The Role of Voltage-gated Sodium Channels in Non-small Cell Lung Cancer

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2012

Thomas M Campbell
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Abstract

The Role of Voltage-gated Sodium Channels in Non-small Cell Lung Cancer

Thomas M Campbell

2012

Faculty of Life Sciences, The University of Manchester

This abstract pertains to a thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences.

Various ion channels are expressed in human cancers where they are intimately involved in proliferation, angiogenesis, migration and invasion. Expression of functional voltage-gated sodium (Na\textsubscript{v}) channels is implicated in the metastatic potential of breast, prostate, colon, cervical and lung cancer cells. However, the cellular mechanisms that regulate Na\textsubscript{v} channel expression in cancer remain largely unknown. Growth factors are attractive candidates; they not only play crucial roles in cancer progression but are also key regulators of ion channel expression and activity in non-cancerous cells. Here, the role of epidermal growth factor receptor (EGFR) signalling and Na\textsubscript{v} channels in non-small cell lung cancer (NSCLC) cell lines have been examined. It is shown that functional expression of Na\textsubscript{v}1.7 promotes invasion in strongly metastatic H460 NSCLC cells. However, in non-invasive A549 NSCLC cells, Na\textsubscript{v}1.7 is completely absent. Inhibition of Na\textsubscript{v}1.7 either pharmacologically by tetrodotoxin (TTX) or genetically by small interfering RNA (siRNA) reduces H460 cell invasion by up to 50%. Whilst EGFR signalling enhances proliferation, migration and invasion of H460 cells, EGFR-mediated upregulation of Na\textsubscript{v}1.7 specifically, is required to promote invasive behaviour in these cells. Examination of Na\textsubscript{v}1.7 expression at the mRNA, protein and functional levels further reveals that EGFR signalling via the ERK1/2 pathway controls transcriptional regulation of Na\textsubscript{v}1.7 expression to promote cellular invasion in NSCLC. The role of Na\textsubscript{v} channels in promoting cancer cell invasion is also unclear. Therefore, the effect of Na\textsubscript{v} channel activity on two likely downstream contributors to cellular invasion, intracellular calcium concentration, [Ca\textsuperscript{2+}], and intracellular pH, pH\textsubscript{i}, have been examined. It is shown that functional expression of Na\textsubscript{v}1.7 likely drives H460 NSCLC cell invasion via H\textsuperscript{+} efflux from the cell in an uncharacterised mechanism potentially involving NHE1, resulting in extracellular acidification of the perimembrane space. However, much more work is needed to understand this Na\textsuperscript{+}-dependent invasive mechanism. Immunohistochemistry (IHC) of patient biopsies confirms the clinical relevance of Na\textsubscript{v}1.7 expression in NSCLC. Thus, Na\textsubscript{v}1.7 has significant potential as a novel target for therapeutic intervention, possibly in conjunction with existing EGFR inhibitors, and/or as a diagnostic/prognostic marker in NSCLC.
Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.
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Acknowledgements

I would like to thank my supervisor, Liz Fitzgerald, for her guidance and encouragement over the last four years. A PhD can feel an awful lot longer and tougher with the wrong supervisor, so I am hugely grateful to have found one who was able to bring the best out of me by wholeheartedly supporting me throughout my project whilst allowing me the room to work in my own way. A big thanks must also go to my industrial supervisor, Martin Main, for his invaluable contribution to my project and also for being a willing participant in lengthy discussions regarding football when I didn’t want to think about science.

I would also like to thank all of the good friends that I have made since starting my PhD in Manchester. You fall into one of two categories – those who understood what I was talking about when you asked me what I was doing and those who didn’t but just nodded and tried to look interested anyway. There are far too many of you to list here, but you know who you are. Special thanks should go to everyone who shared my passion for extended tea/coffee breaks at work, especially Rebecca Brookfield, Andrew Chadburn, Alex Ryan, Katherine Campion and Parini Mankad. At times, I swear they were the only things keeping me sane! My gratitude must also be extended to the former members of my lab group for their help and friendship; Philip Robinson, Laura Roberts, Sarah Etheridge and Lele Song.

I have been fortunate enough to live with many fantastic people during my PhD. All of them have had to endure me excitedly talking gibberish when things were going well, and moping about when things weren’t. It really was great fun living with all of you (even Henry).

Finally, and most importantly, I would like to thank my family for their unconditional love and support, and for accepting the fact that I haven’t been doing a real job for the last four years. Thanks to my parents, to whom I dedicate this thesis, for putting up with me during my write-up, and thanks to my brother, Ed, for always keeping me grounded by making me look bad by comparison.

…I think I might be ready for a job!
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABS</td>
<td>Absorbance</td>
</tr>
<tr>
<td>AIS</td>
<td>Axon initial segment</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ba^{2+}</td>
<td>Ionic barium</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCECF</td>
<td>2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>BK</td>
<td>Bradykinin</td>
</tr>
<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Ionic calcium</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>CAM</td>
<td>Cellular adhesion molecule</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Ca_v</td>
<td>Voltage-gated calcium channel</td>
</tr>
<tr>
<td>CCh</td>
<td>Carbachol</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CUP</td>
<td>Cancer of unknown primary</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGFR-TK</td>
<td>EGFR-tyrosine kinase</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-Ethyl-N-isopropyl)amiloride</td>
</tr>
<tr>
<td>E_m</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed, paraffin-embedded</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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GEF  Guanine nucleotide exchange factor
$G_{\text{max}}$  Maximal conductance
GRB2  Growth factor receptor-bound protein 2
GTP  Guanosine triphosphate
HEK  Human embryonic kidney
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER  Human epidermal growth factor receptor
HRP  Horseradish peroxidase
HT  High-throughput
$I_{\text{Ba}}$  Barium current
ICC  Immunocytochemistry
Ig  Immunoglobulin
IHC  Immunohistochemistry
$I_{\text{max}}$  Maximal current density
$I_{\text{Na}}$  Sodium current
$I_{\text{Na},\text{P}}$  Persistent sodium current
$I-V$  Current-voltage
JAK  Janus kinase
$K_v$  Voltage-gated potassium channel
MAPK  Mitogen-activated protein kinase
MBS  MES-buffered saline
MES  2-(N-Morpholino)ethanesulfonic acid
MMP  Matrix metalloproteinase
mRNA  Messenger RNA
MS  Multiple sclerosis
MβC  Methyl-β-cyclodextrin
n.s.  Not significant
$\text{Na}^+$  Ionic sodium
$[\text{Na}^+]_i$  Intracellular sodium concentration
$Na_v$  Voltage-gated sodium channel
NBF  Neutral buffered formalin
NCX1  Sodium/calcium exchanger 1
NF155/186  Neurofascin-155/186
NGF  Nerve growth factor
NHE1  Sodium/hydrogen exchanger 1
nNa$_v$1.5  Neonatal Na$_v$1.5
nNa$_v$1.7  Neonatal Na$_v$1.7
NOR  Node of Ranvier
NrCAM  Neuronal cell adhesion molecule
NSCLC  Non-small cell lung cancer
OD  Optical density
PAGE  Polyacrylamide gel electrophoresis
PBS  Phosphate-buffered saline
PDGF  Platelet-derived growth factor
PDK1  Phosphoinositide-dependent protein kinase 1
PH  Pleckstrin homology
<table>
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<th>Symbol</th>
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<tr>
<td>pH_e</td>
<td>Extracellular pH</td>
</tr>
<tr>
<td>pH_i</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP₃</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMP</td>
<td>Protein metallophosphatase</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<td>PP</td>
<td>Protein phosphatase</td>
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<td>Physiological saline solution</td>
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<td>Phosphatase and tensin homologue</td>
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</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPTPβ</td>
<td>Receptor protein tyrosine phosphatase β</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SBFI</td>
<td>Sodium-binding benzofuran isophthalate</td>
</tr>
<tr>
<td>SCLC</td>
<td>Small cell lung cancer</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>SHIP</td>
<td>Src homology 2 domain-containing inositol phosphatase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNK</td>
<td>Student-Newman-Keuls</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STX</td>
<td>Saxitoxin</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween-20 Tris-buffered saline</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>V_{50,act}</td>
<td>Voltage for half maximal activation of current</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar ATPase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>V_h</td>
<td>Holding potential</td>
</tr>
<tr>
<td>V_{peak}</td>
<td>Voltage at which I_{max} is elicited</td>
</tr>
<tr>
<td>V_{rev}</td>
<td>Reversal potential</td>
</tr>
<tr>
<td>τ_{act}</td>
<td>Time constant for activation</td>
</tr>
<tr>
<td>τ_{inact}</td>
<td>Time constant for inactivation</td>
</tr>
</tbody>
</table>
Chapter 1:
GENERAL INTRODUCTION
A distinguishing feature of electrically excitable cells is the diversity of ion channels that help to mediate the complex communication between different parts of the cell. Signals are generated by the opening or closing of voltage-dependent ion channels at one point in the cell membrane, resulting in local changes in the membrane potential that causes electric current to flow rapidly to other points in the membrane. By controlling the flux of ions in and out of the cell, electrically excitable cells are able to generate robust signals, action potentials (APs), which propagate from one end of a cell to the other (Catterall 1984; Rutecki 1992). In the case of neurons, these signals can then go on to mediate intercellular communication at the synapse between two cells by controlling the exocytosis of neurotransmitters from the presynaptic cell. These neurotransmitters can then bind to receptors on the neighbouring cell, generating a new AP.

Voltage-gated sodium (Na\textsubscript{v}) channels are responsible for AP creation and propagation in electrically excitable cells such as neurons, myocytes and certain types of glia via the conduction of sodium ions (Na\textsuperscript{+}) into the cell down its electrochemical gradient in response to membrane depolarisation. However, Na\textsubscript{v} channels have also been found to play crucial roles in metastatic behaviours of several cancers derived from non-excitable epithelial cells, where these channels serve no obvious physiological role. These include breast cancer (Roger et al. 2004; Fraser et al. 2005; Brackenbury et al. 2007; Gao et al. 2009; Gillet et al. 2009), prostate cancer (Grimes et al. 1995; Laniado et al. 1997; Grimes and Djamgoz 1998; Diss et al. 2001; Abdul and Hoosein 2002; Bennett et al. 2004; Diss et al. 2005; Nakajima et al. 2009; Yildirim et al. 2012), cervical cancer (Diaz et al. 2007; Hernandez-Plata et al. 2012), colon cancer (House et al. 2010), ovarian cancer (Gao et al. 2010), small cell lung cancer (SCLC) (Pancrazio et al. 1989; Blandino et al. 1995; Onganer and Djamgoz 2005; Onganer et al. 2005), and more recently, non-small cell lung cancer (NSCLC) (Roger et al. 2007). Thus, the acquisition of a more neuronal phenotype via the upregulation of Na\textsubscript{v} channel expression and activity is associated with the development of cancer metastases in a variety of carcinomas. Whilst some progress has been made towards understanding the possible downstream effects of Na\textsubscript{v} channels in cancer, the cellular mechanism(s) that regulate the expression and activity of Na\textsubscript{v} channels in metastatic cancer cells remain unclear.
In addition to their roles in cell growth and cancer progression, cellular growth factors also regulate the expression and activity of numerous ion channels. Of particular note, epidermal growth factor (EGF) enhances Na<sub>v</sub> channel currents in non-cancerous cells, including cardiac myocytes (Liu et al. 2007). Furthermore, in human and rat prostate cancer, functional upregulation of Na<sub>v</sub> channels has been implicated in the pro-invasive response of cells to EGF stimulation (Uysal-Oganer and Djamgoz 2007; Ding et al. 2008), although the cellular mechanism(s) responsible for these effects are unknown. The central theme of this project involves a detailed characterisation of Na<sub>v</sub> channels in human NSCLC, an investigation of the effects of EGF receptor (EGFR) signalling (a well-established and tractable therapeutic target in the treatment of NSCLC) on Na<sub>v</sub> channel expression and activity in NSCLC cells, and an investigation into how Na<sub>v</sub> channel activity may promote enhanced metastatic potential in invasive NSCLC cells.

1.1 Voltage-gated ion channels

Ion channels are pore-forming, transmembrane protein complexes that regulate the flow of ions across biological membranes. Voltage-gated ion channels are a specific class of ion channel that open and close in response to changes in the membrane potential near the channel, allowing for the rapid, voltage-dependent conduction of ions down their electrochemical gradients. They are especially important in electrically excitable cells such as neurons where they directionally propagate electrical signals over large distances, but are common in many types of cell.

Since the advent of voltage-clamp recordings (Hodgkin and Huxley 1952), the three main classes of ion channels that contribute to electrical excitability are classified according to the cation that is selectively conducted across the plasma membrane and the fact that the gating properties of the channels are tightly controlled by the membrane potential. These are voltage-gated sodium (Na<sub>v</sub>), potassium (K<sub>v</sub>) and calcium (Ca<sub>v</sub>) channels. Na<sub>v</sub> channels are responsible for the depolarising phase of an AP in an electrically excitable cell. If enough Na<sub>v</sub> channels open following a change in a cell’s membrane potential, a small but significant number of Na<sup>+</sup> ions will enter the cell, further depolarising the membrane around it. K<sub>v</sub>
channels play a crucial role in returning a depolarised cell to a resting state following an AP, thus restoring the resting membrane potential of the cell. $Na_v$ channels both open and close more rapidly than $K_v$ channels, resulting in an influx of $Na^+$ toward the beginning of an AP and an efflux of $K^+$ toward the end. $Ca_v$ channels are also activated in response to membrane depolarisation. When open, $Ca_v$ channels allow the entry of $Ca^{2+}$ into cells, which, depending on the cell type, results in intracellular processes such as muscular contraction, upregulation of gene expression or the release of hormones or neurotransmitters (Catterall 2011). The targeting of these different voltage-gated ion channels to different regions of electrically excitable cells links the channel with its specific cellular function (Figure 1.1).

All three classes of voltage-gated ion channel share a common structure (the structure of $Na_v$ channels, specifically, shall be considered in more detail later (Figure 1.4)); each contains four transmembrane ‘domains’ and a total of 24 $\alpha$-helices. Each of the four transmembrane domains (homologous subunits for $K_v$ channels or homologous repeat domains for $Na_v$ and $Ca_v$ channels) is composed of six transmembrane $\alpha$-helices, termed S1 through S6, with both the amino- and carboxy- termini located intracellularly. The S4 helix is highly conserved in all voltage-gated channels and acts as the channel’s voltage-sensor. It consists of positively charged residues at regular intervals; a lysine or arginine at every third position (Catterall 1995). These are paired with negative charges from other parts of the channel and when membrane depolarisation reduces the voltage gradient across the membrane, this culminates in an outward rotation of the S4 helix, resulting in a conformational transition that opens the channel, allowing the passage of ions through the pore and down their electrochemical gradients (Bezanilla 2000). The ions are conducted through a pore, which can be broken into two regions. The more extracellular region of the pore is formed by the ‘P-loops’ between the S5 and S6 helices of each domain. They consist of a stretch of 20 amino acids which loop into the membrane between the S5 and S6 helices as a pair of antiparallel $\beta$-strands (Catterall 1995). This region is the narrowest part of the pore and it is the P-loop that provides the ion selectivity filter for the channel. The more cytoplasmic region of the pore is formed by the combined S5 and S6 regions of the four domains.
Figure 1.1: Subcellular localisation of neuronal voltage-gated ion channels. $K_v$ channels, including ERG, predominate in the somatodendritic region of a neuron, but the major part of $K^+\text{ conductance}$ arises from channels in the axon initial segment (AIS). The axonal region of a neuron comprises $Na_v$ and $K_v$ channels, with $Na_v$ channels located at the Nodes of Ranvier (NOR) between myelinated regions of the axon, and a population of $K_v$ channels located at the juxtaparanodal region, under the myelin sheath (green) (Wang et al. 1993). Myelin serves to speed up the conduction of an electrical impulse by forcing it to propagate by saltation. $Ca_v$ channels are localised exclusively to the presynaptic terminal where they mediate the release of neurotransmitters into the synaptic cleft. Such localisations link the particular voltage-gated ion channels to a specific function.
1.2 The voltage-gated sodium (Na\textsubscript{v}) channel family

As discussed, Na\textsubscript{v} channels are one of three major classes of voltage-gated ion channels that mediate cellular electrical excitability. They are responsible for AP initiation and propagation in electrically excitable cells, including nerve, muscle and neuroendocrine cell types (Hodgkin and Huxley 1952; Hille 1986; Marban et al. 1998). They are also expressed at very low levels in non-excitatory cells, where their physiological role remains unclear.

The creation and conduction of APs represents the fundamental means of communication in excitable cells and tissues (Figure 1.2), and Na\textsuperscript{+} entry through Na\textsubscript{v} channels in response to membrane depolarisation is an absolutely critical event in the generation of APs (Brown 1992). At the resting membrane potential of a cell, Na\textsubscript{v} channels are closed. However, if the membrane potential rises to a precisely defined threshold value, they rapidly adopt an open conformation. This results in Na\textsuperscript{+} influx, thus altering the local electrochemical gradient immediately surrounding the channel, which in turn produces a further rise in the membrane potential, causing more Na\textsubscript{v} channels to open. With all available Na\textsubscript{v} channels open, there is an explosive entry of Na\textsuperscript{+} into the cell and a large depolarisation of the cell membrane. This rapid influx of Na\textsuperscript{+} into the cell causes the polarity of the cell membrane to reverse and the Na\textsubscript{v} channels rapidly inactivate before closing. The slower activation of K\textsubscript{v} channels in response to membrane depolarisation results in the efflux of K\textsuperscript{+} from the cell following the rising phase of the AP, bringing about a repolarisation of the cell membrane. After an AP has fired, Na\textsuperscript{+} and K\textsuperscript{+} are actively transported via the Na\textsuperscript{+}-K\textsuperscript{+} pump, returning the membrane potential and electrochemical gradients for the two ions to their resting states. Electrical impulses, initiated by Na\textsubscript{v} channels, are communicated over large distances in electrically excitable cells and tissues via these self-propagating APs, and at the synapse of a neuron the electrical signal is converted to a chemical signal that is then propagated to the postsynaptic cell.
Figure 1.2: Action potential propagation in electrically excitable cells. (A) At the leading edge of an AP, Na\textsubscript{v} channels open, allowing Na\textsuperscript{+} to flow into the cell down its electrochemical gradient. This influx of Na\textsuperscript{+} results in a further depolarisation of the membrane immediately surrounding the channel, thus triggering more Na\textsubscript{v} channels to open and causing the AP to move on. The trailing edge of an AP undergoes a refractory period where subsequent APs cannot be generated. K\textsubscript{v} channels open and Na\textsubscript{v} channel inactivation gates close. The movement of K\textsuperscript{+} out of the cell through the K\textsubscript{v} channels repolarises the cell membrane, restoring the resting potential of the cell. (B) A schematic of a typical AP reveals the role played by ion flux during the propagation of an electrical impulse. When Na\textsubscript{v} channels open in response to a stimulus, the flow of Na\textsuperscript{+} into the cell results in depolarisation of the membrane. If the entry of Na\textsuperscript{+} into the cell is sufficient to drive the membrane potential from -70 mV up to -55 mV (the threshold value for an AP), the process continues. Red lines represent sub-threshold initiations. If the threshold value is reached, the subsequent influx of Na\textsuperscript{+} depolarises the membrane to about +30 mV. The Na\textsubscript{v} channels inactivate and close and K\textsubscript{v} channels open, allowing the membrane to repolarise back towards its resting potential. Since K\textsubscript{v} channels are much slower to open than Na\textsubscript{v} channels, the depolarisation has time to be completed. Repolarisation of the membrane typically overshoots the resting potential to about -90 mV. This hyperpolarisation prevents another AP from firing by raising the threshold for a new stimulus. It also ensures that an impulse is propagated in just one direction. Following hyperpolarisation, the Na\textsuperscript{+}-K\textsuperscript{+} pump returns the membrane to its resting state of -70 mV.
1.3 \( Na_v \) channel discovery and classification

\( Na_v \) channels are the founding members of the ion channel superfamily in terms of both their discovery as a protein and determination of their amino acid sequence. However, in evolutionary terms, the \( Na_v \) channel family is the most recent of the voltage-gated ion channels to have arisen, having evolved from similarly structured \( Ca_v \) channels, which are also monomeric proteins composed of four homologous domains: since protozoa and plants have \( K_v \) and \( Ca_v \) channels but do not appear to possess \( Na_v \) channels, it is believed that \( Na_v \) channels probably evolved from \( Ca_v \) channels (Strong et al. 1993). \( Ca_v \) channels, in turn, probably arose following two rounds of gene duplication from the ancestors of \( K_v \) channels, whose structure comprises four homologous single-domain subunits in a homotetramer. Domains I and III of \( Na_v \) channels are the most similar, as are domains II and IV. Based on these similarities, the ancestral \( Ca_v/Na_v \) channel probably duplicated into a two-subunit channel consisting of domains I-III and II-IV, and this channel then duplicated to result in the structure I, II, III and IV (Hille 1989; Strong et al. 1993). This notion is supported by the finding of a \( Ca_v \) channel \( \alpha \)-subunit isoform, cloned from neonatal skeletal muscle, that possesses only two domains (Malouf et al. 1992). Furthermore, certain \( K_v \) channel protein dimers, constructed by \( \alpha \)-subunit concatenation, are able to form functional channels (Isacoff et al. 1990). The emergence of four-domain \( Na_v \) channels coincided with the evolution of metazoans that had specialised neurons (Anderson and Greenberg 2001), and whilst four-domain \( Na_v \) channels have not been found in prokaryotes, a single-domain \( Na_v \) channel has been identified in the salt-loving bacterium \textit{Bacillus halodurans} (Ren et al. 2001). The channel shares sequence and pharmacological characteristics with \( Ca_v \) channels, suggesting it may be similar to the ancestral single-domain channel from which the \( Ca_v \) and \( Na_v \) channels have arisen.

\( Na_v \) channels have been identified in a variety of animals, including flies, leeches, squid and jellyfish, as well as mammalian and non-mammalian vertebrates (Anderson and Greenberg 2001; Goldin 2002). The biophysical properties, pharmacology, gene organisation and even intron splice sites of invertebrate \( Na_v \) channels are largely similar to the mammalian \( Na_v \) channels, supporting the idea that the primordial \( Na_v \) channel was established before the
evolutionary divergence of the invertebrates from the vertebrates (Plummer and Meisler 1999). Vertebrate α-subunit genes, particularly those encoding the human and rodent channels, are the best characterised to date; all of the different isoforms have been identified and cloned as full length channels in both species. At least 20 exons encode each of the nine Na_v channel α-subunit isoforms (Na_v1.1-1.9) and an evolutionary analysis reveals that they occupy four distinct groups, with members of each group mapping to the same chromosomal segment (Figure 1.3). The four chromosome segments containing the Na_v channel genes are paralogous, and each also contains a cluster of Hox genes, which encode transcription factors involved in the control of developmental patterning (Plummer and Meisler 1999). The genes encoding Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.7 and Na_x are clustered together on chromosome 2 in humans and mice, and form the largest branch of the phylogenetic tree. Na_v1.1, Na_v1.2, Na_v1.3 and Na_v1.7 share a number of characteristics, including sequence similarity, biophysical properties, expression in the nervous system and block by nanomolar concentrations of tetrodotoxin (TTX) (Goldin et al. 2000). Na_x is unusual in that it has sequence differences in functionally important regions, including fewer charged residues in the S4 helix and the absence of the IFM motif that is critical for fast inactivation (George et al. 1992; Goldin 2001). Knockout mouse studies suggest that this channel has a physiological function (associated with salt intake) other than as a voltage-gated channel (Watanabe et al. 2000; Hiyama et al. 2002). A second cluster of genes encoding the Na_v1.5, Na_v1.8 and Na_v1.9 isoforms is located on chromosome 3 in humans and mice. These isoforms also share characteristics, including the fact that they are all classified as TTX-resistant Na_v channels, requiring micromolar concentrations of TTX before they are blocked. The remaining two isoforms, Na_v1.4 (expressed in skeletal muscle) and Na_v1.6 (abundant in the central nervous system), each represent a separate branch of the tree and the genes encoding the channels are on separate chromosomes. The gene for Na_v1.4 is located on chromosome 11 in humans and chromosome 17 in mice, and the gene for Na_v1.6 is located on chromosome 15 in humans and chromosome 12 in mice. These two channels share greater than 85% sequence identity and similar functional properties to the chromosome 2-encoded channels, including TTX sensitivity in the nanomolar concentration range.
Due to the varying sensitivities of the different Na\textsubscript{v} channel isoforms to TTX, Na\textsubscript{v} channels can be grouped into one of two categories: TTX-sensitive and TTX-resistant channels (Table 1.1). Although they are approximately 75% identical in amino acid sequence to the group of channels on chromosome 2, the three TTX-resistant channels located on chromosome 3 (Na\textsubscript{v}1.5, Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9) have amino acid changes that confer varying degrees of TTX resistance. Na\textsubscript{v}1.5, the principal cardiac isoform, has a single amino acid change from phenylalanine to cysteine in the pore region of domain I, which is responsible for a 200-fold reduction in TTX-sensitivity relative to the TTX-sensitive channels (Satin et al. 1992). At the same position in Na\textsubscript{v}1.8 and Na\textsubscript{v}1.9 (both preferentially expressed in peripheral sensory neurons (Akopian et al. 1999; Dib-Hajj et al. 2002)) the residue is serine. This change results in even greater resistance to TTX (Sivilotti et al. 1997). In addition to their sensitivity to TTX, Na\textsubscript{v} channels are often classified according to their tissue distribution. In fact, based on sequence comparisons between Na\textsubscript{v} channels from human and rat, there is greater similarity among the channels from the same tissue than from the same species, indicating that the tissue specialisation of Na\textsubscript{v} channels preceded the separation of at least humans and rats. The tissue-specific diversity also suggests that each Na\textsubscript{v} channel isoform may be optimised either for specialised function or for subcellular localisation in the tissue of origin.
Figure 1.3: Phylogenetic relationships of human Na_v channel α-subunits. The position of the different subtypes in the tree correlates with their chromosomal location.

<table>
<thead>
<tr>
<th>Channel isoform</th>
<th>Distribution</th>
<th>TTX EC_{50} (nM)</th>
<th>TTX sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na_v 1.1</td>
<td>CNS</td>
<td>6 (Clare et al. 2000)</td>
<td>TTX-sensitive</td>
</tr>
<tr>
<td>Na_v 1.2</td>
<td>CNS</td>
<td>12 (Catterall et al. 2005)</td>
<td>TTX-sensitive</td>
</tr>
<tr>
<td>Na_v 1.3</td>
<td>CNS</td>
<td>4 (Chen et al. 2000)</td>
<td>TTX-sensitive</td>
</tr>
<tr>
<td>Na_v 1.4</td>
<td>Skeletal muscle</td>
<td>25 (Chahine et al. 1994)</td>
<td>TTX-sensitive</td>
</tr>
<tr>
<td>Na_v 1.5</td>
<td>Heart</td>
<td>2,000 (Clare et al. 2000)</td>
<td>TTX-resistant</td>
</tr>
<tr>
<td>Na_v 1.6</td>
<td>CNS</td>
<td>2.5 (Burbidge et al. 2002)</td>
<td>TTX-sensitive</td>
</tr>
<tr>
<td>Na_v 1.7</td>
<td>PNS</td>
<td>25 (Klugbauer et al. 1995)</td>
<td>TTX-sensitive</td>
</tr>
<tr>
<td>Na_v 1.8</td>
<td>PNS</td>
<td>60,000 (Akopian et al. 1996)</td>
<td>TTX-resistant</td>
</tr>
<tr>
<td>Na_v 1.9</td>
<td>PNS</td>
<td>200,000 (Rugiero et al. 2003)</td>
<td>TTX-resistant</td>
</tr>
</tbody>
</table>

The three TTX-resistant channels have TTX sensitivity in the micromolar range. CNS, central nervous system; PNS, peripheral nervous system.
1.4 Structure and function of Na\textsubscript{v} channels

The primary function of Na\textsubscript{v} channels is to control the flux of Na\textsuperscript{+} into electrically excitable cells in response to membrane depolarisation. Classical work by Hodgkin and Huxley defined the three key features of Na\textsubscript{v} channels that allow this to happen: voltage-dependent activation, rapid inactivation and selective ion conductance (Hodgkin and Huxley 1952). Since the first Na\textsubscript{v} channel was cloned in 1984 (Noda et al. 1984), a plethora of studies investigating the relationship between Na\textsubscript{v} channel structure and function have allowed us to understand the structural features of Na\textsubscript{v} channels that enable them to possess these three important properties.

Na\textsubscript{v} channels consist of a highly processed α-subunit, associated with one or more auxiliary β-subunits (Figure 1.4). The auxiliary subunits are not essential for channel function, as demonstrated by the synthesis of functional Na\textsubscript{v} channels following injection of only the α-subunit into Xenopus oocytes (Noda et al. 1986; Joho et al. 1990), but do modulate channel gating and regulate the degree of channel expression at the cell surface. As discussed, the pore-forming α-subunit is a single polypeptide, approximately 260 kDa in size, consisting of four homologous domains (I-IV). Each domain comprises six transmembrane α-helices (S1-S6), connected by intracellular and extracellular polypeptide loops. In each of the domains, the voltage-sensor is located in the S4 helix, which is composed of repeated motifs of one positively charged amino acid residue (lysine or arginine) followed by two hydrophobic residues, potentially creating a helical arrangement of positive charges through the membrane. As is the case for all voltage-gated ion channels, the voltage-dependence of activation of Na\textsubscript{v} channels derives from the screwlike rotation of the positively charged S4 helix toward the outer membrane surface, and the concurrent conformational change that opens the channel pore, as a result of an altered electric field across the membrane following membrane depolarisation (Hodgkin and Huxley 1952; Bezanilla 2000). This ‘helical screw’ (Guy and Seetharamulu 1986) model of channel activation is supported by strong evidence (Catterall 2000). Notably, when the positive residues of the S4 helix are neutralised in Na\textsubscript{v} channels, there is a marked reduction in the voltage-dependence of gating (Stuhmer et al. 1989). The region between the S5 and S6 helices of each domain consists of a stretch of 20 amino acids.
which loops into the membrane as a pair of antiparallel $\beta$-strands, forming the extracellular end of the channel pore. This P-loop region is the narrowest part of the pore and provides the ion selectivity filter for the channel. Two important amino acids in analogous positions in all four domains are thought to form the negatively charged outer and inner rings that serve as the selectivity filter and the receptor site for TTX. Mutations of these residues have significant effects on the affinity of TTX to $\text{Na}_v$ channels, and also have marked effects on the selectivity of permeation of monovalent cations through the channel (Heinemann et al. 1992; Schlief et al. 1996; Sun et al. 1997). The linker regions connecting the four domains are all located on the cytoplasmic side of the membrane, as are the amino- and carboxy- termini.

$\text{Na}_v$ channels are subject to regulation at the levels of gene transcription (developmentally regulated and tissue restricted), subunit interaction and post-translational modification. Regulation of $\text{Na}_v$ channels by phosphorylation is complex. Neuronal isoforms contain four consensus sites for protein kinase A (PKA) phosphorylation and one consensus site for protein kinase C (PKC) phosphorylation in the intracellular linker between domains I and II. The cardiac channel has eight candidate consensus PKA sites in the I-II linker, all of which are distinct from the neuronal channels. However, in vivo studies of the expressed cardiac channel demonstrate cyclic adenosine monophosphate (cAMP)-dependent phosphorylation of only two serine residues (Murphy et al. 1996). PKA differentially modulates the function of neuronal and cardiac $\text{Na}_v$ channels, whilst function of the skeletal muscle isoform is not affected by PKA (Smith and Goldin 1992; Smith and Goldin 1996), despite the fact that the channel is an excellent substrate for PKA-mediated phosphorylation (Yang and Barchi 1990). Phosphorylation of sites in the I-II linker of the brain channel reduces current amplitude without significantly affecting gating (Gershon et al. 1992; Li et al. 1993; Murphy et al. 1993; Smith and Goldin 1996; Cantrell et al. 1997; Smith and Goldin 1997), whereas when the cardiac channel is phosphorylated by PKA, the whole-cell conductance increases (Schreibmayer et al. 1994; Murphy et al. 1996; Frohnwieser et al. 1997). This serves to highlight the fact that the importance of $\text{Na}_v$ channel phosphorylation is not always clear, but does point to the I-II linker region as a major site of neuromodulation of $\text{Na}_v$ channels. In addition to alterations in channel gating, phosphorylation of $\text{Na}_v$ channel $\alpha$-subunits by PKA can result in the promotion of intracellular protein trafficking (Chahine et al.
In adrenal chromaffin cells, PKA increases Na\textsubscript{v}1.7 expression in the plasma membrane by changing the balance of intracellular protein trafficking in favour of externalisation from Golgi (Wada et al. 2004). Similarly, in dorsal root ganglion (DRG) neurons, the PKA-mediated increase in Na\textsubscript{v}1.8 current amplitude is prevented by the Golgi blocker, chloroquine, suggesting that outward trafficking is involved (Vijayaragavan et al. 2004). In contrast to PKA, PKC alters the function of all of the mammalian Na\textsubscript{v} channel isoforms by reducing the maximal conductance of the channels and altering gating in an isoform-specific manner (Numann et al. 1991; West et al. 1991; West et al. 1992; Li et al. 1993; Numann et al. 1994; Bendahhou et al. 1995; Qu et al. 1996; Murray et al. 1997; Cantrell and Catterall 2001). The PKC effect is largely attributed to phosphorylation of a highly conserved serine in the intracellular linker between domains III and IV.

All of the Na\textsubscript{v} channel subunits undergo glycosylation. The brain and muscle α-subunits, as well as the auxiliary β1 and β2-subunits, are heavily glycosylated, with up to 40% of the mass being carbohydrate (James and Agnew 1989). In contrast, only 5% of the cardiac α-subunit is sugar by weight (Cohen and Levitt 1993). The addition of highly charged carbohydrates such as sialic acid, a prominent component of the N-linked carbohydrate of Na\textsubscript{v} channels, has been shown to be important in ion permeation and the voltage-dependence of gating of Na\textsubscript{v} channels (Terlau et al. 1991; Chiamvimonvat et al. 1996; Bennett et al. 1997). Furthermore, co-translational glycosylation has been shown to be important for the maintenance of Na\textsubscript{v} channel cell surface expression (Ritchie 1988; Zona et al. 1990).

Inactivation of Na\textsubscript{v} channels is a critical process that occurs within milliseconds of channel opening. Na\textsubscript{v} channels can exist in three distinct states: deactivated (closed), activated (open) and inactivated (closed). Channels in the deactivated state are blocked on their intracellular side by an activation gate, which is removed in response to membrane depolarisation. Inactivation takes place soon after channel activation and involves the spontaneous closing of the channel, generating an inactive channel that will not reopen until the membrane is repolarised. The ability of the channel to inactivate once it is opened is due to the highly conserved intracellular loop that connects domains III and IV. It is referred to as the inactivation gate and consists of a rigid α-helical structure flanked by three key hydrophobic
residues (IFM) that folds into and blocks the Na\(^+\)-conducting pore until the membrane is repolarised. Peptides containing this motif can restore inactivation to Na\(_v\) channels that have a mutated inactivation gate (Eaholtz et al. 1994). In contrast to the short IFM motif that forms the inactivation gate, the receptor for the inactivation gate on the body of the channel may be composed of multiple hydrophobic residues near the intracellular mouth of the channel pore. Inactivation of Na\(_v\) channels is very important in cell physiology as it prevents excessive depolarisation of membranes and it enables APs in electrically excitable cells to be directional because an inactivated channel cannot reopen until the membrane is repolarised. A small fraction of Na\(^+\) current, termed the persistent sodium current (\(I_{Na,P}\)), fails to inactivate significantly with all Na\(_v\) channels, although the size of the \(I_{Na,P}\) is isoform-dependent (Kiss 2008). It can result in prolonged APs and elevated Na\(^+\) levels in electrically excitable cells. Na\(_v\) channel mutations that increase \(I_{Na,P}\) lead to altered cellular excitability and have been implicated in neurological diseases such as epilepsy (Meisler and Kearney 2005). Hypoxia has been shown to enhance \(I_{Na,P}\) in cells (Ju et al. 1996), and more recently, enhanced \(I_{Na,P}\) has been identified as a characteristic of cancer cells which reside in hypoxic environments (Djamgoz and Onkal 2012).

A remarkable feature of monovalent cation-selective channels is their ability to discriminate between ions of the same charge and similar size. The mechanism of K\(^+\) selectivity has been studied extensively, whereas Na\(^+\) selectivity is less well-defined, principally due to a lack of high-resolution 3D structures until very recently (Payandeh et al. 2011; Payandeh et al. 2012; Zhang et al. 2012). As discussed previously, Na\(_v\) channels have a similar structure to K\(_v\) channels and are thought to have evolved from K\(_v\) channels by gene duplications. However, Na\(_v\) channels appear to achieve their selectivity for Na\(^+\) over K\(^+\) by a very different mechanism. In contrast to K\(_v\) channels, the four Na\(_v\) channel P-loops between the S5 and S6 helices of each domain are splayed out, leaving only a short selectivity region (Hille 1975; Lipkind and Fozzard 1994; Yamagishi et al. 1997). In addition, the cation remains mostly hydrated and cation interaction in the Na\(_v\) channel selectivity filter is with flexible side chains of negatively and positively charged residues of the P-loops, as opposed to the more constrained main chain carbonyls (Hille 1971). Four key residues of the selectivity region have been identified as a DEKA motif, consisting of an amino acid from each of the
four P-loops from domains I-IV, respectively. Binding of TTX requires these residues for high affinity (Terlau et al. 1991; Penzotti et al. 1998) and mutations of these residues significantly alter cation permeation and selectivity (Terlau et al. 1991; Chiamvimonvat et al. 1996; Favre et al. 1996; Schlief et al. 1996; Tsushima et al. 1997; Lipkind and Fozzard 2008). In fact, by mutating these inner ring residues from DEKA to EEEE, their counterparts in CaVCa channels, it is possible to produce a Ca^{2+}-selective NaV channel (Heinemann et al. 1992). The precise mechanism of selectivity of Na^+ over K^+ remains unclear, despite such mutational studies. Presumably, NaV channels favour Na^+ over K^+ because the more constricted part of the channel pore is only 5 Å in diameter and so can accommodate Na^+.H_2O, but not K^+.H_2O, which is larger than 5 Å. Some K^+.H_2O does, however, get through owing to the flexibility of the channel, but sodium is strongly selected for.

