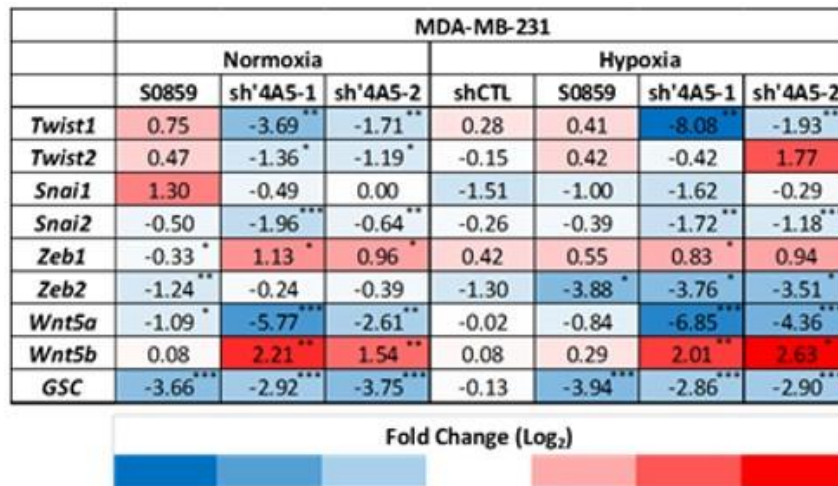


Figure 10.8 Knockdown and inhibition of NDBT altered mRNA expression of many EMT transcription factors in MDA-MB-231.

Expression of *Twist1* was increased by NDBT inhibition but reduced by SLC4A5 knockdown in normoxia and hypoxia. *Twist2* expression was increased by S0859 treatment in normoxia but not hypoxia. SLC4A5 knockdown reduced *Twist2* expression in normoxia. NDBT inhibition but not SLC4A5 knockdown increased and decreased *Snail* expression in normoxia and hypoxia respectively. *Slug*, *Zeb2*, *WNT5a*, and *GSC* expression was reduced by NDBT inhibition or SLC4A5 knockdown in normoxia and hypoxia. Expression of *ZEB1* was increased by NDBT inhibition or SLC4A5 knockdown in normoxia but not hypoxia. Expression of *WNT5b* was increased in normoxia and hypoxia by NDBT inhibition or SLC4A5 knockdown. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

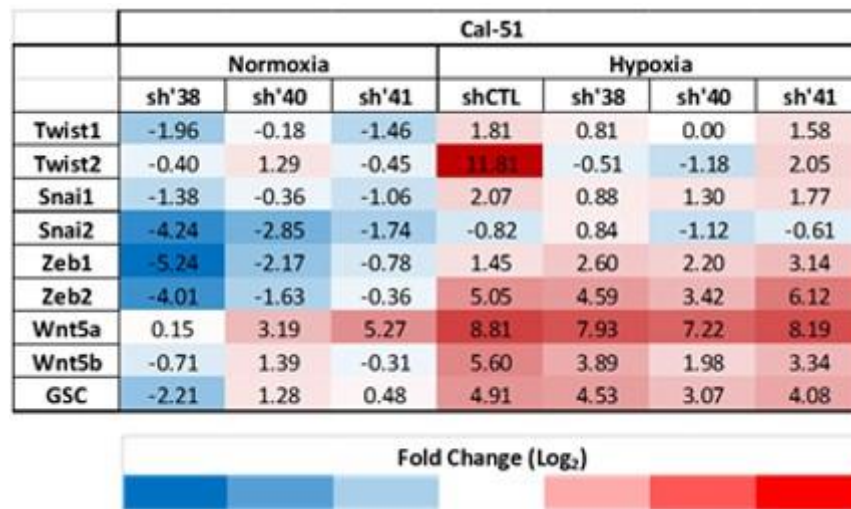


Figure 10.9 Knockdown and inhibition of NDBT altered mRNA expression of many EMT transcription factors in Cal-51.

In normoxia knockdown of SLC4A4 in CAL-51 reduces the expression of: Twist1, Twist2, Snail, Slug, Zeb1, Zeb2, WNT5a, and GSC. SLC4A4 knockdown in normoxia increases the expression of WNT5a. In hypoxia SLC4A4 knockdown reduced the induction of Twist1, Twist2, Snail1, WNT5b, and GSC. Expression of Zeb1 was increased in hypoxia by SLC4A4 knockdown. Expression of Snail2, Zeb2, WNT5b, and GSC were unchanged by SLC4A% knockdown in hypoxia. (P < 0.01). ***, P < 0.001; **, P < 0.01; *, P < 0.05; n = 3.

	Sum-159			
	Normoxia		Hypoxia	
	shCTL	shCTL +S0859	shCTL	shCTL +S0859
Twist1	0.00	0.44	2.13	2.21
Twist2	0.00	-0.99	1.92	-1.31
Snai1	0.00	0.33	2.03	2.31
Snai2	0.00	-0.47	1.82	1.58
Zeb1	0.00	-0.01	2.91	4.76
Zeb2	0.00	-0.12	3.84	3.35
Wnt5a	0.00	-0.59	2.04	0.79
Wnt5b	0.00	-0.32	1.40	0.76
GSC	0.00	-1.30	1.85	1.86

Figure 10.10 NDBT inhibition altered EMT transcription factor mRNA expression in normoxia and hypoxia.

Expression of Twist1 and Snail2 were increased by NDBT inhibition in normoxia. In normoxia expression of Twist2, Slug, Zeb2, WNT5a, WNT5b, and GSC were reduced. Expression of Zeb1 was unchanged. In hypoxia expression of Twist2, Slug, Zeb2, WNT5a, and WNT5b were reduced by S0859 treatment. Expression of Twist1, Snail, and GSC were unchanged by S0859 treatment in hypoxia. Zeb1 expression was increased by NDBT inhibition in hypoxia. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

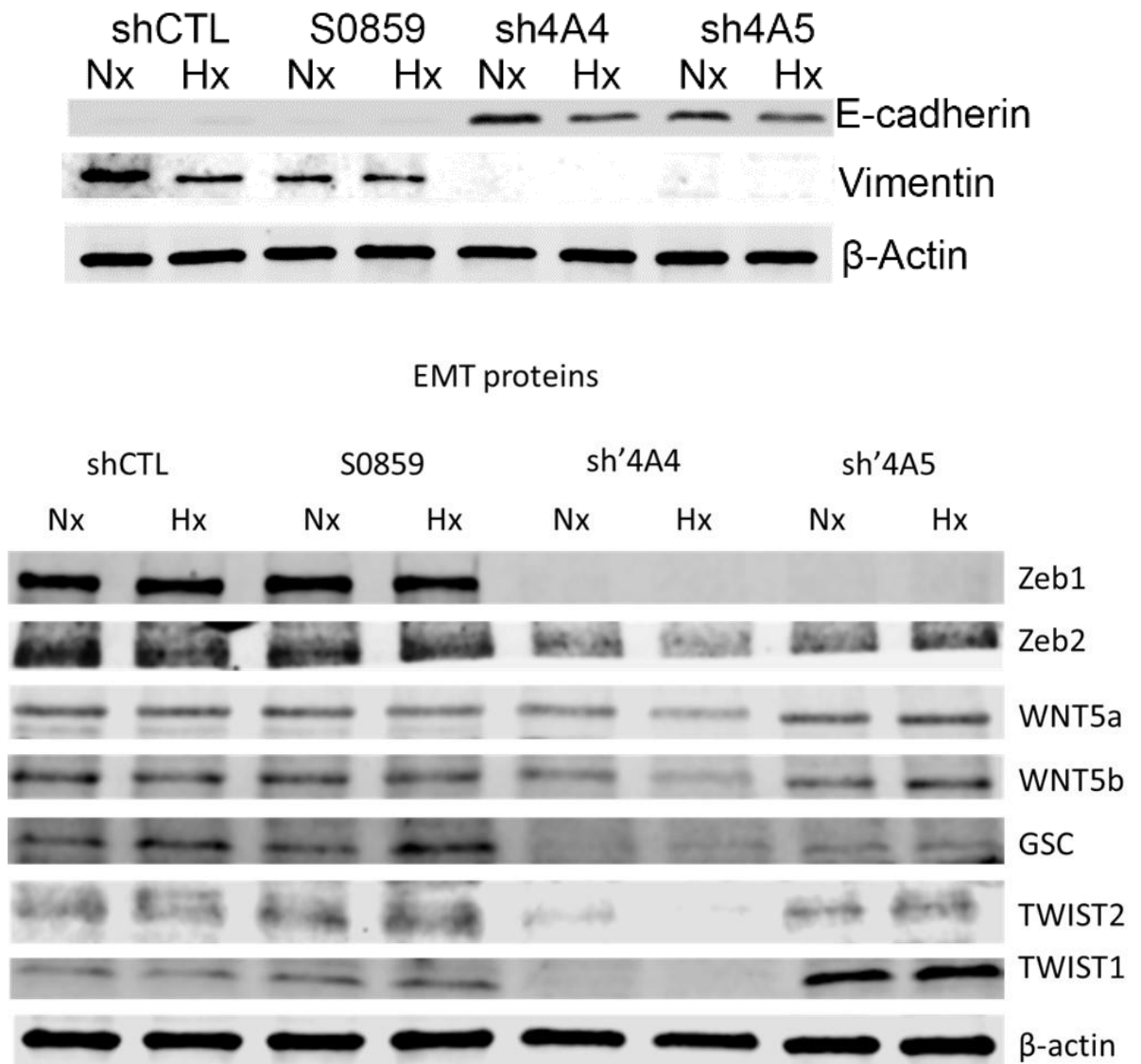


Figure 10.11 Knockdown of NDBTs alters expression of EMT related proteins in HCC-1806.

NDBT knockdown ablated expression of EMT marker Vimentin in normoxia and hypoxia. Additionally, expression of E-cadherin was increased by NDBT knockdown in normoxia and hypoxia. Inhibition of NDBTs by S0859 reduced expression of vimentin. NDBT inhibition did not alter the expression of EMT transcription factor expression in normoxia or hypoxia. Expression of Zeb1 was ablated by SLC4A4 and SLC4A5 knockdown in both normoxia and hypoxia. Expression of Zeb2 was reduced by NDBT knockdown in normoxia and hypoxia. Expression of ZNT5a and WNT5b was unchanged by NDBT knockdown in normoxia and hypoxia. NDBT knockdown reduced the expression of GSC in normoxia and hypoxia. Twist1 and Twist2 expression were reduced by SLC4A4 knockdown in normoxia and hypoxia but increased by SLC4A5 knockdown. n = 3

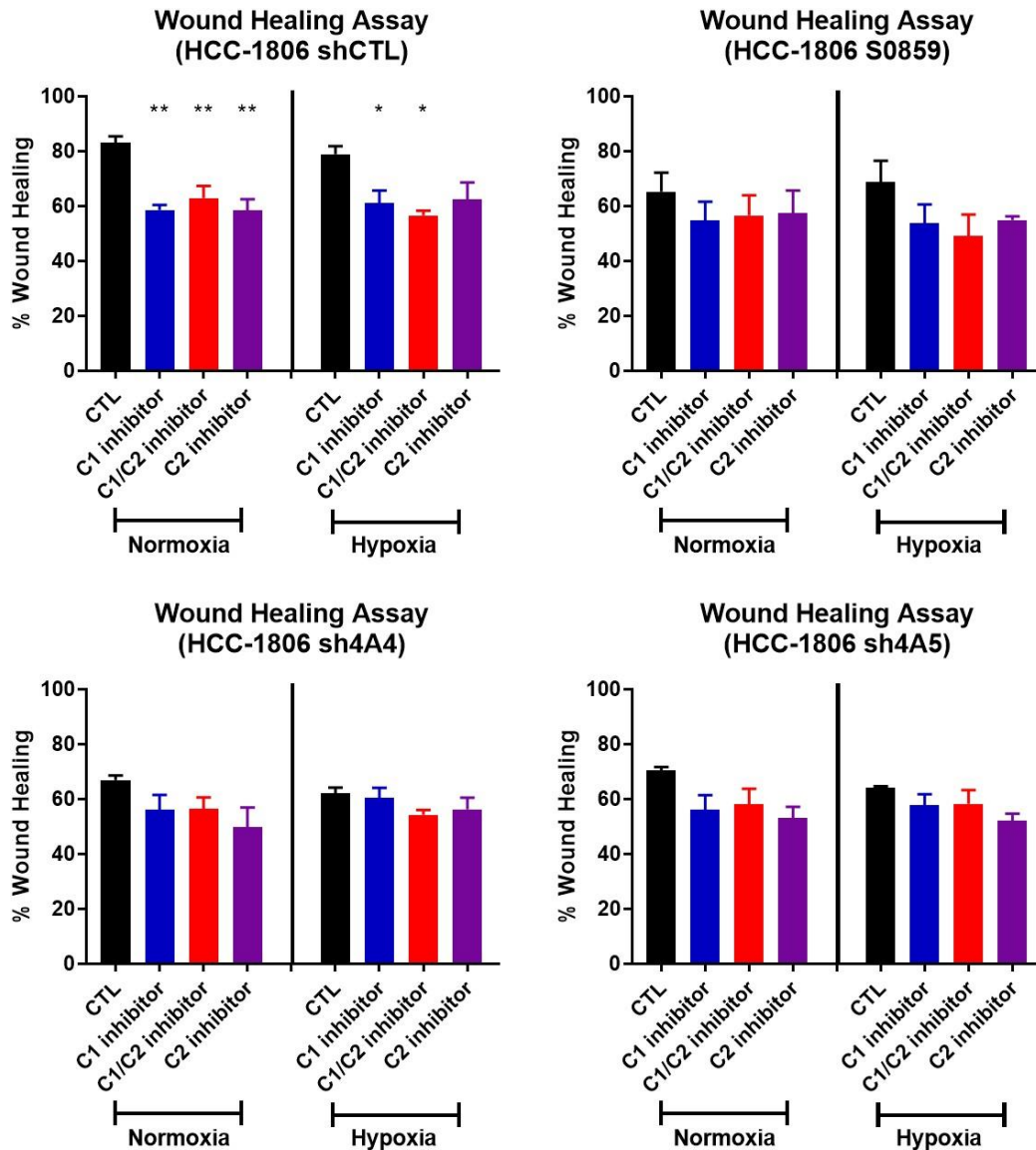


Figure 10.12 Wound healing was not reduced further by mTOR inhibition in combination by NDBT inhibition or knockdown in HCC-1806.

Inhibition of mTOR was undertaken by targeting each isoform separately and also using a pan inhibitor. Wound healing was reduced by 23% by C1 inhibition, 18% by C1/C2 inhibition and 20% by C2 inhibition in normoxia in the control. In hypoxia in the control wound healing was reduced 17% by C1, 22% by C1/2, and 15% by C2 inhibition. In S0859 treated cells mTOR inhibition reduced wound healing however this was not significant. In SLC4A4 and SLC4A5 knockdown cells, inhibition of mTOR did not reduce wound healing in normoxia or hypoxia. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

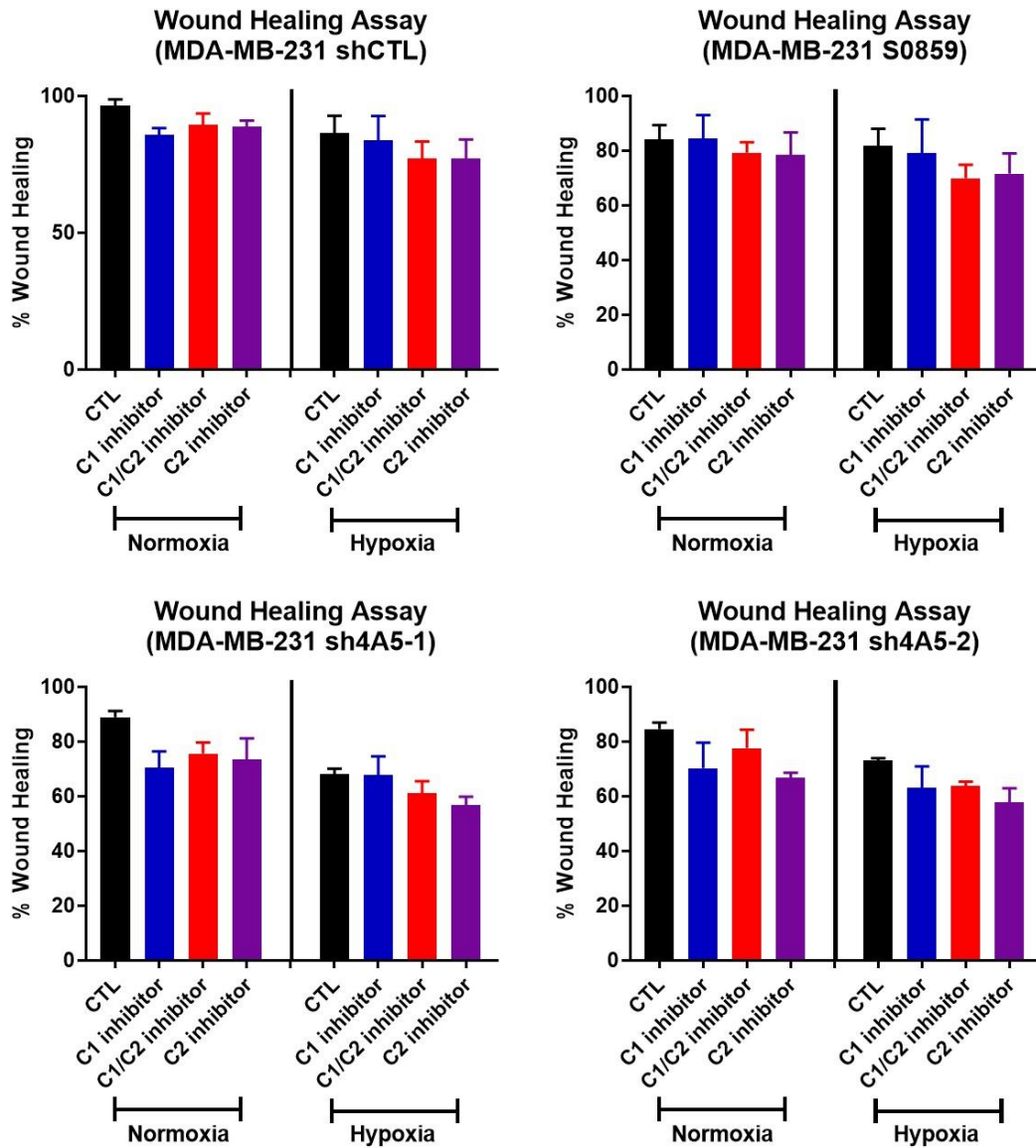


Figure 10.13 Wound healing was not further reduced by mTOR inhibition in combination with NDBT knockdown or inhibition. Furthermore, mTOR inhibition did not reduce migration in control cells. n = 3.

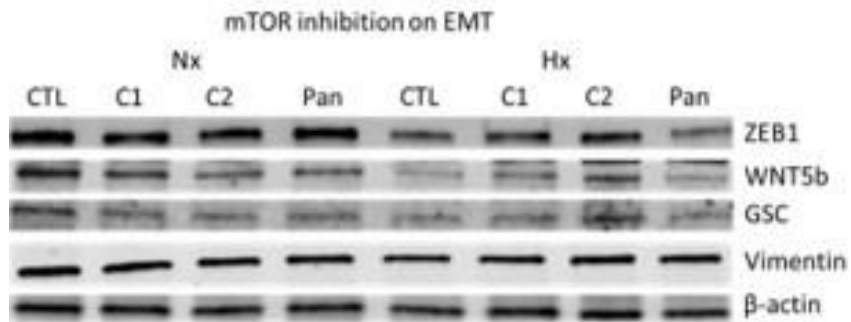


Figure 10.14 mTOR inhibition did not recapitulate effects of NDBT knockdown on HCC-1806 cells.

Expression of Zeb1 was unchanged in normoxia and increased in hypoxia by mTOR inhibition. Similarly expression of WNT5b was unchanged in normoxia but increased in hypoxia by mTOR inhibition. Expression of GSC was reduced in normoxia by mTOR inhibition but unchanged in hypoxia. Vimentin expression was unaltered by mTOR inhibition in normoxia and hypoxia. n = 3

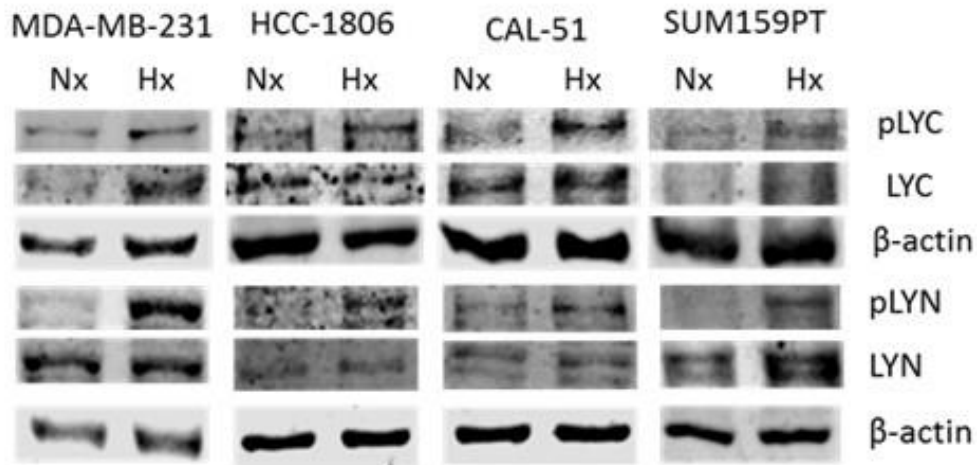


Figure 10.15 Hypoxia increases phosphorylation of LYC and LYN kinases in hypoxia in multiple cell lines.

Expression of pLYC is increased 2 fold in MDA-MB-231, 3 fold in HCC-1806, 4 fold in Cal-51, and 3 fold in SUM-159pt in hypoxia. Total LYC expression was increased 4 fold in MDA-MB-231, unchanged in HCC-1806, increased 30% in Cal-51, and increased 3 fold in SUM-159PT in hypoxia. Expression of pLYN was increased 6 fold in MDA-MB-231, 3 fold in HCC-1806, 2 fold in CAL-51, and 2 fold in SUM-159ot in hypoxia. Total LYN expression was unchanged in MDA-MB-231, HCC-1806, Cal-51, and increased 2 fold in SUM-159PT in hypoxia. n = 3

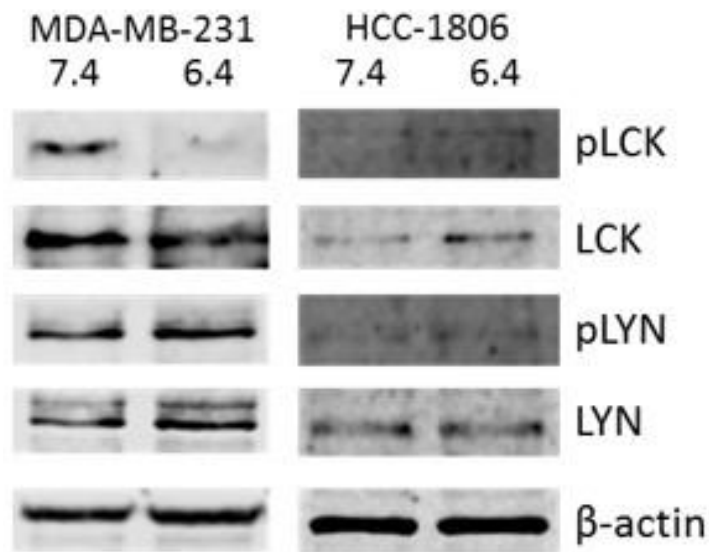


Figure 10.16 Acidosis reduces phosphorylation of LCK but not LYN in MDA-MB-231 but maintained phosphorylation of LCK and LYN in HCC-1806.

Expression of pLCK was ablated in MDA-MB-231 in acidosis but total LCK was unchanged. In HCC-1806 pLCK levels were unchanged by total levels increased in acidosis. Expression of active and total LYN was unchanged by acidosis in MDA-MB-231 and HCC-1806. n = 3

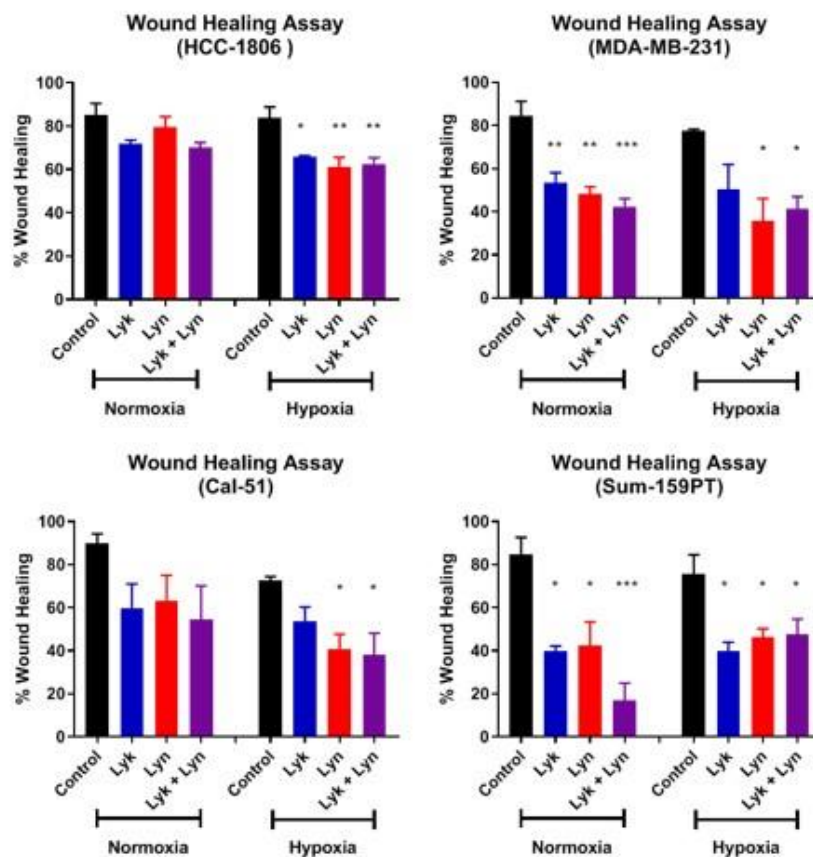


Figure 10.17 Inhibition of LCK or LYN reduced migration and invasion in multiple cell lines in normoxia and hypoxia.

LYC or LYN inhibition did not reduce migration in HCC-1806 in normoxia however, in hypoxia LYC inhibition reduced migration by 20%, LYN by 23% and LYCLYN by 22%. Migration in MDA-MB-231 was reduced 25% and 20% by LYC inhibition, 28% and 30% by LYN inhibition, and 29% and 25% by LYCLYN inhibition in normoxia and hypoxia for each condition respectively. In Cal-51 migration was reduced by 28%, 22%, and 32% in normoxia by LYC, LYN, and LYCLYN inhibition. In hypoxia migration was reduced by 15%, 22% and 24% by LYC, LYN and LYCLYN inhibition in Cal-51. Migration was reduced 40%, 38%, and 65% by LYC, LYN, and LYCLYN inhibition in normoxia. In hypoxia migration was reduced by 30%, 28%, and 25% in SUM-159pt.. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$

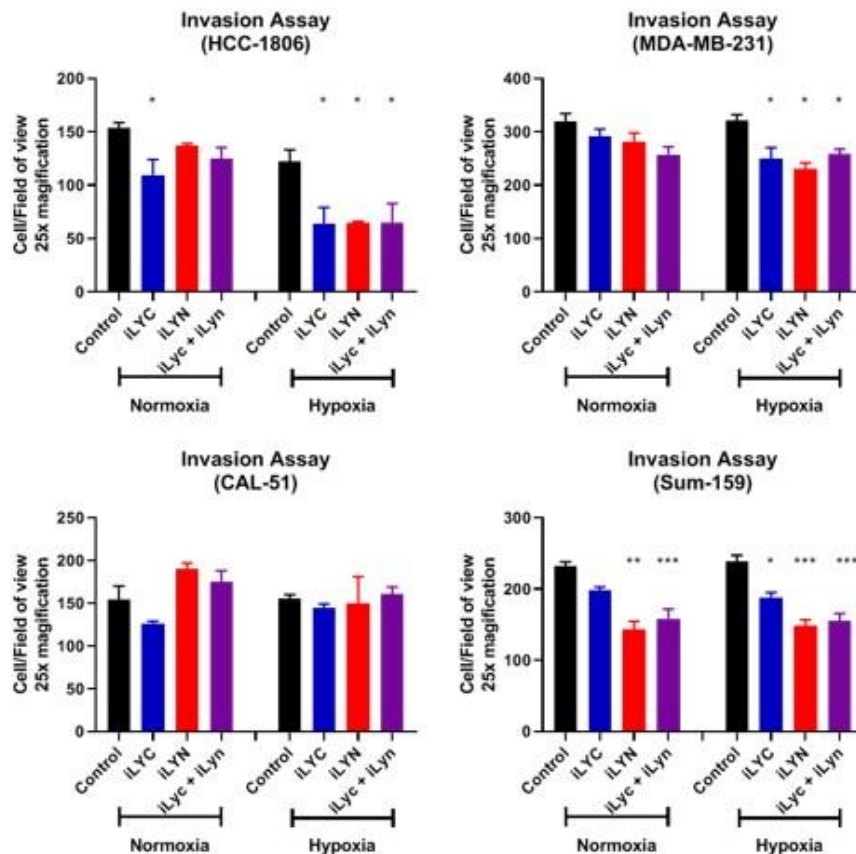


Figure 10.18 Inhibition of LCK or LYN reduces invasion in multiple cell lines in normoxia and hypoxia.