As mentioned previously, the principal reason for many of the poorly-defined structural features of NaV channels to date has been the lack of high-resolution 3D structures. William Catterall’s group were the first to successfully determine the crystal structure of a NaV channel (Payandeh et al. 2011), and this exciting development has shed interesting light on the structural features of NaV channels and how they relate to channel function. The bacterial channel from *Arcobacter butzleri* (NaVAb) was captured in a closed-pore conformation at 2.7 Å resolution. The structure now provides a template for understanding electrical signalling in excitable cells and the actions of drugs at the atomic level, opening up an exciting period of research into NaV channels. Since the crystal structure of this channel was determined, the same channel has also been captured in two potentially inactivated states at 3.5 Å resolution (Payandeh et al. 2012), and the crystal structure of an orthologue of the Na^+-selective channel of bacteria (NaChBac) from *alphaproteobacterium HIMB114* (*Rickettsiales sp.*), termed NaVRh, has also been determined (Zhang et al. 2012).
Figure 1.4: Diagrammatic representation of the Na\textsubscript{v} channel α-subunit. The α-subunit consists of four homologous transmembrane domains (I-IV), each of which contains six transmembrane α-helices (S1-S6). A re-entrant loop between the S5 and S6 helices of each domain forms the ion-selective filter and the extracellular end of the pore. The S4 helix in each domain is the voltage sensor. It contains positively charged residues, and upon membrane depolarisation, the screwlike movement of the S4 helix results in a conformational change in the protein that opens the channel pore. G, sites of probable N-linked glycosylation; P, sites of protein phosphorylation by PKA (circles) and PKC (diamonds); I, sites implicated in forming the inactivation gate. The region located on the intracellular loop between domains III and IV is believed to be the inactivation gate particle of the inactivation gate loop; green circles, the area containing the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion selectivity filter and TTX binding site. Binding sites for α- and β-scorpion toxins (ScTx) and the site of interaction between the α-subunit and β1-subunit are also shown.
1.5 Na\textsubscript{v} channel pharmacology

All of the pharmacological agents that act on Na\textsubscript{v} channels have receptor sites on the α-subunits. At least six distinct receptor sites for neurotoxins and one receptor site for local anaesthetics and related drugs have been identified (Cestele and Catterall 2000).

Amino acid residues in the S6 helices from at least three of the four channel domains (I, III and IV) contribute to the complex drug receptor site for local anaesthetics and related antiepileptic and antiarrythmic drugs, with the S6 helix of domain IV playing the dominant role. The drugs bind to overlapping receptor sites located in the inner cavity of the pore of the channel where they block it in a voltage-dependent manner (Ragsdale et al. 1994; Catterall 2000). Block is enhanced at depolarised potentials, suggesting that local anaesthetics act as allosteric effectors for inactivation gating: when they bind to the channel, they facilitate inactivation (Balser et al. 1996).

Pharmacological competition studies and mutagenesis have defined a number of neurotoxin binding sites in Na\textsubscript{v} channels. Neurotoxin receptor site 1 binds the non-peptide pore blockers TTX and saxitoxin (STX), and the peptide blocker μ-conotoxin (Fozzard and Hanck 1996; Terlau and Stuhmer 1998; Catterall 2000). The receptor sites for these toxins are formed by amino acid residues in the P-loops and immediately on the extracellular side of the P-loops at the outer end of the pore. As discussed previously, a single amino acid residue in the P-loop of domain I accounts for most of the Na\textsubscript{v} channel isoform-specific TTX-sensitivity: an aromatic residue (phenylalanine or tyrosine) at this position confers high TTX affinity, whereas its absence renders the channel TTX-resistant (Satin et al. 1992; Sivilotti et al. 1997). Neurotoxin receptor site 2 binds a family of lipid-soluble toxins which enhance activation of Na\textsubscript{v} channels. These include veratridine, batrachotoxin, aconitine and grayanotoxin. It is thought that the S6 helices of domains I and IV contain the receptor site for batrachotoxin (Cestele and Catterall 2000). Neurotoxin receptor site 3 binds the α-scorpion toxins and the sea anemone toxins, which slow the coupling of Na\textsubscript{v} channel activation to inactivation. These peptide toxins bind to a complex receptor site that includes the extracellular loop between the S3 and S4 helices of domain IV (Rogers et al. 1996; Cestele and Catterall 2000). The fact that
these toxins are capable of slowing current decay by binding to an external site remote from the inactivation gate emphasises the importance of disparate regions of the channel in inactivation gating. Neurotoxin receptor site 4 binds the β-scorpion toxins, which enhance activation of Na$_v$ channels. The receptor site for these toxins include the extracellular loop between the S3 and S4 helices of domain II (Cestele and Catterall 2000). Neurotoxin receptor site 5 binds the complex polyether toxins brevetoxin and ciguatoxin, which are produced naturally by dinoflagellates. The toxins work by lowering the threshold for channel activation. The S5 and S6 helices of domains IV and I, respectively, are implicated in brevetoxin binding (Cestele and Catterall 2000). Finally, neurotoxin receptor site 6 binds δ-conotoxins, which, like the α-scorpion toxins, slow the rate of channel inactivation, resulting in a prolonged AP. The location of receptor site 6 remains unidentified, although there is some evidence to suggest that δ-conotoxins interact with a conserved hydrophobic triad (YFV) located in the extracellular linker between the S3 and S4 helices of domain IV in a position almost identical to that of receptor site 3, which binds the α-scorpion toxins (Leipold et al. 2005).

1.6 Alternative splicing of Na$_v$ channels

The different Na$_v$ channel isoforms are functionally and pharmacologically diverse, suggesting that they may be optimised for specialised functions, as supported by their tissue-specific distribution. The potential for further Na$_v$ channel diversity is increased considerably by alternative mRNA splicing for a specific Na$_v$ channel subtype. In this way, several slightly different Na$_v$ channels with substituted modulatory domains can be generated from a single gene, permitting additional fine-tuning of Na$_v$ channel activity. To date, five major sites for alternative exon usage have been identified, enabling inclusion, exclusion or substitution of amino acid residues in modulatory regions (Figure 1.5). The proportion of differentially spliced transcripts depends on a number of factors, including age of development, the tissue of origin and the presence of modulatory agents such as cAMP (Gustafson et al. 1993; Plummer et al. 1997; Dietrich et al. 1998; Oh and Waxman 1998). However, electrophysiological differences arising from alternative splicing have only been demonstrated for two alternatively spliced forms of Na$_v$1.6 (Dietrich et al. 1998), so it remains unclear to what degree alternative splicing of Na$_v$ channels contributes to their functional diversity.
Figure 1.5: Exon structure of Na$_v$ channels. Thick black bars represent conserved exon boundary positions in all Na$_v$ channel isoforms. Five major sites for alternative exon usage have been identified, enabling inclusion, exclusion or substitution of amino acid residues in modulatory regions of the protein: (1) Domain 1:segment 3 (D1:S3) splicing, (2) Interdomain 1-2 (ID1-2) splicing, (3) Interdomain 2-3 (ID2-3) splicing, (4) Domain 3 (D3) splicing, (5) Domain 4:segment 3 (D4:S3) splicing.
1.7 The auxiliary β-subunits

\( \text{Na}_v \) channels exist as macromolecular complexes \textit{in vivo}; the pore-forming \( \alpha \)-subunit is associated with at least one auxiliary \( \beta \)-subunit in a 1:1 stoichiometry for \( \alpha:\beta \) (Catterall 1992). Five mammalian \( \beta \)-subunits have been identified (\( \beta 1, \beta 1B, \beta 2, \beta 3 \) and \( \beta 4 \)), encoded by four genes (\( SCN1B-SCN4B \)) and named in order of discovery (Table 1.2). With the exception of the \( SCN1B \) splice variant, \( \beta 1B \), the \( \beta \)-subunits are type I transmembrane glycoproteins with an extracellular N-terminus and a cytoplasmic C-terminus (Figure 1.6). In contrast, \( \beta 1B \) lacks a transmembrane domain and so exists as a secreted protein (Patino et al. 2011). As members of the immunoglobulin (Ig) superfamily, these subunits contain a V-set Ig loop in their extracellular/N-terminal domain and are homologous to neuronal cell adhesion molecules (NrCAMs) and the large family of L1 CAMS: they share no homology with their Ca\( _v \) and K\( _v \) channel counterparts (Catterall 2000). The tissue-specific expression profiles of each of the \( \beta \)-subunits are subtly different, but clearly overlapping. As with the \( \alpha \)-subunits, \( \beta \)-subunits are highly expressed in electrically excitable cells. Importantly, however, growing evidence suggests that \( \beta \)-subunits are expressed in a broad range of traditionally non-excitable cells, including stem cells, glia, vascular endothelial cells and carcinoma cells (Diss et al. 2008; Chioni et al. 2009; Andrikopoulos et al. 2011).

The precise functional role of the \( \beta \)-subunits is not fully understood. However, a growing body of work reveals that they can be considered diverse, multifunctional proteins. They are certainly not essential for a functional channel, since \( \text{Na}_v \) channel activity is observed when the \( \alpha \)-subunit is expressed in heterologous systems on its own, indicating that it alone possesses all the necessary structural elements for channel formation. Whilst not essential for channel function, \( \beta \)-subunits modulate channel gating, voltage-dependence and kinetics of \( \alpha \)-subunits, as well as regulate the degree of channel expression at the cell surface. As a result, they do regulate cellular excitability \textit{in vivo}. Numerous studies have demonstrated that all five \( \beta \)-subunits alter gating and kinetics of \( \alpha \)-subunits expressed in heterologous cells (Catterall 2000; Kazen-Gillespie et al. 2000; Qin et al. 2003; Yu et al. 2003). This work started with the initial observations that \( \beta 1 \) and \( \beta 2 \) increase the peak Na\(^+ \) current through \( \text{Na}_v 1.2 \), as well as accelerate inactivation and shift the voltage-dependence of activation and inactivation to more
negative potentials (Isom et al. 1992; Isom et al. 1994; Isom et al. 1995), and the α-subunit of the rat skeletal muscle channel isoform inactivates more slowly than the native channel, whereas channels formed by coexpression of both α- and β-subunits inactivate at a similar rate to the native channel (Ji et al. 1994). However, inconsistencies between different reports documenting current amplitude and modulation suggest that the cell background is a critical consideration when interpreting the data (Moran et al. 2000; Moran et al. 2003; Meadows and Isom 2005). Furthermore, some of the effects of β-subunits on Na⁺ current in heterologous cells, especially *Xenopus* oocytes, do not appear to be reflected *in vivo*; the effects can be subtle and cell type-specific (Chen et al. 2002; Chen et al. 2004; Aman et al. 2009; Patino et al. 2009; Brackenbury et al. 2010).

In addition to their roles in channel modulation, as members of the Ig superfamily of CAMs, β-subunits participate in a number of non-conducting activities where they regulate cell adhesion and cell migration (Isom et al. 1995; Isom 2001; Yu et al. 2003; Brackenbury and Isom 2011). CAMs of the Ig superfamily interact homophilically and heterophilically to transduce signals between adjacent cells or adjacent axons where they participate in, for example, axonal fasciculation, growth cone guidance and nodal formation. It has been shown that CAMs of the L1 family interact homophilically in a *trans* mechanism to encourage cellular aggregation (Hortsch and Bieber 1991; Kamiguchi and Lemmon 1997). Both β1 and β2 participate in *trans*-homophilic adhesion, resulting in cellular aggregation and recruitment of ankyrin and spectrin to points of cell-cell contact (Malhotra et al. 2000). By contrast, β3, in spite of its high homology to β1, does not participate in homophilic adhesion, but mediates heterophilic adhesion (McEwen et al. 2009).

The β-subunits interact heterophilically with several other CAMs and extracellular matrix (ECM) proteins. β1, for example, interacts with β2, contactin, neurofascin-155 (NF155), neurofascin-186 (NF186), NrCAM, tenascin-R and N-cadherin (Kazarinova-Noyes et al. 2001; Ratcliffe et al. 2001; Malhotra et al. 2004; McEwen and Isom 2004). Interaction between β1 and β2, contactin or NF186 increases Na⁺ currents in heterologous systems, suggesting that β-subunit-dependent adhesion may regulate α-subunit function (Kazarinova-Noyes et al. 2001; McEwen et al. 2004). Less is known about the heterophilic interactions of
the other β-subunit isoforms. β2 is known to interact with tenascin-C (Srinivasan et al. 1998) but does not interact with contactin (McEwen et al. 2004), whereas β3, which does not interact with either β1 or contactin, does interact with NF186 (Ratcliffe et al. 2001; McEwen et al. 2009). Although similar studies have not been performed for β1B, it is logical to assume that its heterophilic binding partners are likely similar to those of β1, given that both molecules share an identical Ig domain (Patino and Isom 2010).
Table 1.2: Na\(_v\) channel \(\beta\)-subunit isoforms.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene product</th>
<th>Interacting proteins</th>
<th>Distribution</th>
<th>Disease association</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN1B</td>
<td>(\beta1)</td>
<td>Na(_v)1.1-1.7, ankyrin(B), ankyrin(G), (\beta1, \beta2, ) contactin, K(_v)4.3, NF155, NF186, N-cadherin, NrCAM, tenascin-R, RPTP(\beta)</td>
<td>CNS, PNS, heart, skeletal muscle, glia</td>
<td>Epilepsy, cardiac arrhythmia, cancer</td>
</tr>
<tr>
<td>SCN1B</td>
<td>(\beta1B)</td>
<td>(\beta1)</td>
<td>CNS, PNS, heart, skeletal muscle, glia</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>SCN2B</td>
<td>(\beta2)</td>
<td>Na(_v)1.1, Na(_v)1.2, Na(_v)1.5-1.7, ankyrin(G), (\beta1, \beta2, ) tenascin-C, tenascin-R</td>
<td>CNS, PNS, heart, glia</td>
<td>Altered pain response, cardiac arrhythmia, MS, seizure susceptibility</td>
</tr>
<tr>
<td>SCN3B</td>
<td>(\beta3)</td>
<td>Na(_v)1.1-1.3, Na(_v)1.5, NF186</td>
<td>CNS, PNS, heart, adrenal gland, kidney</td>
<td>Epilepsy, cardiac arrhythmia, traumatic nerve injury</td>
</tr>
<tr>
<td>SCN4B</td>
<td>(\beta4)</td>
<td>Na(_v)1.1, Na(_v)1.2, Na(_v)1.5, (\beta1)</td>
<td>CNS, PNS, heart, skeletal muscle</td>
<td>Huntington’s disease, long-QT syndrome</td>
</tr>
</tbody>
</table>

CNS, central nervous system; MS, multiple sclerosis; NF155, neurofascin-155; NF186, neurofascin-186; NrCAM, neuronal cell adhesion molecule; PNS, peripheral nervous system; RPTP\(\beta\), receptor protein tyrosine phosphatase \(\beta\).
Figure 1.6: Structure of Na\textsubscript{v} channel β-subunits. The pore-forming α-subunit of Na\textsubscript{v} channels is associated with one or more auxiliary β-subunits. β1, β2, β3 and β4 contain an extracellular immunoglobulin (Ig) loop, transmembrane domain and an intracellular C-terminal domain. β1B also contains an Ig loop but has a C-terminus lacking a transmembrane domain and so exists as a soluble, secreted protein (Patino et al. 2011). β2 and β4 are disulphide linked to α-subunits, whereas β1 and β3 associate non-covalently. Mutation sites responsible for causing genetic epilepsy with febrile seizures (GEFS+1), temporal lobe epilepsy (TLE) and Dravet syndrome are located in the extracellular Ig loop of β1. Alternative splicing site for β1B, putative palmitoylation site, ankyrin interaction site, tyrosine phosphorylation site, N-glycosylation sites (Ψ) and α-secretase cleavage site are also marked.
1.8 Cancer

Cancer is a disease of cells in the body and usually develops from a single somatic cell by the process broadly referred to as carcinogenesis. The development of a cancerous phenotype is a complex process involving the accumulation of multiple independent mutations that ultimately leads to deregulation of cell signalling pathways central to the control of cell growth and cell fate (Renan 1993; Bild et al. 2006). This results in a suppressed response to the growth limitations observed in normal cells, leading to the development of a mass clone of cells (a tumour), often with impaired function. The current view of cancer which states that a malignancy arises from a transformation of the genetic material of a normal cell, followed by successive mutations, ultimately leading to the uncontrolled proliferation of progeny cells, can be traced to seminal works of many scientists. Boveri formulated the ‘somatic mutation’ hypothesis of the origin of cancer in 1929 (McKusick 1985), Berenblum and Shubik demonstrated the multistep, sequential nature of carcinogenesis (the initiation, promotion, progression model) (Berenblum and Shubik 1947; Berenblum and Shubik 1949), Knudson demonstrated that an inherited defect in one allele of a protective gene (tumour suppressor) can predispose an individual to cancer (Knudson 1985) and Nowell proposed the clonal evolution model of tumour progression which postulated that when a cell acquires a specific genetic alteration it may develop a proliferative advantage, and clonal expansion of the cell, driven by successive mutation, could lead to tumour progression (Nowell 1976).

The cells and tissues comprising a cancer differ from normal in that there is loss of cell number control, acquisition of a blood supply via angiogenesis, loss of differentiated cell properties and an ability to spread to other sites of the body (invasion and metastasis). It is now widely accepted that the huge catalogue of cancer cell genotypes is ultimately a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1.7): self-sufficiency in growth factors, insensitivity to anti-growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential (avoiding senescence through the activation of telomerase, for example), sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2011). Each of these physiological changes – novel capabilities acquired during tumour development – represents the successful
breaching of an anticancer defence mechanism hardwired into cells and tissues. The most life threatening aspect of cancer is undoubtedly represented by the development of tumour invasion and the ability of cancer cells to metastasise to distant sites where they can form secondary tumours.

It must be emphasised, however, that whilst the successive mutations model of cancer, whereby a cell escapes the growth limitations of normal cells to generate a clonal mass of cells (primary cancer), and then through subsequent genetic alterations becomes progressively more malignant until it is able to metastasise and establish a secondary cancer at a distant site, is applicable to many cases of cancer, it does not always take place like this. The processes of primary and secondary tumourigenesis are distinct mechanisms and there is plenty of evidence to suggest that secondary tumourigenesis can take place without the need for a primary tumour, referred to as cancer of unknown primary (CUP) (Chorost et al. 2004). For example, it is not uncommon for secondary lung cancer to be found in the liver, brain or bone without any sign of a primary cancer in the lung itself (Lobins and Floyd 2007). This therefore suggests that cells can be transformed from normal to metastatic without clonal expansion. As cell metastasis represents the most life threatening aspect of cancer, understanding the mechanisms of secondary tumourigenesis, principally those associated with cell invasion, are particularly important in the fight against it.
Figure 1.7: Acquired capabilities of cancer. Most, if not all cancers acquire the same set of functional capabilities during their development, albeit through various mechanistic strategies. All of these processes closely synergise to sustain carcinogenesis. Processes categorised in one capability often influence other capabilities. For example, epigenetic changes may upregulate expression of growth factor receptors which once stimulated, alter the proliferative and apoptotic responses of a cell.
1.9 Na\textsubscript{v} channels in metastatic cancer

As discussed previously, Na\textsubscript{v} channels are most abundant in electrically excitable cells where they are responsible for AP creation and propagation. More recently, they have also been shown to be functionally expressed in strongly metastatic cancer cell lines derived from non-excitable epithelial tissues, where these channels serve no known physiological role. It has therefore been suggested that the upregulation of Na\textsubscript{v} channels, and the acquisition of a more neuronal cell phenotype, could be an accelerating factor in the development of cancer metastases in carcinomas. However, the cellular mechanisms underlying the transcriptional regulation of Na\textsubscript{v} channel expression in cancer, as well as how Na\textsubscript{v} channel activity may actually promote the development of a more metastatic cancer phenotype, remain unclear.

Expression of Na\textsubscript{v} channels has been reported both in vitro and/or in vivo in a wide range of epithelial cancers, including breast cancer (Roger et al. 2004; Fraser et al. 2005; Brackenbury et al. 2007; Gao et al. 2009; Gillet et al. 2009), prostate cancer (Grimes et al. 1995; Laniado et al. 1997; Grimes and Djamgoz 1998; Diss et al. 2001; Abdul and Hoosein 2002; Bennett et al. 2004; Diss et al. 2005; Nakajima et al. 2009; Yildirim et al. 2012), cervical cancer (Diaz et al. 2007; Hernandez-Plata et al. 2012), colon cancer (House et al. 2010), ovarian cancer (Gao et al. 2010), SCLC (Pancrazio et al. 1989; Blandino et al. 1995; Onganer and Djamgoz 2005; Onganer et al. 2005) and NSCLC (Roger et al. 2007). In all of these studies there has been a marked association of Na\textsubscript{v} channel expression and activity with strongly metastatic cancer cell lines, where inhibition of the channel significantly reduces cell invasion. With the development of invasion and metastases representing the most life threatening aspects of cancer, combined with the fact that Na\textsubscript{v} channel expression appears to be uniquely upregulated in strongly metastatic epithelial cancer cell lines, Na\textsubscript{v} channels may prove to be useful biomarkers and/or tractable drug targets in the diagnosis, prognosis and treatment of certain cancers.

Early studies noted that tumour samples had higher intracellular concentrations of Na\textsuperscript{+} relative to normal tissue and that this had the potential to drive cancer invasion (Cameron et al. 1980). More recently, with Na\textsubscript{v} channels emerging as potential markers of metastatic
progression of epithelial cancer cells, a key experiment showed that increased Na\textsubscript{v} channel expression alone is necessary and sufficient to increase the invasive potential of a set of human cancer cell lines. When the human adult skeletal muscle Na\textsubscript{v} channel isoform, Na\textsubscript{v}1.4, was transiently expressed in both weakly and strongly metastatic human prostate cancer cell lines, there was a significant increase in cell invasion in all of the cell lines tested, and this increased invasive potential could be reduced to control levels by blocking the Na\textsubscript{v} channels with TTX (Bennett et al. 2004).

Individual Na\textsubscript{v} channel α-subunit isoforms generate unique physiological signatures in different cell types and there is often expression of multiple Na\textsubscript{v} channel α-subunit isoforms within a single cell (Diss et al. 2004). When looking at Na\textsubscript{v} channel expression in cancer cells specifically, there is increasing evidence that the cardiac, TTX-resistant Na\textsubscript{v}1.5 isoform and the neuronal, TTX-sensitive Na\textsubscript{v}1.7 isoform are particularly important in the development of a metastatic cancer phenotype. The Na\textsubscript{v}1.5 isoform has been shown to be upregulated in breast cancer (Fraser et al. 2005; Brackenbury et al. 2007; Gao et al. 2009; Gillet et al. 2009; Yang et al. 2012), colon cancer (House et al. 2010), ovarian cancer (Gao et al. 2010) and in T-lymphocyte Jurkat cells (Fraser et al. 2004). Enhanced Na\textsubscript{v}1.7 expression has been associated with prostate cancer (Diss et al. 2001; Diss et al. 2005; Nakajima et al. 2009; Yildirim et al. 2012), cervical cancer (Diaz et al. 2007; Hernandez-Plata et al. 2012) and NSCLC (Roger et al. 2007). In all of these cases it was reported that the expression of Na\textsubscript{v}1.5/Na\textsubscript{v}1.7 is associated with a strongly metastatic cancer phenotype and that inhibition of channel activity markedly reduces cell invasion. Furthermore, there is also an increasing body of evidence to suggest that some Na\textsubscript{v} channel expression events that take place during carcinogenesis may be oncofoetal: that is to say, during cancer progression, neonatal Na\textsubscript{v} channel splice variants are upregulated and it is these embryonic channels that contribute to enhanced cell invasion. If this is the case, it may permit the development of more targeted therapies against Na\textsubscript{v} channels in cancer. A neonatal splice variant of Na\textsubscript{v}1.5 (nNa\textsubscript{v}1.5) is thought to be predominant in the Na\textsubscript{v} channel-dependent invasive behaviour of MDA-MB-231 breast cancer cells, and it has been shown that the targeted inhibition of this splice variant using specific siRNA and polyclonal antibody treatments significantly reduces MDA-MB-231 cell invasion (Brackenbury et al. 2007; Onkal and Djamgoz 2009). The expression of developmentally-
regulated neonatal splice variants has also been demonstrated for Na\textsubscript{v}1.5 in neuroblastoma (Ou et al. 2005) and Na\textsubscript{v}1.7 in prostate cancer (Diss et al. 2001; Diss et al. 2005). However, when investigating which Na\textsubscript{v} channel isoforms were functionally expressed in colon cancer, House et al. reported that the adult variant of Na\textsubscript{v}1.5 appears to be critical for colon cancer invasiveness, whereas the foetal variant plays no role (House et al. 2010).

The available \textit{in vitro} and \textit{in vivo} data taken together are consistent with Na\textsubscript{v} channel expression and activity being a significant driving force for the metastatic progression of epithelial cancers. However, the exact mechanisms by which the functional expression of Na\textsubscript{v} channels may promote invasive behaviours in epithelial cancer cells remain unclear. Analysis of Na\textsuperscript{+} current activation/steady-state inactivation (conductance/availability versus voltage) in several Na\textsubscript{v} channel-expressing cancer cell lines reveals the presence of ‘window currents’ consistent with Na\textsubscript{v} channels being tonically active around the resting membrane potential of these cells, thus permitting an influx of Na\textsuperscript{+}. This would account for the maintained increase in the Na\textsuperscript{+} content of malignant cancer tissue (Cameron et al. 1980; Ouwerkerk et al. 2007). It is logical to assume that an altered intracellular Na\textsuperscript{+} homeostasis, as a result of the aberrant expression of functional Na\textsubscript{v} channels, could have a knock-on effect on a variety of Na\textsuperscript{+}-dependent transport proteins that, in turn, may influence metastatic behaviour. Obvious candidates include proteins associated with Na\textsuperscript{+}/Ca\textsuperscript{2+} and/or Na\textsuperscript{+}/H\textsuperscript{+} exchange. Moreover, Na\textsubscript{v} channel activity may ultimately results in the downstream expression of genes associated with cell motility/invasion, independently from Ca\textsuperscript{2+}/H\textsuperscript{+} flux. House et al. have successfully modelled loss-of-function screens and microarray data to establish a role for Na\textsubscript{v}1.5 as a high level regulator of a colon cancer invasion network involving genes that encompass the Wnt signalling pathway (House et al. 2010). Gillet et al. propose that Na\textsubscript{v}1.5 enhances the invasiveness of MDA-MB-231 breast cancer cells by promoting perimembrane acidification that favours the pH-dependent activity of cysteine cathepsins B and S (Gillet et al. 2009). Subsequently, it has been suggested that Na\textsubscript{v}1.5 could enhance MDA-MB-231 invasion by increasing Na\textsuperscript{+}/H\textsuperscript{+} exchanger type 1 (NHE1)-dependent H\textsuperscript{+} efflux from the cells, thus providing a possible mechanism for extracellular acidification (Brisson et al. 2011).
Alternatively, the role of Na\textsubscript{v} channels in cancer cell metastasis could involve a non-conducting function via direct interactions of Na\textsubscript{v} channel \(\alpha\)- and/or \(\beta\)-subunits with other plasma membrane and/or intracellular proteins. As discussed previously, Na\textsubscript{v} channel \(\beta\)-subunits function as CAMs and regulate cell adhesion and motility. \(\beta\)-subunits are expressed in human breast cancer cell lines, where \(\beta1\) mRNA and protein levels are significantly higher in the weakly metastatic MCF-7 cell line compared with the strongly metastatic MDA-MB-231 cell line, consistent with the greater adhesiveness of MCF-7 cells (Palmer et al. 2008; Chioni et al. 2009). Furthermore, it has also been demonstrated that downregulation of \(\beta1\) expression with siRNA increased the migratory abilities (via reduced cell adhesion) of MCF-7 cells, whereas stable overexpression of \(\beta1\) in MDA-MB-231 cells upregulated \(I_{Na}\) yet reduced cellular motility, suggesting that the cell adhesive effects of \(\beta1\) are independent of changes in cellular excitability (Chioni et al. 2009). This latter study supports the idea that \(\beta\)-subunits are able to function as CAMs independently of the ion-conducting \(\alpha\)-subunits (Isom 2002; Brackenbury and Isom 2008). Conversely, however, in human prostate cancer cells, \(\beta\)-subunits are more highly expressed in the strongly metastatic PC-3 cell line relative to the weakly metastatic LNCaP cell line, therefore suggesting that \(\beta\)-subunits serve different roles in different cells (Diss et al. 2008). For example, upregulation of \(\beta\)-subunit expression would increase cell adhesion but at the same time would promote functional Na\textsubscript{v} channel expression, which would have opposite effects upon metastatic potential. Further work is required to elucidate the non-conducting role of Na\textsubscript{v} channels and the functional significance of \(\beta\)-subunit expression in cancer cell metastasis.

Whilst the downstream mechanisms linking the functional expression of Na\textsubscript{v} channels to the enhanced metastatic behaviour of epithelial cancer cells remain unclear, it is also true that very little is known about how Na\textsubscript{v} channel expression is upregulated in metastatic cancer cell lines in the first place. It has been demonstrated that an activity-dependent positive feedback mechanism involving PKA may play a role in the upregulation of Na\textsubscript{v} channels in strongly metastatic cancer cell lines. PKA has been shown to play an important role in the functional expression of Na\textsubscript{v}1.7 and nNa\textsubscript{v}1.5 in rat prostate cancer Mat-LyLu cells (Brackenbury and Djamgoz 2006) and human breast cancer MDA-MB-231 cells (Chioni et al. 2010), respectively, by mediating activity-dependent positive feedback, thus enhancing the
cells’ metastatic potential through an, as yet, uncharacterised mechanism. If correct, this general mechanism of Na\textsubscript{v} channel-dependent PKA activation could also conceivably lead to the identification of PKA targets that may play a role in the progression of metastases.

Since several cancers are hormone-sensitive (e.g. breast, prostate), and many cancers are dependent on growth factor signalling, a plausible starting point in elucidating the regulatory mechanisms associated with Na\textsubscript{v} channel expression in strongly metastatic cancer cells would be serum factors. Indeed, such factors have been shown to affect Na\textsubscript{v} channel expression and cell motility in strongly metastatic Mat-LyLu and MDA-MB-231 cells (Ding and Djamgoz 2004; Pan and Djamgoz 2008). As regards steroid hormones, there appears to be a direct association with the hormone sensitivity of a cell and Na\textsubscript{v} channel expression: strongly metastatic PC-3M prostate and MDA-MB-231 breast cancer cells are both devoid of classic receptors for androgen and oestrogen, respectively, and both express functional Na\textsubscript{v} channels, whereas corresponding hormone-sensitive, weakly metastatic cancer cells do not express functional Na\textsubscript{v} channels. Interestingly, steroid hormones can also have non-transcriptional roles (Leung et al. 2007; Prossnitz et al. 2008). Such an effect has been demonstrated for the potentiating action of oestrogen on Na\textsubscript{v} channel activity in MDA-MB-231 cells, involving GPR30 (a G-protein-coupled oestrogen receptor) as a cell-surface receptor and decreased cell adhesion as the functional output (Fraser et al. 2010). When cancer cells become hormone-insensitive, they frequently become dependent on growth factors (Santen et al. 2005; Song et al. 2007; Santen et al. 2008), which are well-known modulators of Na\textsubscript{v} channel expression and activity.

1.10 Growth factor regulation of Na\textsubscript{v} channels

The biochemical makeup of extracellular medium has been shown to be a critical factor in both the regulation of Na\textsubscript{v} channel expression and cell motility in strongly metastatic Mat-LyLu and MDA-MB-231 cell lines (Ding and Djamgoz 2004; Pan and Djamgoz 2008). Growth factors are prime candidates for the constituents responsible for these effects as they are well-known modulators of Na\textsubscript{v} channel expression and activity, as well as being key contributors to cancer development and progression. Fibroblast growth factor (FGF) and nerve
growth factor (NGF) signalling regulates \( Na_v \) channel expression and activity in various human and animal cell lines (Toledo-Aral et al. 1995; Akopian et al. 1999; Lei et al. 2001; Lou et al. 2005; Brackenbury and Djamgoz 2007; Goldfarb et al. 2007; Goetz et al. 2009; Laezza et al. 2009). EGFR signalling enhances cell proliferation and motility in many cancer cell types and has also been shown to potentiate \( Na_v \) channel currents in guinea pig ventricular myocytes (Liu et al. 2007). In prostate cancer cells, which are exposed to the highest levels of EGF in the body (Russell et al. 1998; Gann et al. 1999), EGF has been demonstrated to promote cancer progression via enhanced cell invasion (Turner et al. 1996; Kim et al. 1999; Montano and Djamgoz 2004). Moreover, an upregulation of functional \( Na_v \) channel expression has been implicated in the pro-invasive response of prostate cancer cells to EGFR stimulation (Uysal-Onganer and Djamgoz 2007; Ding et al. 2008).

The targeted inhibition of the EGFR is now a first-line NSCLC therapy, with marketed drugs such as Iressa® (gefitinib) used for the treatment of adult patients with high-grade, metastatic NSCLC with activating mutations of EGFR-tyrosine kinase (EGFR-TK) (Lynch et al. 2004; Paez et al. 2004; Cappuzzo et al. 2005; Metro et al. 2006; Domingo et al. 2010; Cataldo et al. 2011). EGFR signalling is now a well-characterised and tractable target in NSCLC treatment, and it may be the case that elevated expression of functional \( Na_v \) channels is one of the downstream consequences of this signalling, contributing to the development of a more metastatic NSCLC phenotype. With multiple growth factors regulating \( Na_v \) channel expression in metastatic cancer cells, \( Na_v \) channels may conceivably prove to be a focal point, common to a number a growth factor signalling pathways, thus making them potentially attractive targets in the treatment of metastatic cancer.

1.11 Epidermal growth factor receptor (EGFR) signalling in NSCLC

One or more members of the family of EGFR genes are overexpressed or otherwise deregulated in virtually all epithelial tumours, including NSCLC. This and related observations on the importance of protein phosphorylation and the discovery that the first identified oncogene, \( v-Src \), encodes a protein kinase led to the selection of the EGFR as the first target of molecular targeted therapy more than 25 years ago (Cohen 2002; Mendelsohn
The EGFR is a cell-surface receptor for members of the EGF family of extracellular protein ligands. It is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases (RTKs): ErbB1 (EGFR, HER1), ErbB2 (Neu, HER2), ErbB3 (HER3) and ErbB4 (HER4) (Hynes and Lane 2005). Receptor-ligand interaction results in RTK homodimerisation, activation of the intrinsic kinase domain and phosphorylation of specific tyrosine residues in the cytoplasmic tail of the receptor. These phosphorylated tyrosine residues then become docking sites for multiple intracellular signalling proteins, which in turn activate downstream signalling cascades, including the MAPK (ERK1/2) pathway, the PI3-K pathway and the JAK/STAT pathway (Figure 1.8).

EGFR ligands activate the MAPK pathway through the binding of GRB2, via its Src homology 2 (SH2) domain, to phosphorylated ErbB receptors, which in turn results in the recruitment of the guanine nucleotide exchange factor (GEF), SOS, to the activated receptor dimer by way of the two src homology 3 (SH3) domains of GRB2 (Henson and Gibson 2006). Activated SOS then promotes the removal of GDP from membrane-associated Ras, leaving it free to bind GTP and become activated. Activated Ras then goes on to activate the protein kinase activity of Raf, which phosphorylates MEK1 and MEK2, which in turn activate ERK1 and ERK2, respectively. Activated ERK is then able to directly control gene expression, amongst numerous other things.

In the PI3-K signalling pathway, EGF triggers the recruitment of PI3-K to the activated ErbB receptors. This is mediated by the binding of SH2 domains in the p85 regulatory subunit of PI3-K to the phosphorylated tyrosine residues in the cytoplasmic tail of the receptor. The catalytic p110 subunit of PI3-K in turn phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2), leading to the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3). A key downstream regulator of PIP3 is AKT. The pleckstrin homology (PH) domain of AKT binds directly to PIP3. Since PIP3 is restricted to the plasma membrane, this results in the translocation of AKT to the plasma membrane. Likewise, the phosphoinositide-dependent protein kinase 1 (PDK1) also contains a PH domain that binds directly to PIP3, causing it also to translocate to the plasma membrane upon activation of PI3-K. The co-localisation of AKT and activated PDK1 results in the former becoming phosphorylated by the latter, leading to
activation of AKT and the subsequent regulation of many downstream effectors, such as transcription factors.