Invasion is reduced LYC inhibition in normoxia by 33% and 50% in hypoxia in HCC-1806. In hypoxia invasion is reduced by 50% by LYN inhibition and 50% by LYCLYN inhibition in HCC-1806. LYC, LYN, or LYCLYN inhibition did not reduce invasion in MDA-MB-231 cells in normoxia. In hypoxia invasion was reduced 16%, 18%, and 15% by LYC, LYN, and LYCLYN inhibition in MDA-MB-231 cells. In Cal51 LYN inhibition reduced migration by 15%, whereas LYN and LYCLYN inhibition increased migration by 20% and 18% in normoxia respectively. Inhibition did not alter invasion in Cal51 in hypoxia. In sum-159pt invasion was reduced 12% by LYC inhibition in normoxia, and 17% in hypoxia. LYN inhibition reduced invasion by 21% in normoxia and 22% in hypoxia. Whereas LYCLYN inhibition reduced invasion by 19%, and 21% in normoxia and hypoxia respectively in Sum159pt. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

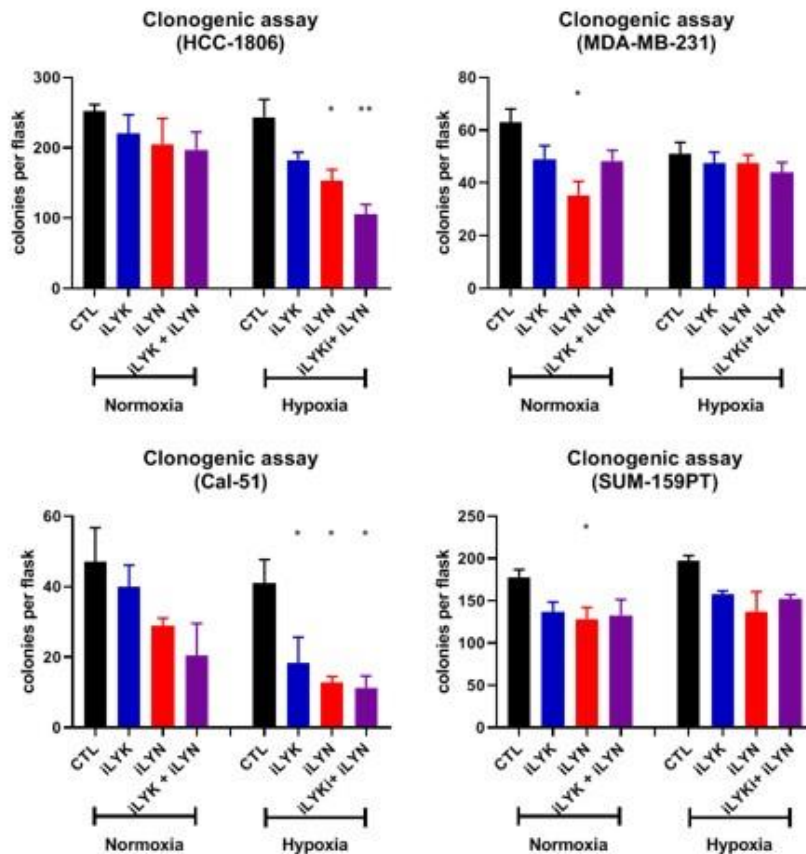


Figure 10.19 Inhibition of LYC and LYN kinases reduced survival in multiple cell lines in normoxia and hypoxia.

Survival was reduced 16%, 17% and 18% by LYC, LYN , and LYCLYN inhibition in normoxia in HCC-1806. In hypoxia survival was reduced by 23%, 30%, and 58% in HCC-1806 by LYC, LYN, and LYCLYN inhibition. In MDA-MB-231 survival was reduced 10%, 23%, and 10% by LYC, LYN, or LYCLYN inhibition in normoxia. Survival was not altered in hypoxia by inhibition in MDA-MB-231. LYN inhibition reduced survival by 7% and 50% in normoxia and hypoxia in Cal51. LYN inhibition reduced survival 35% and 64% in normoxia and hypoxia in Cal-51. LYCLYN inhibition reduced survival by 50% in normoxia and 68% in hypoxia in Cal-51. Survival was reduced by 15%, 17%, and 16% by LYC, LYN, or LYCLYN inhibition respectively in normoxia in Sum159pt. In hypoxia survival was reduced 15%, 19%, and 16% by LYC, LYN, or LYCLYN inhibition in Sum159pt. ($P < 0.01$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

HCC-1806

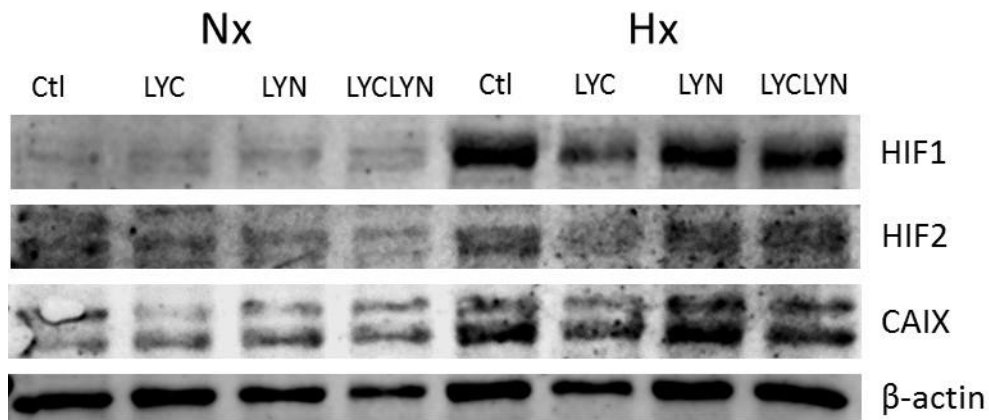


Figure 10.20 Stabilization of HIF-1 α or HIF-2 α and activation of downstream signalling is not altered by LCK or LYN inhibition separately or in combination.

Hypoxic stabilization of HIF-1 α occurred to the same levels in hypoxia despite inhibition of LYC or LYN. Similarly expression of HIF-2 α was stabilized to the same extent regardless of LYC or LYN inhibition in hypoxia. Downstream HIF-1 α signalling was unchanged by LYC or LYN inhibition in hypoxia as expression of CAIX was unchanged. n = 1

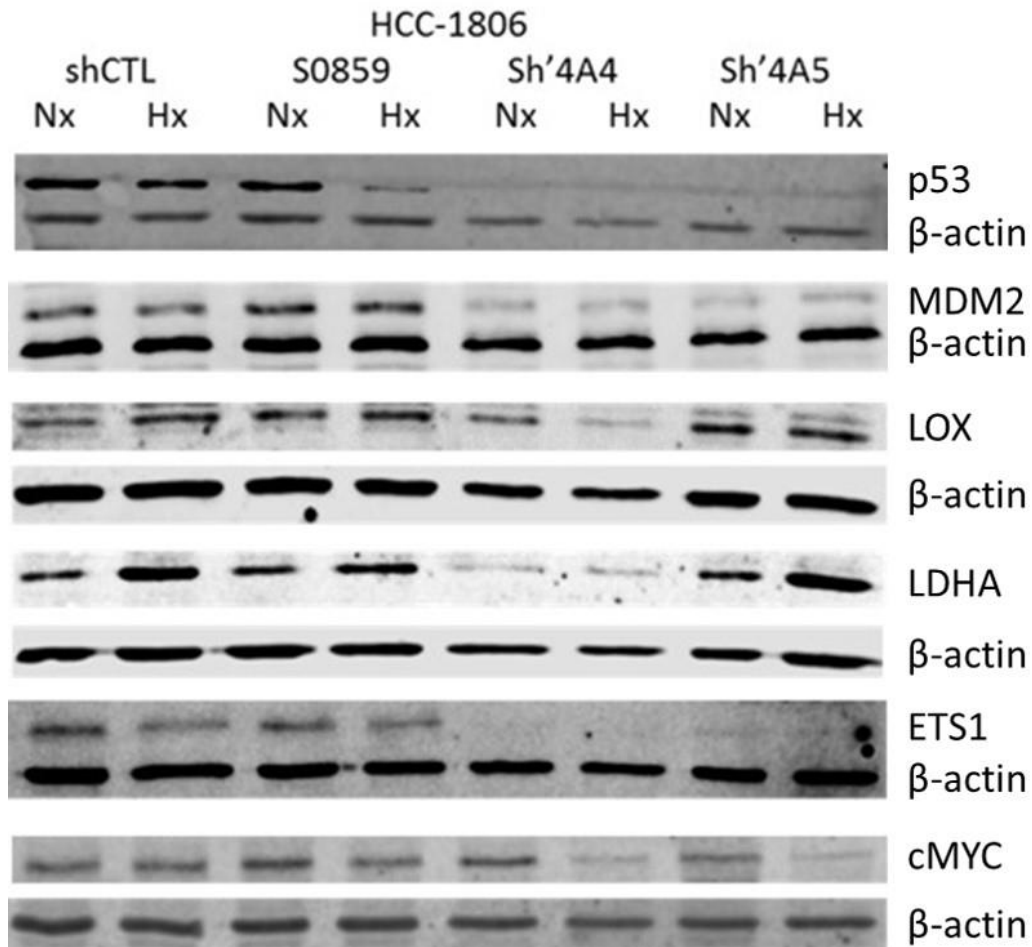


Figure 10.21 NDBT inhibition and knockdown alters intracellular signalling.

Expression of p53 was unchanged in normoxia but reduced by 60% in hypoxia by NDBT inhibition. Knockdown of SLC4A4 or SLC4A5 ablated the expression of p53 in normoxia and hypoxia. Expression of MDM2 was increased by NDBT inhibition by 40% in normoxia and 50% in hypoxia whereas NDBT knockdown reduced MDM2 expression 75% and 70% in normoxia and hypoxia for SLC4A4 knockdown and 85% and 60% for SLC4A5 knockdown. LOX and LDHA expression were unchanged by NDBT knockdown or inhibition in normoxia and hypoxia. NDBT inhibition did not alter ETS1 expression in normoxia or hypoxia. However, SLC4A4 and SLC4A5 knockdown ablated ETS1 expression in normoxia and hypoxia. Expression of cMYC was increased by S0859 treatment in normoxia by 60% but unchanged in hypoxia. Knockdown of SLC4A4 or SLC4A5 did not alter cMYC expression in normoxia but reduced expression by 70% and 65% in hypoxia respectively. n = 3

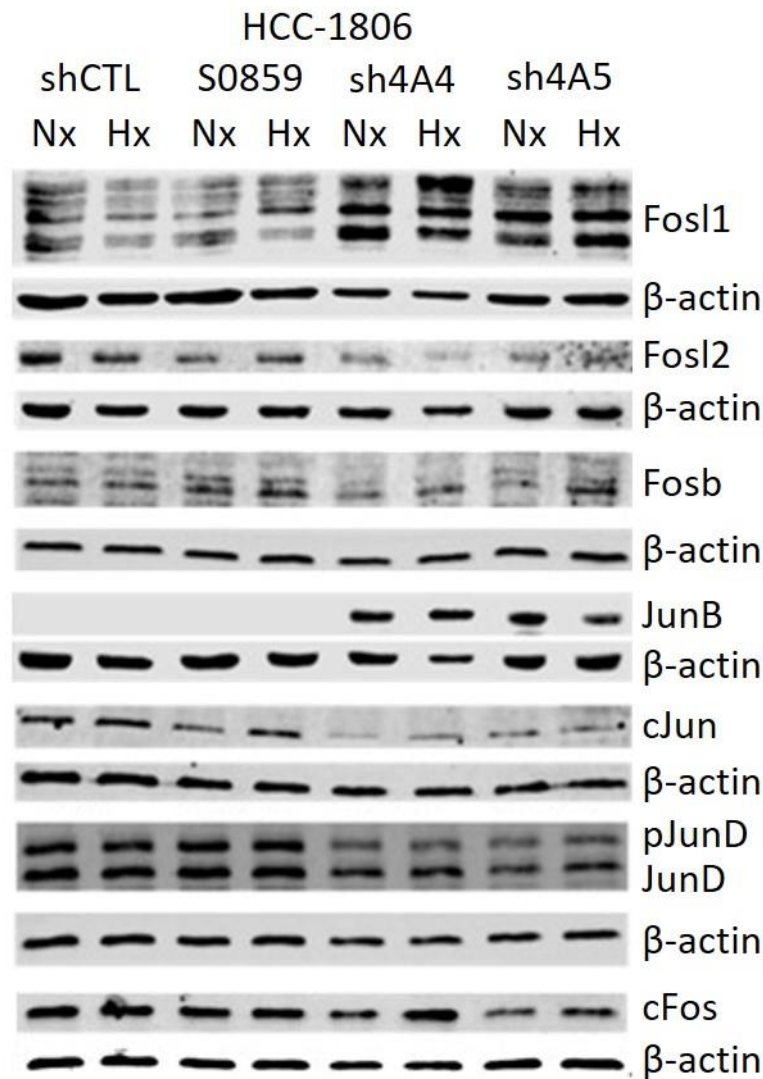


Figure 10.22 NDBT knockdown alters the expression pattern of AP1 subunits in normoxia and hypoxia in HCC-1806.

NDBT inhibition did not expression patterns of AP1 subunits in normoxia or hypoxia. Fosl1 expression was increased by SLC4A4 and SLC4A5 in normoxia and hypoxia by 3 fold, 3.5 fold, 3 fold and 3.2 fold respectively. Fosl2 expression was reduced by 70% and 90% by SLC4A4 knockdown in normoxia and hypoxia. SLC4A5 knockdown reduced Fosl2 expression by 50% and 70% in normoxia and hypoxia. Fosb expression was reduced by 80% and 95% by SLC4A4 knockdown in normoxia and hypoxia respectively. Similarly expression of Fosb was reduced by SLC4A5 knockdown by 70% in normoxia and 20% in hypoxia. Expression of JunB was induced in normoxia and hypoxia by NDBT knockdown compared to zero expression in the control. Expression of cJun was reduced by 90% in normoxia and 75% in hypoxia by SLC4A4 knockdown. SLC4A5 knockdown reduced the expression of cJun by 50%

and 60% in normoxia and hypoxia respectively. Total JunD expression was reduced by 50% by NDBT knockdown in normoxia and hypoxia. Furthermore pJunD expression was reduced by 55% by NDBT knockdown in normoxia and hypoxia. Expression of cFOS was reduced by 60% by SLC4A4 knockdown and 70% by SLC4A5 knockdown in normoxia but expression levels were unchanged by NDBT knockdown in hypoxia. n = 3

10.4 Discussion:

The results in this chapter answer the hypothesis “Knockdown or inhibition of NDBTs will reduce intracellular phospho-signalling” in triple negative breast cancer cells. However, further studies will be needed to fully determine the pathways involved in each of the conditions tested. Overall phospho-signalling was increased in hypoxia and reduced in acidosis. Combining hypoxia and acidosis reduced phospho-signalling. NDBT knockdown reduced phospho-signalling in hypoxia and further in hypoxia and acidosis. Activation of mTOR and subsequently HIF stabilization was reduced resulting no induction of CAIX in hypoxia. Moreover, the lack of HIF signalling resulted in a lack of EMT induction; E-cadherin was increased, vimentin and Zeb1 expression was ablated by NDBT knockdown. Combining mTOR inhibition in combination with NDBT knockdown recapitulated some effects but not completely indicating other intracellular kinases were involved. Investigations into LYC and LYN signalling showed that migration, invasion, survival but not proliferation were reduced by inhibition. Further to this NDBT knockdown was investigated with regards to p53, cMYC, and AP1 signalling. Expression of p53 was totally ablated in normoxia and hypoxia whereas cMYC expression was only reduced in hypoxia. The expression of AP1 subunits was altered by NDBT knockdown.