In the JAK/STAT signalling pathway, JAKs are bound to ErbB receptors and the binding of extracellular ligand to the receptor activates them. With increased kinase activity, they phosphorylate tyrosine residues on the receptor and create sites for interaction with STAT proteins that possess SH2 domains, which are themselves tyrosine-phosphorylated by JAKs. STATs may also be phosphorylated directly by RTKs. The phosphorylated tyrosine residues on the STATs then serve as binding sites for the SH2 domains of other STATs, mediating their dimerisation. The activated STAT dimers then accumulate in the nucleus of the cell and activate transcription of target genes.

EGFRs are known to be highly expressed in a number of malignant cell lines, often correlating with poor prognosis (Ozanne et al. 1986; Baselga and Mendelsohn 1994; Mendelsohn 2003). Thus, the EGFR is an antigen that is relatively specific for some cancer cells. NSCLC cells can produce and release several EGFR ligands (Fujimoto et al. 2005; Volante et al. 2007; Wu et al. 2007), thereby establishing an EGFR autocrine loop and rendering the cells sensitive to inhibition by RTK inhibitors (Fujimoto et al. 2005; Wu et al. 2007; Gazdar and Minna 2008). This ‘autocrine hypothesis’ of cell signalling is not a new phenomenon: it was reported in 1980 that cultured cancer cells can produce a transforming growth factor termed TGF-α, which activates EGFRs (Sporn and Todaro 1980). By producing TGF-α which can activate EGFRs on their own cell surfaces, cancer cells can achieve self-directed growth factor receptor stimulation, thus escaping dependency on external (endocrine or paracrine) sources of growth factors. Furthermore, there is increasing evidence that there is a strong selective pressure on EGFR signalling pathways in lung cancers and that selective targeting of these pathways is the best therapeutic strategy. EGFR mutations may target many regions of the gene, but kinase domain mutations are particularly common in lung cancers (Shigematsu and Gazdar 2006; Sharma et al. 2007). These mutations may play a key role in driving lung tumourigenesis but they also leave lung cancer cells dependent on EGFR signalling pathway activation for growth and survival (Gazdar et al. 2004; Sharma et al. 2007). Therefore, inhibition of EGFRs by selective RTK inhibitors rapidly leads to replicative
senescence and apoptosis. However, since the discovery of the EGFR mutations, it has become clear that tumour response and resistance to RTK inhibitors are influenced by a myriad of factors (Pao et al. 2005; Engelman and Cantley 2006; Riely et al. 2006), making the generation of an effective therapeutic strategy more complicated. EGFR copy number gains are also common in lung cancers and have been shown to occur more frequently together with EGFR mutations than alone (Nomura et al. 2007; Okabe et al. 2007; Dahabreh et al. 2011). This, combined with the fact that autocrine loops and other perturbations of EGFR signalling are frequent in all types of NSCLC, highlights that the signalling pathways associated with EGFR stimulation are crucial in NSCLC progression. Moreover, it emphasises just how versatile tumour cells are in finding ways to activate vital pathways to both survive and ward off extinction, which is further affirmed by the relapse and subsequent drug resistance of lung cancers that had previously responded to EGFR-targeted drugs.

Lung cancer has a particularly high mortality that is predominantly due to the development of metastatic lesions. As discussed previously, functional Na\textsubscript{v} channel expression is associated with the development of a more metastatic cancer phenotype in non-excitable epithelial tumours. Similarly, it has also been demonstrated that the expression of EGFR ligands, which would promote the formation of autocrine loops, is also strongly associated with the development of metastases. Work carried out by Zhang et al. showed that the EGFR ligand, epiregulin, is involved in the development of an invasive cancer phenotype in NSCLC (Zhang et al. 2008). This research was stimulated by their previous work which demonstrated that epiregulin is one of the several highly expressed EGFR ligands in EGFR-mutant NSCLC cells (Fujimoto et al. 2005). Furthermore, the group suggests that approximately two thirds of all NSCLCs express at least one of the EGFR ligands. With both Na\textsubscript{v} channels and components of the EGFR signalling network being implicated in the development of metastases in NSCLC, it is highly probable that self-directed EGFR stimulation of NSCLC cells, through the development of autocrine loops, may drive the expression of Na\textsubscript{v} channels in these cells, thus promoting cell invasion through an, as yet, uncharacterised mechanism. With the problems of relapse and drug resistance in lung cancers that initially respond well to EGFR-targeted therapies, and the fact that Na\textsubscript{v} channel expression may well prove to be a convergent point in a number of growth factor signalling pathways that are upregulated in
cancer, Na\textsubscript{v} channels represent exciting candidates as novel and tractable markers in the diagnosis, prognosis and therapy of epithelial cancers.
Figure 1.8: EGFR signalling pathways. Activation of the EGFR leads to receptor homodimerisation, autophosphorylation of key tyrosine residues and recruitment of several proteins at the intracellular portion of the receptors via their SH2 domains. This results in the activation of downstream signalling cascades, including the MAPK (ERK1/2) pathway (blue), the PI3-K pathway (green) and the JAK/STAT pathway (red). These pathways act in a coordinated manner to promote the transcription of genes associated with a number of cellular behaviours.
1.12 Key project questions

There is growing evidence to suggest that Na\textsubscript{v} channels are uniquely expressed in strongly metastatic cancer cells, derived from non-excitable epithelial cells, and that their expression and activity in these cells contribute to the oncogenic process, driving the development of a more invasive cancer phenotype. However, the cellular mechanisms underlying the transcriptional regulation of Na\textsubscript{v} channel expression in cancer, as well as how Na\textsubscript{v} channel activity promotes the development of a more metastatic cancer phenotype, remain unclear. In an effort to elucidate how Na\textsubscript{v} channel expression is controlled in human NSCLC cells, and how Na\textsubscript{v} channel activity promotes NSCLC invasion, there are several questions that have been addressed in this project:

1. Are Na\textsubscript{v} channels functionally expressed in strongly versus weakly metastatic NSCLC cell lines, and if so, do they contribute to invasive properties?
2. Is there evidence that Na\textsubscript{v} channels are more abundant in cancerous human lung tissue compared with normal, healthy lung tissue?
3. Does EGFR signalling, known to drive NSCLC progression, have any effect on the expression of functional Na\textsubscript{v} channels in metastatic NSCLC cell lines?
4. What are the downstream effects of functional Na\textsubscript{v} channel expression in NSCLC cells?

1.13 Central hypothesis and sub-hypotheses

Based on evidence that Na\textsubscript{v} channels are uniquely expressed in strongly metastatic cancer cell lines, and that EGFR signalling is a key player in NSCLC progression as well as being a known regulator of Na\textsubscript{v} channel expression,

It can be hypothesised that the expression of functional Na\textsubscript{v} channels in NSCLC cells, derived from non-excitable epithelial cells, is a contributory factor in the oncogenic process, resulting in the development of a more invasive cancer
phenotype, and that expression of these Na\textsubscript{v} channels is driven by EGFR signalling.

Sub-hypotheses:

**Na\textsubscript{v} channel expression is elevated in cancerous human lung tissue compared with normal, healthy human lung tissue.**

**Na\textsubscript{v} channel β-subunit expression is low in strongly metastatic NSCLC cells versus weakly metastatic NSCLC cells, contributing to their invasive phenotype.**

**Intracellular Na\textsuperscript{+} is elevated in strongly metastatic cells compared with weakly metastatic cells and this drives cell invasion through changes in intracellular Ca\textsuperscript{2+} levels and/or cellular pH.**

**1.14 Experimental strategy**

A general overview of the experimental approach used to address the key project questions outlined in section 1.12 is represented in Figure 1.9. It was first necessary to screen a broad panel of human NSCLC cell lines for the presence of Na\textsubscript{v} channel currents in order to establish any trend between functional Na\textsubscript{v} channel expression and metastatic potential, and to identify suitable candidate cell lines to use in the project. To this end, the high-throughput IonWorks® electrophysiology platform was utilised to screen 22 human NSCLC cell lines for the presence of Na\textsubscript{v} channel currents. Two cell lines (one strongly metastatic cell line that has detectible Na\textsubscript{v} channel currents and one weakly metastatic cell line that does not have detectible Na\textsubscript{v} channel currents) were selected for more detailed characterisation.

Following characterisation of the Na\textsubscript{v} channel currents present in the strongly metastatic NSCLC cell line using conventional whole-cell patch-clamp electrophysiology, and the determination of the key Na\textsubscript{v} channel isoforms present in the cell lines using qPCR, the general approach employed to address the hypotheses involved setting up functional assays so
that the cellular behaviours (proliferation, migration, invasion and adhesion) of NSCLC cells could be analysed following various treatments. This, combined with molecular and cell biology approaches such as Western immunoblotting, immunocytochemistry, qPCR and transient transfection of siRNA, provided a systematic approach that allowed several of the key project questions to be addressed. Furthermore, in order to establish what role elevated intracellular Na\(^+\), brought about as a result of Na\(_v\) channel expression, might be having on the cellular behaviours of strongly metastatic NSCLC cells, fluorescence measurement of intracellular Na\(^+\), Ca\(^{2+}\) and H\(^+\) was carried out in the presence of suitable pharmacological agents.

In order to demonstrate the clinical relevance of this research, immunohistochemistry was used to determine levels of Na\(_v\) channel expression in normal versus cancerous human lung tissue using well characterised and optimised isoform-specific antibodies.
Figure 1.9: General overview of project hypotheses and experimental strategy.
Chapter 2:
MATERIALS AND METHODS
2.1 Materials

All human NSCLC cell lines were provided by AstraZeneca, UK. Dulbecco’s Modified Eagle’s Medium (DMEM), DMEM/F-12 medium and RPMI 1640 medium with 4.5 g.litre\(^{-1}\) glucose, 4 mM L-glutamine and 110 mg.litre\(^{-1}\) sodium pyruvate added were obtained from Invitrogen (GIBCO), UK. Phosphate-buffered saline (PBS), foetal bovine serum (FBS), penicillin/streptomycin solution and 0.25% trypsin/EDTA solution were obtained from Sigma-Aldrich, UK. All plasticware including plates, dishes and culture flasks were obtained from Corning, UK.

Chemicals and pharmacological agents were obtained from the following sources: veratridine, 5-(N-Ethyl-N-isopropyl)amiloride (EIPA), wortmannin and ouabain (Sigma-Aldrich, UK), tetrodotoxin (TTX), gefitinib and SN-6 (Tocris Bioscience, UK), epidermal growth factor (EGF) and bafilomycin A1 (Millipore, UK), monensin, nigericin and KT5720 (Calbiochem, UK), U0126 (Merck, UK), forskolin (Fisher Scientific, UK).

All oligonucleotide primers for qPCR were designed and manufactured by PrimerDesign, UK. All siRNAs directed against genes of interest and scrambled siRNA sequences were obtained from QIAGEN, UK.

Primary antibodies were obtained from the following sources: anti-Na\(_v\)1.7 for Western immunoblotting (NeuroMab, USA, dilution used 1:1000), anti-Na\(_v\)1.7 for immunocytochemistry (ICC) and immunohistochemistry (IHC) (AstraZeneca, UK, dilution used 1:200), anti-phospho ERK (Sigma-Aldrich, UK, dilution used 1:10,000; Promega, UK, dilution used 1:1000), anti-total ERK (Promega, UK, dilution used 1:1000), anti-phospho AKT and anti-total AKT (Cell Signalling Technology, UK, dilution used 1:1000), anti-phospho PKA, anti-EGFR and anti-NHE1 (Millipore, UK, dilution used 1:1000), anti-pan-caveolin and anti-caveolin 1 (BD Biosciences, UK, dilution used 1:2000), anti-β-actin (Sigma-Aldrich, UK, dilution used 1:5000). FITC-conjugated anti-rabbit IgG secondary antibody was obtained from Jackson Immunoresearch, UK (dilution used 1:1000) and horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgGs were obtained from Dako, UK.
(dilution used 1:2000). Anti-EGF neutralising antibody was obtained from Millipore, UK (concentration used 10 µg.ml⁻¹).

2.2 Cell culture

Strongly metastatic H460 and weakly metastatic A549 NSCLC cells were cultured at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS (v/v) plus 50 U.ml⁻¹ penicillin and 50 µg.ml⁻¹ streptomycin (referred to as ‘complete’ medium). SH-SY5Y neuroblastoma cells were cultured at 37°C, 5% CO₂ in DMEM/F12 medium supplemented with 15% FBS (v/v) plus 50 U.ml⁻¹ penicillin, 50 µg.ml⁻¹ streptomycin and 1% non-essential amino acids (v/v) (Sigma-Aldrich, UK). All other lung cancer cell lines were cultured at 37°C, 5% CO₂ in RPMI 1640 medium supplemented with 10% FBS (v/v) plus 50 U.ml⁻¹ penicillin and 50 µg.ml⁻¹ streptomycin. All cell lines were passaged 2-3 times per week from an 80-90% confluent T75 culture flask to a new T75 flask. Cell detachment was achieved by treatment of the cell monolayer with 0.25% trypsin/EDTA solution at 37°C, 5% CO₂ for ~3 minutes. All treatments were started after incubating the cells in complete medium at least overnight following seeding. All pharmacological agents were dissolved in appropriate solvents and diluted in cell culture medium.

2.3 Experimental solutions

The external bath solution used to isolate inward Na⁺ channel currents in whole-cell patch-clamp experiments contained (in mM): NaCl 140, CsCl 3, MgCl₂ 1, CaCl₂ 10, glucose 11, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5, adjusted to pH 7.3 with NaOH and 320 mOsm.litre⁻¹ with sucrose. The corresponding internal pipette solution contained (in mM): CsCl 135, NaCl 5, HEPES 10, ethylene glycol tetraacetic acid (EGTA) 5, adjusted to pH 7.4 with CsOH and 310 mOsm.litre⁻¹ with sucrose.

The external bath solution used to isolate inward Caᵥ channel currents in whole-cell patch-clamp experiments contained (in mM): TEA-Br 160, KCl 5, MgCl₂ 1, NaHCO₃ 1, glucose 4, HEPES 10, BaCl₂ 5, adjusted to pH 7.4 with Tris-base and 320 mOsm.litre⁻¹ with
sucrose. The corresponding internal pipette solution contained (in mM): CsAsp 140, MgCl$_2$ 2, CaCl$_2$ 0.1, K-ATP 1, HEPES 20, EGTA 5, adjusted to pH 7.2 with CsOH and 310 mOsm.litre$^{-1}$ with sucrose.

Physiological saline solution (PSS) contained (in mM): NaCl 140, KCl 4, MgCl$_2$ 1, CaCl$_2$ 2, glucose 11, HEPES 10, adjusted to pH 7.4 with NaOH and 320 mOsm.litre$^{-1}$ with sucrose. The corresponding internal pipette solution for PSS, when used for electrophysiology, contained (in mM): K-glutamate 125, KCl 20, MgCl$_2$ 1, CaCl$_2$ 0.37, K-ATP 1, HEPES 10, EGTA 1, adjusted to pH 7.2 with KOH and 310 mOsm.litre$^{-1}$ with sucrose.

The extracellular solution used for IonWorks® electrophysiology was simply PBS pH 7.4. The internal solution used for IonWorks® electrophysiology contained (in mM): K-gluconate 100, KCl 40, MgCl$_2$ 3.2, HEPES 10, EGTA 3, adjusted to pH 7.2 with KOH and 310 mOsm.litre$^{-1}$ with sucrose.

2.4 Generation of dilution clones

H460 NSCLC cell dilution clones were generated for the purpose of isolating populations of H460 cells with differing levels of functional Na$_v$ channel expression. They were generated using the following serial dilution protocol. The initial plate setup (Figure 2.1) was generated by first adding 4000 cells in 200 µl of H460 cell suspension to well A1 of a 96-well plate, whilst 100 µl of complete medium was added to all other wells. 100 µl of cell suspension was then transferred from well A1 to B1 and this 1:2 dilution was repeated sequentially for the rest of column 1 on the 96-well plate. 100 µl was discarded from well H1 so that all the wells in the column contained the same final volume. An additional 100 µl of complete medium was added to each of the wells in column 1 to give a final cell suspension volume of 200 µl.well$^{-1}$, before 100 µl of cell suspension was transferred from the wells in column 1 (A1 through H1) to those in column 2 (A2 through H2), and so on, so that a series of 1:2 dilutions was repeated across the whole plate. Again, 100 µl was discarded from each of the wells in the last column so that all 96 wells contained 100 µl of cell suspension. 100 µl of complete medium was added to all 96 wells to bring the final volume of the wells to 200 µl.
Plates were incubated at 37°C, 5% CO₂ and left for 7-10 days. Any wells containing just a single colony were subcultured into larger vessels (24-well plate, then 6-well plate, then T75 culture flask) until there were enough cells to be used for experiments. To increase the likelihood that the cells of a particular colony originated from a single cell, selected cells were recloned a second time using the same protocol. For all experiments in which H460 cell dilution clones were used, parental control H460 cells were also included in order to provide a direct comparison to the standard population of H460 cells.
Figure 2.1: Initial plate setup for cell cloning by serial dilution in 96-well plates. The initial cell inoculum is added to well A1 of the 96-well plate and a 1:2 serial dilution is performed down column 1 of the plate (red arrow) before a second 1:2 serial dilution is performed across the plate from columns 1-12 for all of the rows A-H (blue arrows). The highest cell densities occur in the wells immediately surrounding the A1 position, whereas single colonies are more likely to be found towards the bottom right corner of the 96-well plate. Wells containing just a single colony are subcultured into larger vessels before being recloned a second time to ensure that cells of a particular colony originated from just a single cell.
2.5 Electrophysiology

2.5.1 Whole-cell patch-clamp electrophysiology

Recordings of inward Na\textsubscript{v} channel current, $I_{Na}$, and inward Ca\textsubscript{v} channel current using barium (Ba\textsuperscript{2+}) as the charge carrier, $I_{Ba}$, from wild type H460 and A549 NSCLC cells were made using the whole-cell configuration of the patch-clamp technique (Hamill et al. 1981) using the solutions described in section 2.3. Before recording, cells were plated onto 22 mm square coverslips, pre-coated with 0.01% poly-L-lysine (w/v) (Sigma-Aldrich, UK), and incubated at 37°C, 5% CO\textsubscript{2} for at least 1 hour. Patch pipettes of resistance 2-5MΩ were pulled from thin-walled borosilicate glass tubing (Intracell, UK), fire-polished, filled with the appropriate internal pipette solution and coated with Sigmacote® (Sigma-Aldrich, UK). All experiments were performed at room temperature. When recording currents from cells that had undergone a particular treatment (pharmacological treatment or gene knockdown), recordings from untreated control cells from the same passage number/split as the treated cells were taken too in order to provide a direct comparison to the treated condition.

An Axopatch\textsuperscript{TM} 200B patch-clamp amplifier (Molecular Devices, USA) was used for recordings, which were filtered at 2 kHz and digitised at 5-10 kHz using a Digidata 1322A A/D converter (Molecular Devices, USA). Upon gigaseal formation, cells were held at a potential, $V_h$, of -90 mV for $I_{Na}$ recordings and -80 mV for $I_{Ba}$ recordings, with a holding current less than -50 pA and series resistance less than 10MΩ. Currents were recorded with the cell capacitance compensated and leak currents subtracted using an online P/4 subtraction protocol. Series resistance was compensated up to 80%. Standard current-voltage ($I$-$V$) protocols for $I_{Na}$ recordings used 30 ms sweeps with command voltages from -90 to +80 mV in 5 mV steps, from $V_h$ -90 mV. $I$-$V$ protocols for $I_{Ba}$ recordings used 120 ms sweeps with command voltages from -60 to +70 mV in 5 mV steps, from $V_h$ -80 mV. Current amplitudes were normalised to cell capacitance and expressed as current density (pA.pF\textsuperscript{-1}). Data acquisition and analysis was performed using pCLAMP® (v9.0, Molecular Devices, USA) and Origin® (v8.0, Microcal Software, UK). $I$-$V$ relationships for each cell were fitted with a Boltzmann function as follows:
\[ I = G(V-V_{\text{rev}}) / 1+\exp(-(V-V_{50,\text{act}})/k) \]

where \( V_{\text{rev}} \) is the reversal potential, \( V_{50,\text{act}} \) is the voltage for half maximal activation of current, \( G \) is the conductance and \( k \) is the slope factor.

Conductance-voltage relationships were derived from individual \( I-V \) plots and fitted with a Boltzmann function of the form:

\[ G / G_{\text{max}} = 1 / 1+\exp((V_{50,\text{act}}-V)/k) \]

where \( G_{\text{max}} \) is the maximal conductance.

The peak conductance, \( G \), at each test potential was calculated from the corresponding peak current, \( I \), as follows:

\[ G = I / (V-V_{\text{rev}}) \]

Steady-state inactivation protocols for \( \text{Na}_v \) channel conductance elicited currents following step depolarisations from prepulse potentials between -120 and +40 mV to a test potential of +10 mV (500 ms prepulse, from \( V_h \) -90 mV, in 10 mV steps, with 5 mV steps between prepulse potentials of -80 and 0 mV). Peak current values were normalised to \( I_{\text{max}} \) and the curves fitted with the following Boltzmann function:

\[ I / I_{\text{max}} = 1 / 1+\exp((V-V_{50,\text{act}})/k) \]

where \( I_{\text{max}} \) is the maximal current density at the test potential.

### 2.5.2 Current-clamp electrophysiology

Measurements of the resting membrane potential (\( E_m \)) of wild type H460 and A549 NSCLC cells were made in the whole-cell current-clamp mode using an Axopatch™ 200B patch-clamp amplifier (Molecular Devices, USA), with injected current equal to zero, \( I=0 \). Prior to cell attachment, entry into whole-cell configuration and subsequent recording of \( E_m \), patch pipettes were lowered into extracellular PSS and the ‘pipette offset’ potentiometer
adjusted until the meter read 0 mV. Patch pipettes of higher resistance (5-10MΩ) were used for current-clamp recordings and were pulled from thin-walled borosilicate glass tubing (Intracell, UK), fire-polished, filled with internal pipette PSS and coated with Sigmacote® (Sigma-Aldrich, UK). All experiments were performed in PSS at room temperature. When recording the $E_m$ of cells that had undergone pharmacological treatment, recordings from untreated control cells from the same passage number/split as the treated cells were taken too in order to provide a direct comparison to the treated condition.

**2.5.3 IonWorks® electrophysiology**

High-throughput (HT) electrophysiology was carried out using the IonWorks® Quattro™ Automated Patch Clamp System (Molecular Devices, USA, Figure 2.2). The IonWorks® electrophysiology platform is a plate-based electrophysiology measurement system. It is an integrated platform that consists of computer-controlled fluid handling, recording electronics and processing tools capable of whole-cell voltage-clamp recordings from thousands of individual cells per day. Cell preparations were obtained by resuspending cells in PBS at a concentration of 1x10$^6$ cells.ml$^{-1}$. Electrical seal resistances ranged from a user-defined lower limit of 50MΩ to approximately 250MΩ. Data were rejected if pre-compound current amplitude was less than 50 pA or if the total resistance resulting from the parallel seal and membrane resistance decreased by more than 50% from the pre-compound measurement to the post-compound measurement. Electrical access to the interior of all successfully positioned cells on the 384-well PatchPlate™ (Molecular Devices, USA) was achieved by the addition of the pore-forming antibiotic, amphotericin B, to a common chamber beneath the PatchPlate™. Amphotericin B (10 mg) was added to 100 ml of internal solution (350 µl of DMSO was used to dissolve the amphotericin B prior to addition). To determine the sensitivity of $I_{Na}$ to TTX, cells were transiently voltage-clamped (at -10, 0 and +10 mV) in blocks of 48 wells prior to addition of 1 µM TTX and again after a 2-minute incubation period with 1 µM TTX, which was added from a 96-well polypropylene compound plate (Matrix Technologies, USA). The TTX was added to the compound plate at 3x working concentration because the IonWorks® platform makes three 3.5 µl additions to each well of the PatchPlate™ (buffer alone, then buffer plus cells, then 3x compound). When recording
currents from cells that had undergone a particular pharmacological treatment, recordings from untreated control cells from the same passage number/split as the treated cells were taken too in order to provide a direct comparison to the treated condition.
Figure 2.2: PatchPlate™ used in IonWorks® HT electrophysiology. A single cell is positioned by negative pressure over a pore in the bottom of each well of a specifically designed PatchPlate™ containing 384 wells. The aperture separates two isolated fluid-filled upper and lower chambers. The positioned cells form stable seals over the apertures, impeding electrical flow between the two chambers. A cell membrane pore-forming agent (amphotericin B) is introduced into the lower chamber, creating an electrical pathway through the portion of the cell membrane exposed via the small aperture in each well. An electronics head containing 48 electrodes is positioned in the upper chamber, clamping the cell membrane potential and subsequently recording ionic currents from up to 48 cells in parallel.
2.6 Quantitative PCR

Total RNA extraction was performed from cell lines using the RNeasy® kit (QIAGEN, UK) according to manufacturer’s recommendations. RNA yield and purity were determined by spectrophotometry using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK) and only samples with an A$_{260}$/A$_{280}$ ratio above 2.0 were kept for further experiments (A$_{260}$/A$_{280}$ ratio refers to the optical spectrometer measurement of absorbance at the wavelengths of 260 and 280 nm, respectively). 1 µg of total RNA was reverse transcribed using the Precision™ Reverse Transcription Kit (PrimerDesign, UK) according to manufacturer’s recommendations, and qPCR was performed using cDNA obtained from 25 ng of total RNA. Primers used for qPCR were specific for each gene of interest (Table 2.1, PrimerDesign, UK). The temperature profile was 10 minutes at 95ºC followed by amplification for 50 cycles consisting of 15 seconds at 95ºC and 1 minute at 60ºC. Data collection took place during the 60ºC step of each cycle. An ABI 7500 Sequence Detection System with software v1.2.3 (Applied-Biosystems, UK) was used to perform qPCR. Amplification and detection were carried out in 96-well plates (Applied-Biosystems, UK) with SYBR® green Precision™ 2x qPCR Mastermix (PrimerDesign, UK). All expression data were normalised to 18S rRNA expression, as determined by an initial reference gene screen using the geNorm™ Housekeeping Gene Selection Kit (PrimerDesign, UK), according to manufacturer’s recommendations. For accurate gene expression measurements it is essential to normalise qPCR data for the gene of interest to a fixed reference – one that is not affected by treatments/experimental conditions. Although normalising to a constitutively expressed housekeeping gene is the most common method, there is no universal reference gene that is constant in all experimental situations. Therefore, a panel of six reference genes was screened for all of the experimental conditions that were used to assess expression of particular genes of interest, and analysed using the geNorm™ software. The analysis showed that of the six reference genes screened (18S rRNA, ACT, ATP5B, GAPDH, TOP1 and UBC), 18S rRNA was the best reference gene for accurate normalisation of the qPCR data. Data analysis was performed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001; Cikos et al. 2007). When performing qPCR on cells that had undergone a particular treatment (pharmacological treatment or gene knockdown), qPCR was also performed on untreated control cells from the
same passage number/split as the treated cells in order to provide a direct comparison of mRNA expression to the treated condition. In order to determine whether or not Na\textsubscript{1.7} mRNA levels in strongly metastatic H460 NSCLC cells corresponded to a sufficient degree of mRNA expression that could result in the expression of functional channel protein, qPCR was also performed for all Na\textsubscript{v} channel isoforms on control SH-SY5Y neuroblastoma cells, known to express a number of functional Na\textsubscript{v} channels.
Table 2.1: qPCR primers used for the determination of Na\_\text{v} channel α-subunit, Na\_\text{v} channel β-subunit, NCX1 and NHE1 expression in human NSCLC cell lines.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene product</th>
<th>Forward primer 5’&gt;3’</th>
<th>Reverse primer 3’&gt;5’</th>
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<tr>
<td>SCN1A</td>
<td>Na_1.1</td>
<td>ATGATCTGCCTATTCCAAATTACAA</td>
<td>GGTTCCCACAGTCCTCCCTTA</td>
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<tr>
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<td>Na_1.2</td>
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<td>TCTCTGTCTTGTTATAGGGAAGCCTG</td>
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<tr>
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<td>Na_1.3</td>
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<td>ACATGCACCACAGGATAAAAATAAC</td>
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<tr>
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<td>CTCCAGTGACAAGTGTTGTCAC</td>
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<tr>
<td>SCN8A</td>
<td>Na_1.6</td>
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<td>TCCCCACAATGGAGAGGATGAC</td>
</tr>
<tr>
<td>SCN9A</td>
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<td>TGTAACCTGCCTTCTGTATTGTG</td>
</tr>
<tr>
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</tr>
<tr>
<td>SLC9A1</td>
<td>NHE1</td>
<td>ACAAGTCCCACACCACCACAT</td>
<td>GAGTAGAAGTAGAAGAGGAGCCG</td>
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</tbody>
</table>
2.7 Determination of RNA concentration

RNA concentration and purity were determined by spectrophotometry using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK), according to manufacturer’s recommendations. The optical density (OD)/absorbance (ABS) value was measured at 260 nm versus a blank (no RNA) control (the same buffer as the samples). The mean of three measurements was calculated for each sample and used as the final RNA concentration value. The $A_{260}/A_{280}$ ratio is an indicator of the purity of a nucleic acid sample. An $A_{260}/A_{280}$ ratio above 1.8 suggests that there is little protein contamination of a DNA/RNA sample. Only RNA samples with an $A_{260}/A_{280}$ ratio above 2.0 were retained for further experiments.

2.8 Western immunoblotting

Cells were grown to nearly confluent monolayers in 10 cm Petri dishes, washed in PBS and lysed on ice using 300 µl RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40 (v/v), 0.5% sodium deoxycholate (w/v), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulphate (SDS) (w/v)) with 1x cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche, UK) added. The cell lysates were passed through a 22-gauge syringe needle several times to shear any genomic DNA and centrifuged at 1000 g for 2 minutes. The resulting supernatants were frozen at -80°C at least overnight before total protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific Pierce, UK), according to manufacturer’s recommendations. To determine the total protein concentration of a cell lysate, the optical density of each sample/BCA mixture was measured in triplicate at 562 nm using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK) and compared to samples of bovine serum albumin (BSA) at known concentrations.

Lysate supernatants were incubated at 95°C for 5 minutes with an equal volume of 2x Novex® Tris-Glycine SDS Sample Buffer (Invitrogen, UK), supplemented with 5% BME (v/v), and sample proteins were separated by 7-10% SDS-PAGE for 90-120 minutes (at 100 V until the samples had passed through the stacking gel and then at 160 V for the remaining
time) using the Mini-PROTEAN® 3 Cell apparatus (Bio-Rad, UK). Protein samples were then transferred by electrophoresis (110 V for 90-120 minutes) onto Protran™ nitrocellulose membranes (Whatman, UK). Following protein transfer, nitrocellulose membranes were treated with 0.1% Ponceau S (w/v) (Sigma-Aldrich, UK) in 5% acetic acid (v/v) for 5 minutes to detect protein bands. Membranes were washed 3x in Tris-buffered saline (TBS) with 0.1% Tween-20 (v/v) (TTBS) for 5 minutes per wash before being incubated at room temperature with blocking buffer (5% BSA (w/v) in TTBS) for 1 hour. Following another three 5-minute washes in TTBS, membranes were incubated overnight at 4°C with the appropriate primary antibody, diluted in blocking buffer. Membranes were washed the next day 3x in TTBS for 5 minutes per wash before being incubated at room temperature for 2 hours with the appropriate HRP-conjugated secondary antibody, diluted in blocking buffer. After more extensive washing with TTBS (three 30-minute washes), air-dried membranes were treated with Western Lightning® Enhanced Chemiluminescence (ECL) Reagent (PerkinElmer, UK) and immunoreactive proteins were detected by exposure to autoradiographic film (GE Life Sciences, UK).

When performing Western immunoblotting on cells that had undergone a particular treatment (pharmacological treatment or gene knockdown), blots from untreated control cells from the same passage number/split as the treated cells were also included in order to provide a direct comparison of protein levels to the treated condition. For Western immunoblots against Na1.7, cell lysates from wild type HEK-293 cells were used as a negative control and cell lysates from HEK-293 cells stably transfected with Na1.7 were used as a positive control. For all Western immunoblots, loading controls of β-actin and/or total-protein (when looking at phosphorylated proteins, specifically) were performed in parallel. Densitometry analysis of Western immunoblots was carried out simply to confirm the linearity of blot optical densities and was not used to quantify the data. Any conclusions drawn from Western immunoblot data are simply based on the fact that the protein is either present or not, or that a particular treatment has an effect on protein levels that can be seen clearly with the naked eye.
2.9 Phosphatase treatment of phosphorylated proteins

When it was necessary to test the specificity of antibodies against phosphorylated proteins of interest, cell lysate supernatants were treated with Lambda Protein Phosphatase (PP) (New England BioLabs, UK) in order to serve as a control condition in Western immunoblotting. Lambda PP is a Mn\(^{2+}\)-dependent protein phosphatase with activity towards serine, threonine and tyrosine residues. 64 µl of cell lysate supernatant was mixed with 8 µl of 10x NEBuffer for Protein MettalloPhosphatases (PMP), 8 µl of 10x MnCl\(_2\) (10 mM) and 1 µl (400 units) of Lambda PP. 1x NEBuffer for PMP had the following composition (in mM): NaCl 100, HEPES 50, DTT 2, with 0.01% Brij® 35, pH 7.5 at room temperature. This reaction mixture was incubated at 30ºC for 30 minutes. Following this incubation, the appropriate volume of reaction mixture was incubated at 95ºC for 5 minutes with an equal volume of 2x Novex® Tris-Glycine SDS Sample Buffer (Invitrogen, UK), supplemented with 5% BME (v/v), and Western immunoblotting was performed as described in section 2.8. One unit is defined as the amount of enzyme that hydrolyses 1 nmol of \(p\)-Nitrophenyl phosphate (50 mM) in 1 minute at 30ºC in a total reaction volume of 50 µl.

2.10 Functional assays

2.10.1 Cell proliferation

To analyse cell proliferation, 4000 cells were seeded into each well of a 96-well plate in complete medium plus 1x treatment, as necessary, and cell growth versus untreated control cells was simply monitored by real time in vitro micro-imaging using an IncuCyte™ incubator (Essen BioScience, UK). This device enables the monitoring of cell proliferation by determining cell confluence. Cells were incubated in the IncuCyte™ incubator at 37ºC, 5% CO\(_2\), and live-cell images were taken every 2 hours until 100% cell confluence had been achieved. Data were presented as growth curves showing cell confluence, determined by the IncuCyte™ incubator software and normalised to the same starting point of 0% cell confluence, versus time in hours.
2.10.2 Cell migration

The spreading and migratory capabilities of NSCLC cells were assessed using the scratch-wound assay, which measures the expansion of a cell population on surfaces. Cells were seeded into the wells of a 24-well plate at a concentration of 2.5x10^5 cells.ml⁻¹ and cultured in complete medium to 100% cell monolayers. A linear wound was generated in the monolayer with a sterile 200 μl plastic pipette tip and any cellular debris was removed by washing the wells with PBS. Complete medium plus 1x treatment, as necessary, was added to the wells and the cells were incubated at 37°C, 5% CO₂ in an IncuCyte™ incubator (Essen BioScience, UK). Live-cell images were taken every 2 hours until the wounds were fully closed. Data were presented as wound confluence, determined by the IncuCyte™ incubator software, versus time in hours and always compared to untreated control cells.

2.10.3 Cell invasion

Cell invasion was analysed in 24-well plates containing BD Matrigel™ Invasion Chambers (BD Biosciences, UK, Figure 2.3). The chambers consist of polyethylene terephthalate membrane cell culture inserts perforated with 8 μm diameter pores and covered with a layer of Matrigel™ Basement Membrane Matrix. The upper compartment of this Boyden chamber setup was seeded with 4x10⁴ viable cells in complete medium plus 1x treatment, as required. The lower compartment was filled with cell culture medium plus 1x treatment, as necessary, supplemented with 20% FBS (v/v) to act as a chemoattractant. After 24-48 hours at 37°C, 5% CO₂, the inserts were washed in PBS for 2 minutes and then submerged into ice-cold methanol for 10 minutes to fix the cells, with care being taken to avoid touching the lower surface of the insert where the invasive cells were positioned. The cells were then stained with haematoxylin (Sigma-Aldrich, UK) for 3 minutes and the remaining non-invasive cells were removed from the upper side of the membrane using a cotton bud. Cells that had successfully invaded and were attached to the lower side of the membrane were counted in the whole insert using a 20x objective lens on an Olympus CK2 Inverted Tissue Culture Microscope (Olympus, UK). When measuring the invasive capabilities of cells that had undergone a particular treatment (pharmacological treatment or
gene knockdown), the assay was also performed with untreated control cells from the same passage number/split as the treated cells in order to provide a direct comparison to the treated condition.
Figure 2.3: Cell invasion assay setup. The invasion assay consists of two chambers separated by a porous filter coated with Matrigel™, which mimics the ECM environment of a cell. The cell suspension, comprised of cells in complete medium, is placed in the upper chamber. Medium containing 20% FBS (to act as a chemoattractant) is placed in the lower chamber. Invasive cells are able to digest through the layer of Matrigel™ and migrate through the pores in the filter to the lower surface of the insert. Invasive cells can then be stained and counted to quantify cell invasion.
2.10.4 Cell adhesion

The adhesive properties of NSCLC cells were analysed by assessing their ability to stick to the ECM glycoprotein, fibronectin. 50 µl of fibronectin at a concentration of 20 µg.ml⁻¹ was added to the wells of a 96-well plate and incubated at 37°C, 5% CO₂ for 1 hour. For negative controls, wells were left uncoated. After two washes with 50 µl washing buffer (0.1% BSA (w/v) in serum-free medium), the wells were ‘blocked’ with 50 µl blocking buffer (0.5% BSA (w/v) in serum-free medium) at 37°C, 5% CO₂ for 1 hour. The wells were washed twice again with washing buffer and then the plate chilled on ice whilst 20,000 cells (50 µl of cell suspension at a concentration of 4x10⁵ cells.ml⁻¹) were added to each well. Following a 30-minute incubation at 37°C, 5% CO₂, the plate was agitated on a BT1500 Orbi-Shaker™ MP orbital plate shaker (Benchmark Scientific, USA) at 1500 rpm for 15 seconds. The wells were washed 3x with washing buffer and then the cells were fixed with 4% paraformaldehyde (w/v) for 15 minutes at room temperature. After another two washes with washing buffer, cells were stained with 1x crystal violet (Sigma-Aldrich, UK) for 10 minutes at room temperature. The wells were then washed with water, turned upside down and left to dry completely. Quantification of the number of adhesive cells was achieved by lysing the remaining cells with 2% SDS (w/v) for 30 minutes at room temperature and then measuring the absorbance of the resulting SDS/crystal violet solution, relative to SDS alone, at 585 nm using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK).

2.11 Immunocytochemistry and fluorescence microscopy

3x10⁴ cells were seeded into 6-well plates containing 22 mm square sterile coverslips, pre-coated with 0.01% poly-L-lysine (w/v) (Sigma-Aldrich, UK), and incubated for 24-48 hours at 37°C, 5% CO₂. After three washes with PBS, coverslips were incubated at room temperature in 4% paraformaldehyde (w/v) for 20 minutes. Following a further three washes with PBS, coverslips were treated with 0.1 M glycine/PBS at room temperature for 10 minutes to quench any remaining paraformaldehyde. For immunocytochemistry with plasma membrane permeabilisation, coverslips were treated with 0.5% saponin (w/v) (Sigma-Aldrich, UK) for 10 minutes at room temperature. Coverslips were then washed 3x with PBS and the
cells were ‘blocked’ with 5% BSA (w/v) at room temperature for 1 hour before a further three washes with PBS and incubation with the appropriate primary antibody, dissolved in 0.05% saponin (w/v), at room temperature for 1 hour. Coverslips were washed again 3x with PBS and then incubated with an appropriate FITC-conjugated secondary antibody, dissolved in 0.05% saponin (w/v), at room temperature for 1 hour. Coverslips were washed a further 3x with PBS before addition of 200 ng.ml⁻¹ DAPI chemical stain (Sigma-Aldrich, UK) for 5 minutes at room temperature and in the dark. Coverslips were washed 3x once again with PBS before being mounted onto glass slides with ProLong® Gold Anti-Fade Reagent (Invitrogen, UK) as mountant. Coverslips were left to set overnight in the dark and then sealed with nail varnish. To avoid plasma membrane permeabilisation, the same protocol would be used in the absence of saponin. To confirm antibody specificity, control conditions were adopted. Firstly, to determine the specificity of the primary antibody against Naᵥ1.7 in this experimental protocol, HEK-293 cells were used as negative controls and HEK-293 cells stably transfected with Naᵥ1.7 were used as positive controls. Secondly, to determine levels of non-specific binding of both the primary and secondary antibodies, control treatments were performed that consisted of exactly the same experimental protocol described above using H460 NSCLC cells, but with either the primary antibody (control 1) or secondary antibody (control 2) incubation steps omitted.

Images of cells were acquired using the DeltaVision RT Deconvolution Microscope (Applied Precision, USA) equipped with a 60x oil immersion objective lens and appropriate excitation and emission wavelength filters for FITC and DAPI. The images were collected using a CoolSNAP™ HQ camera (Photometrics, UK) with a Z optical spacing of 0.1 µm in 12 sections. Raw images were then deconvolved using SoftWoRx® software (Applied Precision, USA) and displayed as maximum projections of stacks using NIH Image J software (W.S. Rasband, National Institutes of Health, USA, http://rsbweb.nih.gov/ij/).

2.12 Immunohistochemistry

Prior to performing IHC on tissue samples, formalin-fixed, paraffin-embedded (FFPE) NSCLC cell pellets were utilised for antibody optimisation and were prepared as follows.
Cells were cultured in complete medium to 70-80% confluence in four T175 culture flasks. After washing the cells twice with PBS, the monolayers were covered with 10% neutral buffered formalin (NBF) solution (Sigma-Aldrich, UK) for 5 minutes at room temperature and the cells were then scraped into the NBF, collected into 50 ml centrifuge tubes and stored at 4°C in NBF for 24 hours. The cells were then centrifuged at 1200 rpm for 5 minutes, the supernatant was removed and the cell pellet washed once with PBS before being resuspended in 0.5 ml 80% ethanol (v/v). The cell suspension was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 12,000 rpm for 5 minutes to pellet the cells. The tops of the microcentrifuge tubes were removed and the space inside them was used as a mould to create a disc-shaped cell pellet. This was achieved by removing the 80% ethanol from the cell pellet, resuspending the cells in 350 µl Histogel™ (Richard-Allan Scientific, UK), pre-warmed to 60°C for at least 1 hour, and then transferring this into the microcentrifuge tube caps. The discs were left briefly at -20°C to solidify and were stored in 80% ethanol overnight before being processed into paraffin wax and embedded on a tissue processor (performed by members of the Immunohistochemistry Department at AstraZeneca, Alderley Park, UK).

FFPE blocks were cut into 4 µm sections using a Leica RM2235 Manual Rotary Microtome (Leica Microsystems, UK). These sections were mounted onto glass slides by floating out the sections onto water kept at 45°C by an Electrothermal MH8516 Paraffin Section Mounting Bath (Electrothermal, UK), submerging the slides into the water and removing them vertically from the water whilst collecting the sections. The mounted slides were left to dry at 37°C overnight. The sections were then de-waxed in two changes of xylene for 5 minutes each and rehydrated through graded alcohols (100%, 100% and 70%) for 5 minutes each using a Leica Autostainer XL (Leica Microsystems, UK) before being washed in running tap water for 5 minutes. High temperature antigen retrieval was then carried out using a Milestone RHS-1 Microwave Tissue Processor (Milestone Medical Technologies, USA) using antigen retrieval buffer (Dako, UK) diluted 1:10 with water. The pressure vessel containing the section-covered slides in buffer was heated and made to follow a pre-programmed temperature curve under pressure. On completion of the programme, the vessel was cooled under running tap water for 10 minutes, the lid removed and the slides washed thoroughly under running tap water. Slides to be stained were then placed on a LabVision
Autostainer 360 (Thermo Fisher Scientific, UK). The staining protocol consisted of a pre-wash with 0.05% TTBS (v/v) followed by an endogenous peroxidase block with 3% H₂O₂ (w/v) at room temperature for 10 minutes. After another wash with TTBS, Serum-Free Protein Block (Dako, UK) was applied for 15 minutes followed by the appropriate primary antibody, diluted in TTBS, for 1 hour at room temperature. Slides were washed again with TTBS and then incubated with ChemMate Envision™+ System-HRP (DAB+) anti-rabbit secondary antibody (Dako, UK) for 30 minutes at room temperature. Slides were washed again with TTBS and then incubated with DAB Peroxidase Substrate Solution (Dako, UK) for 10 minutes at room temperature before a final rinse with water.

Sections were then returned to the Leica Autostainer XL where they were lightly counterstained with haematoxylin (Sigma-Aldrich, UK) for 2 minutes, washed with water, placed in Shandon® Bluing Reagent (Thermo Fisher Scientific, UK) for 30 seconds, washed again with water and dehydrated through graded alcohols (70%, 100% and 100%) for 5 minutes each before being cleared in two changes of xylene for 5 minutes each. Finally, coverslips were added to the slides using the xylene-based mounting media, Poly-Mount® (Polysciences, USA), and left to dry overnight before they could be viewed under any conventional light microscope. Mounted slides consisting of FFPE samples of H460 cell mouse xenografts and human lung and prostate tissue were provided by the Histology Department at AstraZeneca, Alderley Park, UK, and were treated in exactly the same way as previously described. For all IHC experiments, negative control treatments were also performed and consisted of exactly the same experimental protocol described above, but with the primary antibody incubation step omitted.

2.13 Sucrose gradient fractionation

H460 NSCLC cells were cultured in complete medium to 80-100% confluence in T175 culture flasks. After washing the cells twice with PBS, 10 ml of PBS was added to each flask and the cells were scraped into the PBS, collected into 15 ml centrifuge tubes and centrifuged at 2000 rpm for 5 minutes to pellet the cells. Following centrifugation, the supernatant was removed and the cells were resuspended in 2 ml of 0.32 M sucrose in PBS and aliquotted into
1.5 ml microcentrifuge tubes. The cells were then stored at -80°C until they were needed in sucrose gradient fractionation studies.

When needed, cells were thawed on ice, added to a 15 ml centrifuge tube with PBS so that the total volume was 10 ml, and centrifuged at 2000 rpm for 5 minutes to pellet the cells. Following centrifugation, the supernatant was removed and the pelleted cells were lysed with 900 µl of MBS buffer (25 mM MES pH 6.5, 500 mM NaCl) plus 1% Triton™ X-100 (v/v) at 4°C. Lysates were passed through a 22-gauge syringe needle several times to shear any genomic DNA. The lysate was then mixed with 900 µl of 90% sucrose in MBS/Triton™ X-100 (w/v) to give a lysate mixture with a final sucrose mass concentration (w/v) of 45%. This was placed at the bottom of a 5 ml polyallomer centrifuge tube (Beckman Coulter, UK) on ice. The lysate mixture was carefully overlaid with 1.5 ml of 30% sucrose/MBS/Triton™ X-100 (w/v), followed by 1.5 ml of 5% sucrose/MBS/Triton™ X-100 (w/v), ensuring no disruption to the menisci between layers in the sucrose gradient. Gradients were spun at 38,500 rpm (140,000 g) in an Optima L-90K Ultracentrifuge (Beckman Coulter, UK) using an SW55 rotor (Beckman Coulter, UK) for 16 hours at 4°C. Post-centrifugation, 250 µl fractions were carefully taken from the top to the bottom of the gradient and analysed by Western immunoblotting using appropriate antibodies.

To precipitate protein from the collected fractions, the fractions were incubated with one volume of 100% trichloroacetic acid (TCA) (w/v) to four volumes of sample for 30 minutes at 4°C. Samples were centrifuged at 14,000 rpm for 5 minutes at 4°C and the resulting pellets were washed twice with 200 µl ice-cold acetone, ensuring that the pellets remained intact. Pellets were dried at 60°C for 10 minutes in order to drive off the acetone before being resuspended in 100 µl of 2x Novex® Tris-Glycine SDS Sample Buffer (Invitrogen, UK), supplemented with 5% BME (v/v), ready for analysis by Western immunoblotting. When fractions were analysed by Western immunoblotting, 20 µl of each sample was incubated at 95°C for 5 minutes before being loaded onto an SDS-PAGE gel. The same volume of each fraction was analysed each time. Control blots for pan-caveolin, as well as caveolin 1, specifically, were also run for each sucrose gradient fractionation experiment in order to
identify fractions enriched in caveolin, and thus in possession of caveolae-containing membranes.

2.14 Fluorescence measurement of intracellular ions

2.14.1 Fluorescence measurement of intracellular sodium ions

Intracellular Na$^+$ concentration, [Na$^+$]$_i$, was monitored in NSCLC cells using the ratiometric fluorescent dye, SBFI (Molecular Probes, UK). Cells were cultured in complete medium plus 1x treatment, as necessary, in 35 mm FluoroDish™ glass-bottomed dishes (World Precision Instruments, USA) at 37°C, 5% CO$_2$ for 24 hours. Following this incubation period, cells were washed with PSS and then incubated at room temperature in the dark for 3 hours in PSS containing 10 µM SBFI-AM plus 1x treatment, as required (Figure 2.5). The cells were then washed twice with PSS before being incubated at room temperature for a further 30 minutes in PSS plus 1x treatment, as required. Dishes were analysed using a Nikon Eclipse TE2000-S Inverted Microscope (Nikon, UK) equipped with a CoolSNAP™ HQ camera (Photometrics, UK). Excitation light at the two excitation wavelength maxima of SBFI (340 and 380 nm) was chopped by a monochromator (Cairn OptoScan, UK). The excitation protocol was a 50 ms excitation at each wavelength every four seconds. Fluorescence emission at 510 ± 20 nm was detected by a photomultiplier tube (PMT) (Hamamatsu Photonics, Japan) using 3x3 binning. The analogue signal of the PMT was digitised at a sampling frequency of 2 kHz. Autofluorescence was negligible. Background fluorescence, determined at 340 and 380 nm from a cell-free area of the dish after the loading period and wash, were subtracted. Calibration of [Na$^+$]$_i$ was performed in each cell. This was achieved by perfusing two calibration solutions (10 and 20 mM Na$^+$) onto the cells in the presence of the sodium ionophore, monensin, the rationale being that there is a linear relationship between [Na$^+$]$_i$ and the 340/380 fluorescence ratio of SBFI, and so the use of two calibration solutions would allow accurate [Na$^+$]$_i$ values to be determined from 340/380 fluorescence ratios. These solutions had the following composition (in mM): NaCl + KCl 150, HEPES 5, EGTA 10, monensin 0.01, adjusted to pH 7.4 with NaOH and 320 mOsm.litre$^{-1}$ with sucrose. Data are
presented as $[\text{Na}^+]_i$ (in mM) and were calibrated by plotting a linear graph of 340/380 fluorescence ratio versus calibration solution $[\text{Na}^+]$ (Figure 2.4A).

2.14.2 Fluorescence measurement of intracellular calcium ions

Intracellular $\text{Ca}^{2+}$ concentration, $[\text{Ca}^{2+}]_i$, was monitored in NSCLC cells using the ratiometric fluorescent dye, Fura-2 (Molecular Probes, UK). Cells were plated onto 22 mm square coverslips, pre-coated with 0.01% poly-L-lysine (w/v) (Sigma-Aldrich, UK), and incubated at 37°C, 5% CO$_2$ for 24-48 hours in complete medium. Following this incubation period, cells were washed with PSS and then incubated at room temperature in the dark for 30 minutes in PSS containing 4 µM Fura-2-AM (Figure 2.5). The cells were then washed twice with PSS before being incubated at room temperature for a further 30 minutes in PSS. The glass coverslip to which the cells were adhered formed the base of a gravity-fed perfusion chamber, continually perfused with PSS. Liquid access to the perfusion chamber was controlled by automatic valves (Harvard Apparatus, UK), which allowed for the rapid switching of solutions. All treatments were prepared at 1x concentration by diluting stock solutions into PSS. Coverslips were analysed using a Nikon Eclipse TE2000-S Inverted Microscope (Nikon, UK) equipped with a CoolSNAP™ HQ camera (Photometrics, UK). Excitation light at the two excitation wavelength maxima of Fura-2 (340 and 380 nm) was chopped by a monochromator (Cairn OptoScan, UK). Background-subtracted 340 and 380 nm fluorescence images were captured using 3x3 binning, with fluorescence emission at 510 ± 20 nm detected by a PMT (Hamamatsu Photonics, Japan). The analogue signal of the PMT was digitised at a sampling frequency of 0.2 Hz. Autofluorescence was negligible. Data are presented as 340/380 fluorescence ratio traces.

2.14.3 Fluorescence measurement of intracellular pH

Intracellular pH was monitored in NSCLC cells using the pH-sensitive, ratiometric fluorescent dye, BCECF (Molecular Probes, UK). Cells were plated onto 22 mm square coverslips, pre-coated with 0.01% poly-L-lysine (w/v) (Sigma-Aldrich, UK), and incubated at 37°C, 5% CO$_2$ for 24-48 hours in complete medium. Following this incubation period, cells
were washed with PSS and then incubated at room temperature in the dark for 30 minutes in PSS containing 3 µM BCECF-AM (Figure 2.5). The cells were then washed twice with PSS before being incubated at room temperature for a further 30 minutes in PSS. The glass coverslip to which the cells were adhered formed the base of a gravity-fed perfusion chamber, continually perfused with PSS. Liquid access to the perfusion chamber was controlled by automatic valves (Harvard Apparatus, UK), which allowed for the rapid switching of solutions. All treatments were prepared at 1x concentration by diluting stock solutions into PSS. Coverslips were analysed using a Nikon Eclipse TE2000-S Inverted Microscope (Nikon, UK) equipped with a CoolSNAP™ HQ camera (Photometrics, UK). Excitation light at the two excitation wavelength maxima of BCECF (440 and 490 nm) was chopped by a monochromator (Cairn OptoScan, UK). Background-subtracted 440 and 490 nm fluorescence images were captured using 3x3 binning, with fluorescence emission at 530 ± 20 nm detected by a PMT (Hamamatsu Photonics, Japan). The analogue signal of the PMT was digitised at a sampling frequency of 0.2 Hz. Autofluorescence was negligible. Calibration of intracellular pH was performed in each cell. This was achieved by perfusing cells with high-K⁺ solutions buffered to known pH values and containing the ionophore nigericin. Nigericin acts as a K⁺/H⁺ exchanger in the cell membrane and thus, if [K⁺]ₑ = [K⁺]ᵢ then pHₑ = pHᵢ. The high concentration of K⁺ in the extracellular environment (similar to its intracellular concentration) ensures that the pHᵢ value for the cells being calibrated is the same pH as that for each of the buffered calibration solutions. Stock calibration solution had the following composition (in mM): KCl 140, CaCl₂ 1, MgSO₄ 1, HEPES 20. Each individual calibration solution was made by dividing stock calibration solution into aliquots and adjusting each one to the required pH value (5.5, 6.5, 7.0, 7.5 and 8.5) with NaOH. Nigericin was then added to each solution to give a final working concentration of 5 µM. Cells were perfused with PSS for a few minutes after loading normally with BCECF. The superfusate was then switched between the different calibration solutions, allowing enough time for pHᵢ to reach equilibrium in each solution. Data are presented as pHᵢ and were calibrated by plotting a graph of 490/440 fluorescence ratio versus calibration solution pH (Figure 2.4B).
Figure 2.4: SBFI and BCECF calibration plots. (A) Representative linear calibration plot for the ratiometric fluorescent dye, SBFI, used to determine $[\text{Na}^+]_i$ in cells from 340/380 fluorescence ratios. (B) Representative non-linear calibration plot for the ratiometric fluorescent dye, BCECF, used to determine $\text{pH}_i$ in cells from 490/440 fluorescence ratios.
2.15 Transient transfection of siRNA

1x10^5 cells were seeded into each well of a 24-well plate in 0.5 ml of complete medium. siRNA (37.5 ng; 1.5 µl of a 2 µM stock) directed against a particular gene of interest (Table 2.2, QIAGEN, UK) was diluted in 100 µl of cell culture medium without serum. 3 µl of HiPerFect Transfection Reagent (QIAGEN, UK) was then added to the diluted siRNA and mixed by brief vortexing. After a 10-minute incubation at room temperature, the transfection complexes were added dropwise to the cells to give a final siRNA concentration of 5 nM (Figure 2.5). The cells were incubated at 37ºC, 5% CO_2 for 24 hours, after which experiments could be performed on them. For all experiments utilising siRNA transfection, control experiments were performed in parallel and involved the transfection of scrambled siRNA sequences in order to rule out any effect of the transfection process itself not associated with the targeted knockdown of proteins of interest.

Table 2.2: mRNA sequences to which siRNAs are directed for the targeted knockdown of Na_v1.7, β1 and NHE1.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene product</th>
<th>Target mRNA sequence 5'&gt;3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN9A</td>
<td>Na_v1.7</td>
<td>TCGGATAGTGAATACAGCAAA</td>
</tr>
<tr>
<td>SCN1B</td>
<td>β1</td>
<td>CACATTGAGGTAGTGACAAA</td>
</tr>
<tr>
<td>SLC9A1</td>
<td>NHE1</td>
<td>CAAGCTCAACCGTTAATAAA</td>
</tr>
</tbody>
</table>

The chemically synthesised siRNAs are transfected into cells and an RNA-induced silencing complex (RISC) is assembled, resulting in mRNA transcripts with a complementary sequence to the bound siRNA being cleaved, thereby preventing translation into protein.
Figure 2.5: Mechanisms of loading cells with ratiometric fluorescent dyes and gene silencing with siRNA. Ratiometric fluorescent dyes used to measure \([\text{Na}^+]_i\), \([\text{Ca}^{2+}]_i\) and \(pH_i\) (SBFI, Fura-2 and BCECF, respectively) are attached to acetoxy methyl (AM) ester groups. This results in an uncharged molecule that can permeate cell membranes and therefore enter cells. Once inside the cell, the AM groups are removed by cellular esterases, thus preventing the dye from leaving the cell. Chemically synthesised short interfering RNA (siRNA) directed against a gene of interest is transfected into cells. An RNA-induced silencing complex (RISC) is assembled, siRNAs unwind, and a single strand of RNA remains bound to the RISC. The RISC then targets mRNA transcripts that have a complementary sequence to the bound RNA and cleaves the homologous mRNA, preventing translation.
2.16 Cell toxicity assay

In some instances it was necessary to test the cytotoxic effects of certain compounds in order to ensure that the final working concentration used in experiments did not affect cell viability. First, 2x10^5 cells were seeded into 6-well plates in complete medium and incubated at 37°C, 5% CO₂ for 24 hours. The media was then removed from the wells and the cells were washed with PBS. Complete medium plus a range of concentrations of the compound to be tested were added to the appropriate wells and cells were incubated at 37°C, 5% CO₂ for 48 hours. The wells were washed 3x with PBS and then the cells were fixed with 4% paraformaldehyde (w/v) for 15 minutes at room temperature. After another two washes with PBS, cells were stained with 1x crystal violet (Sigma-Aldrich, UK) for 10 minutes at room temperature. The wells were then washed with water, turned upside down and left to dry completely. Quantification of cell number was achieved by lysing the remaining cells with 2% SDS (w/v) for 30 minutes at room temperature and then measuring the absorbance of the resulting SDS/crystal violet solution, relative to SDS alone, at 585 nm using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, UK). Toxic effects of compounds could then be inferred by comparing absorbance values obtained with specific compound concentrations with those obtained with untreated control cells.

2.17 Data analysis

All data are presented as the mean ± standard error of the mean (S.E.M.), unless otherwise stated. Statistical significance was determined using unpaired Student’s t-test or one-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) post hoc correction, as appropriate, with 95% confidence limits. The Kolmogorov-Smirnov test was used to confirm normal distribution of data. All statistical analysis was carried out using GraphPad Prism® software (v5.0, GraphPad Software, USA).
Chapter 3:
RESULTS – Characterisation of $\text{Na}_v$ channel currents in NSCLC cell lines
3.1 Introduction

Lung cancer is the most commonly diagnosed cancer and the most common cause of cancer death worldwide, mostly as a consequence of the development of metastases (Parkin et al. 2005). The development of metastases consists of a complex series of events in which one of the key steps is the acquisition by cancer cells of an invasive phenotype. This relies predominantly on their ability to degrade basement membranes and the ECM (Gupta and Massague 2006) by various extracellular proteases such as matrix metalloproteinases (MMPs) (Egeblad and Werb 2002) or cysteine cathepsins (Mohamed and Sloane 2006). It has been demonstrated that Na_v channels are abnormally expressed in non-excitile cancer cells of epithelial origin, and appear to be strongly associated with disease progression: there is a marked association of functional Na_v channel expression with metastatic potential in a number of epithelial cancers (Diss et al. 2005; Fraser et al. 2005; Onganer et al. 2005; Roger et al. 2007; Gao et al. 2010; House et al. 2010; Hernandez-Plata et al. 2012).

Na_v channels are voltage-dependent transmembrane protein complexes that, once opened, allow for the net movement of extracellular Na^+ into cells. Na_v channels consist of a pore-forming α-subunit associated with one or more auxiliary β-subunits. The β-subunits are not essential for channel function but they do modulate channel gating and the degree of α-subunit expression at the cell surface. The Na_v channel family is composed of nine different α-subunits (Na_v1.1-Na_v1.9) and five different β-subunits (β1B and β1-β4). All of them have been cloned, functionally expressed and characterised (Catterall et al. 2005). Two α-subunit isoforms, Na_v1.5 and Na_v1.7, appear to play major roles in the development of a more invasive cancer phenotype in several non-excitile epithelial cancers. Breast, colon and ovary cancer cells specifically overexpress the TTX-resistant, cardiac Na_v1.5 α-subunit isoform (Fraser et al. 2005; Gao et al. 2010; House et al. 2010), whereas in NSCLC, cervical and prostate cancer cells, functional expression of the TTX-sensitive, neuronal Na_v1.7 isoform is predominantly upregulated (Diss et al. 2005; Diaz et al. 2007; Roger et al. 2007; Hernandez-Plata et al. 2012).
3.2 Aims

The main aim of the studies conducted in this chapter was to establish whether metastatic NSCLC cell lines express functional Na\textsubscript{v} channels, and if so, whether they contribute to the invasive properties of these cells. The specific aims were as follows:

1. To establish whether functional Na\textsubscript{v} channels are expressed in strongly versus weakly metastatic NSCLC cell lines.
2. To determine which Na\textsubscript{v} channel isoforms are expressed in selected NSCLC cell lines.
3. To establish whether Na\textsubscript{v} channel expression/activity contributes to NSCLC cell behaviours – proliferation, migration, invasion and adhesion.

3.3 Results

3.3.1 Strongly metastatic NSCLC cell lines express functional Na\textsubscript{v} channels whereas weakly metastatic cell lines do not

As discussed above, the expression of functional Na\textsubscript{v} channels correlates with invasive behaviours in many epithelial cancer cells. In order to assess the role of Na\textsubscript{v} channels in NSCLC, it was first necessary to identify suitable model cell lines that would allow for a direct comparison of Na\textsubscript{v} channels in strongly versus weakly metastatic NSCLC. To this end, 22 NSCLC cell lines were tested for the presence of functional Na\textsubscript{v} channels using a high-throughput (HT) IonWorks® electrophysiology platform to record inward Na\textsubscript{v} channel currents, \(I_{\text{Na}}\) (Figure 3.1). Of the cell lines tested, five (H460, H23, H1703, H358 and HX147) were shown to express functional Na\textsubscript{v} channels. Of these, the H1703 cell line was shown to express functional TTX-resistant Na\textsubscript{v} channels, whilst \(I_{\text{Na}}\) in the other four cell lines was sensitive to inhibition by 1 µM TTX (TTX-sensitive). All five of the NSCLC cell lines that possess detectible Na\textsubscript{v} channel currents are considered strongly metastatic cell lines, showing an invasive phenotype \textit{in vitro} (pers. comm. February 2009, members of the Oncology Department at AstraZeneca, Alderley Park, UK). Therefore, based on this initial screen there
appears to be a strong positive correlation between functional Na\textsubscript{v} channel expression and metastatic potential amongst cell lines of NSCLC.

On the basis of this preliminary screen, two NSCLC cell lines were selected for further study. Consistent with earlier work (Roger et al. 2007), the H460 cell line, shown to have the largest \(I_{\text{Na}}\) amplitude by IonWorks\textsuperscript{®} analysis, was selected as the model for a strongly metastatic NSCLC cell line which possesses functional Na\textsubscript{v} channels. The A549 cell line was selected as the model for a weakly metastatic NSCLC cell line which has no detectible Na\textsubscript{v} channel currents. In agreement with the IonWorks\textsuperscript{®} data and Roger et al. (2007), conventional whole-cell patch-clamp electrophysiology recordings also confirmed the presence of robust, inward \(I_{\text{Na}}\) in H460 cells (\(I_{\text{max}} = -30.7 \pm 3.8\) pA.pF\(^{-1}\)), compared with a complete lack of \(I_{\text{Na}}\) in the weakly metastatic A549 cells (Figure 3.2). The biophysical properties of the \(I_{\text{Na}}\) elicited in the H460 cells were consistent with established characteristics of Na\textsubscript{v} channel currents in other systems (Figure 3.3 and Table 3.1). In addition to Na\textsuperscript{+} influx via Na\textsubscript{v} channels, Ca\textsuperscript{2+} signalling has also been implicated in the modulation of metastases in several cancers (Amuthan et al. 2002; Huang et al. 2004; Mycielska and Djamgoz 2004; Liao et al. 2006; Wang et al. 2010). Thus, aberrant expression of Ca\textsubscript{v} channels in non-excitable cancer cells of epithelial origin, already shown to express functional Na\textsubscript{v} channels, could theoretically promote the development of a more invasive cancer phenotype. However, there was a complete lack of \(I_{\text{Ba}}\) in both the H460 (\(n=15\)) and A549 (\(n=15\)) cells, tested using conventional whole-cell patch-clamp electrophysiology, therefore suggesting that there is no functional expression of Ca\textsubscript{v} channels in either the strongly or weakly metastatic NSCLC cell lines.

Next, functional assays for cell proliferation, migration and invasion were performed on untreated control H460 and A549 cells (Figure 3.4). The strongly metastatic H460 cells were more proliferative and, predictably, more invasive than the weakly metastatic A549 cells. Notably, however, the migratory capabilities of the A549 cells were greater than those for the H460 cells. Cell migration and invasion are distinct cellular processes (Friedl and Wolf 2003; Sahai 2005). In order for a cancer cell to be invasive, it must first be able to digest the ECM surrounding it before it can migrate to secondary sites. Thus, the greater invasive capabilities of the H460 cells made them more suitable as a model for cell metastases in subsequent
experiments whereas the A549 cells, despite being more migratory, were good models for weakly metastatic NSCLC.
Figure 3.1: Average $I_{\text{max}}$ recordings for TTX-inhibitable Na$^+$ currents in NSCLC cell lines. (A) 22 NSCLC cell lines were screened for the presence of functional Na$_v$ channels using the HT IonWorks® electrophysiology platform. The average $I_{\text{max}}$ for inward $I_{\text{Na}}$, measured at three voltages (-10, 0 and +10 mV) and shown to be TTX-inhibitable by addition of either 1 or 50 µM TTX, as well as uncharacterised outward current, are displayed for each cell line tested. Of the five NSCLC cell lines that were shown to express functional Na$_v$ channels, only one (H1703) expressed TTX-resistant channels. In the H1703 cell line, 1 µM TTX was unable to abolish $I_{\text{Na}}$ whereas in the four other cell lines, 1 µM TTX completely abolished it. 50 µM TTX successfully inhibited $I_{\text{Na}}$ in the H1703 cell line ($n \geq 40$ for all cell lines). (B) Representative IonWorks® current traces from H460 cells before and after addition of 1 µM TTX.
Figure 3.2: Detection of Na\textsubscript{v} channel currents in strongly metastatic H460 and weakly metastatic A549 cells using conventional whole-cell patch-clamp electrophysiology. (A) Average current density-voltage (I-V) plots for inward $I_{\text{Na}}$ in H460 (open squares, $n=17$) and A549 (closed squares, $n=16$) cell lines using Na\textsubscript{v} channel-specific electrophysiological solutions ($P<0.001 (***)$; Student’s $t$-test). (B) Representative current traces from H460 and A549 cells. Currents were evoked using 30 ms depolarising steps in 5 mV intervals (-90 to +70 mV), from $V_h$ -90 mV.
Figure 3.3: Time constants for activation and inactivation of Na\textsubscript{v} channel currents in H460 cells. (A) Time constant for activation of $I_{Na}$ between potentials of -30 and +60 mV. (B) Time constant for inactivation of $I_{Na}$ between potentials of -25 and +60 mV. As inactivation of $I_{Na}$ is biphasic, both fast (initial phase) and slow (later phase) inactivation rates could be calculated. Insets show the regions of the current traces from which $\tau_{act}$ and $\tau_{inact}$ were calculated ($n=17$ for both parameters).

Table 3.1: Biophysical properties of Na\textsubscript{v} channel currents in H460 cells.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{max}$ (pA.pF\textsuperscript{-1})</td>
<td>-30.7 ± 3.8</td>
</tr>
<tr>
<td>$V_{50,act}$ (mV)</td>
<td>-7.9 ± 1.9</td>
</tr>
<tr>
<td>$V_{rev}$ (mV)</td>
<td>+59.8 ± 5.1</td>
</tr>
<tr>
<td>$K$</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>$V_{peak}$ (mV)</td>
<td>+10</td>
</tr>
<tr>
<td>$\tau_{act}$ (ms)</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>$\tau_{inact}$ (ms)</td>
<td>2.60 ± 0.33 (fast)</td>
</tr>
<tr>
<td></td>
<td>9.83 ± 1.09 (slow)</td>
</tr>
</tbody>
</table>

$I_{max}$, maximal current density; $V_{50,act}$, voltage for half maximal activation of current; $V_{rev}$, reversal potential; $k$, slope factor; $V_{peak}$, voltage at which $I_{max}$ is elicited; $\tau_{act}$, time constant for activation; $\tau_{inact}$, time constant for inactivation.
Figure 3.4: Comparison of cell behaviours between H460 and A549 cells. (A) H460 (open squares) and A549 (closed squares) cell proliferation, recorded over 144 hours on 96-well plates, measured as % confluence and analysed using the IncuCyte™ imaging system (n=16 for both cell lines). (B) H460 (open squares) and A549 (closed squares) cell migration, recorded over 58 hours using the scratch-wound assay on 24-well plates, measured as % wound confluence and analysed using the IncuCyte™ imaging system (n=8 for both cell lines). (C) H460 and A549 cell Matrigel™ invasion, recorded after 48 hours (n=9 for both cell lines, P<0.001 (***) Student’s t-test). Inset shows representative images of stained H460 and A549 cells in invasion assay inserts following an invasion assay.
3.3.2 Blocking Na\textsubscript{v} channels inhibits invasion of metastatic H460 cells

Given the established link between the functional expression of Na\textsubscript{v} channels and invasive behaviour in cancerous cells of epithelial origin, the next criterion to satisfy before a more detailed investigation into the putative involvement of Na\textsubscript{v} channels in NSCLC disease progression could take place was whether manipulating Na\textsuperscript{+} influx through Na\textsubscript{v} channels using the Na\textsubscript{v} channel-specific blocker, TTX, and the Na\textsubscript{v} channel-specific opener, veratridine, has any effect on H460 NSCLC cell invasion. Whole-cell patch-clamp electrophysiology confirmed that 0.5 µM TTX was sufficient to abolish $I_{Na}$ in H460 cells, whilst 50 µM veratridine specifically enhanced the persistent sodium current, $I_{Na,P}$ (Figure 3.5A). Application of either 0.5 µM TTX, or, 50 µM veratridine had no effect on H460 cell proliferation or migration (Figure 3.5B and C). However, invasion was significantly reduced in the presence of TTX (39.7 ± 7.2%), and enhanced in the presence of veratridine (19.7 ± 4.7%) (Figure 3.5D). To examine further the influence of Na\textsubscript{v} channel expression and activity on H460 cell invasion, two H460 dilution clones were generated, one with high Na\textsubscript{v} channel expression (termed ‘high’) and the other with low Na\textsubscript{v} channel expression (termed ‘low’) (Figure 3.6). Comparison of relative invasion between the two dilution clones indicated that the ‘high’ dilution clone was 25.6 ± 4.4% more invasive than control H460 cells, whereas the ‘low’ dilution clone was 40.5 ± 3.7% less invasive than control cells. Together, these findings indicate that functional expression of Na\textsubscript{v} channels is crucial in promoting invasion of strongly metastatic H460 NSCLC cells, without influencing either cell proliferation or migration.