Intracellular signalling in hypoxia resulted in an overall increase in phospho-signalling. Many of the downstream transcriptional changes in hypoxia occur due to the stabilization of the HIF transcription factors¹⁴⁴. It has been shown that under hypoxic conditions many proteins are activated that are involved in survival, migration, and invasion⁶⁰⁷. FAK has been shown to be activated in hypoxia, as hypoxic cells are more invasive and migratory activation of this protein was expected^{284,608}. Additionally, FAK is under the control of HIF signalling via RhoA-ROCK1 expression⁶⁰⁹. FAK localises to cellular adhesions, whereby it signals for focal adhesion assembly and disassembly resulting in directed migration⁶¹¹. Moreover, mTOR signalling is required for HIF stabilization. In hypoxia I have shown that PRAS40 phosphorylation is increased. The phosphorylation of PRAS40 by AKT signalling at T246 relieves inhibition of mTORc1 signalling⁶¹⁰. It has been shown previously that STAT5a activation is increased in hypoxia in breast cancer cells⁶¹¹. This increased activation increases its binding and transcriptional activity⁶¹¹. ERK phosphorylation has also been shown to be upregulated in hypoxia⁶¹². ERK and AKT phosphorylation is required for HIF signalling *in-vitro* and *in-vivo*⁶¹³.

I have shown that acidosis results in an overall downregulation of signalling after 48h. Investigations into the signalling effects of acidosis focused primarily on extracellular acidosis²¹⁵. It has been shown that extracellular acidosis induces ERK signalling via increased ROS. It has been shown that ERK upregulation is dependent on p38 signalling. However, after 2h activation was reduced to below neutral condition levels⁶¹⁴. Activation of ERK was also shown to be dependent on ASIC1 and resulted in NFκβ signalling in response to acidosis⁵⁸². CREB is upregulated downstream of p38 signalling and is dependent on ROS signalling⁵⁰⁰. CREB signalling is sustained upon return to neutral pH after acidic shock²¹⁵. Investigations into intracellular acidosis have shown that mTORc1 signalling is reduced²⁷⁷. This reduction is sensed by the TSC2 complex and knockdown of TSC2 results in no reduction in mTOR signalling in acidosis²⁷⁷. Furthermore, LAMP2 is upregulated in acidosis and is localised to lysosomes; loss of LAMP2 in acidosis is lethal²⁷⁷. I have shown that CREB signalling is maintained in acidosis and with NDBT knockdown indicating an important role in acidosis signalling. Recently, investigations into a acidosis signature have been undertaken⁴⁹⁹. It was shown that lactic acidosis induced more genes than hypoxia with lactic acidosis favouring genes responsible for aerobic respiration and hypoxia favouring glycolysis⁴⁹⁹. G-protein coupled receptor signalling and cellular catabolism were the most highly enriched pathways in lactic acidosis⁴⁹⁹. When lactic acidosis and hypoxia were combined, transcription factors ATF3, YY1, CPEB2/3, SAP18, SREBF2 were upregulated whereas pro-apoptotic genes were downregulated⁴⁹⁹. It was subsequently shown that acidosis induced more genes than lactic acidosis with most genes in the acidosis signature not altered in lactic acidosis⁴⁹⁹. RNA seq analysis on MDA-MB-231 in neutral or acidic condition has shown large changes in genes associated with RNA processing and RNA splicing⁶¹⁵. RNA binding proteins had an enrichment of AU-rich motifs in transcripts that were upregulated in low pH⁶¹⁵. It was shown that these events were independent of HIF in hypoxia⁶¹⁵.

I have shown that many RTKs are reduced by NDBT knockdown. I have shown that FGFr2 expression is increased by NDBT knockdown. FGFr2 downregulation is associated with poor prognosis⁶¹⁶. Nuclear localised FGFr2 negatively regulates HIF1/2 cell invasion. FGFr2 blocks the binding of p300 to HIF-1α by binding to the transactivation domain of HIF-1α⁶¹⁶. It has been shown that the expression of VEGFr2 is reduced in acidosis and that stimulation with VEGF does not induce canonical AKT signalling in acidosis compared to neutral conditions⁶¹⁷. Furthermore, anti-angiogenic therapies failed to halt proliferation in acidic conditions⁶¹⁸. Expression of PDGFR is linked to aggressiveness and the triple negative

subtype⁶¹⁹. Both SLC4A4 and SLC4A5 knockdowns ablated expression of PDGFr alpha in both normoxia and hypoxia. PDGF-D expression has been linked to increased enhanced HIF-1 α activity, CAIX induction, extracellular acidosis, and matriptase shedding in prostate cancer thus making the cells more aggressive⁶²⁰. High levels of PDGFRa activation have been linked to shorter metastasis free survival⁶²¹. Expression of total ALK and pALK were reduced by NDBT knockdown compared to control. ALK expression has been linked to poor prognosis and this increase is due to copy number polysomy of chromosome 2 or gene amplifications⁶²². Ablation of total and active ALK indicates that the cells may be less aggressive. The RTK cMET is the putative metastasis RTK⁶²³. High expression of cMET has poor clinopathological and prognostic significance in breast cancer⁶²⁴. HGF signalling via cMET has been shown to regulate proliferation, migration, and metastasis⁶²⁵.

Previous studies into the knockdown of SLC4A4 indicated that the resulting phenotype was unable to stabilize HIF-1A in hypoxic conditions^{454,626}. Knockdown of mTOR has been shown to ablate the stabilization of HIF in hypoxia, thus it was hypothesised that this was a potential mechanistic cause⁴⁵⁸. Thus, the intracellular acidosis induced via NDBT knockdown inhibited the activation of mTOR and ablated HIF stabilization in hypoxia. Inhibition of NDBTs via addition of S0859 had no effect on the expression of total mTOR in both normoxic and hypoxic conditions. As mTOR activation was not inhibited by S0859 treatment no effect was seen on HIF stabilization.

EMT has been shown to be induced by hypoxia in breast cancer. Relative abundance of EMT transcription factor mRNA was increased in hypoxia for many of the genes in each cell lines in line with HIF-1A/HIF2 regulating their expression^{321,388,627,628}. In HCC-1806 mRNA patterns mirrored the protein expression. The largest effects seen were with Vimentin and E-cadherin, well known markers of EMT^{66,629}. ZEB1 was the most severely affected EMT transcription factor by both *SLC4A4* and *SLC4A5* knockdown. ZEB1 has been shown to be sensitive to extracellular acidosis in AT1 cells²⁶⁷. The Expression of WNT5a and WNT5b were less altered than the others. This may be due to β -catenin and the WNT pathway being less sensitive to extracellular acidosis. Levels of β -catenin were unchanged in the signalling array in acidosis. Expression of goosecoid was ablated by knockdown. Interestingly expression of GSC is increased in hypoxia, indicating possible HIF transcriptional regulation. Overall, knockdown of NDBT is reducing the expression of a large number of EMT markers and thus this is a likely mechanism by which migration and invasion were reduced by NDBT knockdown.

Little data is available on LYN in the literature with regards to LYN and EMT in cancer. LYN knockdown was shown to not alter the colony forming effects in prostate cancer cells⁶³⁰. In Ewing's sarcoma targeting LYN reduces metastasis⁶³¹. Higher Lyn expression has been linked to highly aggressive TNBC. In addition, Lyn expression was linked to reduced patient survival⁵⁹². Hypoxia was shown to increase levels of pLYC in both MDA-MB-231 and MCF-7 cell lines. Subsequently it was shown that Lck phosphorylates I κ B α in both MCF-7 and MDA-231 cells. However, LYC and FYN were shown not to be able to. The authors propose that Lck-mediated NF κ B activation through tyrosine phosphorylation of I κ B α subsequently stimulates uPA secretion, ultimately controlling the motility of breast cancer cells⁶³². Lyc was shown to be required for MEK-1-dependent ERK1/2 phosphorylation and uPA secretion through tyrosine phosphorylation of EGF receptor. Upon reoxygenation from hypoxia inhibition of LYC resulted in reduced wound healing⁶³³. My data suggests that hypoxia alone infers sensitivity to LCK inhibition. Another group has shown that Lck is required for the acute response to hypoxia of human T lymphocytes as it confers O₂ sensitivity on Kv 1.3 channels. These channels are important regulators of the T cell activation complex⁶³⁴. Lck has been shown to be a binding partner of SQSTM1 and is regulated by redox signalling⁶³⁵.

EMT regulation has been shown to be important in breast cancer metastasis⁶³⁶. However, the process is regulated by a plethora of transcription factors and most carcinomas undergo only partial EMT⁶³⁷. Signalling by mTOR is well known to control EMT. LYN has been shown to be implicated in breast cancer. LYN regulates proliferation and survival. LYN was shown to have an inverse relationship with E-cadherin expression and knockdown of Lyn by siRNA increased E-cadherin mRNA and protein levels in breast and bladder cancer cell lines. Furthermore knockdown of LYN reduced expression of vimentin and fibronectin in multiple cell line. They showed that LYN regulates the expression of E-cadherin via activation of the Vav1-Rac1-PAK1 resulting in the translocation of slug and snail to the nucleus⁶³⁸. Activation of mTOR is known to facilitate cell migration and invasion via the downstream activation of S6k1 and 4E-BP1^{639,640}. Thus our data in HCC-1806 was in concordance with the literature in this regard. Knockdown of NDBTs resulted in a reduction in mTOR activation and subsequently reduced migration. The data on MDA-MB-231 indicates that an alternative pathway may also be involved. Also others have shown that MDA-MB-231 is resistant to the mTOR inhibitor CCI-779⁶⁴¹. Furthermore investigations into the use of mTOR inhibitors showed that MDA-MB-231 were resistant to all the PI3K pathway inhibitors⁶⁴². Our data indicates that intracellular acidification sensitises MDA-MB-

231 to mTOR inhibition. Due to the large volume of changes induced by intracellular acidosis and that only HCC-1806 was used on the array it is difficult to determine a mechanism. However, overexpression of S6K1 and expression of phosphorylated Akt were shown to be predictors of sensitivity to treatment. Furthermore total expression of mTOR, p70S6K, AKT, and ERK1/2 were predictive of sensitivity in some cancers. The complex feedback loops which the mTOR pathway is connected to makes determination of a mechanism difficult⁶⁴³.

Hypoxia commonly induces p53 protein expression in an oxygen tension and time dependant manner⁶⁴⁴. Hypoxia results in the stabilization of p53, however, HIF-1 α signalling alone is not sufficient for p53 accumulation⁶⁴⁵. HIF-1 α suppresses MDM2, the p53 negative regulator that ubiquitinates and blocks nuclear export of p53 to the proteasome for degradation⁶⁴⁶. This downregulation of MDM2 expression in hypoxia has been shown to be due to p38 signalling⁶⁴⁷. Tumours with wild type p53 are more likely to have lactic acidosis⁴⁹⁹. It has been shown that there is a differential response between hypoxia or hypoxia and acidosis with regards to p53 accumulation²⁶³. Hypoxia and acidosis increased p53 expression greater than hypoxia alone²⁶³. Furthermore it has been shown that hypoxia plus acidosis induces HIF-1 α target gene expression than hypoxia alone⁶⁴⁸. However, loss of p53 function in the presence of acidosis does not enhance HIF-1 α target expression⁶⁴⁸. P53 signalling in acidosis has been shown to increase the activation of the pentose phosphate pathway and glucominolysis²⁶².

I have shown that LDHA expression is unchanged by NDBT knockdown. LDHA has been shown to be upregulated in acidosis and hypoxia. Lactate has been shown to be an inducer of transcription. LDHA itself is under the transcriptional control of many transcription factors such as: HIF1, cMYC, FOXM1, and KLF4⁶⁴⁹. Many stimuli can induce LDHA expression including: lactate, cAMP, and oestrogen⁶⁴⁹. Expression of lactate is a poor prognostic maker in breast cancer⁶⁵⁰. Although LDHA expression was expected to be reduced by NDBT knockdown due to the ablation of HIF1 signalling, due to the complex regulation of LDHA expression it is likely being compensated for by other transcription factors.

LOX is a well-known downstream target of HIF signalling. High expression of LOX is a poor prognostic marker. I have shown that knockdown of NDBTs does not alter expression of LOX. However, this is intracellular LOX and expression of secreted LOX was not undertaken. LOX is under the transcriptional control of other transcription factors other than HIF such

as NFK β . LOX has been shown to be involved in invasion and metastasis. Thus as the NDBT knockdown cells still express some LOX the cells maintain some invasive potential.

I have shown that cMYC expression is reduced by NDBT knockdown in normoxic and hypoxic conditions. It has been shown that acidosis reduced c-MYC protein by reducing mRNA levels⁶⁵¹. Expression of GPR65 is required for the reduction in cMYC in acidosis⁶⁵¹. Hypoxia modulates MYC expression in a HIF dependant context⁶⁵². MYC plays an important role in cell cycle progression and therefore induces many anabolic pathways. However, under hypoxic conditions ATP production is reduced and cell cycle progression is inhibited. HIF and cMYC share many target genes but have opposite effects on cell proliferation, mitochondrial biogenesis, and DNA repair. Induction of HIF-1 α suppresses cell proliferation. HIF-1 α stabilization results in cell cycle arrest by inhibiting c-Myc transcriptional activity⁶⁵³. HIF-1 α binds to Sp1 via it's PASb domain, resulting in c-Myc displacement from Sp1 complexes and decreased c-Myc promoter interaction⁶⁵³. However, HIF-2 α activity results in the stability of cMYC. In HIF-2 α , phosphorylation of threonine 324 residue in the PASb domain blocks the interaction with Sp1⁶⁵⁴. Alternatively, HIF-2 α forms a complex with Max resulting in the stabilization of the MYC/MAX complex⁶⁵⁴. Knockdown of NDBTs ablates HIF-2 α expression resulting in reduced stabilization of cMYC.

ETS1 has been shown to be upregulated in hypoxia⁶⁵⁵. Knockdown of HIF-1 α reduced expression of ETS1⁶⁵⁵. Conversely knockdown of ETS1 in hypoxia inhibited the upregulation of many HIF regulated genes such as CA9⁶⁵⁵. Downstream transcriptional targets of ETS1 include many proteins involved in the regulation of intracellular pH⁶⁵⁶. Extracellular acidosis upregulates the expression of ETS1⁶⁵⁶. It has been shown that inhibiting the mTOR pathway reduces the expression of ETS1⁶⁵⁷. Thus the loss of ETS1 is likely a downstream effect of HIF ablation in hypoxia and loss of mTOR activation.

The AP1 transcription factor controls a wide variety of biological processes and is known to functionally cooperate in hypoxia induced transcriptional regulation⁶⁵⁸. Many AP-1 subunits are upregulated in hypoxic conditions. Signalling by the MAPK pathway have been involved in AP-1 and HIF signalling⁶⁵⁸. Additionally JNK is also a well-known target to be activated in hypoxia, which controls proliferation and stress response⁶⁵⁹. Extracellular acidosis has been shown to increase AP-1 activity⁶⁶⁰. Expression of c-jun and c-fos are activated for 2h after exposure to acidosis but by 3h expression levels return to normal⁶⁶⁰. Activation of AP-1 in acidosis has been shown to play a role of the chronic activation of protein kinase C in

acidosis⁶⁶⁰. As NDBT knockdown was chronic it likely that the reduction in the AP-1 subunit expression is due in part to the increased intracellular acidosis.

10.5 Conclusion:

I have shown that intracellular acidosis induced by NDBT knockdown or inhibition reduces intracellular phospho-signalling. This reduction in phospho-signalling resulted in large downstream expression changes; activation of mTOR was reduced and expression of other proteins also reduced, resulting in hypoxic HIF stabilization being ablated. This lack of HIF stabilization and mTOR signalling in turn, inhibited the induction of EMT in the NDBT knockdown cell lines. Inhibition of the most perturbed signalling proteins by NDBT knockdown or inhibition recapitulated some of the functional effects on migration, and invasion seen by NDBT knockdown. However, the extent to which migration and invasion was reduced by NDBT knockdown was far greater than that produced by inhibition of these proteins. The data in this chapter provides a strong rationale for further investigating the role of NDBTs knockdown or inhibition. Cellular metabolism plays a significant role and AMPKa activation was altered. Metabolic changes are closely associated with metastasis and therefore the role of NDBT knockdown or inhibition on metabolism will be investigated.

11 Chapter 6: Effect of NDBT knockdown on cell metabolism

11.1 Introduction:

Metabolic changes in cancer are numerous and required for the tumour to adapt to the different environmental stresses common within the tumour microenvironment¹⁴⁴.

Alterations in metabolism was an early observation in the study of cancer⁶⁶¹. Determining exactly how these metabolic functions are reprogrammed in cancer cells and how to exploit these changes for therapeutic gain are important for the field of cancer research⁶⁶¹.

The role of metabolic changes within cancer is to reprogram pathways to aid in cell survival under stress or facilitate heightened proliferation⁶⁶¹. This is achieved by altering three systems: Redox balance, bioenergetics, and biosynthesis⁶⁶¹. Currently there are therapeutics targeted towards metabolites or enzyme resulting impaired tumour growth⁶⁶². The most notable examples of these include: folate analogues Methotrexate, Anti-metabolites such as purine analogues 6-mercaptopurine (6-MP) or 5-fluorouracil (5-FU) the uracil analogue, Gemcitabine or cytarabine are incorporated into the DNA and inhibit DNA polymerases⁶⁶².

Reactive oxygen species are intracellular that contain oxygen these species include: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$)⁶⁶¹. Production of ROS occurs in the mitochondrial and in the cytosol. O_2^- is produced by the reduction of oxygen, this is then converted to H_2O_2 by dismutase 1 or 2⁶⁶¹. Subsequent detoxification of H_2O_2 occurs via peroxiredoxins via oxidation of their active-site cysteines⁶⁶¹. Alternatively other enzymes can detoxify H_2O_2 such Glutathione peroxidases via reduction of glutathione or catalase which reduces H_2O_2 in the cytosol⁶⁶¹. Under conditions of stress where antioxidant capabilities are insufficient to reduce ROS accumulation H_2O_2 can interact with ferrous ions or cuprous ions and become $OH\cdot$ ⁶⁶¹. This results in the damage of lipids, proteins and DNA by oxidation. To maintain ROS levels the cell contains multiple sources of NADPH⁶⁶³.

Until recently ROS were considered a lethal metabolic by product produced during respiration and protein folding. However, under controlled levels ROS plays significant roles

in cellular signalling and homeostasis⁶⁶¹. Low levels of H₂O₂ and other ROS can positively regulate proliferation and survival by reversibly oxidising cysteine residues in proteins⁶⁶⁴.

Mitochondria are the primary sources of ROS. ROS are produced during oxidative metabolism through the one-electron reduction of molecular oxygen (O₂), to form a superoxide anion⁶⁶⁵. Within both the mitochondria and cytosol superoxide dismutase's convert superoxide into hydrogen peroxide. Mitochondrial complexes I⁶⁶⁶, II⁶⁶⁷, and III⁶⁶⁸ contain sites for the generation of superoxide. Whereas complexes I and II produce ROS into the inner mitochondrial matrix, complex III can produce ROS onto both sides of the mitochondrial membrane⁶⁶¹. The ROS produced into the intermembrane space has a greater likelihood of acting as a signalling molecule than that produced by complex I and II due to the access to the cytosol⁶⁶¹. Other respiratory chain enzymes have been shown to produce ROS⁶⁶⁹.

The rate of production of ROS by the mitochondria varies⁶⁶¹. Factors such as: concentration of mitochondria in the cell, respiratory rate, mitochondrial inner membrane potential, and post-translational modifications or cellular damage, all alter the number of electron carriers in the mitochondria ready to interact with oxygen⁶⁶¹. Experiments *in-vitro* have estimated ~2% of all oxygen consumption results in the production of superoxide⁶⁷⁰. However, as less is known about *in-vivo* regulation of mitochondrial function it is difficult to make estimates of superoxide levels *in-vivo*⁶⁶¹.

Hypoxia has a paradoxical effect on ROS expression⁶⁷¹. It would be expected that as oxygen tension decreases the mitochondria would produce less ROS as there is lower oxygen substrate for the mitochondria. However, mitochondrial production of ROS is increased in hypoxia and it is most likely a regulator of many cellular responses to hypoxia⁶⁷¹. Expression of ROS during hypoxia from complex III results in a cytosolic signal that stabilizes HIF⁶⁷². This stabilization occurs via ROS inhibition of PHD proteins⁶⁷³. Additionally ROS signals such that it aids in cell survival during hypoxia⁶⁷⁴. To conserve energy during hypoxia the cells switch to less ATP intensive functions⁶⁷⁴. One major consumer of cellular ATP is the Na/K-ATPase. During hypoxia increased ROS reversibly suppresses Na/K-ATPase activity by signalling for rapid endocytosis⁶⁷⁴. Redox signals from mitochondria in hypoxic cells also regulate post-translational responses including the activation of AMP kinase (AMPK)⁶⁷⁴. Hypoxia-induced ROS signals lead to release of intracellular calcium stores⁶⁷⁴. This amplifies Ca²⁺ signalling within the cell, in turn activating AMPK⁶⁷⁴.

It has been observed that tumours display a high rate of glucose consumption in oxygenated conditions has been validated in many tumours⁶⁷⁵. While taking up large quantities of glucose and producing lactate as a by-product does produce ATP, the major advantage of this is that it produces precursor molecules for anabolic pathways⁸⁰. This was demonstrated using inhibitors of pyruvate kinase and subsequently failing to inhibit tumorigenesis⁶⁷⁶. Moreover, mitochondrial metabolism is necessary for cancer cell proliferation and tumorigenesis, despite high glycolytic rates many tumours generate the majority of their ATP through mitochondrial functions⁶⁷⁷. The exception to this are tumours bearing mutations in mitochondrial enzymes SDH and FH⁶⁷⁸. Furthermore amino acids and fatty acids can supply substrates to the TCA cycle to sustain mitochondrial function. The breakdown of fatty acids by beta oxidation produces acetyl CoA and NADH and FADH₂. Glutamate and α -ketoglutarate fuels the TCA cycle through glutaminolysis of glutamine⁶⁶¹. Other amino acids such as isoleucine, valine, and leucine can also be converted and enter TCA cycle⁶⁶¹. This flexibility of substrate input allows cancer cells to respond to microenvironment changes during tumour growth⁶⁷⁹.

During hypoxic conditions cancer cells must adapt to survive. However the mitochondrial electron transport chain can continue to function at oxygen levels of 0.5%⁶⁸⁰. Moreover this occurs by cells using glutamine to fuel oxidative ATP production⁶⁸¹. When nutrient deprivation occurs cancer cells adapt by reducing their demand for ATP. This maintains the ATP/ADP balance such that unfavourable biological reactions can occur. The actions of mTOR drives the anabolic demands of the cells and is inhibited by hypoxia and reduced amino acid levels⁶⁶¹. Reduced mTOR activity drives autophagic flux, providing an intracellular glutamine supply to sustain mitochondrial function⁴⁵⁶. If reducing ATP demand is not sufficient to maintain ATP/ADP ratio, the rise in ADP levels activates adenylate kinase, an enzyme that buffers the fall in ATP levels by converting two ADP molecules into AMP and ATP⁶⁸². The rise in AMP during nutrient deprivation triggers the activation of AMPK, which activates catabolic pathways like fatty acid oxidation to stimulate ATP production⁶⁸³.

Anabolic pathways are essential to cancer metabolism as they produce macromolecules required for cell division and growth. The pathways generally involve the acquisitions of nutrients from the extracellular space and conversion into intermediates by metabolic pathways. Biosynthesis of protein, lipids, and nucleic acids is under the control of PI3K-mTOR signalling. mTOR signalling is stimulated in the presence of amino acids and activates

protein synthesis via effects on translation and ribosome biogenesis⁴⁵⁶. Nonessential amino acids are produced through transamination reactions, which transfer the amino group from glutamate to a ketoacid. Proliferating cancer cells take up glutamine and convert it to glutamate through a variety of deamidation and transamidation reactions, most notably the mitochondrial amidohydrolase glutaminase⁶⁸⁴. Together, these enzymes generate a large intracellular glutamate pool available for nonessential amino acid synthesis. Both glutamine uptake and glutaminase activity are stimulated by mTORC1, providing glutamate for transamination reactions and/or maintenance of the TCA cycle, which also contributes to amino acid synthesis⁶⁸⁵. Furthermore, when the intracellular glutamine supply exceeds the cell's demands, glutamine can be exported in exchange for essential amino acids to stimulate mTORC1 and protein synthesis⁶⁸⁶. Thus, growth conditions in which glutamine and essential amino acids are abundant enable mTORC1-mediated activation of protein synthesis.

11.2 Hypothesis:

I hypothesise that NDBT knockdown or inhibition will alter cellular metabolism resulting in a higher dependence on OXPHOS than glycolysis. To investigate this hypothesis I will undertake the following aims:

1. *Quantify the effect of NDBT knockdown or inhibition in normoxia and hypoxia on total ATP levels*
2. *Quantify the effect of NDBT knockdown or inhibition on extracellular lactate levels in normoxia and hypoxia*
3. *Investigate the effect of NDBT inhibition or knockdown on glycolytic enzyme mRNA expression*
4. *Determine the effect of inhibition or knockdown of NDBTs on expression of PKM2*
5. *Investigate the effect of NDBT inhibition or knockdown on total mitochondrial expression and mitochondrial membrane polarity in normoxia and hypoxia.*
6. *Determine the role of NDBT knockdown or inhibition on ROS levels in normoxia, hypoxia, neutral, and acidic conditions.*
7. *Investigate the effect of knockdown or inhibition of NDBTs on expression mitochondrial localised proteins.*
8. *Investigate the effect of NDBT knockdown or inhibition on mitochondrial morphology in normoxia or hypoxia.*

11.3 Results:

11.3.1 ATP production, Lactate levels, and Mitochondria

Expression of activated pAMPKa was reduced in hypoxia and acidosis by NDBT knockdown. Data from the RTK phosphor-signalling was interrogated to provide further information regarding metabolism (Fig. 11.1). pAMPKa1 expression was reduced 50% by S0859 treatment, 65% by SLC4A4 knockdown and 67% by SLC4A5 knockdown. When combined with acidic conditions expression of pAMPKa1 was reduced by 70% with S0859 treatment, 73% with SLC4A4 knockdown and 75% with SLC4A5 treatment. Similarly to AMPKa1 AMPKa2 activation also reduced by NDBT knockdown in hypoxia. Treatment with S0859 reduced pAMPKa2 expression by 45% in hypoxia, SLC4A4 knockdown reduced expression by 73% and SLC4A5 knockdown reduced expression by 75%. Combination of acidosis and hypoxia reduced pAMPKa2 expression even further when S0859 was added to 70%. Acidosis had no additive effect to pAMPKa expression with NDBT knockdown in hypoxia. Expression remained at 73% and 75% for SLC4A4 and SLC4A5 respectively. These data indicate that AMPKa1 activation is sensitive to hypoxia, acidosis, NDBT knockdown and NDBT inhibition whereas AMPKa2 activation is sensitive to hypoxia, NDBT knockdown, and NDBT inhibition but not acidosis. $n = 1$. To investigate the role of NDBTs and pH regulation in cellular metabolism we initially investigated total ATP levels in the cell. Cells utilize ATP as the primary energy source and as such any changes in metabolism is likely to impact ATP levels. All cells were counted prior to the assay to ensure no changes were due to cell number. AMPKa1 is phosphorylated when ATP levels are reduced therefore it is hypothesized the NDBT knockdown cells would have higher ATP levels than the control. NDBT inhibition reduced ATP by 30% NBCe-1 knockdown by 40% and NBCe-2 knockdown by 55% in normoxia. Hypoxia reduced the ATP production of the control by 20%. The pattern of ATP levels were similar to the results in normoxia. NDBT inhibition reduced levels by 20%, NBCe-1 knockdown by 40% and NBCe-2 knockdown by 55% (Fig. 11.2).

To determine the role of NDBT knockdown or inhibition on metabolism we investigated lactate excretion (Fig. 11.3). As lactate is passively exported out the cell it would also be reflective of intracellular lactate levels as well. With ATP levels being reduced by knockdown it was worth investigating if this lead to a shift to more glycolysis. Hypoxia led

to a 40% increased expression of lactate in the control as expected. NDBT knockdown lead to a reduction in lactate levels in both normoxia (45% and 50%) and hypoxia (70% and 65%). This reduction was greater in hypoxia. NDBT inhibition did reduce lactate levels in normoxia (20%) but overall lactate levels did rise in hypoxia similarly to the control; the expression in the inhibition was still lower than the control (25%).

Further investigations into the glycolysis pathway were undertaken to determine if NDBT knockdown altered dependence on this pathway. Six enzymes in the glycolysis pathway were investigated, the three rate limiting enzymes, and three putative acid sensitive members (Fig. 11.4). Expression of Hexokinase mRNA was increased 8 fold, 4 fold, and 8.3 fold by NDBT inhibition, SLC4A4, and SLC4A5 knockdown respectively in normoxia. Expression was reduced 50%, increased 60% and increased 90% compared to the hypoxic control by NDBT inhibition, SLC4A4, and SLC4A5 knockdown respectively in hypoxia. Expression of Phosphofructo kinase 1 mRNA was upregulated 4 fold in normoxia by NDBT inhibition but unchanged in hypoxia. SLC4A4 knockdown did not alter mRNA levels in normoxia but an 11 fold increase was seen in hypoxia. SLC4A5 knockdown increased phosphofructo kinase 1 mRNA expression 16 fold in normoxia and 43 fold in hypoxia. Inhibition of NDBTs with S0859 increased pyruvate kinase M1/2 mRNA expression 10 fold and 3 fold in normoxia and hypoxia respectively. Knockdown of SLC4A4 reduced mRNA expression 30% and 90% in normoxia and hypoxia respectively. Knockdown of SLC4A5 reduced mRNA expression 10% and 70% in normoxia and hypoxia respectively. Expression of Aldolase A mRNA expression was increased in normoxia 5 fold, 5 fold and 4.5 fold by NDBT inhibition, SLC4A4 knockdown and SLC4A5 knockdown respectively. In hypoxia expression was increased 6 fold 5.8 fold, and 6 fold by NDBT inhibition, SLC4A4 knockdown and SLC4A5 knockdown respectively. (E) Expression of Phosphoglycerate kinase 1 mRNA was increased 4 fold, 5 fold, and 7.3 fold by NDBT inhibition, SLC4A4, and SLC4A5 knockdown respectively in normoxia. Expression was increased 1.7 fold, 5 fold and increased 11 fold compared the hypoxic control by NDBT inhibition, SLC4A4, and SLC4A5 knockdown respectively in hypoxia. Expression of Enolase 1 mRNA was increased 1.9 fold, 2.3 fold, and 5.3 fold by NDBT inhibition, SLC4A4, and SLC4A5 knockdown respectively in normoxia. Expression was increased 10%, 4 fold and increased 3 fold compared the hypoxic control by NDBT inhibition, SLC4A4, and SLC4A5 knockdown respectively in hypoxia. Thus Aldolase A, Phosphoglycerate 1, Enolase 1, and Hexokinase are important for the NDBT knockdown cells to survive. However, with a reduction in expression of Pyruvate kinase M1/2 occurring in NDBT knockdown it may be at this point that glycolysis is being limited.