As discussed earlier, Na\textsubscript{v} channels are normally expressed in excitable cells where they are activated by depolarisation of the cell membrane in response to an AP. Epithelial cells, however, are not electrically excitable cells and do not generate APs. In other invasive cancer cells of epithelial origin, the existence of $I_{Na,P}$ ‘window’ currents at the resting membrane potential, $E_{m}$, of the cell have been hypothesised to result in sufficient Na\textsuperscript{+} influx to promote invasive cell behaviours (Roger et al. 2007; Gillet et al. 2009). Here too, overlapping plots of conductance-voltage and availability-voltage relationships for H460 cells clearly indicates the presence of a ‘window’ current between membrane potential values of -40 and -10 mV, where the Na\textsubscript{v} channels in the cells are partially activated and not fully inactivated (Figure 3.7A).
Consistent with this, current clamp measurements revealed that the $E_m$ was significantly more depolarised in the H460 cells ($E_m = -27.4 \pm 1.2$ mV) compared with the A549 cells ($E_m = -36.1 \pm 3.0$ mV) (Figure 3.7B). Moreover, application of 0.5 µM TTX to H460 cells hyperpolarised the $E_m$ to $-37.0 \pm 1.1$ mV (very similar to the $E_m$ value for the weakly metastatic A549 cells). Conversely, application of 50 µM veratridine caused a small but significant depolarisation of the $E_m$ (-23.0 ± 1.3 mV). Therefore, at the $E_m$ of H460 cells, persistent Na\(^+\) influx via partially activated, TTX-sensitive Na\(_v\) channels is possible. Thus, elevated [N\(+\)]\(_i\) would be predicted in H460 versus A549 cells. Indeed, as expected, [N\(+\)]\(_i\) measured in H460 cells was approximately two-fold higher than that in A549 cells (22.3 ± 4.3 and 9.7 ± 3.2 mM, respectively, Figure 3.7C). Furthermore, [N\(+\)]\(_i\) was decreased following treatment of H460 cells with 0.5 µM TTX (10.8 ± 2.8 mM). Treatment of H460 cells with 50 µM veratridine did result in a slight increase in [N\(+\)]\(_i\) compared with the control H460 cells (28.8 ± 4.0 mM), as predicted, but this was not significant.
Figure 3.5: Blocking Na\textsubscript{v} channels inhibits invasion of strongly metastatic H460 cells. (A) Effect of 0.5 µM TTX (n=5) and 50 µM veratridine (n=6) on \(I_{\text{Na}}\) elicited by a depolarisation to +10 mV (\(V_{\text{peak}}\)), from \(V_h\) -90 mV, in H460 cells. (B) Effect of 0.5 µM TTX (closed squares) and 50 µM veratridine (open circles) on H460 cell proliferation, recorded over 120 hours on 96-well plates, measured as % confluence and analysed using the IncuCyte™ imaging system (n=8 for all conditions). (C) Effect of 0.5 µM TTX (closed squares) and 50 µM veratridine (open circles) on H460 cell migration, recorded over 58 hours using the scratch-wound assay on 24-well plates, measured as % wound confluence and analysed using the IncuCyte™ imaging system (n=8 for all conditions). (D) Effect of 0.5 µM TTX and 50 µM veratridine on H460 cell Matrigel™ invasion, recorded after 48 hours. Also included are the invasion assay results for two H460 cell dilution clones with different levels of Na\textsubscript{v} channel expression, ‘high’ and ‘low’ (Figure 3.6) (n=9 for all conditions, \(P<0.05\) (*), \(P<0.01\) (**); one-way ANOVA; SNK correction).
Figure 3.6: Characterisation of H460 cell dilution clones. (A) $I_{\text{max}}$ recordings from 15 H460 cell dilution clones, recorded using an HT IonWorks® electrophysiology platform ($n \geq 37$ for all dilution clones). (B) Repeated $I_{\text{max}}$ recordings from five H460 cell dilution clones, recorded using an HT IonWorks® electrophysiology platform ($n \geq 46$ for all dilution clones). (C) Average $I$-$V$ plots for $I_{\text{Na}}$ in H460 cell dilution clones with high Na$_v$ channel activity (‘high’/dilution clone #7, open squares, $n=9$) and low Na$_v$ channel activity (‘low’/dilution clone #12, closed squares, $n=10$) ($P<0.05$ (*); Student’s $t$-test). Inset shows representative current traces from the ‘high’ and ‘low’ H460 cell dilution clones. Currents were evoked using 30 ms depolarising steps in 5 mV intervals (-90 to +70 mV), from $V_h$ -90 mV. (D) Relative Matrigel™ invasion, recorded after 48 hours, for the ‘high’ and ‘low’ H460 cell dilution clones ($n=9$ for both dilution clones, $P<0.001$ (***) Student’s $t$-test).
Figure 3.7: Strongly metastatic H460 cells have a higher concentration of intracellular sodium than weakly metastatic A549 cells. (A) Overlay of conductance-voltage (closed squares, n=17) and availability-voltage/steady-state inactivation (closed circles, n=10) relationships for H460 cells. The ‘window’ generated by overlaying the two plots, between potentials -40 to -10 mV, indicates that at these $E_m$ values, Na$_v$ channels in the H460 cells are partially activated and not fully inactivated. (B) Average $E_m$ values recorded from H460 cells (n=10), A549 cells (n=8), H460 cells treated with 0.5 µM TTX (n=10) and H460 cells treated with 50 µM veratridine (n=6) (P<0.05 (*), P<0.01 (**), P<0.001 (***) ; one-way ANOVA; SNK correction). The $E_m$ value for the untreated H460 cells falls in the middle of the ‘window’ shown in (A). (C) [Na$^+$]$_i$ values for H460 cells, A549 cells and H460 cells treated with either 0.5 µM TTX or 50 µM veratridine, monitored using the ratiometric fluorescent dye, SBFI (n=5 for all conditions, P<0.01 (**); one-way ANOVA; SNK correction). (D) Representative images of SBFI fluorescence in H460 and A549 cells at 510 ± 20 nm.
3.3.3 The \( \text{Na}_v \)1.7 \( \alpha \)-subunit is the predominant channel expressed in H460 cells

\( \text{Na}_v \) channels exist as pore-forming \( \alpha \)-subunits usually associated with one or more auxiliary \( \beta \)-subunits. To date, nine \( \alpha \)-subunit and five \( \beta \)-subunit isoforms (including the soluble \( SCNIB \) splice variant, \( \beta 1B \)) have been identified. Of these, \( \text{Na}_v \)1.1, 1.2, 1.3, 1.4, 1.6 and 1.7 are considered TTX-sensitive (\( EC_{50} = 4-25 \text{ nM} \)), whereas the remaining isoforms (\( \text{Na}_v \)1.5, 1.8 and 1.9) are TTX-resistant (\( EC_{50} = 2-200 \text{ µM} \)). Thus, the observed sensitivity of \( I_{\text{Na}} \) in H460 NSCLC cells to 0.5 µM TTX suggests that the channels responsible for the currents in these cells are TTX-sensitive \( \text{Na}_v \) channel isoforms. To determine the isoform(s) predominantly expressed in the H460 cell line, expression of all six TTX-sensitive \( \alpha \)-subunits, as well as all four of the membrane-associated auxiliary \( \beta \)-subunits, was analysed in both the H460 and A549 cell lines. To this end, mRNA levels for each subunit isoform were determined by qPCR (Figure 3.8A). Notably, in the weakly metastatic A549 cells, mRNA levels for all six \( \alpha \)-subunits were low. In the strongly metastatic H460 cells, levels of \( \text{Na}_v \)1.1, 1.2, 1.3, 1.4 and 1.6 were also low. However, the neuronal \( \text{Na}_v \)1.7 isoform was expressed at approximately four-fold higher levels in H460 versus A549 cells. Comparison with \( \text{Na}_v \) channel subunit expression in SH-SY5Y neuroblastoma cells, known to express a number of functional \( \text{Na}_v \) channel isoforms, suggested that \( \text{Na}_v \)1.7 mRNA levels in H460 cells are consistent with levels necessary for functional expression.

Having determined that the \( \text{Na}_v \)1.7 gene (\( SCN9A \)) is strongly expressed in H460 cells, levels of \( \text{Na}_v \)1.7 protein were determined in both the H460 and A549 cells. Western immunoblotting using a \( \text{Na}_v \)1.7-specific primary antibody detected a band of approximately 240 kDa in H460 cells and in control HEK-293 cells stably expressing \( \text{Na}_v \)1.7 (Figure 3.8B). This band was absent in wild type HEK-293 cells and nearly undetectable in A549 cells and in H460 cells that had been treated with siRNA directed against \( \text{Na}_v \)1.7. Similarly, immunolabelling of \( \text{Na}_v \)1.7 with a primary antibody directed against a C-terminal intracellular epitope of \( \text{Na}_v \)1.7 was much stronger in the \( \text{Na}_v \)1.7-positive HEK-293 and H460 cells than in the wild type HEK-293 and A549 cells (Figure 3.8C). In both the stably transfected HEK-293-\( \text{Na}_v \)1.7 and H460 cells there was a punctate distribution of \( \text{Na}_v \)1.7 protein, with high levels of staining at the plasma membrane. Further qPCR analysis of \( \text{Na}_v \)1.7 mRNA expression was
carried out on the two H460 cell dilution clones characterised in Figure 3.6 (Figure 3.8D). As predicted, the level of Na\textsubscript{v}1.7 expression was significantly different between the two dilution clones (the ‘low’ dilution clone expressed 60.8 ± 2.2% less Na\textsubscript{v}1.7 mRNA than the ‘high’ dilution clone), supporting the idea that the TTX-sensitive Na\textsubscript{v}1.7 α-subunit isoform is uniquely upregulated in strongly metastatic H460 NSCLC cells compared with weakly metastatic A549 NSCLC cells, and that this is the channel responsible for the detectible \(I_{Na}\) in H460 cells.

Interestingly, qPCR analysis of Na\textsubscript{v} channel subunit expression also showed that levels of β-subunit mRNA were consistently low in the H460 cells, whereas in the A549 cells the β1-subunit was very highly expressed (approximately eight-fold higher in A549 cells versus H460 cells). Notably, other studies have reported high levels of Na\textsubscript{v} channel β-subunit expression in weakly metastatic cancer cells (Chioni et al. 2009). There is growing evidence to suggest that Na\textsubscript{v} channel β-subunits, and in particular the β1 isoform, function as cell adhesion molecules (CAMs), and so the loss or downregulation of these auxiliary subunits may also contribute to the development of a more invasive cancer phenotype (Isom 2001; Isom 2002; Brackenbury and Isom 2008; Chioni et al. 2009; Patino and Isom 2010; Brackenbury and Isom 2011). A putative role for β1 in NSCLC cell adhesion was confirmed using adhesion assays, as outlined in section 2.10.4 (Figure 3.9). A549 cells were significantly more adhesive on fibronectin-coated plates than H460 cells (\(A_{585}\) values of 0.244 ± 0.008 and 0.104 ± 0.003, respectively). When expression of β1 was knocked down by 67.2 ± 3.0% using siRNA directed against β1 mRNA, A549 cell adhesion on fibronectin-coated plates was reduced significantly (\(A_{585} = 0.181 ± 0.003\)). The reduced adhesive properties of the A549 cells as a result of siRNA-induced knockdown of β1 expression corresponded to a 26.6 ± 4.6% increase in A549 cell invasion. Thus, β1-subunits appear to function as CAMs in weakly metastatic A549 NSCLC cells, contributing to the low invasive potential of these cells.
Figure 3.8: The Na,1.7 α-subunit is predominantly expressed in H460 cells. (A) mRNA expression, assessed by qPCR, for all TTX-sensitive Na, channel α-subunits and all auxiliary β-subunits in H460 and A549 NSCLC cells, and in SH-SY5Y neuroblastoma cells, known to express a number of functional Na, channel isoforms (n=6 for all primer sets in all cell lines, \( P<0.05 \) (*); one-way ANOVA; SNK correction). (B) Representative Western immunoblots showing the expression of Na,1.7 (upper) and β-actin (lower) proteins in negative (wild type HEK-293) and positive (stably transfected HEK-293-Na,1.7) control cells, and in A549 cells, H460 cells and H460 cells transfected with 5 nM siRNA directed against Na,1.7 (n=3 for all blots). (C) Representative ICC images showing cellular localisation of Na,1.7 channel protein in negative (wild type HEK-293) and positive (stably transfected HEK-293-Na,1.7) control cells, and in H460 and A549 cells, using a Na,1.7-specific antibody recognising a C-terminal intracellular epitope of Na,1.7. CTL 1, H460 cells with no primary antibody incubation; CTL 2, H460 cells with no secondary antibody incubation (n≥10 for all cell lines, scale bars: 10 \( \mu \)m). (D) qPCR showing relative Na,1.7 mRNA levels in the ‘high’ and ‘low’ H460 dilution clones (n=6 for both dilution clones, \( P<0.01 \) (**); Student’s \( t \)-test). Inset shows representative Western immunoblots demonstrating the expression of Na,1.7 (upper) and β-actin (lower) proteins in the ‘high’ and ‘low’ H460 dilution clones (n=3 for both blots).
Figure 3.9: Downregulation of $\beta_1$-subunit expression reduces adhesion and enhances invasion of A549 cells. (A) qPCR showing relative effect of 5 nM siRNA directed against the Na$_v$ channel $\beta_1$-subunit on $\beta_1$ mRNA expression in A549 NSCLC cells versus untreated A549 cells and A549 cells transfected with a scrambled siRNA sequence ($n=6$ for all conditions, $P<0.001$ (***) ; one-way ANOVA; SNK correction). (B) Cell adhesion data, analysed by crystal violet staining and A$_{585}$ measurement, for H460 cells, A549 cells and A549 cells treated with 5 nM siRNA directed against $\beta_1$, both in the presence and absence of fibronectin ($n=8$ for all conditions, $P<0.01$ (**), $P<0.001$ (***) ; one-way ANOVA; SNK correction). Inset shows representative images of stained H460, A549 and $\beta_1$-siRNA-treated A549 cells in adhesion plates prior to cell lysis and A$_{585}$ measurement. (C) Effect of 5 nM siRNA directed against $\beta_1$ on A549 cell Matrigel™ invasion, recorded after 48 hours ($n=9$ for both conditions, $P<0.05$ (*); Student’s t-test).
3.3.4 Downregulation of Na\textsubscript{1.7} expression inhibits H460 cell invasion

To confirm the predominant role of Na\textsubscript{1.7} in H460 NSCLC cells, siRNA directed against Na\textsubscript{1.7} mRNA was used for specific knockdown of this isoform. The efficacy of the siRNA in targeting SCN9A transcripts was assessed by qPCR (Figure 3.10A). Compared with a scrambled siRNA sequence, transfection with the Na\textsubscript{1.7} siRNA resulted in a 74.8 ± 1.4\% reduction in Na\textsubscript{1.7} mRNA expression, as well as reduced levels of Na\textsubscript{1.7} channel protein, as shown earlier (Figure 3.8B). Furthermore, this knockdown of Na\textsubscript{1.7} expression resulted in a complete loss of $I_{Na}$ in H460 cells (Figure 3.10B). Crucially, this siRNA-induced reduction of Na\textsubscript{1.7} expression and activity resulted in a 50.2 ± 1.8\% inhibition of H460 cell invasion (Figure 3.10C). Thus, specific loss of functional Na\textsubscript{1.7} channel protein significantly reduces the invasive capabilities of strongly metastatic H460 NSCLC cells.
Figure 3.10: The Na\textsubscript{v}1.7 \(\alpha\)-subunit drives invasion of H460 cells. (A) qPCR showing relative effect of 5 nM siRNA directed against Na\textsubscript{v}1.7 on Na\textsubscript{v}1.7 mRNA expression in H460 cells versus untreated H460 cells and H460 cells transfected with a scrambled siRNA sequence \((n=6\) for all conditions, \(P<0.001\) (***)\; one-way ANOVA; SNK correction). (B) Average \(I-V\) plots for \(I_{Na}\) in untransfected H460 cells (open squares, \(n=10\)), H460 cells transfected with scrambled siRNA (closed squares, \(n=10\)) and H460 cells transfected with 5 nM Na\textsubscript{v}1.7-siRNA (closed circles, \(n=8\)). Inset shows representative current traces from untransfected H460 cells and H460 cells transfected with 5 nM Na\textsubscript{v}1.7-siRNA. Currents were evoked using 30 ms depolarising steps in 5 mV intervals (-90 to +70 mV), from \(V_h\) -90 mV. (C) Effect of 5 nM Na\textsubscript{v}1.7-siRNA on H460 cell Matrigel\textsuperscript{TM} invasion, recorded after 48 hours \((n=9\) for all conditions, \(P<0.001\) (***)\; one-way ANOVA; SNK correction).
3.4 Discussion

3.4.1 Summary of results

The data presented in this chapter demonstrate that there is functional expression of the neuronal Na\textsubscript{v}1.7 Na\textsubscript{v} channel isoform in strongly metastatic H460 NSCLC cells. These TTX-sensitive channels drive cell invasion without having any effect on the proliferative or migratory capabilities of the cells. Conversely, weakly metastatic A549 NSCLC cells do not express Na\textsubscript{v} channel α-subunits, but instead express considerably higher levels of β1-subunit, resulting in enhanced cell adhesion.

3.4.2 Technical considerations

Whilst experimental design has been robust and thorough with all necessary controls implemented, the following technical considerations must be highlighted:

1. For electrophysiological recordings, liquid junction potentials may exist. Liquid junction potentials occur when two solutions of different constituent concentrations are in contact with each other (intracellular pipette solution with extracellular bath solution, or, intracellular pipette solution with the cytosol of an impaled cell, for example). They arise because different ionic species move at different speeds through the solute, depending on their size and the concentration gradient of the ion at the solution interface, thereby creating a slight separation of charges. Therefore, in order to minimise recording errors as a result of these charge separations, liquid junction potentials should be compensated for.

2. Steady-state inactivation protocols involved exposing cells to prepulse potentials (-120 to +40 mV) for a duration of 500 ms prior to a test potential of +10 mV ($V_{\text{peak}}$). A 500 ms prepulse potential was deemed to be of sufficient duration to allow the Na\textsubscript{v} channels to reach steady-state prior to exposing them to $V_{\text{peak}}$, and ensured that the cells were not under too much stress. However, it may be the case that a 500 ms prepulse is
not actually long enough to guarantee that steady-state has been reached. After all, cell membranes are at their resting potential, $E_m$, continuously under physiological conditions.

3. For qPCR experiments, only one constitutively expressed housekeeping gene was used as a fixed reference for data normalisation (18S rRNA). Generally, it is advised that at least two housekeeping genes should be used for the normalisation of expression data for a particular gene of interest. However, the geNorm™ analysis for six human housekeeping genes (18S rRNA, ACT, ATP5B, GAPDH, TOP1 and UBC), outlined in section 2.6, revealed that expression of all of these genes, with the exception of 18S rRNA, is affected by many of the treatments used in the expression experiments conducted in this project. Therefore, it was decided that just 18S rRNA should be used as a housekeeping gene for qPCR experiments.

### 3.4.3 Discussion of results

Functional expression of Na$_v$1.7 has previously been shown in strongly metastatic cancer cell lines of epithelial origin and in cancerous primary cell lines and biopsies, but not in non-cancerous biopsies or weakly metastatic cell lines (Diss et al. 2001; Diss et al. 2005; Diaz et al. 2007; Roger et al. 2007; Uysal-Onganer and Djamgoz 2007; Gao et al. 2010; Hernandez-Plata et al. 2012). This suggests that Na$_v$1.7 expression is a potential marker of metastatic cancer.

The identification of Na$_v$1.7 expression and activity as necessary for invasion of strongly metastatic NSCLC cells agrees with earlier work that has established a crucial role of Na$_v$ channels – particularly Na$_v$1.5 and Na$_v$1.7 – in metastatic behaviours of several cancers, including NSCLC (Laniado et al. 1997; Abdul and Hoosein 2002; Bennett et al. 2004; Onganer et al. 2005; Brackenbury et al. 2007; Diaz et al. 2007; Roger et al. 2007; Uysal-Onganer and Djamgoz 2007; Gao et al. 2009; Gillet et al. 2009; Gao et al. 2010; House et al. 2010). The presence of Na$_v$1.7 mRNA in H460 NSCLC cells was reported by Roger and colleagues (Roger et al. 2007). However, its predominant role in Na$_v$ channel-dependent
invasion of these cells has not been shown before. In breast cancer, the Na$_v$ channel β1-subunit is reported to act as a CAM, being highly expressed in non-invasive cells but not in strongly metastatic cells which exhibit reduced cell adhesion (Brackenbury and Isom 2008; Chioni et al. 2009). Consistent with such reports, a concurrent downregulation of β1 (highly expressed in non-invasive A549 cells) and upregulation of Na$_v$1.7 mRNA is reported in H460 cells, which together likely drive H460 cells towards a more metastatic phenotype.

An influx of Na$^+$ into strongly metastatic H460 cells via Na$_v$1.7 appears to promote cell invasion, without influencing cell proliferation or migration; the Na$_v$ channel-specific blocker, TTX, and opener, veratridine, inhibit and promote H460 cell invasion, respectively. Similarly, the selective knockdown of Na$_v$1.7 expression results in the specific inhibition of H460 cell invasion. In agreement with others (Roger et al. 2007; Gillet et al. 2009), this project presents evidence for continuous influx of Na$^+$ via partial activation of Na$_v$1.7 at the $E_m$ of H460 cells. Block of Na$_v$1.7 activity hyperpolarises $E_m$ and reduces [Na$^+$]$_i$, whereas enhanced channel opening depolarises $E_m$ and raises [Na$^+$]$_i$. Thus, there appears to be a positive feedback mechanism whereby the partial opening of Na$_v$1.7 at $E_m$ allows Na$^+$ influx, which in turn keeps $E_m$ sufficiently depolarised to maintain a continuous influx of Na$^+$. Under physiological conditions, this persistent Na$^+$ influx is sufficient to maintain the invasive capability of cells.

The generation of two H460 cell dilution clones, one with high Na$_v$ channel activity and the other with low Na$_v$ channel activity, also supports a role for Na$_v$1.7 in H460 cell invasion. The benefit of using the two generated dilution clones is that they are from exactly the same cell line and so only differ in their levels of endogenous Na$_v$1.7 expression, thus providing a controlled system in which to analyse Na$_v$ channel expression and its effect on cell behaviour. Therefore, the fact that the ‘high’ clone was significantly more invasive than the ‘low’ clone supports the hypothesis that functional Na$_v$ channel expression alone is sufficient to promote cell invasion in non-excitable NSCLC cells.

It can therefore be proposed that the aberrant expression of Na$_v$ channels in NSCLC cells specifically promotes cell invasion through the influx of extracellular Na$^+$ into the cells,
without affecting cell proliferation or migration. However, the fact that preventing Na\(^+\) influx through Na\(_v\)1.7 only results in a 40-50% reduction in cell invasion indicates that Na\(_v\) channels are not the whole story; they contribute significantly to the invasive capabilities of H460 cells but do not account for all of it. It is also important to remember that cellular migration and invasion are two distinct processes (Friedl and Wolf 2003; Sahai 2005): migration is defined as the amoeboid-like movement of cells, whereas invasion involves the physical breakdown of ECM proteins by membrane-tethered or secreted protease enzymes, enabling cells to invade local tissue and circulatory systems to migrate and form secondary tumours. The fact that Na\(_v\) channels appear to specifically promote the invasive capabilities of epithelial cancer cells suggests that they ultimately influence the expression and/or activity of these proteases.

Very little is known about the transcriptional regulation of Na\(_v\) channel expression in metastatic cancers, the clinical relevance of Na\(_v\) channel expression in NSCLC, or, ultimately, the downstream effect(s) of elevated [Na\(^+\)]\(_i\) on cancer cell invasion, and so these areas comprise the remainder of the project. Having identified Na\(_v\)1.7 as a key player in the invasive properties of H460 NSCLC cells, its clinical relevance in NSCLC was next investigated.
Chapter 4:
RESULTS – Analysis of Na\textsubscript{v}1.7 expression in lung cancer tumour samples by immunohistochemistry
4.1 Introduction

There is increasing \textit{in vitro} evidence that the functional expression of Na\textsubscript{v} channels in non-excitatory cancer cells of epithelial origin is associated with cancer progression through the development of a more invasive/metastatic cancer phenotype. However, what can be seen in the lab does not always translate to what is actually happening physiologically and so a demonstration of the clinical relevance of this field of research through solid \textit{in vivo} evidence is very important: the vast majority of studies have made use of immortalised cell lines and as a result it is often not clear how the findings relate to cancerous tissue. Early studies noted that tumour samples had higher intracellular concentrations of Na\textsuperscript{+} relative to normal tissue and that this had the potential to drive cancer invasion (Cameron et al. 1980). More recently, with Na\textsubscript{v} channels emerging as potential markers of metastatic progression of epithelial cancer cells, there is mounting evidence that Na\textsubscript{v} channel activity (Na\textsuperscript{+} influx) promotes cancer metastases \textit{in vivo} (Abdul and Hoosein 2002; Diss et al. 2005; Fraser et al. 2005; Diaz et al. 2007; House et al. 2010; Hernandez-Plata et al. 2012; Yildirim et al. 2012).

Immunohistochemistry (IHC) is a strong analytical tool that allows for the detection of proteins of interest in cells of a biological tissue section using specific antibodies. It provides evidence for the \textit{in vivo} expression of proteins in tissues and can therefore support or undermine a proposed physiological role for a particular protein of interest. IHC has been used to demonstrate the \textit{in vivo} expression of Na\textsubscript{v} channels in metastatic tumours of breast (Fraser et al. 2005), prostate (Abdul and Hoosein 2002; Diss et al. 2005), cervical (Hernandez-Plata et al. 2012) and colon cancer (House et al. 2010). In all cases, there was a marked difference in the expression of Na\textsubscript{v} channels between cancerous and healthy, normal tissues. To date, there is no accessible information on the \textit{in vivo} expression of Na\textsubscript{v} channels in human NSCLC. Therefore, for Na\textsubscript{v} channels to be considered as tractable therapeutic and/or diagnostic targets in metastatic NSCLC, an unequivocal demonstration of the clinical relevance of these channels in metastatic lung cancer is needed.
4.2 Aims

The aim of this study was to establish whether Na\textsubscript{v}1.7 expression is enhanced in cancerous lung tissue versus normal, healthy lung tissue. In order to address this, the following steps were undertaken:

1. Optimisation of a Na\textsubscript{v}1.7-specific antibody for use in IHC.
2. Determination of Na\textsubscript{v}1.7 expression in cancerous and normal-matched lung tissue using IHC.
3. Determination of whether Na\textsubscript{v}1.7 expression is particularly associated with high-grade/metastatic tumours using IHC.

4.3 Results

4.3.1 Optimisation of an anti-Na\textsubscript{v}1.7 antibody for use in IHC

Before analysis of Na\textsubscript{v}1.7 expression in human lung tissue samples could be carried out, it was first necessary to identify a suitable anti-Na\textsubscript{v}1.7 primary antibody for use with formalin-fixed, paraffin-embedded (FFPE) tissue preparations. To this end, five anti-Na\textsubscript{v}1.7 antibodies (AstraZeneca), selected based upon both their ability to generate bands of the appropriate molecular weight of ~260 kDa by Western immunoblotting and their performance in immunocytochemistry (ICC) experiments, were tested on FFPE samples of wild type HEK-293 cells (lacking Na\textsubscript{v}1.7) and stably transfected HEK-293 cells expressing Na\textsubscript{v}1.7 (HEK-293-Na\textsubscript{v}1.7) using the IHC methodology outlined in section 2.12 (Figure 4.1). A comparison of immunoreactivity for the five antibodies is summarised in Table 4.1. Antibodies AbAZS00013 and AbAZS00018 resulted in background staining in the negative cell lines, suggesting non-specific binding of the antibody. Antibodies AbAZS00015 and AbAZS00017 produced staining in the positive cell line that was weak and localised to the cell nucleus, respectively. The AGGH2013 antibody resulted in no staining in the negative cell line with strong, consistent cytoplasmic and membrane staining in the positive cell line. Thus, AGGH2013
performed best in its ability to distinguish between Na\textsubscript{v}1.7-positive and Na\textsubscript{v}1.7-negative cell lines and was therefore selected as the lead antibody for further optimisation.

Having identified the AGGH2013 antibody as the most suitable anti-Na\textsubscript{v}1.7 antibody for use in Na\textsubscript{v}1.7 channel protein detection in FFPE tissue samples, the same approach was used to confirm Na\textsubscript{v}1.7 expression in FFPE preparations of weakly metastatic A549 and strongly metastatic H460 NSCLC cell lines (Figure 4.2). It was previously shown in Chapter 3 that Na\textsubscript{v}1.7 is more highly expressed in the invasive H460 cell line compared with non-invasive A549s. Consistent with the qPCR, Western immunoblot and ICC data, Na\textsubscript{v}1.7 immunoreactivity was minimal in the A549 cell line, whereas there was strong staining in all the H460 cells. Negative controls (no primary antibody incubation) indicated no staining at all. Further evidence for the specificity of the AGGH2013 antibody to Na\textsubscript{v}1.7, as well as for the high level of Na\textsubscript{v}1.7 expression in the strongly metastatic H460 cell line, was provided by performing the same IHC experiment on FFPE preparations of H460 cells that had been transfected with siRNA directed against Na\textsubscript{v}1.7. As predicted, these siRNA-treated H460 cells showed reduced levels of immunoreactivity compared with untreated H460 cells.

Before analysis of Na\textsubscript{v}1.7 expression in human lung tissue was performed, the AGGH2013 antibody was first tested on FFPE mouse xenografts of H460 cells in order to confirm immunoreactivity against Na\textsubscript{v}1.7 in an \textit{in vivo} model of lung cancer and to ensure that there was no non-specific binding of the antibody with proteins/structures derived from the mouse host (Figure 4.3). As predicted, there was strong staining of the H460 cells in the tumour. Furthermore, the staining was unique to the H460 cells in the tumour with no staining of mouse stromal cells or regions of tissue necrosis within the tumour. Negative controls, consisting of no primary antibody incubation, were performed on the xenografts and show no staining at all. These data show that the AGGH2013 anti-Na\textsubscript{v}1.7 antibody is specific for Na\textsubscript{v}1.7 channel protein in FFPE tissue samples, demonstrating no non-specific cross-reactivity \textit{in vivo}. The next step, therefore, was to use the antibody to perform IHC experiments on cancerous human lung tissue and normal-matched human lung tissue in order to establish if there is differential expression of Na\textsubscript{v}1.7 between the two tissue types.
Figure 4.1: Immunodetection of Na\textsubscript{1.7} channel proteins in Na\textsubscript{1.7}-positive (+ve) and Na\textsubscript{1.7}-negative (-ve) HEK-293 cell lines. High temperature antigen retrieval was performed in low pH and pH 9.0 antigen retrieval buffers for all antibodies and it was determined that results were better when performed in low pH antigen retrieval buffer. Brown regions are areas of primary antibody immunoreactivity as a result of a brown-coloured precipitate forming at the antigen site following HRP activity on DAB Peroxidase Substrate Solution. For all of the tested antibodies there was a marked difference in the level of staining between the two cell lines, suggesting strong immunoreactivity between the antibodies and the Na\textsubscript{1.7} channel protein present in the positive cell line ($n=3$ for all antibodies on both cell lines, scale bars: 200 µm).
Table 4.1: Analysis of antibody suitability for use in Na$_v$1.7 channel protein detection in FFPE tissue samples.

<table>
<thead>
<tr>
<th>Anti-Na$_v$1.7 antibody</th>
<th>+ve versus –ve controls</th>
<th>Localisation</th>
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<tbody>
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<td>AbAZS00013</td>
<td>Strong staining in +ve controls, staining of nucleus in –ve controls</td>
<td>Strong nuclear staining in both cell lines, little membrane staining</td>
</tr>
<tr>
<td>AbAZS00015</td>
<td>Clean background in –ve controls but weak staining in +ve controls</td>
<td>Cytoplasmic staining, no membrane staining</td>
</tr>
<tr>
<td>AbAZS00017</td>
<td>Very clean background in –ve controls, consistent staining in +ve controls</td>
<td>Cytoplasmic staining with some nuclear staining</td>
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<tr>
<td>AbAZS00018</td>
<td>Very strong, consistent staining in +ve controls but more background in –ve controls than AbAZS00017</td>
<td>Strong cytoplasmic staining with some membrane and nuclear staining</td>
</tr>
<tr>
<td>AGGH2013</td>
<td>Very clean background in –ve controls, strong staining in +ve cells</td>
<td>Strong cytoplasmic staining with some appearing to display membrane staining, no nuclear staining</td>
</tr>
</tbody>
</table>

All five antibodies resulted in a marked difference in the level of staining between the Na$_v$1.7-positive (+ve) and Na$_v$1.7-negative (-ve) HEK-293 cell lines. However, only the AGGH2013 antibody was selected for further optimisation based on a clean background in the negative cell line and a strong cytoplasmic and membrane staining in the positive cell line. The other antibodies were rejected based on background staining in the negative cell line, weak staining in the positive cell line and/or staining confined to the nucleus of the cells.
Figure 4.2: Immunodetection of Na\textsubscript{v}1.7 channel proteins in NSCLC cell lines. FFPE A549 cells (A), H460 cells and H460 cells treated with siRNA directed against Na\textsubscript{v}1.7 (B) were incubated with AGGH2013 anti-Na\textsubscript{v}1.7 primary antibody and immunoreactivity against Na\textsubscript{v}1.7 was observed. Brown regions are areas of primary antibody immunoreactivity as a result of a brown-coloured precipitate forming at the antigen site following HRP activity on DAB Peroxidase Substrate Solution. No immunoreactivity was observed in the negative controls (Neg), where the primary antibody was omitted from the experiments ($n=3$ for all cell lines, scale bars: 200 µm).
Figure 4.3: Immunodetection of Na\textsubscript{v}1.7 channel proteins in H460 cell mouse xenografts. (A) FFPE mouse xenografts of human H460 NSCLC cells were incubated with AGGH2013 anti-Na\textsubscript{v}1.7 primary antibody and immunoreactivity against Na\textsubscript{v}1.7 was observed. Lipids (#), unstained mouse stroma (►) and unstained regions of tissue necrosis (*) can be clearly identified. Brown regions are areas of primary antibody immunoreactivity as a result of a brown-coloured precipitate forming at the antigen site following HRP activity on DAB Peroxidase Substrate Solution. (B) Negative control experiments for FFPE mouse xenografts of human H460 NSCLC cells in which the primary antibody step was omitted from the IHC protocol. No immunoreactivity was observed when the primary antibody was omitted from the experiments (n=2 for both experiments, scale bars: 200 µm).
4.3.2 Differential expression of Na$_{\text{v}}$1.7 in cancerous and normal-matched human lung tissue

Having optimised the AGGH2013 anti-Na$_{\text{v}}$1.7 antibody for the immunodetection of Na$_{\text{v}}$1.7 channel protein in FFPE tissue samples, in order to demonstrate the in vivo relevance of this research the antibody was used to examine the levels of Na$_{\text{v}}$1.7 expression in cancerous and normal-matched human lung tissue (Figure 4.4). Assuming upregulation of functional Na$_{\text{v}}$1.7 expression is indeed a marker for the development of invasive NSCLC in vivo, analysis of Na$_{\text{v}}$1.7 expression in healthy versus diseased lung tissue is predicted to demonstrate a clear difference between the two tissue types.

On account of the difficulty in acquiring patient lung cancer samples, only three were tested. In all three of the patient samples, the overall level of staining in the normal, healthy tissue was low, whereas staining in the cancerous tissues was increased. The staining in the cancerous lung tissue appeared to be cytoplasmic with some clear immunoreactivity at the plasma membrane too. Furthermore, immunostaining revealed distinct expression of Na$_{\text{v}}$1.7 on the luminal surface of lung tissue, something also noted in IHC experiments performed on colon cancer tissue (House et al. 2010). The most convincing evidence to suggest that Na$_{\text{v}}$1.7 expression is upregulated in cancerous lung tissue comes from the cancerous tissue obtained from patient 3 (Figure 4.4B, P3). In this sample there is clearly disorganised tumour tissue of the lung (left) bordering healthy tissue (right). Not only is the boundary between the two tissue types clear when looking at tissue morphology, there is also a marked difference in the level of immunoreactivity against Na$_{\text{v}}$1.7 between the two tissues. IHC analysis of human lung tissue therefore confirms the earlier data in cell lines. Thus, these cell lines (H460 and A549) appear to be a reasonable model, lending weight to findings in subsequent chapters.

I next asked whether an increase in Na$_{\text{v}}$1.7 expression might accompany the transition from low- to high-grade tumours. Unfortunately, different staged lung cancer samples could not be obtained. However, FFPE preparations of low- and high-grade prostate cancer tumours, known to be associated with high expression of Na$_{\text{v}}$1.7 (Diss et al. 2001; Diss et al. 2005; Nakajima et al. 2009; Yildirim et al. 2012), were available. Using the AGGH2013 anti-Na$_{\text{v}}$1.7
antibody, I found that in the low-grade prostate cancer samples, Na\textsubscript{v}1.7 immunostaining was very low, to the point where no staining could be detected (Figure 4.5). However, in the two high-grade prostate cancer samples, staining was markedly increased. Based on the earlier observations that upregulated Na\textsubscript{v} channel expression is associated with the development of invasive cell behaviours in NSCLC cell lines and that formation of metastases is a hallmark of late-stage cancer, these data support a role for Na\textsubscript{v} channels in the development of an invasive cancer phenotype \textit{in vivo}. 
Figure 4.4: Immunodetection of Na\(_{\text{v}}\)1.7 channel proteins in cancerous human lung tissue and normal-matched human lung tissue. FFPE samples of normal human lung tissue (A) and cancerous human lung tissue (B) from three separate patients (P1-P3) were incubated with AGGH2013 anti-Na\(_{\text{v}}\)1.7 primary antibody and immunoreactivity against Na\(_{\text{v}}\)1.7 was observed. Brown regions are areas of primary antibody immunoreactivity as a result of a brown-coloured precipitate forming at the antigen site following HRP activity on DAB Peroxidase Substrate Solution. The black regions in the samples are deposits of carbon and are common in preparations of lung tissue (\(n=2\) for both tissue types, scale bars: 200 µm).
Figure 4.5: Immunodetection of Na\(_{\text{v}}\)1.7 channel proteins in low-grade and high-grade human prostate cancer samples. FFPE samples of a low-grade prostate cancer tumour and two high-grade prostate cancer tumours were incubated with AGGH2013 anti-Na\(_{\text{v}}\)1.7 primary antibody and immunoreactivity against Na\(_{\text{v}}\)1.7 was observed. Brown regions are areas of primary antibody immunoreactivity as a result of a brown-coloured precipitate forming at the antigen site following HRP activity on DAB Peroxidase Substrate Solution (n=2 for all three tumour samples, scale bars: 200 µm).
4.4 Discussion

4.4.1 Summary of results

The data presented in this chapter demonstrate that there is a marked association of Na_v1.7 protein with cancerous human lung tissue compared with normal, healthy human lung tissue. Furthermore, the presence of Na_v1.7 protein appears to be associated with high grade/late stage carcinomas, as demonstrated with human prostate cancer samples.