Expression of PKM2 protein expression was investigated to determine if protein levels were dropping in line with mRNA levels. The reductions in PKM expression in hypoxia make it the most likely candidate for the reductions in ATP levels in the knockdown cell lines. Inhibition of NDBTs by S0859 did not reduce PKM2 expression in normoxia but reduced expression by 50% in hypoxia. Knockdown of SLC4A4 did not reduce PKM2 expressions in either normoxia nor hypoxia. PKM2 expression was reduced by 85% in normoxic and 70% in hypoxic conditions by SLC4A5 knockdown. The mismatch between mRNA and protein levels could be an indication of that the mRNA is for PKM1/2 whereas only PKM2 protein expression was indicated (Fig. 11.5).

Further investigations into the mitochondria were required to fully understand the changes in metabolism. The reduction in ATP levels were too large for the effect to be glycolysis alone, therefore mitochondria were stained using a mitochondrial lipid dye and imaged by confocal microscopy (Fig. 11.6). Inhibition increased mitochondrial content in normoxia 2 fold but reduced content in hypoxia by 10%. Knockdown of SLC4A4 increased mitochondrial content 2 fold in normoxia and hypoxia. Knockdown of SLC4A5 increased mitochondrial content by 1.6 fold in normoxia and 3 fold in hypoxia. Further to this the ratiometric dye JC-12 was used to determine mitochondrial action potential and thus mitochondrial health (Fig. 11.7). The dye exhibits an emission shift depending upon if the dye is in monomeric or aggregate form. If the mitochondrial pore complex opens and the dye is exported out of the mitochondria it is indicative of a loss mitochondrial membrane polarity and thus mitochondrial health being damaged. The increased expression of green JC-12 in normoxia and hypoxia by NDBT knockdown inhibition indicates that the cells have increased number of mitochondria but that this mitochondria is less healthy and potentially damaged. These results may indicate a loss of control of mitophagy induced by NDBT knockdown and inhibition. This would explain why new mtDNA is lower in the control and S0859 treated cells but total mitochondria and JC-12 expression is higher in the knockdown cells. To determine if components required for mitochondrial function were also being increased in response to NDBT knockdown mRNA expression of CAV β was investigated. CAV β is a mitochondrial localised carbonic anhydrase. Expression of CAV β followed the same pattern as that seen with JC-12 and the total mitochondrial stain. S0859 treated cells has no increase in normoxia but 3 fold increase in hypoxia. SLC4A4 knockdown increased expression 4 fold in normoxia and hypoxia. SLC4A5 knockdown increased expression 5 fold in normoxia and 18 fold in hypoxia (Fig. 11.7).

ROS are produced at many places within the cell but particularly in the mitochondria. It has been shown that hypoxia and acidosis independently lead to increases in ROS. However, with a lack of pH control it was expected ROS would be even higher. However, as mtDNA copy number is reduced with NDBT knockdown it was uncertain to if this would be the case (Supp Fig). NDBT knockdown or inhibition lead to an increase in ROS in normoxia (Fig. 11.8). Inhibition lead to a 2 fold increase, NCBe-1 knockdown a 4.3 fold increase, and NCBe-2 knockdown a 3.8 fold increase. In normoxia and acidosis NDBT knockdown also lead to an increase in ROS, however it was lower than just the normoxic conditions. Inhibition lead to a 3.6 fold increase, NCBe-1 a 2 fold increase and NCBe-2 a 2.5 fold increase. Hypoxia alone lead to a 1.5 fold increase in ROS expression in the control. Inhibition lead to a 4 fold increase, NCBe-1 a 4.2 fold increase, and NCBe-2 lead to a 3 fold increase. Hypoxia combined with acidosis lead to an increase in ROS. Inhibition lead to a 4.2 fold increase, NCBe-1 a 3.7 fold increase, and NCBe-2 a 3 fold increase.

As ATP production and lactate levels were reduced and mitochondrial health compromised investigations into alternative pathways from anabolic intermediates were undertaken to determine how the cells were responding to NDBT knockdown (Fig 11.10). Glutamine is an amino acid used by cancer cells to increase survival and proliferation under conditions of metabolic stress. NDBT inhibition reduced growth by 35% in normoxia and 30% in hypoxia. SLC4A4 did not significantly increase growth in normoxia but increased growth by 1.3 fold in hypoxia. SLC4A5 knockdown increased growth by 20% in normoxia and 34% in hypoxia. These data indicate that S0859 is incurring off target effects compared to NDBT knockdown. Growth in the glutamine free media indicates the NDBT knockdown lines are fuelled by an alternative metabolite.

Blotting was also undertaken for key proteins in other anabolic proteins (Sup Fig.). FDPS is involved in sterol biosynthesis. Expression was unchanged by NDBT knockdown and inhibition. Cystathionine γ -Lyase is an enzyme involved in the metabolism of sulphur containing amino acids. NDBT knockdown ablated the expression of CYT indicating that in those cells metabolism of these amino acids is impaired. This would indicate less hydrogen sulphide is produced. ATG3 is a member of the autophagy complex. NDBT knockdown reduced ATG3 expression indicating autophagy is impaired. These data are in line with the mitophagy data produced. SHMT2 is the mitochondria localised serine hydroxymethyltransferases. Expression was reduced slightly indicating that the NDBT knockdown cells may not be dependent on this pathway. TIA1 is a stress and apoptosis

related protein. NDBT knockdown drastically increased the expression of this protein in both normoxia and hypoxia. This protein localises to stress granules and repress translation in potentially damaging conditions. Due to the large variety of metabolic proteins and the number of proteins involved, metabolomics or RNA seq would be the most pertinent method of instigating the metabolic effects induced by NDBT knockdown.

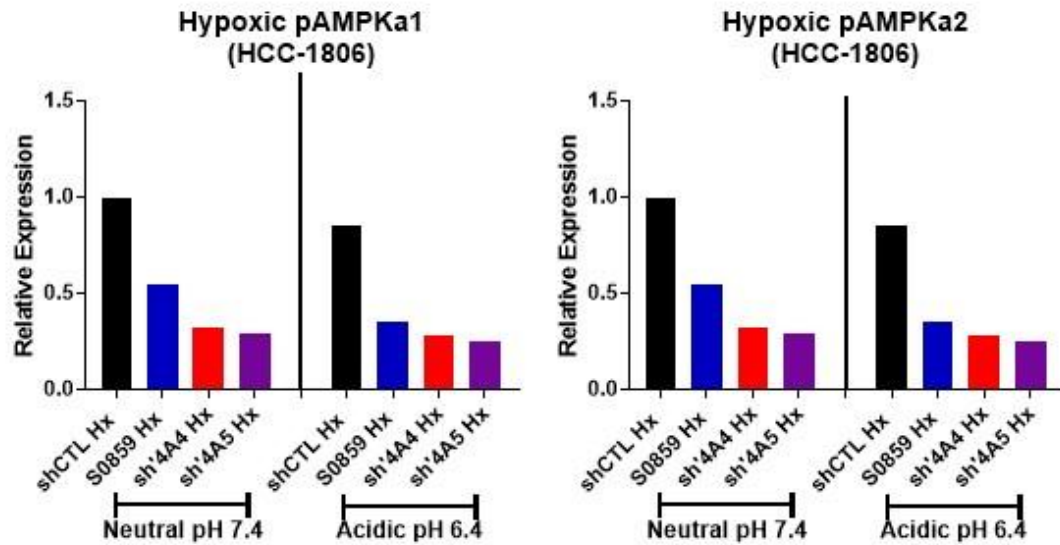


Figure 11.1 Knockdown or inhibition of NDBT reduces activation of AMPKα1/2 in hypoxia and acidosis.

Knockdown or inhibition of NDBT reduces the expression of active AMPKα1 and AMPKα2 in hypoxia and acidosis. (A) Treatment with S0859 reduced AMPKα1 activation by 45% in neutral conditions, in acidic conditions activation was reduced by 65%. Knockdown of SLC4A4 reduced activation by 70% in neutral conditions and by 70% in acidic conditions. Knockdown of SLC4A5 reduced activation by 73% in neutral conditions and by 75% in acidic conditions. (B) Treatment with S0859 reduced AMPKα2 activation by 48% in neutral conditions, in acidic conditions activation was reduced by 63%. Knockdown of SLC4A4 reduced activation by 70% in neutral conditions and by 70% in acidic conditions. Knockdown of SLC4A5 reduced activation by 73% in neutral conditions and by 75% in acidic conditions. n = 1.

X

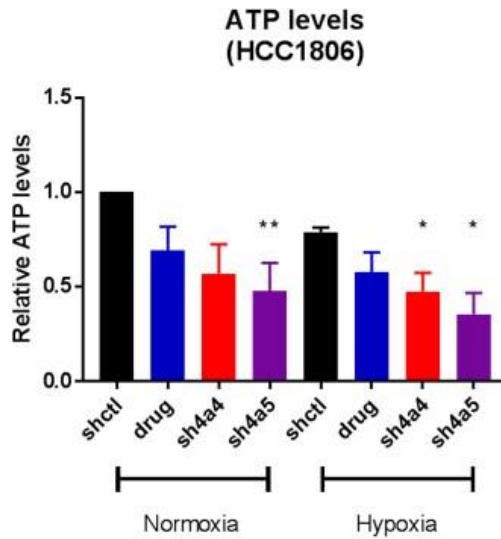


Figure 11.2 Knockdown of NBDT or inhibition of NBDT reduces ATP production in HCC-1806.

In normoxia and hypoxia NBDT inhibition or knockdown reduced intracellular ATP levels. Hypoxia total ATP levels were reduced slightly in the control compared to normoxic conditions. NBDT inhibition reduced ATP levels by 26% in normoxia and 20% in hypoxia. SLC4A4 knockdown reduced ATP levels in normoxia by 37% and 30 in hypoxia. SLC4A5 knockdown reduced ATP levels by 53% in normoxia and by 42% in hypoxia. Error bars, SEM. **, $P < 0.01$; *, $P < 0.05$; $n = 3$

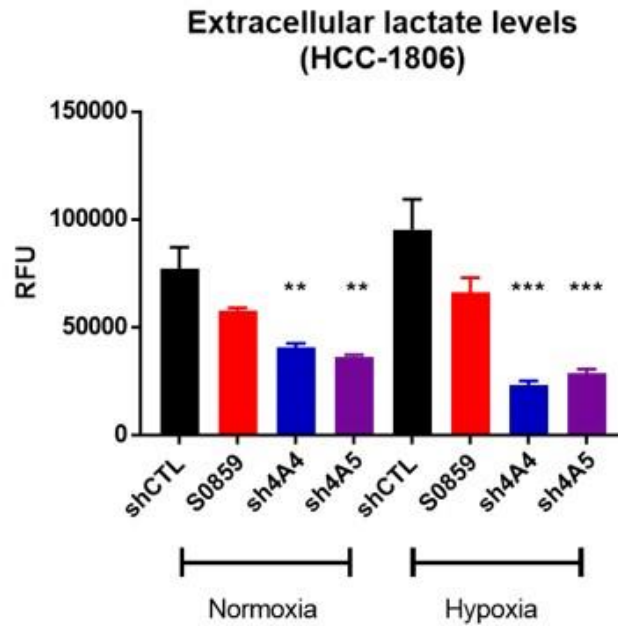


Figure 11.3 Extracellular lactate levels are reduced by NDBT knockdown or inhibition in HCC-1806.

NDBT knockdown or inhibition reduced expression levels of lactate in both normoxic and hypoxic conditions. Inhibition with S0859 reduced extracellular lactate levels by 24% in normoxia and 30% in hypoxia. SLC4A4 knockdown reduced extracellular lactate levels by 34% in normoxia and by 81% in hypoxia. Knockdown of SLC4A5 reduced extracellular lactate by 37% in normoxia and by 76% in hypoxia. Both the control and S0859 cells saw increased lactate levels compared to the normoxic conditions whereas the NDBT knockdown lines did not. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

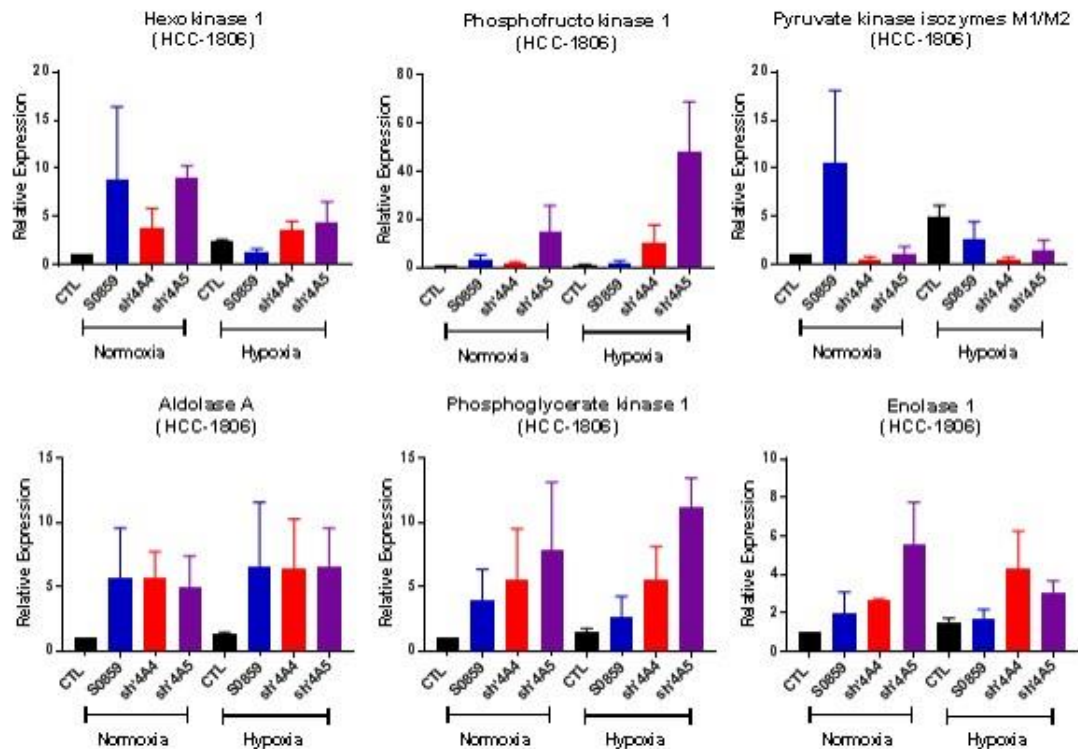


Figure 11.4 Glycolytic enzyme mRNA expression. Expression of glycolytic enzymes mRNA expression was altered by NDBT knockdown or Inhibition.

Enzymes were split into two groups, Rate limiting enzymes and acidosis sensitive cell lines. NDBT inhibition increased the mRNA expression of PFK, PKM, Aldolase A, PGK1, and Enolase. Whereas knockdown of NDBTs increased the expression of HK, PFK, Aldolase A, PGK1, and Enolase.