4.4.2 Technical considerations

Whilst experimental design has been robust and thorough with all necessary controls implemented, the following technical considerations must be highlighted:

1. Access to human lung and prostate tissue samples was a limiting factor in these IHC experiments. As a result, the n numbers for the experiments conducted in this chapter are relatively small. Nevertheless, the high consistency of the data obtained herein support all of the conclusions drawn from it.

2. A limitation of the experimental approach adopted in this chapter is that it is difficult to discern plasma membrane localisation of Na_v1.7 protein in individual cells. In order for Na_v1.7 to be functional as an ion channel it must first be successfully addressed at the plasma membrane, and so a clear demonstration of this would have been desirable. Instead, any identification of plasma membrane localisation of Na_v1.7 was subjective, and IHC analysis was only really able to indicate relative levels of global Na_v1.7 expression in cells/tissues.
4.4.3 Discussion of results

The aim of this study was to establish whether Na\textsubscript{v}1.7 expression is enhanced in cancerous lung tissue versus normal, healthy lung tissue. Taken together, the *in vivo* data are consistent with the *in vitro* findings regarding both increased Na\textsubscript{v} channel expression with lung cancer progression (metastasis) and the molecular identity of the candidate Na\textsubscript{v} channel (Na\textsubscript{v}1.7). The strong positive association of Na\textsubscript{v}1.7 expression with cancerous lung tissue suggests that expression of this channel *in vivo* may well represent an important event in the progression of lung cancer to the metastatic mode. At the very least, it appears to be a strong tumour marker in the lung, as has been demonstrated with Na\textsubscript{v} channels in other cancerous epithelial tissues (Abdul and Hoosein 2002; Diss et al. 2005; Fraser et al. 2005; House et al. 2010; Hernandez-Plata et al. 2012). Interestingly, immunostaining revealed that there was distinct expression of Na\textsubscript{v}1.7 on the luminal surface of cancerous lung tissue, perhaps facilitating the development of an aggressive/invasive cancer phenotype in cells with readily available conduits through which to metastasise. Similar results have been presented for Na\textsubscript{v}1.5 in colon cancer (House et al. 2010).

An investigation into Na\textsubscript{v}1.7 expression in prostate cancer tumours reveals that there is a marked upregulation of Na\textsubscript{v}1.7 protein levels in prostatic epithelial cells at the transition from low-grade to high-grade cancer. This is consistent with my previous findings, which independently showed that Na\textsubscript{v}1.7 mRNA transcripts are considerably enhanced in strongly metastatic H460 NSCLC cells compared with weakly metastatic A549 NSCLC cells. Since Na\textsubscript{v}1.7 protein levels have been shown to increase in epithelial tissues/structures with the development and progression of both NSCLC and prostate cancer, upregulated Na\textsubscript{v}1.7 expression is likely to be localised specifically to the epithelial cancer cells, putatively driving their development towards a more metastatic/invasive phenotype.

In conclusion, the results of the IHC analysis investigating Na\textsubscript{v}1.7 channel protein expression in cancerous versus normal-matched lung tissues, and in low-grade versus high-grade prostate cancer tissues, show that Na\textsubscript{v}1.7 is upregulated during epithelial cancer progression *in vivo*. Combined with the data presented previously, this suggests that Na\textsubscript{v}1.7
may be a clinically relevant protein that potentiates a series of cell behaviours integral to the metastatic cascade. If this is the case, Na\textsubscript{v}1.7 represents a very attractive, tractable target in the diagnosis, prognosis and treatment of metastatic epithelial cancers. Having developed a greater understanding of the functional expression of Na\textsubscript{v} channels in metastatic NSCLC, and confirmed that the H460 and A549 NSCLC cell lines are suitable, clinically relevant models for Na\textsubscript{v} channel-driven NSCLC disease progression, the focus of the project shifted to the regulation of functional Na\textsubscript{v}1.7 expression in NSCLC.
Chapter 5:
RESULTS – EGFR regulation of Na$_v$1.7 expression in metastatic NSCLC
5.1 Introduction

Whilst the abnormal functional expression of Na$_v$ channels has been reported in a wide range of invasive epithelial cancers, the cellular mechanisms underlying the regulation of Na$_v$ channel expression and activity in cancer cells remain unclear. The biochemical makeup of extracellular medium has been shown to be a critical factor in both Na$_v$ channel expression and cell motility in strongly metastatic epithelial cancer cell lines (Ding and Djamgoz 2004; Pan and Djamgoz 2008). Therefore, prime candidates for the constituent of extracellular medium that may be responsible for these effects on metastatic cancer cells are growth factors, particularly as they are known both as regulators of Na$_v$ channel expression and key contributors to cancer development and progression (Toledo-Aral et al. 1995; Turner et al. 1996; Akopian et al. 1999; Kim et al. 1999; Lei et al. 2001; Montano and Djamgoz 2004; Lou et al. 2005; Brackenbury and Djamgoz 2007; Goldfarb et al. 2007; Liu et al. 2007; Goetz et al. 2009; Laezza et al. 2009). Mutations in components of growth factor signalling pathways are prominent in cancer, and cancer cells have been shown to produce and release their own growth factors, thereby establishing so-called autocrine loops of growth factor signalling in order to grow and survive (Sporn and Todaro 1980; Fujimoto et al. 2005; Volante et al. 2007; Wu et al. 2007). Thus, growth factor receptor stimulation and the resulting downstream signalling cascades are a logical starting point in attempting to elucidate the cellular mechanisms underlying the transcriptional control of Na$_v$ channel expression in metastatic cancer cells.

FGF and NGF signalling have long been known to regulate the expression and activity of Na$_v$ channels in a number of human and animal cell lines (Toledo-Aral et al. 1995; Akopian et al. 1999; Lei et al. 2001; Lou et al. 2005; Brackenbury and Djamgoz 2007; Goldfarb et al. 2007; Goetz et al. 2009; Laezza et al. 2009). However, in the context of metastatic cancer, EGFR signalling, known to drive cancer progression via enhanced cell invasion (Turner et al. 1996; Kim et al. 1999; Montano and Djamgoz 2004), has been directly implicated in the Na$_v$ channel-driven invasive behaviour of prostate cancer cell lines (Uysal-Onganer and Djamgoz 2007; Ding et al. 2008), which are exposed to high levels of EGFR ligand (Russell et al. 1998; Gann et al. 1999).
In NSCLC, drugs targeting the EGFR are well-characterised late-stage treatments, owing to the fact that enhanced EGFR expression, the acquisition of EGFR mutations, EGFR copy number gains and the formation of EGFR autocrine loops are all associated with the development of a more metastatic cancer phenotype and a poor prognosis (Sporn and Todaro 1980; Ozanne et al. 1986; Baselga and Mendelsohn 1994; Mendelsohn 2003; Gazdar et al. 2004; Fujimoto et al. 2005; Nomura et al. 2007; Okabe et al. 2007; Sharma et al. 2007; Volante et al. 2007; Wu et al. 2007; Gazdar and Minna 2008; Zhang et al. 2008; Dahabreh et al. 2011). Based on the available evidence, it is logical to hypothesise that Na\textsubscript{v} channel expression may be one consequence of deregulated EGFR signalling in metastatic NSCLC cells, thereby promoting cell invasion through an, as yet, uncharacterised mechanism. If this is the case, with the problems of relapse and drug resistance in lung cancers that had initially responded well to EGFR-targeted therapies, combined with the fact that Na\textsubscript{v} channel expression may well prove to be common focal point in a number of growth factor signalling pathways that are upregulated in cancer, Na\textsubscript{v} channels could well represent novel and tractable markers in the diagnosis, prognosis and therapy of metastatic carcinomas.

5.2 Aims

The main aim of the studies conducted in this chapter was to establish whether EGFR signalling, known to drive NSCLC progression, controls the functional expression of Na\textsubscript{v} channels in metastatic NSCLC, and if so, whether it contributes to the invasive properties of NSCLC cells. The specific aims were as follows:

1. To determine whether EGFR signalling regulates the functional expression of Na\textsubscript{v}1.7 in metastatic NSCLC cells, and if so, to identify the specific downstream signalling cascade(s) involved.

2. To determine whether EGFR signalling affects metastatic NSCLC cell behaviours (proliferation, migration and invasion), and if so, whether such effects are mediated via Na\textsubscript{v}1.7.
5.3 Results

5.3.1 EGFR signalling enhances functional expression of Na\textsubscript{v}1.7 in H460 cells

Having established a clinically relevant link between Na\textsuperscript{+} influx into H460 NSCLC cells via Na\textsubscript{v}1.7 and cell invasion, the following question was addressed: how is Na\textsubscript{v}1.7 expression and activity upregulated in these strongly metastatic cells? As discussed previously, it is well known that growth factor signalling plays critical roles in cancer progression, and even in non-cancerous cells ion channel expression and activity are both regulated by growth factor signalling (Toledo-Aral et al. 1995; Blair and Marshall 1997; Akopian et al. 1999; Lei et al. 2001; Lou et al. 2005; Goldfarb et al. 2007; Liu et al. 2007; Woodall et al. 2008; Goetz et al. 2009; Laezza et al. 2009). In prostate cancer cell lines, upregulation of functional Na\textsubscript{v} channel expression has been implicated in their pro-invasive response to EGFR stimulation (Uysal-Onganer and Djamgoz 2007; Ding et al. 2008). Furthermore, EGFR signalling is recognised as being particularly important in NSCLC, with targeted therapies against the EGFR and its downstream components now a mainstay of NSCLC treatment (Cappuzzo et al. 2005; Metro et al. 2006; Domingo et al. 2010; Cataldo et al. 2011).

To assess the potential role of EGFR signalling in the regulation of Na\textsubscript{v}1.7-driven H460 cell invasion, initial experiments used qPCR to determine Na\textsubscript{v}1.7 mRNA levels in H460 cells following treatments to manipulate EGFR signalling (Figure 5.1A). H460 cells were first serum starved for 24 hours, followed by incubation for a further 24 hours in the presence of 100 ng.ml\textsuperscript{-1} EGF, or simply serum starved for the full 48 hours. Control cells were maintained in the presence of serum throughout. Serum starvation reduced Na\textsubscript{v}1.7 mRNA levels by 81.2 ± 1.1% relative to the control cells, whereas addition of EGF for 24 hours post-serum starvation returned Na\textsubscript{v}1.7 expression to control levels. Treatment of control H460 cells with the EGFR inhibitor, gefitinib (1 µM), for 24 hours reduced Na\textsubscript{v}1.7 expression by 90.7 ± 1.9%. Similarly, co-application of EGF and gefitinib to serum starved cells also significantly reduced Na\textsubscript{v}1.7 expression (65.0 ± 3.5%), suggesting that gefitinib abolishes the EGF-mediated upregulation of Na\textsubscript{v}1.7 expression seen in the serum starved cells. These robust changes in Na\textsubscript{v}1.7 mRNA
levels in H460 cells in response to EGFR signalling were also mirrored by changes in Na_v,1.7 protein levels, as demonstrated by Western immunoblotting (Figure 5.1B). Equivalent functional analysis could not be carried out in serum starved cells since serum starvation adversely affected the H460 cells, particularly membrane integrity, such that whole-cell patch-clamp electrophysiology recordings proved extremely difficult. Consequently, to determine the effects on Na_v channel function, electrophysiological analysis of I_{Na} had to be performed on H460 cells maintained in the presence of serum (complete medium). Treatment of H460 cells with EGF for 24 hours had only a negligible effect on I_{max} (Figure 5.1C), presumably because EGFR-mediated signalling is already close to maximal in these cells owing to the presence of growth factors in the extracellular medium (Ding and Djamgoz 2004; Pan and Djamgoz 2008). However, when EGFR signalling was inhibited either with 1 µM gefitinib, or, 10 µg.ml^{-1} of an anti-EGF neutralising antibody, I_{Na} was significantly reduced, resulting in I_{max} values of -1.5 ± 0.1 and -1.4 ± 0.4 pA.pF^{-1}, respectively, compared with -8.9 ± 0.9 pA.pF^{-1} for controls. There were no effects on the biophysical properties of the Na_v channel currents (Table 5.1).

The concurrent effects on Na_v,1.7 mRNA, protein and I_{Na} suggest that EGFR signalling controls the transcriptional regulation of Na_v,1.7 in strongly metastatic H460 NSCLC cells, and hence the number of functional channels at the cell surface. However, growth factor signalling does also induce post-translational modification of ion channels at the cell surface; NGF-mediated phosphorylation of neuronal Ca_v channels by ERK1/2 (Martin et al. 2006; Woodall et al. 2008), or the functional regulation of Na_v channel currents by FGF (Laezza et al. 2009), for example. Moreover, trafficking of Na_v channels to the cell surface would also be expected to have a similar timecourse as any gating effects (Leterrier et al. 2010). To rule out the possibility that stimulation of the EGFR might also induce acute effects on Na_v,1.7 channels in H460 cells, the effect of a 10-minute gefitinib treatment on I_{Na} was investigated (Figure 5.1D). These data clearly demonstrate that there were no acute effects of gefitinib on I_{Na} in the H460 cells. Thus, it is concluded that EGFR signalling in H460 cells controls transcriptional regulation of Na_v,1.7 rather than post-translational modification and/or trafficking of the expressed channel protein.
Figure 5.1: Endogenous EGF tonically upregulates functional expression of Na\textsubscript{v}1.7 in H460 cells. (A) qPCR showing relative effect of serum starvation for 48 hours, stimulation with 100 ng.ml\textsuperscript{-1} EGF for 24 hours post-serum starvation (24 hours), treatment with 1 \textmu M gefitinib for 24 hours in the presence of serum, and co-application of 100 ng.ml\textsuperscript{-1} EGF and 1 \textmu M gefitinib for 24 hours post-serum starvation (24 hours) on Na\textsubscript{v}1.7 mRNA expression in H460 cells (n=6 for all conditions, \(P<0.001\) (***) ; one-way ANOVA; SNK correction). (B) Representative Western immunoblots showing the expression of Na\textsubscript{v}1.7 (upper) and \(\beta\)-actin (lower) proteins in H460 cells that had been serum starved (SS) for 48 hours, treated with 100 ng.ml\textsuperscript{-1} EGF for 24 hours post-serum starvation (24 hours) or treated with 1 \textmu M gefitinib (Gef) for 24 hours in the presence of serum (n=3 for both blots). (C) Average I-V plots for \(I_{Na}\) in untreated H460 cells (open squares, n=10) and H460 cells treated with either 100 ng.ml\textsuperscript{-1} EGF (closed squares, n=11), 1 \textmu M gefitinib (open circles, n=12) or 10 \textmu g.ml\textsuperscript{-1} anti-EGF neutralising antibody (closed circles, n=10) for 24 hours in the presence of serum (\(P<0.001\) (***) ; one-way ANOVA; SNK correction). (D) Average I-V plots for \(I_{Na}\) in untreated H460 cells (open squares, n=10) and H460 cells treated with 1 \textmu M gefitinib for 10 minutes (closed squares, n=11). Insets for I-V plots show representative current traces. Currents were evoked using 30 ms depolarising steps in 5 mV intervals (-90 to +70 mV), from \(V_{h}\) -90 mV.
Table 5.1: Biophysical properties of Na_v channel currents in H460 cells following EGFR stimulation/inhibition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$I_{\text{max}}$ (pA.pF^{-1})</th>
<th>$V_{50,\text{act}}$ (mV)</th>
<th>$V_{\text{rev}}$ (mV)</th>
<th>$k$</th>
<th>$V_{\text{peak}}$ (mV)</th>
<th>$\tau_{\text{act}}$ (ms)</th>
<th>$\tau_{\text{inact}}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>-8.9 ± 0.9</td>
<td>-7.8 ± 1.7</td>
<td>58.6 ± 4.0</td>
<td>6.9 ± 0.7</td>
<td>+10</td>
<td>0.30 ± 0.02</td>
<td>9.91 ± 0.98 (f)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.54 ± 0.30 (s)</td>
</tr>
<tr>
<td>EGF</td>
<td>-10.5 ± 0.8</td>
<td>-8.2 ± 1.9</td>
<td>59.8 ± 5.1</td>
<td>6.3 ± 0.7</td>
<td>+10</td>
<td>0.31 ± 0.02</td>
<td>9.04 ± 0.77 (f)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.40 ± 0.22 (s)</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>-1.5 ± 0.1</td>
<td>-9.6 ± 2.1</td>
<td>58.0 ± 3.7</td>
<td>9.6 ± 0.8</td>
<td>+10</td>
<td>0.34 ± 0.03</td>
<td>9.28 ± 0.97 (f)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.15 ± 0.31 (s)</td>
</tr>
<tr>
<td>$\alpha$-EGF Ab</td>
<td>-1.4 ± 0.4</td>
<td>-9.7 ± 2.4</td>
<td>63.7 ± 5.4</td>
<td>11.9 ± 0.9</td>
<td>+10</td>
<td>0.35 ± 0.02</td>
<td>9.98 ± 1.05 (f)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.25 ± 0.33 (s)</td>
</tr>
</tbody>
</table>

$I_{\text{max}}$, maximal current density; $V_{50,\text{act}}$, voltage for half maximal activation of current; $V_{\text{rev}}$, reversal potential; $k$, slope factor; $V_{\text{peak}}$, voltage at which $I_{\text{max}}$ is elicited; $\tau_{\text{act}}$, time constant for activation; $\tau_{\text{inact}}$, time constant for inactivation; (f), fast inactivation; (s), slow inactivation.
5.3.2 EGFR signalling promotes H460 cell invasion via upregulation of functional Na\textsubscript{v}1.7 expression

As expected, treatment of H460 NSCLC cells with EGF enhanced both cell proliferation and migration, whilst treatment with gefitinib had the opposite effect (Figure 5.2A and B). However, as predicted from the earlier findings (Chapter 3), this EGFR-driven promotion of cell proliferation and migration is not mediated through Na\textsubscript{v}1.7 activity; co-application of EGF and TTX had exactly the same effect on H460 cell proliferation and migration as EGF treatment alone. In contrast, however, the pro-invasive response of H460 cells to EGFR stimulation does appear to be mediated primarily through Na\textsubscript{v} channel activity (Figure 5.2C). Treatment with EGF enhanced H460 cell invasion by 38.0 ± 5.0%, whereas gefitinib reduced invasion by 44.3 ± 10.0%. Co-application of TTX largely blocked EGF-mediated invasion such that invasion was inhibited by 22.7 ± 4.5%.

A working concentration of 1 µM gefitinib was consistently used in order to inhibit the EGFR in H460 cells. This concentration was selected based on the supplier’s recommendation and on information available in published literature. However, in order to confirm that this was a suitable concentration to use, providing a satisfactory level of EGFR inhibition, dose-response experiments were performed using gefitinib concentrations ranging from 0 to 10 µM (Figure 5.3). Western immunoblotting against phospho-ERK demonstrated a dose-dependent inhibition of ERK1/2 signalling, with levels of phosphorylated ERK1/2 protein being noticeably reduced in the presence of 0.25 µM gefitinib. At 1 µM gefitinib, bands corresponding to phospho-ERK were extremely faint. Similarly, qPCR experiments demonstrated a dose-dependent reduction in Na\textsubscript{v}1.7 mRNA expression in response to gefitinib treatment, with significant reduction of Na\textsubscript{v}1.7 expression first observed at 0.5 µM gefitinib. Finally, invasion assays confirmed that 1 µM gefitinib was significantly more effective at inhibiting H460 cell invasion than 0.25 µM gefitinib. Therefore, based on these dose-response experiments, it was concluded that a working concentration of 1 µM gefitinib appears to be a suitable concentration for the inhibition of EGFR signalling in H460 NSCLC cells. The fact that levels of phosphorylated AKT remained unchanged, even at the highest gefitinib concentrations, demonstrates that the PI3-K signalling pathway is constitutively active in
H460 cells, and therefore not effectively blocked by EGFR antagonists. This supports work performed by another group (Janmaat et al. 2003) and suggests that the development of a more invasive cancer phenotype in strongly metastatic H460 NSCLC cells, through the functional expression of Na\textsubscript{v}1.7, is likely controlled by ERK1/2 signalling (MAPK signalling pathway) rather than PI3-K signalling.
Figure 5.2: Endogenous EGF specifically promotes H460 cell invasion via an upregulation of functional Na,1.7 expression. (A) Effect of 0.5 µM TTX, 100 ng.ml⁻¹ EGF, 1 µM gefitinib and 100 ng.ml⁻¹ EGF/0.5 µM TTX co-treatment on H460 cell proliferation, recorded over 120 hours on 96-well plates, measured as % confluence and analysed using the IncuCyte™ imaging system (n≥8 for all conditions). (B) Effect of 0.5 µM TTX, 100 ng.ml⁻¹ EGF, 1 µM gefitinib and 100 ng.ml⁻¹ EGF/0.5 µM TTX co-treatment on H460 cell migration, recorded over 58 hours using the scratch-wound assay on 24-well plates, measured as % wound confluence and analysed using the IncuCyte™ imaging system (n=8 for all conditions). (C) Effect of 0.5 µM TTX, 100 ng.ml⁻¹ EGF, 1 µM gefitinib and 100 ng.ml⁻¹ EGF/0.5 µM TTX co-treatment on H460 cell Matrigel™ invasion, recorded after 48 hours (n=9 for all conditions, P<0.05 (*), P<0.01 (**); one-way ANOVA; SNK correction).
Figure 5.3: Dose-dependent inhibition of the ERK1/2 signalling pathway by gefitinib in H460 cells and its effect on Na\scriptscriptstyle{v}1.7 expression and cell invasion. (A) Representative Western immunoblots showing the levels of phosphorylated and total ERK and AKT, and β-actin proteins in H460 cells treated with different concentrations of gefitinib (0, 0.05, 0.1, 0.25, 0.5, 1, 5 and 10 µM) for 3 hours, following serum starvation overnight and before stimulation with 100 ng.ml\textsuperscript{-1} EGF for 5 minutes (n≥2 for all blots). (B) qPCR showing relative Na\scriptscriptstyle{v}1.7 mRNA levels in H460 cells treated with different concentrations of gefitinib (0, 0.1, 0.25, 0.5, 1 and 5 µM) for 24 hours (n≥5 for all concentrations, P<0.01 (**), P<0.001 (***); one-way ANOVA; SNK correction). (C) Relative Matrigel\textsuperscript{TM} invasion, recorded after 48 hours, for H460 cells treated with different concentrations of gefitinib (0, 0.25 and 1 µM) (n=6 for all concentrations, P<0.01 (**), P<0.001 (***); one-way ANOVA; SNK correction).
5.3.3 EGFR-mediated upregulation of Na,1.7 involves ERK1/2 signalling

Having demonstrated that EGFR stimulation of strongly metastatic H460 NSCLC cells drives cell invasion via functional expression of Na,1.7, the signalling pathways downstream of the EGFR were next examined.

Two likely candidates downstream of EGFR stimulation are the ERK1/2 pathway and the PI3-K pathway, both of which have been shown to modulate ion channels in excitable cells (Adams et al. 2000; Qiu et al. 2003; Black et al. 2008; Woodall et al. 2008; Smani et al. 2010; Stamboulian et al. 2010; Persson et al. 2011) and to be intimately involved in cancer progression (Stahl et al. 2004; Li et al. 2005; Fremin and Meloche 2010; Keshet and Seger 2010). The gefitinib dose-response experiments (Figure 5.3) suggest that the ERK1/2 pathway is inhibited in H460 cells following EGFR block, whereas the PI3-K pathway remains active. Thus, EGFR-mediated upregulation of Na,1.7 expression is more likely to involve ERK1/2 rather than PI3-K signalling. To confirm this, H460 cells were pretreated for 24 hours with either the MEK inhibitor, U0126 (10 µM), or the PI3-K inhibitor, wortmannin (100 nM). Treatment with U0126 reduced Na,1.7 mRNA expression by 81.8 ± 4.2% compared with controls, whereas treatment with wortmannin had no effect (Figure 5.4A). The efficacy of both signalling pathway inhibitors was confirmed by Western immunoblotting against the phosphorylated forms of the downstream targets of MEK (phospho-ERK) and PI3-K (phospho-AKT) (Figure 5.4B). Screening for the functional effects of U0126 and wortmannin on $I_{Na}$ in H460 cells was initially conducted using the HT IonWorks® electrophysiology platform (Figure 5.4C). Pretreatment of H460 cells for 24 hours with U0126 inhibited the $I_{max}$ by 44% from -0.39 ± 0.05 to -0.22 ± 0.02 nA. In contrast, treatment with wortmannin had no effect on $I_{max}$. The inhibitory effect of U0126 was confirmed using conventional whole-cell patch-clamp electrophysiology recordings, where U0126 reduced $I_{max}$ by 47% from -9.40 ± 0.71 to -4.96 ± 0.79 pA.pF$^{-1}$ (Figure 5.4D).

Consistent with the effects on Na,1.7 mRNA levels and $I_{Na}$, H460 cell invasion was also inhibited following treatment with U0126 (42.4 ± 2.2%), whereas wortmannin was without effect (Figure 5.4E). Importantly, no additive effect was observed when combining
U0126 and TTX treatments, implying overlapping/converging effects. Taken together, these data suggest that EGFR-mediated ERK1/2 signalling upregulates functional expression of Na\textsubscript{v}1.7, which in turn drives invasion of H460 NSCLC cells.
Figure 5.4: EGFR stimulation regulates functional expression of Na\(_{\text{v}}\)1.7 and subsequent H460 cell invasion via the ERK1/2 signalling cascade. (A) qPCR showing the relative effect of treatment of H460 cells with 10 µM U0126 or 100 nM wortmannin for 24 hours on Na\(_{\text{v}}\)1.7 mRNA expression (\(n=6\) for all conditions, \(P<0.05\) (*); one-way ANOVA; SNK correction). (B) Representative Western immunoblots showing levels of phosphorylated and total ERK (upper), and phosphorylated and total AKT (lower) proteins in untreated H460 cells and in H460 cells following treatment with either 10 µM U0126 or 100 nM wortmannin for 24 hours (\(n=3\) for all blots). (C) \(I_{\text{max}}\) recordings from untreated H460 cells and H460 cells treated with either 10 µM U0126 or 100 nM wortmannin for 24 hours, recorded using an HT IonWorks® electrophysiology platform (\(n\) numbers represent number of cells with a TTX-inhibitable \(I_{\text{Na}}/\text{total number of cells tested}\)). (D) Average \(I-V\) plots for \(I_{\text{Na}}\) in untreated H460 cells (open squares, \(n=9\)) and H460 cells treated with 10 µM U0126 for 24 hours (closed squares, \(n=11\)) (\(P<0.01\) (**); Student’s \(t\)-test). Inset shows representative current traces from untreated H460 cells and H460 cells treated with 10 µM U0126 for 24 hours. Currents were evoked using 30 ms depolarising steps in 5 mV intervals (-90 to +70 mV), from \(V_{\text{h}}\) -90 mV. (E) Effect of 10 µM U0126, 100 nM wortmannin, 10 µM U0126/0.5 µM TTX co-treatment and 100 nM wortmannin/0.5 µM TTX co-treatment on H460 cell Matrigel™ invasion, recorded after 48 hours (\(n=9\) for all conditions, \(P<0.05\) (*), \(P<0.01\) (**); one-way ANOVA; SNK correction).
5.4 Discussion

5.4.1 Summary of results

The data presented in this chapter demonstrate that EGFR signalling regulates functional expression of Na\(_{\text{v}}\)1.7 in metastatic H460 NSCLC cells at the level of gene transcription, with no observed post-translational effects. This EGFR-mediated Na\(_{\text{v}}\)1.7 expression is regulated by the ERK1/2 signalling cascade downstream of the EGFR. Stimulation of the EGFR in H460 cells promotes cell proliferation, migration and invasion, with cell invasion, specifically, shown to be influenced by EGFR-mediated Na\(_{\text{v}}\)1.7 expression.

5.4.2 Technical considerations

Whilst experimental design has been robust and thorough with all necessary controls implemented, the following technical considerations must be highlighted:

1. When investigating the additivity of combined treatments on cell invasion, it is important to consider the possibility that individual treatments may have already achieved a maximum level of invasion inhibition. If this is the case, any conclusions drawn from combined treatments (stating no additive effect) may be overinterpreted. With this in mind, in order to fully understand the effect of combined treatments on cell invasion, it would be sensible to use multiple drug concentrations (i.e. sub-saturation doses) rather than just a single drug concentration.

2. Liquid junction potentials (see section 3.4.2).

3. qPCR housekeeping genes (see section 3.4.2).
5.4.3 Discussion of results

Following the initial characterisation of Na\textsubscript{1.7} in strongly metastatic H460 NSCLC cells, and confirming its clinically relevant role in NSCLC cell invasion, it has been shown that EGFR signalling via the ERK1/2 signalling pathway controls transcriptional regulation of Na\textsubscript{1.7} to promote cellular invasion in H460 cells. Thus, I identify Na\textsubscript{1.7} as a novel downstream effector of the EGFR signalling pathway with potential as a target for therapeutic intervention and/or as a diagnostic/prognostic marker in metastatic NSCLC.

To date, there has been little characterisation of the mechanisms which drive functional expression of Na\textsubscript{v} channels in cancerous cells (Uysal-Onganer and Djamgoz 2007; Ding et al. 2008; Fraser et al. 2010). To address this critical issue, I focussed on the role of EGFR signalling, which not only plays key roles in NSCLC (Mendelsohn 2003; Fujimoto et al. 2005; Volante et al. 2007) but also regulates expression and activity of various ion channels, including Na\textsubscript{v} channels (Liu et al. 2007; Uysal-Onganer and Djamgoz 2007; Ding et al. 2008). In H460 cells, EGFR signalling via ERK1/2 tonically upregulates the functional expression of Na\textsubscript{1.7} to enhance Na\textsuperscript{+} influx and hence cell invasion. Since NSCLC cells can produce and release several EGFR ligands (Fujimoto et al. 2005; Volante et al. 2007; Wu et al. 2007), thereby establishing an EGFR autocrine loop, it is possible that this EGFR-driven expression of Na\textsubscript{1.7} is self-directed. Whilst proliferation, migration and invasion of H460 cells are all promoted by EGFR stimulation, only invasion is driven by EGFR-mediated enhancement of Na\textsubscript{1.7} activity. Since block of the EGFR, ERK1/2 and Na\textsubscript{1.7} inhibit invasion by ~40-50% without any additive effects of combined treatments, it can be inferred that EGFR/ERK-mediated invasion in these cells occurs exclusively via upregulation of Na\textsubscript{1.7}. However, since this pathway promotes only ~40-50% of cell invasion, other cellular factors must also be involved. VEGF, FGF and PDGF all play roles in NSCLC (Ballas and Chachoua 2011) and are linked with Na\textsubscript{v} channel function (Hilborn et al. 1998; Goldfarb et al. 2007; Andrikopoulos et al. 2011). Whilst further work is obviously required to establish the relevance of these growth factors, it is conceivable that Na\textsubscript{v}1.7 could be a convergence point in several cancer pathways.
Whilst transcriptional upregulation of Na\textsubscript{v}1.7 is necessary to promote sufficient expression of the channel at the cell surface, it is important to remember that it is the influx of Na\textsuperscript{+} which appears to be essential for Na\textsubscript{v} channel-dependent cell invasion, and although inhibition of the EGFR, ERK1/2 and Na\textsubscript{v}1.7 consistently reduced invasion by \(\sim 40-50\%\), the effects on \(I_{\text{Na}}\) varied from complete loss of current (TTX) to 47\% inhibition (U0126). This implies that not all available channels need to be functional to allow sufficient Na\textsuperscript{+} influx for invasion. Indeed, as discussed previously (Chapter 3), I find evidence for the continuous influx of Na\textsuperscript{+} via partial activation of Na\textsubscript{v}1.7 at the \(E_m\) of H460 cells. Under physiological conditions, therefore, this persistent Na\textsuperscript{+} influx is sufficient to maintain the invasive capability of cells. Endogenous modulators of Na\textsubscript{v}1.7, e.g. ERK1/2, could thus provide an exquisite mechanism for fine-tuning of EGFR-mediated invasion in NSCLC. Similarly, other transport proteins that allow Na\textsuperscript{+} influx might also be expected to promote invasive behaviour in NSCLC via an uncharacterised mechanism. In MDA-MB-231 breast cancer cells, Na\textsubscript{v}1.5 is postulated to be functionally coupled with NHE1, which extrudes H\textsuperscript{+} to cause extracellular acidification, thereby providing an optimal environment for the pH-dependent activity of cysteine cathepsins and proteolysis of the ECM (Gillet et al. 2009; Brisson et al. 2011). Na\textsuperscript{+} influx has also been suggested to impact on intracellular Ca\textsuperscript{2+} homeostasis, primarily through reversal of NCX activity (Blaustein and Lederer 1999; Andrikopoulos et al. 2011), which would have numerous cellular effects, including altered gene expression.

Regardless of how raised [Na\textsuperscript{+}], promotes cell invasion, I have nevertheless demonstrated unequivocally that functional expression of Na\textsubscript{v}1.7 is associated with increased invasive potential of NSCLC cell lines. Whilst small molecule EGFR inhibitors such as gefitinib and erlotinib have significant therapeutic benefit in many patients with advanced NSCLC, drug resistance remains a major cause of chemotherapy failure (Lynch et al. 2004; Paez et al. 2004; Domingo et al. 2010; Cataldo et al. 2011). My evidence that EGFR-dependent invasion of strongly metastatic H460 cells relies on Na\textsubscript{v}1.7 activity suggests that drugs targeting this novel downstream effector could be used in conjunction with existing therapies such as EGFR inhibitors. Na\textsubscript{v} channel-specific blockers are already in clinical use as local anaesthetics, and more recently, Na\textsubscript{v}1.7-specific inhibitors are being developed for the treatment of pain (Dib-Hajj et al. 2009; Clare 2010; Ghelardini et al. 2010; Chowdhury et al. 2011).
2011; Theile and Cummins 2011). Given its undoubted functional significance and marked upregulation in tumour tissue, \( \text{Na}_v 1.7 \) represents an important new target for therapeutic intervention and/or as a diagnostic/prognostic marker in NSCLC. Having gained a greater understanding of the transcriptional regulation of \( \text{Na}_v 1.7 \) in strongly metastatic NSCLC, the downstream effects of \( \text{Na}_v 1.7 \) expression, and how this promotes an invasive cancer phenotype, were next investigated.
Chapter 6:
RESULTS – The role of Na\textsubscript{v} channels in promoting NSCLC cell invasion
6.1 Introduction

The abnormal expression of Na\textsubscript{v} channels has been reported in a wide range of invasive epithelial cancers, and the findings presented in Chapter 5 support a role for functional expression of Na\textsubscript{v} channels in the pro-invasive response to EGFR signalling in NSCLC. However, the precise downstream effects of Na\textsubscript{v} channel expression, and how Na\textsuperscript{+} influx contributes to cell invasion, remain unclear.

Characterisation of $I_{Na}$ in Chapter 3 indicates that Na\textsubscript{v} channels are tonically active at the $E_m$ of strongly metastatic H460 NSCLC cells, allowing a small but continuous influx of Na\textsuperscript{+} into the cells. This could therefore account for the maintained increase in the Na\textsuperscript{+} content of malignant cancer tissue (Cameron et al. 1980; Ouwerkerk et al. 2007). There is little evidence that Na\textsuperscript{+} itself can act directly as a second messenger to influence the invasive properties of cancer cells. However, it is possible that elevated [Na\textsuperscript{+}]	extsubscript{i} has indirect effects that impact upon cellular processes that promote invasion. For example, changes in [Na\textsuperscript{+}]	extsubscript{i} could influence Na\textsuperscript{+}-dependent transport proteins such as NCX1 and NHE1 to alter levels of intracellular Ca\textsuperscript{2+} and H\textsuperscript{+}, respectively, and this, combined with downregulation of $\beta$-subunit expression (Chapter 3) might promote cell invasion.

Ca\textsuperscript{2+} is a vital second messenger in practically every cell type, impacting on numerous Ca\textsuperscript{2+}-dependent functions e.g. gene expression, cell differentiation, cell movement. Thus, not surprisingly, Ca\textsuperscript{2+} signalling has been implicated in the modulation of metastases in several cancers (Amuthan et al. 2002; Huang et al. 2004; Mycielska and Djamgoz 2004; Liao et al. 2006; Wang et al. 2010). Furthermore, changes in the expression profiles of ion channels and ion pumps/transporters that are associated with Ca\textsuperscript{2+} flux in cancer cells have been proposed to play important roles in carcinogenesis (Monteith et al. 2007; Roderick and Cook 2008; Parkash and Asotra 2010). Ca\textsuperscript{2+} signalling can be mediated by influx of Ca\textsuperscript{2+} through the plasma membrane and/or release from internal stores (Berridge et al. 1998; Berridge et al. 2003; Clapham 2007). Ca\textsuperscript{2+} influx can occur via many different mechanisms, including Ca\textsubscript{v} channels, receptor-operated Ca\textsuperscript{2+} channels and store-operated Ca\textsuperscript{2+} channels (Berridge et al. 1998). Moreover, a rise in intracellular Ca\textsuperscript{2+} levels can also result from a reversal of
sodium/calcium exchanger (NCX) activity, known to take place in cardiac myocytes and epithelial cells in response to elevated intracellular Na\(^+\) (Blaustein and Lederer 1999; Andrikopoulos et al. 2011).

What little is known about how functional \(\text{Na}_v\) channel expression might promote the development of an invasive cancer phenotype appears to point to pH as a key downstream factor. Gillet et al. propose that \(\text{Na}_v1.5\) enhances the invasiveness of MDA-MB-231 breast cancer cells by promoting perimembrane acidification, thus favouring the pH-dependent activity of the proteolytic enzymes, cysteine cathepsins B and S (Gillet et al. 2009). Subsequently, it has been suggested that \(\text{Na}_v1.5\) could enhance MDA-MB-231 invasion by increasing \(\text{Na}^+/\text{H}^+\) exchanger type 1 (NHE1)-mediated H\(^+\) efflux from the cells, thus providing a mechanism for extracellular acidification (Brisson et al. 2011). *In vitro*, elevated NHE1 expression is observed in cancer cell lines at sites of focal ECM degradation where its activity promotes cell invasion via acidification of the extracellular environment (Bourguignon et al. 2004; Cardone et al. 2005; Stock and Schwab 2006; Stock et al. 2007; Stuwe et al. 2007; Stock and Schwab 2009; Busco et al. 2010; Brisson et al. 2012). These data have also been translated into an *in vivo* setting where NHE1 co-localises with structures that resemble invadopodia in human clinical breast cancer samples, and following intra-arterial injection of melanoma cells into mice, a significant decrease in the number of liver metastases was reported in cells transfected with siRNA directed against NHE1, relative to the control (Stock et al. 2007; Busco et al. 2010).

PKA has been shown to play a role in both the externalisation of \(\text{Na}_v1.7\) from Golgi (Wada et al. 2004) and the functional expression of \(\text{Na}_v1.7\) and \(\text{nNa}_v1.5\) in rat prostate cancer Mat-LyLu cells (Brackenbury and Djamgoz 2006) and human breast cancer MDA-MB-231 cells (Chioni et al. 2010), respectively. It is postulated that PKA mediates an activity-dependent positive feedback mechanism whereby \(\text{Na}^+\) influx enhances \(\text{Na}_v\) channel expression and in turn, \(\text{Na}_v\) channel activity (\(\text{Na}^+\) influx) stimulates PKA. \(\text{Na}_v\) channel-mediated stimulation of PKA could phosphorylate downstream effectors that promote cell invasion. For example, the localisation and activity of vacuolar-ATPase (V-ATPase), responsible for cellular H\(^+\) extrusion, is regulated by PKA (Alzamora et al. 2010). The increased activity of
PKA as a result of enhanced Na$_v$ channel expression in cancer cells could therefore promote extracellular acidification via V-ATPase activity, which plays a role in promoting metastatic behaviours (Fais et al. 2007).

6.2 Aims

The aim of the studies conducted in this chapter was to determine how Na$_v$1.7 activity promotes NSCLC cell invasion. The specific aims were as follows:

1. To establish whether elevated [Na$^+$]$_i$ alone is able to promote NSCLC invasion.
2. To determine whether inhibition of other proteins associated with Na$^+$ transport have any effect on NSCLC invasion.
3. To test whether altering [Na$^+$]$_i$ in NSCLC cells has any effect on [Ca$^{2+}$]$_i$ and/or pH$_i$.
4. To determine whether PKA activity is influenced by Na$_v$ channel activity, and if so, to establish whether or not PKA promotes NSCLC cell invasion.
5. To examine whether proteins that are identified as being potentially important in Na$_v$ channel-mediated NSCLC invasion are co-localised in lipid rafts.

6.3 Results

6.3.1 Raising the level of intracellular Na$^+$ independently of Na$_v$1.7 promotes NSCLC invasion

With previous work showing that strongly metastatic H460 NSCLC cells experience a persistent influx of Na$^+$ at their $E_m$ via Na$_v$1.7, and that inhibition or knockdown of Na$_v$1.7 significantly reduces the invasive capabilities of H460 cells, it is logical to assume that the ion flux made possible through the functional expression of Na$_v$1.7 directly impacts on invasive cellular mechanisms. This therefore raises the following question: do you specifically need the Na$_v$ channel or will increased [Na$^+$]$_i$ via any means be sufficient? This was addressed by simply treating both H460 (Figure 6.1A) and A549 (Figure 6.1B) NSCLC cells with
monensin, a Na\textsuperscript{+}-selective ionophore that serves as an ideal tool by which to raise [Na\textsuperscript{+}]\textsubscript{i} (Mollenhauer et al. 1990). Treatment of H460 cells with 10 µM monensin enhanced cell invasion by 80.3 ± 19.4%. Similarly, monensin-treatment of the weakly metastatic A549 cells, already shown to be particularly non-invasive and lacking functional Na\textsubscript{v}1.7, enhanced cell invasion by 33.1 ± 5.1%. Thus, even in the absence of Na\textsubscript{v}1.7, cell invasion can be enhanced simply by increasing [Na\textsuperscript{+}]\textsubscript{i}. Furthermore, co-treatment of A549 cells with monensin and siRNA directed against the Na\textsubscript{v} channel β1-subunit resulted in even greater levels of cell invasion (52.3 ± 4.8%), adding more support for a role of β1 in NSCLC cell adhesion. A role for [Na\textsuperscript{+}]\textsubscript{i} in dictating the invasive capabilities of NSCLC cells was further supported by the fact that treatment of H460 cells with 100 nM ouabain, a selective Na\textsuperscript{+}/K\textsuperscript{+}-ATPase inhibitor, also induced a small but significant increase in their ability to invade (20.4 ± 2.4%, Figure 6.1C). Together, these data suggest that simply raising [Na\textsuperscript{+}]\textsubscript{i}, regardless of whether or not it is brought about through Na\textsubscript{v} channel activity, is sufficient to promote invasion of NSCLC cells. Thus, the following question is raised: how does increased cytoplasmic Na\textsuperscript{+} promote NSCLC cell invasion? Based on the hypothesis that elevated [Na\textsuperscript{+}]\textsubscript{i} has indirect effects on intracellular Ca\textsuperscript{2+} and/or pH homeostasis to drive cell invasion, both of these potential endpoints were next investigated.
Figure 6.1: The Na\(^+\) ionophore, monensin, and the Na\(^+\)/K\(^+\)-ATPase inhibitor, ouabain, drive invasion of NSCLC cells. (A) Effect of 10 µM monensin on H460 cell Matrigel\(^\text{TM}\) invasion, recorded after 48 hours (\(n=6\) for both conditions, \(P<0.001\) (***)\; Student’s \(t\)-test). (B) Effect of 10 µM monensin and 10 µM monensin/5 nM β1-subunit siRNA co-treatment on A549 cell Matrigel\(^\text{TM}\) invasion, recorded after 48 hours (\(n=6\) for all conditions, \(P<0.01\) (**), \(P<0.001\) (***)\; one-way ANOVA; SNK correction). (C) Effect of 100 nM ouabain on H460 cell Matrigel\(^\text{TM}\) invasion, recorded after 48 hours (\(n=6\) for both conditions, \(P<0.05\) (*)\; Student’s \(t\)-test).
6.3.2 Elevated intracellular Na\(^+\) does not affect intracellular Ca\(^{2+}\) signalling in NSCLC cells

One possible mechanism by which elevated [Na\(^+\)]\(_i\) could influence the invasive capabilities of NSCLC cells is through an indirect effect on intracellular calcium concentration, [Ca\(^{2+}\)]\(_i\). Influx of Na\(^+\) into cells has been proposed to mediate Ca\(^{2+}\) influx, or, inhibit its removal from cells, by slowing or reversing sodium/calcium exchanger, NCX, activity (Rosker et al. 2004; Eder et al. 2005), a phenomenon known to take place in cardiac myocytes and epithelial cells in response to elevated [Na\(^+\)]\(_i\) (Blaustein and Lederer 1999; Andrikopoulos et al. 2011). Elevated [Ca\(^{2+}\)]\(_i\) could then potentially result in the expression of genes associated with cell invasion or potentiate the release of proteolytic enzymes by exocytosis (Mycielska et al. 2003), thereby promoting the development of metastases. Before any detailed characterisation of [Ca\(^{2+}\)]\(_i\) levels in NSCLC cells could be carried out, it was first necessary to determine whether NCX has any influence on NSCLC cell invasion. qPCR confirmed that NCX1 mRNA is expressed in H460 cells (Figure 6.2A). However, when H460 cells were treated with 10 µM SN-6, an NCX1-specific blocker selective for its reverse mode (Niu et al. 2007), there was no effect on cell invasion (Figure 6.2B), suggesting that NCX1 in reverse mode plays no role in this cell behaviour. Nevertheless, when H460 cell invasion was analysed in the presence of 0.1 mM EGTA (Figure 6.2B), a Ca\(^{2+}\)-selective chelator that sequesters extracellular Ca\(^{2+}\), thereby lowering the driving force for Ca\(^{2+}\) entry into cells, there was a significant reduction in cell invasion (36.6 ± 6.9%). Thus, extracellular Ca\(^{2+}\) and its movement into strongly metastatic H460 cells may be an important factor in H460 cell invasion. However, application of EGTA is a very non-specific method and does not give any indication of how Ca\(^{2+}\) influx occurs, whether it is Na\(^+\)-dependent, or its putative effect on invasion and its effect on other cellular behaviours such as proliferation and migration, both of which are Ca\(^{2+}\)-sensitive. It is highly likely that chelation of extracellular Ca\(^{2+}\) impacts on numerous cellular processes that influence cell invasion. To gain a more detailed insight into possible changes in [Ca\(^{2+}\)]\(_i\) in response to altered [Na\(^+\)]\(_i\), Ca\(^{2+}\) imaging was performed.

Fura-2, a ratiometric Ca\(^{2+}\)-sensitive fluorescent dye, was used to record changes in [Ca\(^{2+}\)]\(_i\) levels in weakly metastatic A549 versus strongly metastatic H460 NSCLC cells.
Neither the A549 nor H460 cells exhibited any spontaneous Ca\(^{2+}\) oscillations. However, both the A549 and H460 cells responded to 10 µM carbachol (CCh) and 100 µM ATP with an increase in [Ca\(^{2+}\)]\(_i\), whilst only the A549 cells responded to 10 µM bradykinin (BK) – control responses (Figure 6.3A and B). Next, the effects of Na\(_v\)1.7 activity were assessed using veratridine and TTX, followed by control CCh/ATP treatments to ensure that the cells were responding normally. The results demonstrated that treatment of H460 cells with either 50 µM veratridine or 1 µM TTX for ~10 minutes had no effect on [Ca\(^{2+}\)]\(_i\) levels (Figure 6.3C). To rule out the possibility that Na\(_v\) channel activity might enhance [Ca\(^{2+}\)]\(_i\) levels in H460 cells following stimulation of Ca\(^{2+}\) entry by other means, [Ca\(^{2+}\)]\(_i\) levels were recorded in H460 cells in response to treatment with CCh, either with no pre-treatment or following pre-treatment with TTX for ~10 minutes (Figure 6.3D). Pre-treatment of H460 cells with TTX had no effect on [Ca\(^{2+}\)]\(_i\) levels induced by CCh treatment. In fact, manipulation of [Na\(^+\)]\(_i\) had very little effect at all on [Ca\(^{2+}\)]\(_i\) in H460 cells, as demonstrated by treatment of the cells with 10 µM monensin. The vast majority of H460 cells tested (87%) showed little or no change in [Ca\(^{2+}\)]\(_i\) in response to monensin treatment (Figure 6.4A and B). Only 9% of H460 cells demonstrated a moderate increase in [Ca\(^{2+}\)]\(_i\) in response to monensin (Figure 6.4C), whilst only 4% of H460 cells showed a large increase in [Ca\(^{2+}\)]\(_i\) in response to monensin (Figure 6.4D). The magnitudes of the [Ca\(^{2+}\)]\(_i\) changes in H460 cells in response to monensin treatment were determined subjectively by eye and it was concluded that overall there was no real effect. The fact that such a small proportion of H460 cells responded to elevated [Na\(^+\)]\(_i\) with an increase in [Ca\(^{2+}\)]\(_i\), and that manipulation of Na\(_v\) channel activity had no effect at all on it, suggests that Ca\(^{2+}\) signalling is not the key promoter of H460 cell invasion downstream of elevated [Na\(^+\)]\(_i\). Based on IonWorks® electrophysiology screens, approximately 82% of H460 cells have robust \(I_{Na}\) compared with 13% of cells that demonstrate moderate to robust changes in [Ca\(^{2+}\)]\(_i\) following treatment with monensin. Nevertheless, there could be a small sub-population of cells whose Ca\(^{2+}\) signalling is affected downstream of enhanced Na\(^+\) influx. As has been alluded to in the work focussing on the role of NHE1 in H460 cell invasion, however, localised changes in pH appears to be the most likely candidate for the downstream consequence of increased [Na\(^+\)]\(_i\) that could promote the development of an invasive phenotype in NSCLC.
Figure 6.2: The reverse mode of NCX1 does not contribute to the invasive properties of H460 cells. (A) Relative mRNA expression, assessed by qPCR, for Na\textsubscript{v}1.7 and NCX1 in H460 cells (\(n=6\) for both primer sets, \(P<0.05\) (*); Student’s \(t\)-test). (B) Effect of 10 \(\mu\)M SN-6 and 0.1 mM EGTA on H460 cell Matrigel\textsuperscript{TM} invasion, recorded after 48 hours (\(n=6\) for all conditions, \(P<0.01\) (**); one-way ANOVA; SNK correction).
Figure 6.3: Na\textsubscript{v} channel activity has no effect on \([\text{Ca}^{2+}]_i\) in H460 cells. (A) Effect of 10 µM CCh, 10 µM BK and 100 µM ATP on 340/380 fluorescence ratio in A549 cells, recorded using Fura-2 (n=45). (B) Effect of 10 µM CCh, 10 µM BK and 100 µM ATP on 340/380 fluorescence ratio in H460 cells, recorded using Fura-2 (n=36). (C) Effect of 50 µM veratridine and 1 µM TTX on 340/380 fluorescence ratio in H460 cells, recorded using Fura-2 (n=81). (D) Effect of 10 µM CCh, with and without a 1 µM TTX pre-treatment, on 340/380 fluorescence ratio in H460 cells, recorded using Fura-2 (n=49). Displayed traces are representative. Histograms show mean peak 340/380 fluorescence ratios for each treatment.
Figure 6.4: Monensin has a minimal effect on \([\text{Ca}^{2+}]_i\) in H460 cells. Effect of 10 µM monensin on 340/380 fluorescence ratio in H460 cells, recorded using Fura-2 \((n=107)\). A represents 66% of monensin-treated cells, B represents 21% of monensin-treated cells, C represents 9% of monensin-treated cells and D represents 4% of monensin-treated cells. Displayed traces are representative. Histograms show mean peak 340/380 fluorescence ratios.
In a recent study in MDA-MB-231 human breast cancer cells it was reported that the sodium/hydrogen exchanger, NHE1, is an important regulator of extracellular acidification via H⁺ efflux. This in turn results in activation of extracellular cysteine cathepsin proteases and hence cell invasion. It was concluded that NHE1 activity is increased by Na⁺ influx via Naᵥ1.5 (Gillet et al. 2009; Brisson et al. 2011). To determine whether NHE1 is a mediator of cell invasion in NSCLC, mRNA and protein levels for NHE1 in strongly metastatic H460 cells were determined and the effect of NHE1 inhibition, alone or in combination with Naᵥ1.7 inhibition, on H460 cell invasion was investigated.

Not only is NHE1 expressed in H460 cells, as demonstrated by qPCR, it is expressed more strongly than Naᵥ1.7 (Figure 6.5A). Furthermore, expression of NHE1 in H460 cells appears to be unrelated to Naᵥ1.7 expression, as demonstrated by the fact that NHE1 mRNA levels are unaffected by treatment of H460 cells with either 1 µM TTX or 5 nM siRNA directed against Naᵥ1.7. Work performed by Chiang and colleagues has shown that NHE1 expression and activity in cervical cancer cells is upregulated by EGFR signalling (Chiang et al. 2008). As shown in Chapter 5, EGFR signalling in H460 cells directly controls the functional expression of Naᵥ1.7 and subsequent cell invasion. Like with Naᵥ1.7, expression of NHE1 at both the mRNA and protein levels also appears to be under the control of EGFR signalling in H460 cells. The mRNA levels for NHE1 increased by 54.8 ± 14.0% following treatment with 100 ng.ml⁻¹ EGF for 24 hours, whereas NHE1 expression decreased by 42.6 ± 6.0% following 24-hour treatment with 1 µM gefitinib. Interestingly, the Western immunoblot results (Figure 6.5B) suggest that NHE1 protein is expressed more highly in weakly metastatic A549 cells compared with the invasive H460 cells, contrary to what might be predicted from the literature. If NHE1 activity is therefore shown to play an important role in NSCLC cell invasion, this would add support to the hypothesis that functional Naᵥ channel expression (not present in the A549 cells) is perhaps the key factor in the development of an invasive cancer phenotype.
To test whether NHE1 can promote H460 cell invasion, H460 cells were treated with the NHE1-specific inhibitor, EIPA. A working concentration of 10 µM EIPA was used based on the results of cell toxicity assays (Figure 6.5C); higher concentrations of EIPA (>10 µM) proved toxic to H460 cells. Inhibition of NHE1 using 10 µM EIPA reduced H460 cell invasion by 39.8 ± 2.1% (Figure 6.5D), demonstrating that NHE1 activity also plays a significant role in the promotion of a metastatic phenotype in these cells. Importantly, the co-inhibition of NHE1 and Na_v1.7 with combined EIPA and TTX treatments had no additive effect on cell invasion, suggesting a possible shared mechanism in the promotion of the invasive capabilities of H460 cells by these two proteins. Similarly, it could infer that there are two mechanisms that are independent of each other but convergent on a common point downstream. This raises the possibility that, like in MDA-MD-231 breast cancer cells (Brisson et al. 2011), enhanced activity of NHE1 and subsequent H^+ efflux from H460 cells may drive cell invasion, and this may be dependent upon Na^+ influx via Na_v1.7. However, this model appears to be counterintuitive physiologically; Na^+ influx via Na_v1.7 would slow down NHE1 activity. In addition to permitting H^+ efflux from cells, activity of NHE1, like Na_v1.7, also brings extracellular Na^+ into the cell, thereby raising [Na^+]. If simply raising [Na^+], is the crucial factor in driving cell invasion, as suggested by treatment of NSCLC cells with monensin and ouabain, then NHE1 may simply be another conduit for Na^+ influx. Therefore, either the activity of NHE1 or Na_v1.7 could allow a threshold level of [Na^+] to be reached, necessary to drive invasion via an uncharacterised mechanism. However, the fact that NHE1 is strongly expressed in A549 as well as H460 cells, whereas Na_v1.7 is expressed in H460 cells only, suggests that Na_v1.7 is the key player in the Na^+-dependent promotion of cell invasion. Another possibility is that the presence of both proteins is necessary in order to generate sufficient Na^+ influx to provide a threshold level of [Na^+] that in turn impacts on the invasive properties of the cell.

Regardless of the role of NHE1 in NSCLC cell invasion, it is well established that its activity results in H^+ efflux from cells and this has been shown to offer a significant advantage to metabolically active cancer cells, promoting their invasive capabilities (Brisson et al. 2012). Based on the preliminary findings in this present study and the evidence that NHE1 is thought to function downstream of Na_v1.5 activity in strongly metastatic MDA-MB-231 breast cancer
cells to promote cell invasion (Brisson et al. 2011), I next tested whether NHE1 functions downstream of Na,1.7 in H460 NSCLC cells, using pH$_i$ measurements.
Figure 6.5: NHE1 is strongly expressed in H460 cells and promotes cell invasion. (A) qPCR showing relative effect of stimulation with 100 ng.ml⁻¹ EGF for 24 hours, treatment with 1 µM gefitinib for 24 hours, treatment with 1 µM TTX for 24 hours and treatment with 5 nM siRNA directed against Naᵥ1.7 on NHE1 mRNA expression in H460 cells. Also included on the plot for comparison is the level of Naᵥ1.7 mRNA expression in H460 cells (n=8 for all conditions, P<0.05 (*), P<0.001 (***) ; one-way ANOVA; SNK correction). (B) Representative Western immunoblots showing the expression of NHE1 (upper) and β-actin (lower) proteins in A549 and H460 cells, and in H460 cells that had been treated with 100 ng.ml⁻¹ EGF or 1 µM gefitinib (Gef) for 24 hours (n=3 for all blots). (C) Dose-response cell toxicity assay to test the effect of 5-100 µM EIPA, applied for 48 hours, on H460 cell viability. Based on these results, a concentration of 10 µM EIPA was applied to H460 cells for a period of 48 hours in order to determine what effect inhibition of NHE1 has on the invasive capabilities of H460 cells (n=3 for all conditions, P<0.001 (***) ; one-way ANOVA; SNK correction). (D) Effect of 10 µM EIPA and 10 µM EIPA/1 µM TTX co-treatment on H460 cell Matrigel™ invasion, recorded after 48 hours (n=9 for all conditions, P<0.001 (***) ; one-way ANOVA; SNK correction).
6.3.4 Elevated intracellular Na\(^+\) results in intracellular alkalinisation of NSCLC cells

With experimental results suggesting that elevated [Na\(^+\)]\(_i\) has very little effect on Ca\(^{2+}\) signalling in strongly metastatic H460 NSCLC cells, combined with the fact that NHE1 appears to play a major role in H460 cell invasion in a mechanism potentially linked to Na\(_v\)1.7 activity, pH seems likely to be the key factor that renders H460 cells highly invasive and, therefore, strongly metastatic. In order to test this hypothesis further, the effect that manipulation of [Na\(^+\)]\(_i\) has on the intracellular pH, pH\(_i\), of NSCLC cells was measured using the pH-sensitive ratiometric fluorescent dye, BCECF.

Calibration of pH\(_i\) using high-K\(^+\) buffered solutions containing nigericin (section 2.14.3) showed that the pH\(_i\) values for H460 and A549 cells at rest were approximately 7.3 and 6.9, respectively (Figure 6.6). As the strongly metastatic H460 cells have a more alkaline pH\(_i\) compared with the weakly metastatic A549 cells, it is logical to assume that they have a more acidic extracellular pH, pH\(_e\). This preliminary result therefore fits with the hypothesis that invasive cell lines of NSCLC have a more acidic pH\(_e\), putatively providing a more favourable environment for extracellular protease activity, necessary for ECM digestion and subsequent invasion. Moreover, elevated pH\(_i\) alone is a well established characteristic of invasive tumour cells that is thought to encourage metastatic progression (Webb et al. 2011). I next determined the effects of elevated [Na\(^+\)]\(_i\), Na\(_v\)1.7 activity and NHE1 activity on pH\(_i\) in H460 cells.

In all pH experiments, a preliminary test treatment of 60 mM acetate was applied to the cells in order to elicit the control response of intracellular acidification, followed by recovery to resting pH\(_i\) upon removal of acetate. This response confirmed successful loading of cells with BCECF and that the cells were responding normally, prior to application of test treatments. Raising [Na\(^+\)]\(_i\) by monensin treatment caused alkalinisation of pH\(_i\) in both H460 and A549 cells; the pH\(_i\) of H460 cells changed from 7.29 ± 0.01 to 7.75 ± 0.04 (Figure 6.7A), whilst the pH\(_i\) of A549 cells changed from 6.91 ± 0.02 to 7.55 ± 0.04 (Figure 6.7B). This is
consistent with a decrease in pH, showing that Na\textsuperscript{+} influx is sufficient to promote extracellular acidification of NSCLC cells.

Following treatment of H460 cells with 50 µM veratridine, there was a change in pH\textsubscript{i} from 7.30 ± 0.02 to 7.61 ± 0.03 (Figure 6.8A), whereas treatment of H460 cells with 1 µM TTX resulted in a shift of pH\textsubscript{i} from 7.29 ± 0.02 to 6.97 ± 0.02 (Figure 6.8B). Thus, Na\textsubscript{v} channel activity has an indirect effect on pH\textsubscript{i}, consistent with increased Na\textsubscript{v} channel activity promoting intracellular alkalisation/extracellular acidification of the cells. By contrast, treatment of A549 cells with veratridine and TTX predictably resulted in no changes at all to pH\textsubscript{i} (Figure 6.8C). These data confirm that Na\textsubscript{v}1.7 influences pH\textsubscript{i} and hence pH\textsubscript{e} in strongly metastatic H460 NSCLC cells, and raises the following question: is Na\textsubscript{v}1.7’s influence on pH homeostasis in H460 cells mediated via NHE1 activity?

Whilst treatment of A549 cells with veratridine and TTX had no effect on pH\textsubscript{i}, inhibition of ubiquitously expressed NHE1 in these cells with 10 µM EIPA resulted in a change in pH\textsubscript{i} from 6.90 ± 0.02 to 6.54 ± 0.03 (Figure 6.9A). Similarly, when H460 cells were treated with 10 µM EIPA, there was an intracellular acidification of the cells from a pH\textsubscript{i} value of 7.33 ± 0.02 to 6.88 ± 0.03 (Figure 6.9B). The effect of blocking NHE1 activity on the pH\textsubscript{i} of H460 cells is therefore identical to the effect of blocking Na\textsubscript{v}1.7 activity. However, like with cell invasion, the effects that inhibiting NHE1 and Na\textsubscript{v}1.7 have on pH\textsubscript{i} in H460 cells are not additive; treatment of H460 cells with EIPA and EIPA/TTX combined resulted in a change in pH\textsubscript{i} from 7.35 ± 0.01 to 6.94 ± 0.03 and 7.36 ± 0.02 to 6.95 ± 0.03, respectively (Figure 6.9C). This finding seems to support the hypothesis proposed by Brisson and colleagues which states that Na\textsubscript{v} channel activity is crucial in promoting cell invasion via NHE1-dependent H\textsuperscript{+} efflux (Brisson et al. 2011). Both the analysis of NSCLC cell invasion and pH\textsubscript{i} changes have confirmed that the effects of Na\textsubscript{v}1.7 and NHE1 activity are not additive in either of these outputs and that Na\textsubscript{v}1.7 is possibly a key regulator of NHE1 function in an unknown mechanism that promotes H\textsuperscript{+} efflux, extracellular acidification, and subsequent cell invasion. Furthermore, the slower kinetics for changes in pH\textsubscript{i} following veratridine/TTX treatment versus EIPA treatment of H460 cells suggests that Na\textsubscript{v}1.7 activity is upstream of something else; if Na\textsubscript{v}1.7 activity had a direct effect on pH\textsubscript{i}, an instant response would be expected. The
fact that monensin treatment does not exhibit similar slow kinetics to veratridine treatment can possibly be explained by the fact that it results in a large, rapid manipulation of [Na⁺], whereas under physiological conditions only a persistent and localised ‘trickle’ of Na⁺ influx through Naᵥ channels takes place.

Whilst this hypothesis is supported by much of the experimental data, it doesn’t explain why A549 cells are non-invasive despite expressing functional NHE1 that appears to contribute to intracellular alkalisation/extracellular acidification by a similar order of magnitude to those present in H460 cells. Despite lacking Naᵥ1.7, NHE1-mediated H⁺ efflux still takes place in A549 cells, yet A549 cells are significantly less invasive than H460 cells. Furthermore, the fundamental flaw of the above hypothesis is that it cannot be rationalised physiologically: if the activity of both proteins results in Na⁺ influx, how can Naᵥ1.7 possibly impact on NHE1? It should lower the driving force for NHE1 activity. It is clear that NHE1 activity impacts on pH homeostasis in NSCLC cells, as expected, thereby providing a mechanism for the extracellular acidification of strongly metastatic cells, but it is unclear where Naᵥ1.7 fits in, and on its own, cannot account for the invasive phenotype of A549 cells.

It could be that Na⁺ influx via NHE1 is the important role of this protein in NSCLC cell invasion and that the more immediate H⁺ efflux is less so. Maybe the combined activity of Naᵥ1.7 and NHE1 is required in order to generate sufficient Na⁺ influx (a convergent point) to obtain a threshold level of [Na⁺], that impacts indirectly on pH regulatory proteins to promote further intracellular alkalisation/extracellular acidification via an alternative mechanism (PKA upregulation of V-ATPase activity, for example). If this were the case, it would explain why A549 cells were not invasive despite expressing functional NHE1, and provides a physiologically plausible mechanism for Na⁺-dependent cell invasion. The different kinetics following inhibition of Naᵥ1.7 and NHE1 could be explained by the simple fact that this more indirect mechanism of NHE-1-dependent pH regulation would be masked by the more immediate effect of blocking NHE1 activity on cellular pH in H460 cells.
Figure 6.6: Calibration of pH<sub>i</sub> in H460 and A549 cells. High-K<sup>+</sup> buffered solutions containing nigericin were perfused onto H460 (A) and A549 (C) NSCLC cells and the resulting 490/440 fluorescence ratio, measured using BCECF, was recorded. These 490/440 ratios were then plotted against the pH of the buffered solutions in order to calibrate the pH<sub>i</sub> and determine the resting pH<sub>i</sub> values for H460 (B) and A549 (D) cells (n ≥ 62 for both cell lines).
Figure 6.7: Monensin results in intracellular alkalinisation of H460 and A549 cells. Effect of 10 µM monensin on pH\textsubscript{i} in H460 (A, \(n=39\)) and A549 (B, \(n=34\)) cells, recorded using BCECF. Displayed traces are representative. Histograms show mean change in pH\textsubscript{i} from the baseline.
Figure 6.8: Na\textsubscript{v} channel activity results in intracellular alkalinisation of H460 cells. (A) Effect of 50 μM veratridine on pH\textsubscript{i} in H460 cells, recorded using BCECF (n=48). (B) Effect of 1 μM TTX on pH\textsubscript{i} in H460 cells, recorded using BCECF (n=53). (C) Effect of 50 μM veratridine and 1 μM TTX on pH\textsubscript{i} in A549 cells, recorded using BCECF (n=47). Displayed traces are representative. Histograms show mean change in pH\textsubscript{i} from the baseline.
Figure 6.9: NHE1 activity results in intracellular alkalinisation of H460 and A549 cells. (A) Effect of 10 µM EIPA on pH$_i$ in A549 cells, recorded using BCECF ($n=35$). (B) Effect of 10 µM EIPA on pH$_i$ in H460 cells, recorded using BCECF ($n=32$). (C) Effect of 10 µM EIPA and 10 µM EIPA/1 µM TTX co-treatment on pH$_i$ in H460 cells, recorded using BCECF ($n=64$). Displayed traces are representative. Histograms show mean change in pH$_i$ from the baseline.
As discussed previously, a potential mechanism that could result in the extracellular acidification of NSCLC cells in response to elevated \([Na^+]_i\) is via vacuolar H\(^+\)-ATPase (V-ATPase) activity. V-ATPase, responsible for actively pumping protons out of cells, has been identified as a viable anti-cancer target (Fais et al. 2007), and its localisation is reported to be controlled by PKA (Alzamora et al. 2010). Furthermore, there is evidence in breast and prostate cancer cell lines that PKA activity can be directly influenced by elevated \([Na^+]_i\) as a result of Na\(_v\) channel activity, which in turn upregulates Na\(_v\) channel expression via an activity-dependent positive feedback mechanism (Brackenbury and Djamgoz 2006; Chioni et al. 2010). Therefore, any increased activity of PKA as a result of enhanced Na\(_v\) channel and/or NHE1 expression and/or activity in NSCLC cells could promote extracellular acidification through enhanced V-ATPase activity, thereby driving cell invasion. Preliminary experiments to determine whether PKA activity could be influenced by Na\(_v\)1.7 or NHE1 activity showed that treatment of H460 cells with monensin, TTX, veratridine or EIPA had no effect on levels of active, phosphorylated PKA in the cells, as determined by Western immunoblotting (Figure 6.10A). This therefore suggests that changes in \([Na^+]_i\) in H460 cells have no effect on PKA activity and thus, presumably, on V-ATPase activity. In fact, by analysing changes in the pH\(_i\) of H460 cells following treatment of the cells with the V-ATPase inhibitor, bafilomycin A1 (Figure 6.10B), it was demonstrated that V-ATPase plays no role in H460 cells. Application of 500 nM bafilomycin A1 to H460 cells had no effect on the pH\(_i\) of the cells, suggesting its lack of expression and/or activity. However, with a lack of qPCR and Western immunoblot data, it cannot be concluded that V-ATPase is not expressed in H460 cells and a great deal more work can be done to determine what role, if any, this pH regulator might play in NSCLC cell invasion.
Figure 6.10: PKA and V-ATPase play no role in Na\(^+\)-mediated H460 cell invasion. (A) Representative Western immunoblots showing the levels of phosphorylated PKA (upper) and β-actin (lower) proteins in H460 cells following various cell treatments. H460 cells were treated with Lambda PP in order to dephosphorylate any phosphorylated amino acid residues, thus confirming antibody specificity. 50 µM forskolin and 500 nM KT5720 were used to upregulate and downregulate pPKA levels, respectively. 10 µM monensin, 1 µM TTX, 50 µM veratridine and 10 µM EIPA were used to see what effect manipulation of [Na\(^+\)]\(_i\) had on pPKA levels in H460 cells (n=4 for both blots). (B) Effect of 500 nM bafilomycin A1 on pH\(_i\) in H460 cells, recorded using BCECF (n=57). Displayed trace is representative. Histogram shows mean change in pH\(_i\) from the baseline.
6.3.5 Na\textsubscript{v}1.7 and NHE1 co-localise in caveolin-rich lipid rafts

Preliminary work appears to point to changes in pH homeostasis as the most likely downstream effect of altered [Na\textsuperscript{+}]\textsubscript{i} via Na\textsubscript{v}1.7 activity that promotes invasive behaviours in NSCLC cells. I have demonstrated unequivocally that there is an influx of Na\textsuperscript{+} into strongly metastatic H460 NSCLC cell lines that express Na\textsubscript{v}1.7, that this somehow results in H\textsuperscript{+} efflux and that NHE1 also plays a role in the pH-dependent invasion of strongly metastatic H460 cells, whether through the generation of a threshold level of [Na\textsuperscript{+}]\textsubscript{i} with Na\textsubscript{v}1.7, or as a downstream consequence of Na\textsubscript{v}1.7 activity. However, this fails to explain what Na\textsuperscript{+} is actually doing. The hypothesis that intracellular Na\textsuperscript{+} might upregulate PKA activity to both sustain activity-dependent positive feedback of Na\textsubscript{v}1.7 expression and promote extracellular acidification of NSCLC cells via V-ATPase activity was not supported by the experimental evidence. Therefore, whatever its role may be, NHE1 appears to be a particularly important protein in Na\textsubscript{v}1.7-dependent extracellular acidification of strongly metastatic NSCLC cells. Among all pH regulators, NHE1 has attracted substantial attention in the field of cancer research and provides a mechanism by which cancer cells can adapt to their high metabolic H\textsuperscript{+} production (Gatenby et al. 2007). Indeed, NHE1 has been shown to play a predominant role in the extracellular acidification of tumour cells and to be involved in the invasive process (Bourguignon et al. 2004; Cardone et al. 2005; Stock and Schwab 2006; Stock et al. 2007; Stuwe et al. 2007; Stock and Schwab 2009; Busco et al. 2010; Brisson et al. 2011; Brisson et al. 2012).

Brisson and colleagues have demonstrated that Na\textsubscript{v}1.5 co-localises with NHE1 in caveolae in MDA-MB-231 human breast cancer cells (Brisson et al. 2011). If Na\textsubscript{v}1.7 and NHE1 share a common mechanism in the promotion of NSCLC invasion, co-localisation of the two proteins could also be expected to take place in strongly metastatic H460 NSCLC cells. In H460 cells, abnormally expressed Na\textsubscript{v}1.7 does indeed co-localise with NHE1 in caveolin-rich lipid rafts, potentially accommodating a functional interaction between the two proteins within caveolae. This was demonstrated using sucrose gradient fractionation experiments (Figure 6.11). Anti-caveolin immunoreactivity (both caveolin 1 and pan-caveolin) was observed at the 5/30% sucrose boundary along with Na\textsubscript{v}1.7 and NHE1. As expected, there
were also non-raft populations of Na\textsubscript{v}1.7 and NHE1 in the 45\% sucrose fractions. The EGFR on the otherhand was solely present in non-raft fractions. It has been shown that the EGFR does reside in detergent-free lipid raft preparations (Macdonald and Pike 2005), but these experiments have demonstrated that the receptor does not localise to Triton™ X-100-sensitive lipid raft fractions, nor does it co-localise with caveolin, or indeed, with Na\textsubscript{v}1.7 or NHE1. As demonstrated previously, both Na\textsubscript{v}1.7 and NHE1 expression are controlled by EGFR signalling in H460 cells. However, aside from that there is no requirement for the EGFR to co-localise with either of these proteins, whereas if the activity of NHE1 were dependent on the activity of Na\textsubscript{v}1.7, or the resulting Na\textsuperscript{+} influx from both proteins stimulated a common downstream mechanism, the close proximity and/or functional interaction of the two proteins would be desirable. This data, combined with the fact that NHE1 expression is ubiquitous, yet only functional Na\textsubscript{v} channel expression is present in strongly metastatic NSCLC cells, and that inhibition of either Na\textsubscript{v}1.7 or NHE1 in H460 cells reduces cell invasion whilst inhibition of both does not have an additive effect, appears to demonstrate that Na\textsubscript{v}1.7 channels and NHE1 antiporters are mechanistic partners in H460 NSCLC cell invasiveness. This interaction putatively takes place in caveolae, or more specifically, caveolae within invadopodia, thereby allowing for localised extracellular acidification and targeted ECM degradation (Busco et al. 2010).