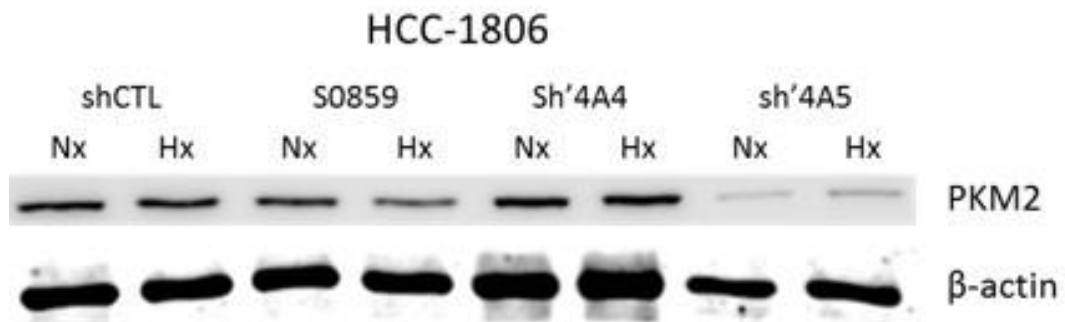


Figure 11.5 Knockdown of SLC4A5 reduced PKM2 expression.

Inhibition of NDBTs reduced PKM2 expression 50% in hypoxia but not normoxia. Knockdown of SLC4A4 did not alter expression of PKM2 expression. Knockdown of SLC4A5 reduced expression of PKM2 by 85% in normoxia and 70% in hypoxia. $n = 3$

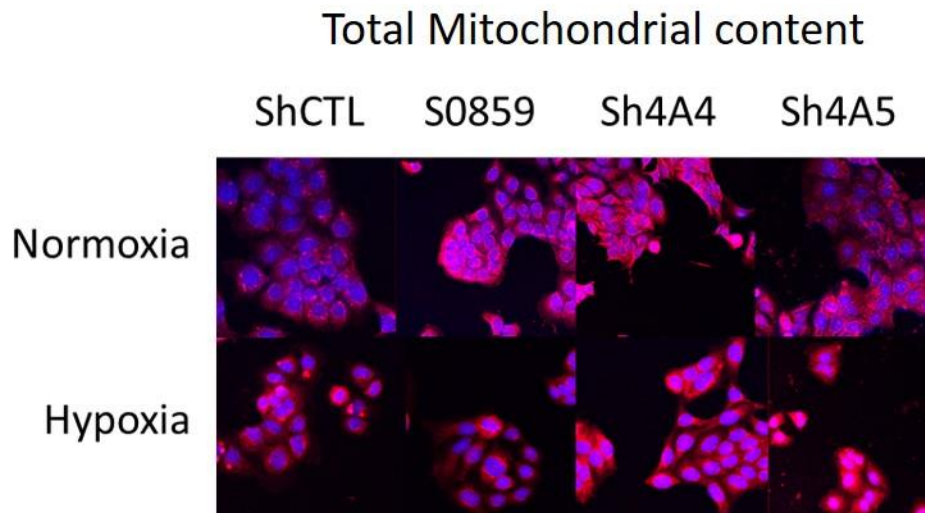


Figure 11.6 Knockdown of NDBT increases total mitochondrial content in normoxia and hypoxia.

Inhibition or knockdown of NDBT increases total mitochondrial content in normoxia and hypoxia. Pink = mitochondria, blue = nucleus. Inhibition increased mitochondrial content in normoxia 2 fold but reduced content in hypoxia by 10%. Knockdown of SLC4A4 increased mitochondrial content 2 fold in normoxia and hypoxia. Knockdown of SLC4A5 increased mitochondrial content by 1.6 fold in normoxia and 3 fold in hypoxia. n = 3.

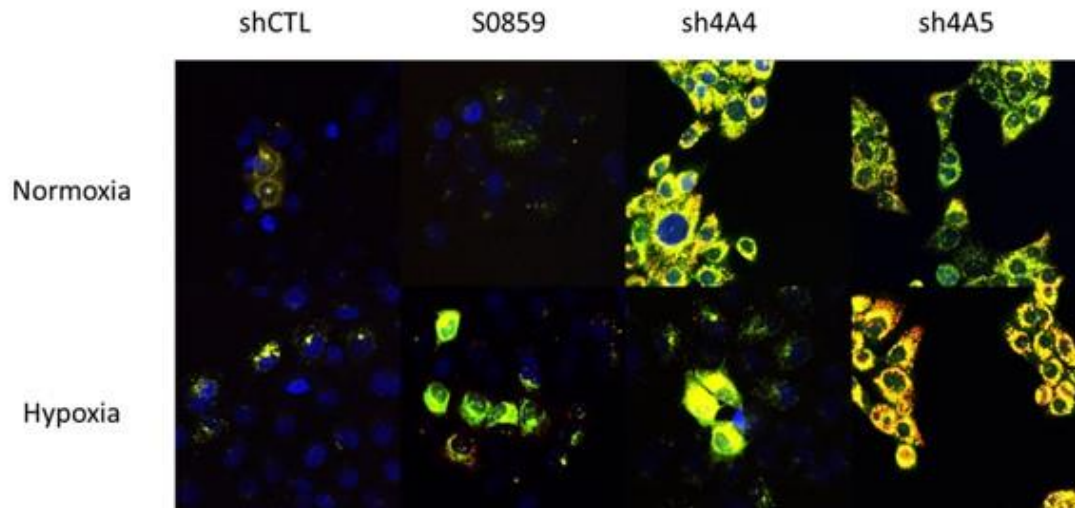


Figure 11.7 Mitochondrial polarity is changed by NDBT knockdown.

Inhibition and knockdown of NDBTs altered mitochondrial membrane polarity. Orange fluorescence indicate living cells with dye congregating inside the mitochondrial membrane, green fluorescence indicate early apoptotic cells due to an opening of mitochondrial permeability transition pore. Control cells displayed almost no green fluorescence in normoxia and hypoxia. Inhibition of S0859 slightly altered membrane polarity in normoxia but significantly increased polarity in hypoxia. Knockdown of SLC4A4 and SLC4A5 both significantly increased membrane polarity in normoxia and hypoxia. $n = 3$

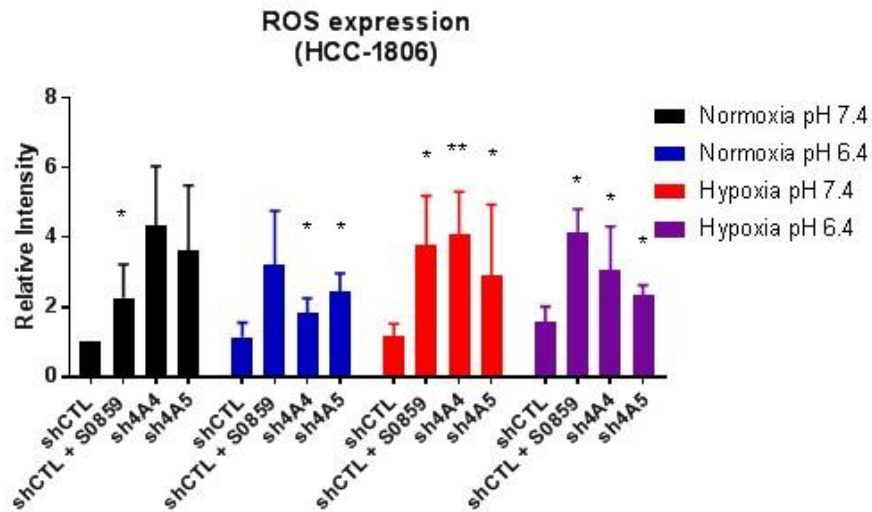


Figure 11.8 ROS levels are increased with NDBT knockdown and inhibition in hypoxia and acidosis in HCC-1806.

Knockdown or inhibition of NDBTs increased the levels of ROS in hypoxia and acidosis. Acidosis in normoxia increased ROS expression 20% in the control. Inhibition increased ROS expression by 3.2 fold, SLC4A4 knockdown by 2 fold, and SLC4A5 2.6 fold compared to the normoxic control. Hypoxic conditions increased ROS expression by 20% in the control. Inhibition increased ROS expression by 4 fold, SLC4A4 knockdown by 4.2 fold, and SLC4A5 3.6 fold compared to the normoxic control. Hypoxic and acidosis conditions combined increased ROS expression by 70% in the control. Inhibition increased ROS expression by 4.4 fold, SLC4A4 knockdown by 3.8 fold, and SLC4A5 3.6 fold compared to the normoxic control. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$

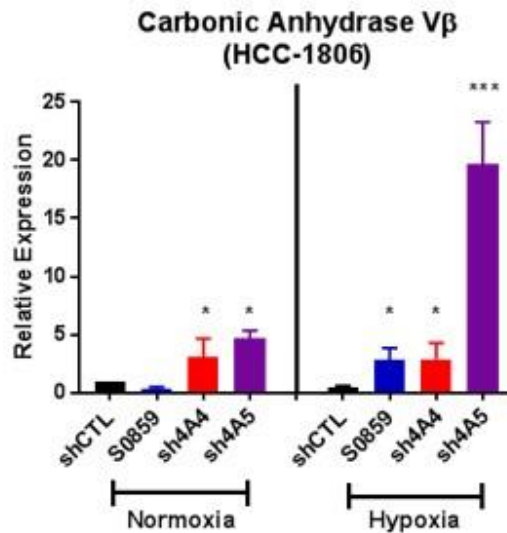


Figure 11.9 Carbonic Anhydrase V β expression is increased by NDBT knockdown in normoxia and hypoxia.

NDBT knockdown and inhibition increased CAV β mRNA expression in normoxia and hypoxia. Inhibition reduced CAV β expression by 50% in normoxia and increased expression 3 fold in hypoxia. SLC4A4 knockdown increased expression 3.2, and 3 fold in normoxia and hypoxia respectively. SLC4A5 knockdown increased expression 4, and 18 fold in normoxia and hypoxia respectively. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$

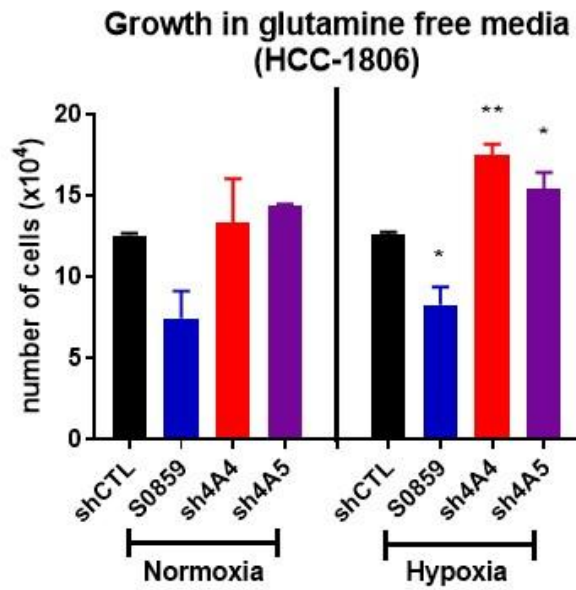


Figure 11.10 Knockdown of NDBT increases the growth of HCC-1806 cells in hypoxia.

NDBT inhibition reduced growth in glutamine free media whereas NDBT knockdown increased growth in normoxia and hypoxia. Inhibition reduced growth by 35% in normoxia and 30% in hypoxia. SLC4A4 did not significantly increase growth in normoxia but increased growth by 1.3 fold in hypoxia. SLC4A5 increased growth by 20% in normoxia and 34% in hypoxia. Error bars, SEM. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; $n = 3$.

11.4 Discussion:

The results in this chapter answers the hypothesis “NDBT knockdown or inhibition will alter cellular metabolism resulting in a higher dependence on OXPHOS” in triple negative breast cancer cells. However, further studies are required for further elucidation of metabolic changes and mechanisms involved. Knockdown or inhibition of NDBTs reduced ATP levels and extracellular lactate levels but increased overall levels of mitochondria. However, the increased mitochondria displayed a loss of membrane polarity and increased ROS indicating a lack of function.

I have shown AMPKa1 and AMPKa2 activation are reduced by NDBT knockdown and inhibition in normoxia and further reduced in hypoxia (fig. 11.1). AMPK is a master regulator of cellular energy homeostasis⁶⁸⁷. AMPK is phosphorylated and activated by depletion of ATP stores⁶⁸⁸. Cellular stresses such as hypoxia have also been shown to activate AMPKa⁶⁸⁹. Downstream signalling from AMPKa results in activation of glycolysis and fatty acid oxidation to replenish ATP store⁶⁸³. Many of these pathways produce H^+ ions and CO_2 as a by-product induce metabolic acidosis⁶⁸³. It has been shown that extracellular acidosis reduces AMPK activation and this modules the autophagy response independently of mTOR another pH sensitive protein⁶⁹⁰. This extracellular acidosis subsequently lead to a downregulation of AMPK regulated pathways such as autophagy⁶⁹⁰. Acidosis has been shown to inhibit glycolysis and as such NHE1 is a downstream target of AMPK resulting in NHE1 activation and regulation of pH⁶⁹¹. Similarly it has been shown that AMPKa2 activation results in the recruitment of NHE5 to the cell surface to regulate intracellular pH⁶⁹².

I have shown that ATP levels are reduced in NDBT knockdown or inhibited cells in normoxia and hypoxia (fig. 11.2). Hypoxia results in a mismatch between ATP demand and production⁴⁸⁵. To adapt this environmental change and keep bioenergetics levels stable, hypoxic cells downregulate protein translation and the activity of high energy consuming proteins such as the mitochondrial Na-K-ATPase⁶⁹³. Cells respond to hypoxic stress by activating AMPK⁶⁸⁸. However, AMPK activation is inhibited by acidosis⁶⁹⁰. Thus it is likely the lowered ATP levels are due to the NDBT knockdown or inhibition cells inability to respond as AMPK is inhibited by intracellular acidosis.

I have shown that extracellular lactate levels are reduced by NDBT knockdown or inhibition in normoxia and hypoxia (fig. 11.3). Lactate levels have been shown to be increased in hypoxia due to increased glycolysis⁶⁹⁴. Glycolysis has been shown to be inhibited by acidosis and high lactate levels⁶⁹⁵. The glycolysis pathway is rate limited by three enzymes: PKM1/2, hexokinase 1, and phosphofructokinase 1⁶⁹⁶. These proteins are upregulated in hypoxia⁶⁹⁷. I have shown that mRNA expression of hexokinase 1 and phosphofructokinase 1 are upregulated whereas PKM1/2 is reduced by NDBT knockdown and inhibition in normoxia and hypoxia (Fig. 11.4). Therefore it is likely that the reduction in lactate levels is due to the limiting effect of PKM1/2 expression in these cell lines. I have shown that Aldose A, phosphoglycerate kinase 1, and enolase 1 mRNA expression is increased by NDBT knockdown or inhibition in normoxia and hypoxia (Fig. 11.5). It has been shown that these glycolytic enzymes are upregulated in acidic and hypoxic conditions⁴⁹⁹. Many of these proteins have large pH range optima⁶⁹⁸⁻⁷⁰⁰. Protons have been shown to be inhibitors of key rate limiting glycolytic enzymes such as phosphofructokinase⁷⁰¹. NDBT knockdown or inhibition increased expression of these acid sensitive enzymes in line with the induced intracellular acidosis. I have shown that PKM2 protein expression is reduced by NDBT knockdown and inhibition in hypoxia. PKM2 is a direct target of HIF-1 α ⁷⁰². It has been shown that under acidosis cells revert from PKM2 to PKM1 and undertake aerobic glycolysis⁶⁹⁵. It is likely that inhibition of PKM2 is the mechanism by which lactate levels are reduced.

I have shown that NDBT knockdown or inhibition increases total mitochondrial expression in normoxia and hypoxia (Fig. 11.6). AMPK activation positively regulates mitochondria biogenesis⁶⁸⁷. Mitochondria switch morphology towards fission away from fusion to reduce ROS production in hypoxia. Moreover, mitochondrial mass is reduced under hypoxia⁷⁰³. This is regulated by HIF-1 α signalling and the downstream target BNIP3⁷⁰⁴. Acidosis has been shown to switch cell metabolism from glycolysis to OXPHOS⁷⁰⁵. It has been shown that autophagy is reduced in acidosis. I have shown that CREB expression is increased in the NDBT knockdown cell lines. CREB regulates many mitochondrial gene expression⁷⁰⁶. Hypoxia selectively activates CREB gene transcription⁷⁰⁷. Thus the increase in mitochondria in NDBT knockdown and inhibition is likely due a combination of loss HIF-1 α induced autophagy and an increase in CREB signalling.

I have shown that NDBT knockdown or inhibition results in a perturbation of mitochondrial membrane potential in normoxia and hypoxia (fig. 11.7). In glioma cell lines it has been

shown that the sensitivity to hypoxia is determined by mitochondrial function⁷⁰⁸. Hypoxia sensitive cell lines are unable to maintain a stable mitochondrial membrane potential and ATP levels whereas hypoxia insensitive cell lines were⁷⁰⁸. Therefore, it is likely the increased number of mitochondria is not an increase in active mitochondria and many are non-functional. It has been shown that extracellular acidosis induces mitochondrial dysfunction, increased intracellular ROS level, mitochondrial proton pump opening and ROS dependent cell death⁷⁰⁹. It has been shown that mitochondrial ROS production in acidosis is in part regulated by AKT activation⁷¹⁰. The mitochondrial proton pump is pH sensitive and requires a proton gradient to function⁷¹¹. The outer mitochondrial membrane is permeable to H⁺ however, inner membrane is impermeable⁷¹². Trafficking of the proton pump in acidosis is regulated by CREB and SRC signalling⁷¹³. Thus this lack of activation is a driving force behind the reduced levels of ATP in NDBT knockdown cell line (Fig 11.2).

I have shown that survival in low glutamine conditions is unchanged by NDBT knockdown in normoxia or hypoxia⁶⁷¹. It has been shown that glutamine metabolism is regulated by HIF1 α and SIAH2 signalling⁷¹⁴. Hypoxia induces a switch to glutamine metabolism from oxidative to reductive carboxylation⁷¹⁵. Acidosis, similarly switches glutamine metabolism to reductive carboxylation⁷¹⁶. The usage of glutamine is second to glucose as a carbon source in the body and is used as the main carbon source in cell culture. Thus it was hypothesised that reduced glutamine would kill NDBT knockdown cells as they exhibit reduced glycolysis. Thus NDBT knockdown cells must be sourcing carbon from an alternative source or the high glucose media used may be masking the results.

I have shown that NDBT knockdown increases the expression of mitochondria localised carbonic anhydrase CAV β in normoxia and hypoxia with changes correlating to changes in total mitochondrial mass. Acidosis has been shown to increase the expression of other mitochondria localised genes such as BCL-2⁵⁸⁵. This increased expression of CAV β is likely to facilitate pH homeostasis in the increased number of mitochondria.

11.5 Conclusion

I have shown that knockdown or inhibition of NDBT perturbs metabolic function in normoxia and hypoxia. Total ATP, and extracellular lactate levels were reduced by NDBT knockdown or inhibition. To compensate for this expression of mitochondria were increased subsequently leading to increased ROS levels. However, the mitochondria displayed an altered and more fractured morphology indicating a loss of function. The data in this chapter provides evidence that the effects of intracellular acidosis induced by NDBT knockdown or inhibition are systemic and severely perturb cellular function.

12 Chapter 7: Conclusion and future work

This work has investigated the role of NDBTs in TNBC. The data has shown that knockdown of NDBTs reduces migration and invasion *in vitro* and metastasis *in vivo*. These reductions in invasion and metastasis are due to an increase in intracellular acidification that occurs when NDBTs are knocked down or inhibited. Intracellular acidosis subsequently results in the loss of mTOR activation which is crucial for the stabilization of HIF in hypoxia. The loss of HIF stabilization reduced the induction of EMT in the knockdown lines and perturbed metabolic function, primarily in the glycolysis pathway resulting in lower levels of ATP in the cells. The data indicates that targeting NDBTs would be a viable therapeutic target to reduce breast cancer metastasis and recurrence.

S0859, the small molecular inhibitor chosen during this study showed promising results initially. Upon treatment of the cells with this drug migration and invasion was reduced. However, due to the difficulties of using S0859 in-vivo it was not taken forward into the full colonisation study. S0859 was rapidly cleared from the mice circulation and high doses were fatal. Furthermore, many of the protein expression changes induced by NDBT knockdown were not induced by S0859 treatment. This may be due to a S0859 treatment only temporarily increasing intracellular acidosis. Potentially the effects of S0859 may be due to its ability to hit many other targets other than the NDBTs.

Future directions for this project could take two distinct routes.

Firstly they could investigate the roles of other members of the NDBT family or other proteins involved in the regulation of cellular pH. The study which produced preliminary data for this project indicated that knockdown of SLC4A9 reduces invasion and metastasis. However, this study did not further investigate the molecular mechanisms by which this occurs. As knockdown of SLC4A4 and SLC4A5 resulted in some similar and differential effects with regards to EMT transcription factors (ZEB1 and Twist 2 respectively) it is likely that SLC4A9 may affect a distinct set of EMT transcription factors as well. Furthermore other NDBT members were also highly upregulated in hypoxia in some cell lines and this is likely to be similar in other tumour types. Thus it may be that other NDBTs or pH regulatory proteins are more important in differing tissue types. One more possibility is that a double knockdown could be produced. It possible that the double knockdown would be synergistic as the cell may become even acidic.

Secondly, new molecularly targeted therapies could be developed against the NDBTs. Currently S0859 would not be viable clinically and attempts to produce derivatives with similar effects have produced lacklustre results. A consortium has proposed producing new compounds and screening for efficacy. Ideally more targeted inhibitors could be produced specific for each individual NDBTs. With regards to other methods of targeting the NDBTs, the data indicates that producing a hypoxia activated prodrug would be a viable approach to increasing selectivity. This would allow for the targeting of only the hypoxic tumour regions whilst additionally reducing potential side effects. Additionally, if a specific inhibitor of the NDBTs were produced, if used in a neoadjuvant setting it may be useful to reduce recurrence and metastasis as migration and invasion would be reduced.

Moreover, had time constraints not halted the project further experiments were planned to add weight to current results and investigate potential alternative mechanisms.

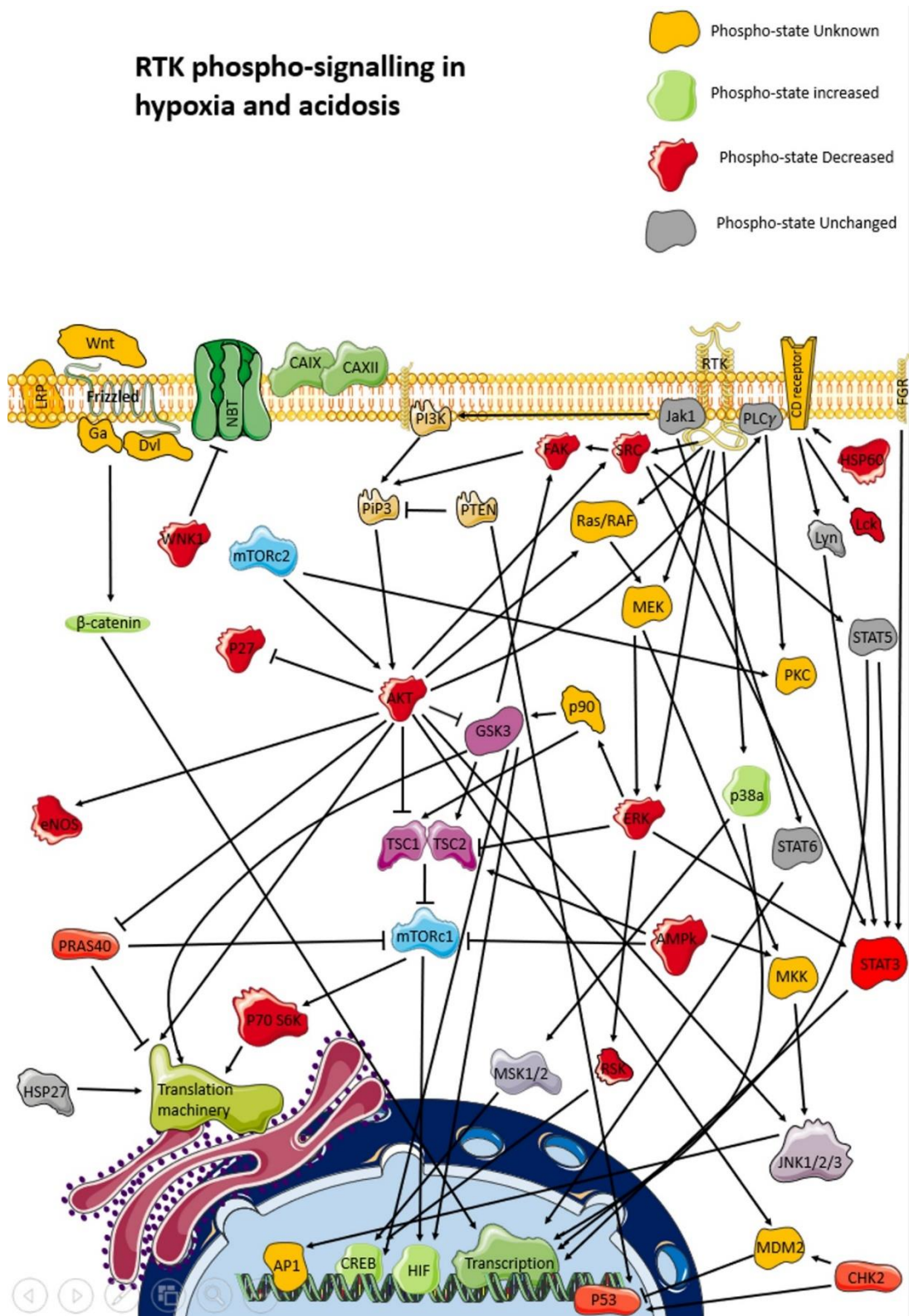


Figure 13.1 Schematic diagram linking regulation of phospho-array proteins

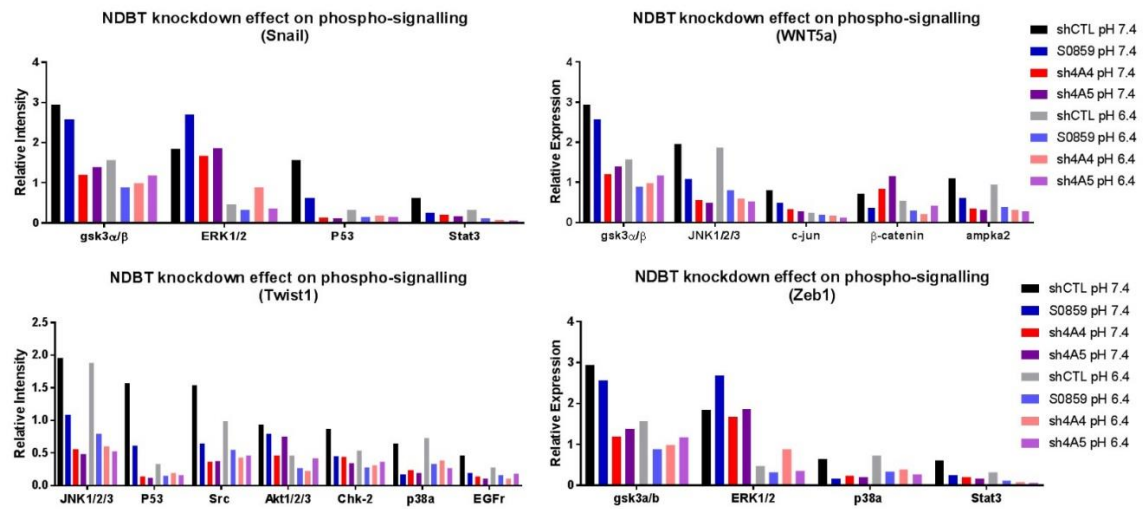


Figure 13.2 NDBT knockdown reduces the phosphorylation of multiple proteins related to EMT regulation

Protein	Molecular Weight (KDa)
AE4	100
AKT	60
ALK	220
ATF4	49
ATG3	40
CAIX	55
cFOS	62
cJUN	48
cMET	140
cMYC	65
CREB	43
E-cadherin	135
EGF	6
EGFr	175
ETS1	52
FAK	125
FDPS	48
Fgfr2	145
FOSB	48
FOSL1	40
FOSL2	45
Glut-1	55
GSC	40
Hgf	85
HIF-1 α	125
HIF-1 β (ARNT)	90
HIF-2 α	125
Integrin- β	115
JUNB	43
JUND	42
LDHA	37
LOX	56
Luciferase	75
LYC	56
LYN	56
MAPK	38
MDM2	55
MTOR	250
NBCe-1	125
NBCe-2	140
NBCn-1	150
P53	53
pALK	220
pcMET	140
Pdgfr- α	190
PKM2	60
pLYC	56
pLYN	56

pMTOR	250
SHMT2	52
Slug	30
Snail	29
TIA1	44
Twist1	26
Twist2	26
VEGFr2	210
Vimentin	55
WNT5a	45
WNT5b	45
ZEB1	200
ZEB2	200
β -actin	42

Figure 13.3 Protein molecular weights

Gene target	shRNA name	shRNA sequence
SLC4A4	TRCN0000043138	CCGGCCGGCTTTGTTGGTCACTATACTCGAGTATAG TGACCAACAAAGCCGGTTTTTG
SL4A4	TRCN0000043140	CCGGCCCAGAGTATTGCGCAACTATCTCGAGATAGT TGGCAAATACTCTGGGTTTTTG
SLC4A4	TRCN0000043142	CCGGGCCAATGTTTAACCACAGTATCTCGAGATACT GTGGTTAAACATTGGCTTTTTG
SLC4A5	TRCN0000414761	CCGGATCGCAGGAATTGATGAATTTCTCGAGAAATTC ATCAATTCCTGCGATTTTTTG
SLC4A5	TRCN0000442365	CCGGACCGCTCCTTAGCTGACATTGCTCGAGCAATGT CAGCTAAGGAGCGGTTTTTTG
SLC4A5	TRCN0000038175	CCGGCCCTCTTTACAGAGATGGATACTCGAGTATCCA TCTCTGTAAAGAGGGTTTTTG

Figure 13.4 Lentiviral shRNA transduction particle sequences

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