If the co-localisation of Na\textsubscript{v}1.7 and NHE1 in caveolae is an important part of a mechanistic association of the two proteins in the promotion of a more invasive cancer phenotype in NSCLC cells, then disruption of this association should reduce the invasive properties of the cells. In order to test whether or not the presence of caveolin-rich lipid rafts in the cell membrane of H460 NSCLC cells is required for effective cell invasion, H460 cells were depleted of cholesterol by incubation with 1 mM methyl-β-cyclodextrin (MβC) for the duration of an invasion assay, thereby disrupting lipid raft structures in the cells, and cell invasiveness was analysed. A working concentration of 1 mM MβC was used based on the results of cell toxicity assays (Figure 6.12A). Generally, it is recommended that a concentration of 5 mM MβC should be used for cholesterol depletion work in vitro. However, when applied for 48 hours or just one hour, this concentration of MβC significantly reduced H460 cell viability and promoted cell death. When treated with just 1 mM MβC for 48 hours,
however, no effect on H460 cell viability was observed. Therefore, in order to analyse the effect of cholesterol depletion on H460 cell invasion, cells were treated with 1 mM MβC for the duration of the invasion assay. This resulted in a 57.4 ± 2.5% reduction in cell invasion compared to untreated H460 cells (Figure 6.12B), suggesting that the co-localisation of Na\textsubscript{v}1.7 and NHE1 within caveolae could be functionally important in H460 cell metastasis.
Figure 6.11: Na\textsubscript{v}1.7 and NHE1 co-localise in caveolin-rich lipid rafts. Representative Western immunoblots showing sucrose gradient membrane fractionation results for Na\textsubscript{v}1.7, NHE1 and EGFR. Membrane fractionation on a sucrose gradient reveals that Na\textsubscript{v}1.7 and NHE1 are co-localised in buoyant fractions 5-7, immediately at the 5/30% sucrose boundary. These fractions represent caveolae-containing membranes, as shown by enrichment in caveolin. The EGFR is only present in non-raft fractions (n=6 for all blots).
Figure 6.12: Cholesterol depletion and subsequent lipid raft disruption inhibits H460 cell invasion. (A) Effect of 5 mM and 1 mM MβC, applied for either 48 hours or one hour, on H460 cell viability, analysed using the cell toxicity assay. Based on these results, a concentration of 1 mM MβC was applied to H460 cells for a period of 48 hours in order to determine the effect cholesterol depletion has on the invasive capabilities of H460 cells (n=3 for all conditions, *P*<0.05 (*), ***P*<0.001 (***); one-way ANOVA; SNK correction). (B) Effect of 1 mM MβC on H460 cell Matrigel™ invasion, recorded after 48 hours (n=6 for both conditions, *P*<0.001 (**)); Student’s *t*-test).
6.3.6 Expression of Na\textsubscript{v}1.7, β1 and NHE1 in H460 cells is non-reciprocal

Having demonstrated a role for Na\textsubscript{v}1.7 and NHE1 (ion flux), as well as the auxiliary β1-subunit (adhesion), in NSCLC cell invasion, I investigated whether expression of one of these proteins impacted on expression of another. Reciprocal expression of proteins represents a simple mechanism to ensure that different proteins are expressed concomitantly, often because they have roles in a common cellular mechanism and so need to be present together spatially and/or temporally. Previous studies have shown that stable expression of β1 in MDA-MB-231 breast cancer cells results in increased neonatal Na\textsubscript{v}1.5 (nNa\textsubscript{v}1.5) expression (Chioni et al. 2009). Furthermore, Na\textsubscript{v} channel activity can result in gene expression, most notably through a positive feedback mechanism involving PKA (Brackenbury and Djamgoz 2006; Chioni et al. 2010). In support of previous data, qPCR analysis revealed that expression levels for Na\textsubscript{v}1.7, NHE1 and β1 differ greatly in H460 NSCLC cells (Figure 6.13A). In contrast to observations from other cancer cell lines, treatment of H460 cells with siRNA directed against each of the three proteins of interest (Na\textsubscript{v}1.7, NHE1 and β1) demonstrated that knocking down expression of one of the three proteins has no significant effect on expression of the other proteins (Figure 6.13B).
Figure 6.13: Expression of Na<sub>v</sub>1.7, β1 and NHE1 in H460 cells is non-reciprocal. (A) mRNA levels, assessed by qPCR, for Na<sub>v</sub>1.7, β1 and NHE1 in H460 cells (n=6 for all primer sets, \( P<0.01 \) (**), \( P<0.001 \) (***) ; one-way ANOVA; SNK correction). (B) Normalised mRNA expression, assessed by qPCR, for Na<sub>v</sub>1.7 (white), β1 (grey) and NHE1 (black) in H460 cells following treatment of the cells with siRNA directed against either Na<sub>v</sub>1.7, β1 or NHE1. All data is normalised to the untreated control condition for each of the proteins being analysed (n=9 for all conditions with all primer sets, \( P<0.05 \) (*), \( P<0.001 \) (***) ; one-way ANOVA; SNK correction).
6.4 Discussion

6.4.1 Summary of results

The data presented in this chapter demonstrate that elevated intracellular sodium alone is able to promote NSCLC cell invasion. Elevated intracellular sodium in metastatic H460 NSCLC cells, as a result of aberrant expression of Na\(_{\text{v}}\)1.7, does not have a significant effect on intracellular calcium signalling. However, it does result in intracellular alkalinisation, and thus presumably, extracellular acidification of H460 cells to drive cell invasion via an unknown mechanism potentially involving NHE1. Na\(_{\text{v}}\)1.7 and NHE1 co-localise in caveolin-rich lipid raft membrane fractions, supporting the hypothesis that they may be functionally coupled in the promotion of NSCLC cell invasion.

6.4.2 Technical considerations

Whilst experimental design has been robust and thorough with all necessary controls implemented, the following technical considerations must be highlighted:

1. For the majority of cell invasion assays conducted in this project, cell proliferation assays were conducted as well using the same treatments. This was done in order to ensure that any effect on the invasive capabilities of cells was not simply the result of significant changes in the proliferative capabilities of the cells over the duration of the invasion assay (48 hours). For those invasion assays in which parallel proliferation assays were not performed, it may be the case that a small amount of any effect on cell invasion is the result of changes in cell proliferation. However, the duration of the invasion assay was selected so that cells would have a sufficient amount of time to invade without undergoing too much proliferative activity.

2. Fluorescence measurement of intracellular Ca\(^{2+}\) and pH using the ratiometric fluorescent dyes, Fura-2 and BCECF, respectively, monitor global intracellular changes in the levels of Ca\(^{2+}\) and H\(^+\). As a result, these experiments lack a degree of
sensitivity and are unable to detect very subtle, localised changes in ion concentrations, where physiologically relevant adjustments may be taking place. This should be considered when interpreting Ca\(^{2+}\)/pH imaging data.

3. When investigating the reciprocal expression of proteins in a cell, one should consider the possibility that the time-scale of reciprocity might not be the same for all proteins. In the experiments performed in this project, mRNA levels for proteins of interest were measured following a 24-hour incubation with siRNA directed against another protein of interest in order to determine whether or not the expression of two proteins is reciprocal. In order to fully understand these relationships and draw strong conclusions from the data, it may be necessary to increase the duration of siRNA treatment.

4. qPCR housekeeping genes (see section 3.4.2).

5. Additivity of combined treatments on cell invasion (see section 5.4.2).

6.4.3 Discussion of results

Functional expression of Na\(_v\) channels in non-excitable epithelial cancer cells may be considered a marker of a metastatic cancer phenotype. The functional expression of Na\(_v\)1.7 in H460 NSCLC cells results in an elevated [Na\(^+\)]\(_i\) compared with weakly metastatic A549 cells which do not express Na\(_v\) channels. This increased [Na\(^+\)]\(_i\) promotes cell invasion. However, the downstream effects of raised [Na\(^+\)]\(_i\) and the identity of the molecular target(s) regulated by Na\(_v\) channel activity that may promote NSCLC invasion remain unclear. The studies conducted in this chapter suggest that Na\(_v\) channel activity promotes H460 cell invasion through alkalisation of the intracellular environment, and thus putative acidification of the extracellular environment, in a mechanism that may involve NHE1.

NHE1 is ubiquitously expressed (Orlowski and Grinstein 2004; Loo et al. 2012), and like Na\(_v\)1.7, its expression in H460 NSCLC cells appears to be regulated by EGFR signalling. Blocking NHE1 in H460 cells inhibits cell invasion by the same degree as blocking Na\(_v\)1.7
activity, yet blocking both NHE1 and Naᵥ1.7 simultaneously does not result in further reduction in cell invasion, suggesting a common mechanism (Brisson et al. 2011). This is supported by evidence which demonstrates that NHE1 expression appears to be higher in weakly metastatic A549 cells, which lack Naᵥ1.7, compared with H460 cells, yet they are significantly less invasive. The fact that NHE1 expression is ubiquitous suggests that on its own, it does not drive cell invasion, but rather something else is promoting its activity, or, something else is needed alongside NHE1 in order to promote cell invasion.

The hypothesis proposed by Brisson and colleagues (Brisson et al. 2011) states that elevated \([\text{Na}^+]_i\) in NSCLC cells, brought about through whatever means (monensin or ouabain \textit{in vitro} and functional Naᵥ channel expression physiologically, for example), somehow promotes enhanced NHE1 activity, which in turn drives cell invasion (an interdependent mechanism). The role of NHE1-mediated H⁺-efflux in metastases has been demonstrated many times, with elevated NHE1 expression known to take place in cancer cells, namely at sites of focal ECM degradation where it reportedly promotes cell invasion through the generation of an acidic extracellular environment (Bourguignon et al. 2004; Cardone et al. 2005; Stock and Schwab 2006; Stock et al. 2007; Stuwe et al. 2007; Stock and Schwab 2009; Busco et al. 2010; Brisson et al. 2012). It is widely accepted that pHₑ is lower in many tumours than in corresponding normal tissue, and provides an optimum environment for cell metastasis (Rofstad et al. 2006). Therefore, NHE1-mediated H⁺ efflux from H460 cells in response to elevated \([\text{Na}^+]_i\) via functional Naᵥ1.7 expression enables the cells to achieve a more acidic pHₑ, and therefore, a more invasive cancer phenotype. Whilst much of the experimental evidence supports a role for NHE1 activity downstream of Na⁺ influx into H460 cells via Naᵥ1.7 activity, however, this cannot be rationalised physiologically; elevated \([\text{Na}^+]_i\) would be expected to lower the driving force for Na⁺/H⁺ exchange via NHE1. Moreover, NHE1 makes a similar contribution to pH homeostasis in non-invasive A549 cells as it does in strongly invasive H460 cells, without the need for Naᵥ channel expression and without enhancing their metastatic phenotype, suggesting that NHE1-dependent H⁺ efflux alone is not sufficient to drive cell invasion. Therefore, with no coherent way of modeling this relationship, another hypothesis must be proposed.
An alternative hypothesis states that Na\textsubscript{v}1.7 and NHE1 most likely exert their effects on pH independently and that the two mechanisms contribute to pH regulation and hence invasion: NHE1 via direct changes in pH and Na\textsubscript{v}1.7 via, as yet, unknown pH effectors. However, it is clear that NHE1 alone is not sufficient to promote cell invasion, otherwise A549 cells would also be invasive, and that Na\textsubscript{v}1.7 and NHE1 appear to share a common mechanism in the pH-dependent promotion of H460 cell invasion. Therefore, Na\textsubscript{v}1.7 activity is crucial but it is unclear what its precise role is. Na\textsuperscript{+} influx into NSCLC cells by whatever means promotes intracellular alkalinisation, and thus presumably, extracellular acidification, and so Na\textsuperscript{+} influx alone can clearly contribute to cell invasion by altering pH. In metastatic H460 cells, evidently Na\textsubscript{v}1.7 is the key player for Na\textsuperscript{+} influx in this context. The model which best satisfies the experimental data and can be rationalised physiologically therefore states that, whilst NHE1 behaves as expected (H\textsuperscript{+} efflux), the combined Na\textsuperscript{+} influx through Na\textsubscript{v}1.7 and NHE1 is sufficient to obtain a threshold level of [Na\textsuperscript{+}]\textsubscript{i}, that impacts indirectly on pH regulatory proteins in a mechanism separate from direct H\textsuperscript{+} efflux by NHE1, and that inhibiting either Na\textsubscript{v}1.7 or NHE1 results in a sub-threshold level of [Na\textsuperscript{+}]\textsubscript{i} (the proteins function independently but converge on a common point downstream). Clearly, however, more work is required in order to understand any relationship between Na\textsubscript{v}1.7 and NHE1 in the promotion of NSCLC cell invasion.

Contrary to observations presented by other groups (Brackenbury and Djamgoz 2006; Chioni et al. 2010), the evidence presented in this chapter does not support a role for Na\textsubscript{v} channel-dependent regulation of PKA activity in H460 NSCLC cells. This is unfortunate, as successful demonstration of Na\textsuperscript{+}-dependent activity of PKA in these cells would have supported the independent pathway/convergent point hypothesis by identifying a protein downstream of elevated [Na\textsuperscript{+}]\textsubscript{i}, that could impact on cellular pH, e.g. via V-ATPase activity. Whilst it appears that V-ATPase plays no role in H\textsuperscript{+} efflux from H460 cells, a great deal more work can be done to confirm this. I simply show that the V-ATPase inhibitor, bafilomycin A1, failed to alter pH\textsubscript{i} in H460 cells. For a more complete analysis of V-ATPase activity in H460 cells, expression studies and treatment with different pharmacological agents should be performed. Work presented by Supino and colleagues demonstrates the antimetastatic effect of
a small molecule V-ATPase inhibitor, both in vitro and in vivo, on H460 cells (Supino et al. 2008), thus highlighting the need for further investigation in this area.

Na\textsubscript{v} channel activity and the resulting rise in [Na\textsuperscript{+}]\textsubscript{i} has a minimal effect on Ca\textsuperscript{2+} signalling in H460 cells. The fact that monensin treatment increased [Ca\textsuperscript{2+}]\textsubscript{i} in only a small subset of H460 cells suggests that the effect of elevated [Na\textsuperscript{+}]\textsubscript{i} on Ca\textsuperscript{2+} signalling is not great enough to have any meaningful influence on the invasive properties of the cells, particularly given that Na\textsuperscript{+} influx through Na\textsubscript{v} channels is much smaller and that treatment of the cells with veratridine and TTX had no effect at all on [Ca\textsuperscript{2+}]\textsubscript{i}. However, it must not be forgotten that only a tiny percentage of cells in a tumour need to respond in a pro-invasive manner in order to make a difference in cancer. It is possible that some aspects of NSCLC metastases depend on Ca\textsuperscript{2+} signalling as a result of elevated [Na\textsuperscript{+}]\textsubscript{i}. Moreover, subtle changes in [Ca\textsuperscript{2+}]\textsubscript{i} levels are difficult to pick up in these global measurements, and in reality, very localised changes in [Ca\textsuperscript{2+}]\textsubscript{i} may have huge effects on cell behaviour. That said, however, the data clearly shows that elevated [Na\textsuperscript{+}]\textsubscript{i} unequivocally leads to alkalinisation of pH\textsubscript{i} in H460 cells, and thus acidification of pH\textsubscript{e}, supporting a role for altered pH homeostasis in Na\textsubscript{v} channel-driven NSCLC cell invasion. It may be the case that the Na\textsubscript{v} channel-dependent acidification of the extracellular environment is greater in particular membrane locations, such as caveolae, leading to a specific microenvironment with a lower pH than that observed globally. Both Na\textsubscript{v}1.7 and NHE1 were shown to co-localise in caveolin-rich lipid raft membrane fractions, supporting the idea that they share a common invasive mechanism, localised to isolated membrane structures. Regardless of whether Na\textsubscript{v}1.7 and NHE1 are functionally coupled, the fact that they reside together in invadopodia-like fractions suggests that they are important in NSCLC cell invasion. A growing body of work is shedding light on the role played by invadopodia (actin-rich membrane protrusions formed by invasive cancer cells) in cancer cell invasion (Reshkin et al. 2012; Sibony-Benyamini and Gil-Henn 2012; Yamaguchi 2012), with NHE1 now recognised as an important component (Busco et al. 2010).

Proteolytic digestion of ECM proteins is a necessary process in tumour cell invasion and is ultimately what distinguishes cell invasion from cell migration. In order to digest the ECM, cancer cells express extracellularly active proteases that can be associated with the
membrane or released as soluble forms into the surrounding environment. Work performed by Gillet et al. showed that ~75% of H460 cell invasion is regulated by secreted cysteine cathepsin enzymes, and that some of these enzymes (CatB, CatS and CatL) are, at least partially, regulated by Na\textsubscript{v} channel activity (Gillet et al. 2009). The enzymes were shown to be more active in more acidic extracellular environments, brought about via Na\textsubscript{v} channel activity, whilst there was no effect on levels of enzyme expression or secretion. These observations provide support for the findings in this chapter regarding Na\textsubscript{v} channel-mediated H\textsuperscript{+} efflux in H460 cells and its importance in driving the invasive process. However, an investigation into the effect of H\textsuperscript{+} efflux on the activity of secreted proteases was beyond the scope of the project.

The proposed hypothesis for Na\textsubscript{v} channel-mediated invasion of H460 cells assumes that the observed intracellular alkalinisation of the cells as a result of elevated [Na\textsuperscript{+}]\textsubscript{i} results in a concomitant acidification of the extracellular environment/pericellular space. Indeed, this has already been demonstrated for H460 cells (Gillet et al. 2009). However, regardless of whether or not extracellular acidification takes place to the extent required to drive invasion via secreted protease activity, intracellular alkalinisation of invasive tumour cells alone is known to enhance metastatic progression, principally via the enhancement of directed migration (Webb et al. 2011). Nevertheless, owing to the fact that the metastatic mechanisms associated with intracellular alkalinisation of cancer cells appear to affect their migratory abilities, and that Na\textsubscript{v} channel-dependent enhancement of NSCLC metastatic behaviour has been shown to impact on invasion specifically, a model of enhanced metastatic potential via extracellular acidification is favoured.

What the evidence herein shows is that elevated [Na\textsuperscript{+}]\textsubscript{i} in strongly metastatic H460 NSCLC cells, as a result of functional Na\textsubscript{v}1.7 expression, promotes cell invasion via H\textsuperscript{+} efflux and subsequent extracellular acidification. It is unclear, however, how Na\textsuperscript{+} influx via Na\textsubscript{v}1.7 promotes this extracellular acidification. The evidence could be interpreted to show that, in agreement with others (Brisson et al. 2011), NHE1-dependent H\textsuperscript{+} efflux may be a downstream effect of Na\textsubscript{v}1.7 activity, and that Na\textsubscript{v}1.7 and NHE1 are functionally coupled in an unknown mechanism localised to caveolae, tightly controlled by EGFR signalling. What is perhaps
more likely, however, is that the EGFR-regulated expression of Na\textsubscript{v}1.7 and NHE1 independently contribute to pH regulation and that the combined Na\textsuperscript{+} influx via the two proteins also drives changes in pH homeostasis via an uncharacterised Na\textsuperscript{+}-dependent mechanism. If any relationship exists between these proteins then the precise dynamics of their interaction could be even more crucial in areas of the membrane directly involved in ECM degradation, such as invadopodia. A lot more work is required in order to gain an understanding of how Na\textsubscript{v}1.7 and NHE1 mediate NSCLC invasion.
Chapter 7:
GENERAL DISCUSSION
From the outset of this project, the overarching motive has been to gain a greater understanding of how the expression of functional Na\textsubscript{v} channels is regulated in metastatic cell lines of NSCLC, and how the presence of these Na\textsubscript{v} channels in non-excitable epithelial cells potentiates the development of a more invasive cancer phenotype. Na\textsubscript{v} channels have been found to play crucial roles in metastatic behaviours of several cancers derived from non-excitable epithelial cells, where these channels serve no obvious physiological role. These include breast cancer (Roger et al. 2004; Fraser et al. 2005; Brackenbury et al. 2007; Gao et al. 2009; Gillet et al. 2009), prostate cancer (Grimes et al. 1995; Laniado et al. 1997; Grimes and Djamgoz 1998; Diss et al. 2001; Abdul and Hoosein 2002; Bennett et al. 2004; Diss et al. 2005; Nakajima et al. 2009; Yildirim et al. 2012), cervical cancer (Diaz et al. 2007; Hernandez-Plata et al. 2012), colon cancer (House et al. 2010), ovarian cancer (Gao et al. 2010), SCLC (Pancrazio et al. 1989; Blandino et al. 1995; Onganer and Djamgoz 2005; Onganer et al. 2005), and more recently, NSCLC (Roger et al. 2007). Thus, the acquisition of a more neuronal phenotype via the upregulation of Na\textsubscript{v} channel expression and activity is associated with the development of cancer metastases in a variety of carcinomas. Whilst some progress has been made towards understanding the possible downstream effects of Na\textsubscript{v} channels in cancer, there is still a great deal to learn. Furthermore, the cellular mechanism(s) that regulate the expression and activity of Na\textsubscript{v} channels in metastatic cancer cells remain unclear.

To this end, a strategy was adopted that allowed the regulation, characterisation, localisation and function of Na\textsubscript{v} channels in NSCLC cells, as well as their putative role in the promotion of NSCLC invasion and other cellular behaviours, to be studied. Moreover, the clinical relevance of Na\textsubscript{v} channels in NSCLC was investigated in the hope that they may prove to be useful biomarkers and/or tractable drug targets in the diagnosis, prognosis and/or treatment of certain epithelial cancers.
7.1 Na\textsubscript{v}1.7 is functionally expressed in the strongly metastatic H460 NSCLC cell line and promotes cell invasion

The initial stage of the project involved screening for Na\textsubscript{v} channel currents in a panel of NSCLC cell lines using the IonWorks® electrophysiology platform, primarily to enable selection of model cell lines for further characterisation and experimentation. From this screen, H460 cells were identified as a suitable model for strongly metastatic NSCLC cells that express functional Na\textsubscript{v} channels and A549 cells were selected as a suitable model for weakly metastatic NSCLC cells that lack Na\textsubscript{v} channel activity. Whilst the presence of Na\textsubscript{v}1.7 mRNA in strongly metastatic H460 cells, and its lack of expression in weakly metastatic A549 cells, had already been reported by Roger and colleagues (Roger et al. 2007), I am the first to demonstrate its predominant role in Na\textsubscript{v} channel-dependent invasion of H460 cells. The identification of Na\textsubscript{v}1.7 activity as necessary for invasion of strongly metastatic NSCLC cells supports earlier work that has established a crucial role of Na\textsubscript{v} channels – particularly Na\textsubscript{v}1.5 and Na\textsubscript{v}1.7 – in metastatic behaviours of several cancers, including NSCLC (Laniado et al. 1997; Abdul and Hoosein 2002; Bennett et al. 2004; Onganer et al. 2005; Brackenbury et al. 2007; Diaz et al. 2007; Roger et al. 2007; Uysal-Onganer and Djamgoz 2007; Gao et al. 2009; Gillet et al. 2009; Gao et al. 2010; House et al. 2010). Despite the fact that developmentally-regulated neonatal splice variants of Na\textsubscript{v}1.5 and Na\textsubscript{v}1.7 have been reported in several epithelial cancer cell lines (Diss et al. 2001; Diss et al. 2005; Ou et al. 2005; Brackenbury et al. 2007; Onkal and Djamgoz 2009), putatively driving Na\textsubscript{v} channel-dependent cell invasion, an investigation into the proportion of Na\textsubscript{v}1.7 that is neonatal/adult in H460 cells was not undertaken in this project. This is because, unlike Na\textsubscript{v}1.5, Na\textsubscript{v}1.7 exists as mixed populations of neonatal/adult splice variants in healthy human dorsal root ganglia (DRGs) (Raymond et al. 2004), and so even if nNa\textsubscript{v}1.7 predominated in NSCLC and proved to be a tractable drug target, this negates the need for the development of a targeted therapy against nNa\textsubscript{v}1.7 in the treatment of cancer.

In support of work carried out by other groups (Roger et al. 2007; Gillet et al. 2009), characterisation of Na\textsubscript{v}1.7 activity and its effect on cell behaviours revealed that at the $E_m$ of H460 cells there is continuous influx of Na$^+$ via partial activation of the channel, and that this
has the ability to drive invasion of the cells without influencing cell proliferation or migration. Furthermore, there appears to be a positive feedback mechanism whereby the partial opening of Na\(_{\text{v}}\)1.7 at \(E_m\) allows \(Na^+\) influx, which in turn keeps \(E_m\) sufficiently depolarised to maintain a continuous influx of \(Na^+\), thus perpetuating the cycle and providing a continual driving force for \(Na^+\)-dependent cell invasion under physiological conditions.

The experimental evidence presented herein suggests that the aberrant expression of Na\(_{\text{v}}\)1.7 in NSCLC cells specifically promotes cell invasion through the influx of extracellular \(Na^+\) into the cells, without affecting other cell behaviours. However, inhibition of \(Na^+\) influx through Na\(_{\text{v}}\)1.7 only ever results in a 40-50% reduction in cell invasion. Therefore, whilst it contributes significantly to the invasive capabilities of H460 cells, the functional expression of Na\(_{\text{v}}\) channels certainly does not account for all of it. There are likely numerous other cellular factors/mechanisms promoting an invasive phenotype in H460 cells. The fact that the functional expression of Na\(_{\text{v}}\)1.7 specifically impacts on cell invasion without influencing cell migration suggests that whatever downstream effect Na\(_{\text{v}}\)1.7 activity is having in H460 cells, it ultimately results in enhanced extracellular protease activity. Cellular migration and invasion are two distinct processes (Friedl and Wolf 2003; Sahai 2005), with invasion specifically requiring the physical breakdown of ECM proteins by membrane-tethered or secreted protease enzymes, enabling cells to invade local tissues and circulatory systems to migrate and form secondary tumours. The fact that Na\(_{\text{v}}\) channels specifically promote the invasive capabilities of epithelial cancer cells suggests that their activity ultimately influences the expression and/or activity of these proteases.

7.2 The auxiliary β1-subunit is strongly expressed in the weakly metastatic A549 NSCLC cell line where it functions as a CAM

In breast cancer, the Na\(_{\text{v}}\) channel β1-subunit is reported to act as a CAM, being highly expressed in non-invasive cells but not in strongly metastatic cells which exhibit reduced cell adhesion (Brackenbury and Isom 2008; Chioni et al. 2009). Consistent with such reports, there is a marked difference in β1 expression between strongly and weakly metastatic NSCLC cells, with the weakly metastatic A549 cells showing high expression of the auxiliary subunit
compared with the strongly metastatic H460 cells. I demonstrate that β1 does indeed function as a CAM in NSCLC cells, enabling cells to be significantly more adhesive on fibronectin surfaces. There is still much to learn about the role of Na\textsubscript{v} channel β-subunits in the development and progression of epithelial cancers. However, based on the evidence, it is logical to hypothesise that a concurrent downregulation of β1 and upregulation of Na\textsubscript{v}1.7 in NSCLC cells together drive malignantly transformed cells towards a more metastatic phenotype.

7.3 Na\textsubscript{v}1.7 is expressed in human lung cancer samples

The strong positive association of Na\textsubscript{v}1.7 expression with cancerous human lung tissue demonstrates its clinical relevance and suggests that its expression in vivo may well represent an important event in the progression of lung cancer to the metastatic mode. As a minimum, it appears to be a strong tumour marker in the lung, as has been demonstrated with Na\textsubscript{v} channels in other cancerous epithelial tissues (Abdul and Hoosein 2002; Diss et al. 2005; Fraser et al. 2005; House et al. 2010; Hernandez-Plata et al. 2012). Moreover, I demonstrate a marked upregulation of Na\textsubscript{v}1.7 protein levels in prostatic epithelial cells at the transition from low-grade to high-grade cancer, which is characterised predominantly by the development of metastases, thus supporting the hypothesis that Na\textsubscript{v}1.7 expression is considerably enhanced in strongly metastatic versus weakly metastatic NSCLC cells and may potenti ate a series of cell behaviours integral to the metastatic cascade. An unequivocal demonstration of the clinical relevance of this work is extremely important if Na\textsubscript{v} channels are to be considered as potential future targets in the diagnosis, prognosis and treatment of metastatic lung cancer. Therefore, the immunohistochemistry experiments performed in this project represent an important piece of work in the field of Na\textsubscript{v} channels in NSCLC that successfully bridges the gap from in vitro to in vivo.

On account of the difficulty in acquiring patient lung cancer samples, only three were tested. Similarly, only three FFPE preparations of low- and high-grade prostate cancer tumours, also known to be associated with high expression of Na\textsubscript{v}1.7 (Diss et al. 2001; Diss et al. 2005; Nakajima et al. 2009; Yildirim et al. 2012), were tested for the same reason and
because different staged lung cancer samples were not available. For a complete investigation into \( \text{Na}_\text{v}1.7 \) expression in human lung cancer and its putative association with metastatic disease, a much broader analysis needs to be performed using multiple patient samples of cancerous and normal-matched lung tissue at different stages of the disease.

7.4 *Expression of functional Na\(_v\)1.7 in H460 cells is tightly regulated by EGFR signalling working via the ERK1/2 signalling cascade*

I report for the first time that expression of functional \( \text{Na}_\text{v}1.7 \) is a novel downstream effect of ERK1/2 signalling in H460 cells that specifically promotes cell invasion and thus the generation of a more metastatic cancer phenotype (Figure 7.1). To date, there has been little characterisation of the mechanisms which drive functional expression of \( \text{Na}_\text{v} \) channels in cancerous cells (Uysal-Onganer and Djamgoz 2007; Ding et al. 2008; Fraser et al. 2010). The data presented herein proposes that EGFR ligand, known to be produced and released by NSCLC cells to establish a so-called EGFR autocrine loop and self-directed EGFR stimulation (Fujimoto et al. 2005; Volante et al. 2007; Wu et al. 2007), binds to the EGFR on the cell surface of H460 cells, triggering transcription of \( \text{Na}_\text{v}1.7 \) via the ERK1/2 signalling cascade without having any acute effects on \( \text{Na}_\text{v}1.7 \) activity, which in turn enhances the invasive properties of the cells via elevated \([\text{Na}^+]_i\).

Throughout the course of this project H460 cell invasion was consistently inhibited by \(~40-50\%\) following block of the EGFR, ERK1/2 or \( \text{Na}_\text{v}1.7 \), and additive effects of combined treatments were not observed. This suggests that EGFR/ERK-mediated cell invasion occurs exclusively via expression of functional \( \text{Na}_\text{v}1.7 \). As highlighted previously, the fact that this pathway only ever promotes 40-50\% of H460 cell invasion implies that other cellular factors must also be involved in the invasive cascade. Having demonstrated a clear role for EGFR signalling in the promotion of NSCLC cell invasion, it is logical to hypothesise that other growth factor signalling pathways might contribute to the remaining 50-60\%. VEGF, FGF and PDGF all play roles in NSCLC (Ballas and Chachoua 2011) and are linked with \( \text{Na}_\text{v} \) channel function (Hilborn et al. 1998; Goldfarb et al. 2007; Andrikopoulos et al. 2011) so may conceivably promote NSCLC cell invasion too, either through upregulation of \( \text{Na}_\text{v} \) channels or
independently of it. Moreover, this project has only focussed on EGF as a ligand to the EGFR. Many other growth factors stimulate the EGFR, e.g. TGF-α and epiregulin (Harris et al. 2003), and so there is plenty of scope for further work into the role of growth factor signalling pathways in Na, channel-dependent NSCLC metastasis. It is possible that Na,1.7 could be a convergent point in several of these pathways. If this is the case, Na,1.7 would prove to be a very attractive drug target against metastatic NSCLC. In addition, despite the fact that small molecule EGFR inhibitors such as gefitinib and erlotinib have significant therapeutic benefit in many patients with advanced NSCLC, drug resistance, whether inherited or acquired, remains a major cause of chemotherapy failure (Lynch et al. 2004; Paez et al. 2004; Domingo et al. 2010; Cataldo et al. 2011). With EGFR-dependent invasion of strongly metastatic H460 cells relying on Na,1.7 activity, drugs targeting this novel downstream effector could be used in conjunction with existing therapies such as EGFR inhibitors.

Whilst inhibition of the EGFR, ERK1/2 and Na,1.7 consistently reduced H460 cell invasion by ~40-50%, the effects on $I_{Na}$ varied from complete loss of current (TTX) to 47% inhibition (U0126). I propose that this is because not all available channels need to be functional to allow sufficient Na$^+$ influx for invasion, and that under physiological conditions the persistent Na$^+$ influx brought about through the partial activation of Na,1.7 at the $E_m$ of H460 cells is sufficient to maintain the invasive capabilities of the cells. Endogenous modulators of Na,1.7, e.g. ERK1/2, could thus provide an exquisite mechanism for fine-tuning of EGFR-mediated invasion in NSCLC.
Figure 7.1: EGFR-driven expression of functional Na\textsubscript{v}1.7 exclusively promotes H460 cell invasion. In H460 NSCLC cells, the EGFR-regulated expression of functional Na\textsubscript{v}1.7 promotes cell invasion whilst EGFR-regulated cell proliferation and migration are independent of Na\textsubscript{v}1.7 activity.
7.5 Elevated $[\text{Na}^+]_i$ via functional expression of Na\textsubscript{1.7} promotes H460 cell invasion via extracellular acidification of the cell

Whilst a small subset of H460 cells may respond to elevated $[\text{Na}^+]_i$ with very subtle changes in $[\text{Ca}^{2+}]_i$, the experimental evidence presented in this project does not support a role for remodelled Ca\textsuperscript{2+} signalling as a downstream consequence of Na\textsuperscript{+} influx via Na\textsubscript{1.7} in these cells. Rather, it suggests that Na\textsubscript{1.7} activity promotes H460 cell invasion through acidification of the extracellular environment in a mechanism that potentially involves NHE1.

The fact that blocking NHE1 in H460 cells inhibits cell invasion by the same degree as blocking Na\textsubscript{1.7}, yet blocking the two simultaneously has no additive effect on inhibition of cell invasion, suggests a shared mechanism (Brisson et al. 2011). Furthermore, it can be postulated that Na\textsubscript{1.7} activity and the resulting rise in $[\text{Na}^+]_i$ is upstream of NHE1-dependent H\textsuperscript{+} efflux from the cell in this mechanism. NHE1 expression is ubiquitous (Orlowksi and Grinstein 2004; Loo et al. 2012) and actually appears to be higher in weakly metastatic A549 cells compared with H460 cells. If NHE1 alone were able to successfully drive cell invasion, then why are A549 cells not at all invasive? It must be that something else is promoting activity of NHE1 and the resulting H\textsuperscript{+} efflux drives cell invasion, or, something else is required in addition to NHE1 to promote cell invasion. Based on the evidence, I propose that in addition to NHE-1 mediated H\textsuperscript{+} efflux from H460 cells, Na\textsubscript{1.7} and NHE1 function independently, serving as conduits through which Na\textsuperscript{+} influx into the cells can occur in order to trigger a Na\textsuperscript{+}-dependent invasive mechanism that ultimately results in further acidification of the extracellular environment. The fact that there is no additive effect when simultaneously blocking Na\textsubscript{1.7} and NHE1 on H460 cell invasion implies that both proteins are needed in order to generate sufficient Na\textsuperscript{+} influx for a threshold level of $[\text{Na}^+]_i$ to be reached. Once a threshold level is obtained, Na\textsuperscript{+} drives invasion via an uncharacterised mechanism independent of direct H\textsuperscript{+} efflux via NHE1, resulting in further extracellular acidification of the cell. The putative Na\textsuperscript{+}-dependent upregulation of PKA activity, resulting in upregulation of V-ATPase activity and subsequent H\textsuperscript{+} efflux, could be just such a mechanism. However, preliminary work does not support a role for PKA or V-ATPase in H460 cell invasion. That said, this mechanism should not be ruled out completely as a great deal more work can be
done in this area. The alternative model, which states that Na_v channel activity promotes the NHE1-dependent H^+ efflux from strongly metastatic cells cannot be rationalised physiologically. Clearly, a great deal more work is required in order to fully understand what relationship, if any, exists between Na_v1.7 and NHE1 in the promotion of NSCLC invasion.

Whatever the relationship, a role for NHE1 in metastases has been demonstrated many times, primarily at sites of focal ECM degradation (Bourguignon et al. 2004; Cardone et al. 2005; Stock and Schwab 2006; Stock et al. 2007; Stuwe et al. 2007; Stock and Schwab 2009; Busco et al. 2010; Brisson et al. 2012). I report that NHE1 is present in caveolin-rich lipid rafts in H460 cells where it co-localises with Na_v1.7. Not only does this support the idea of a shared mechanism for these two proteins, it also suggests that the Na_v channel-dependent acidification of the extracellular environment is greater in particular membrane locations, such as caveolae, leading to a specific microenvironment with a lower pH than that observed globally. Regardless of whether Na_v1.7 and NHE1 are functionally coupled, the fact that they reside together in invadopodia-like fractions suggests that they are important in NSCLC cell invasion.

Assuming that H^+ efflux and extracellular acidification is indeed the downstream consequence of functional expression of Na_v1.7 in strongly metastatic NSCLC cells, this must ultimately impact on the proteolytic digestion of ECM proteins: it is a necessary process in tumour cell invasion and is fundamentally what distinguishes cell invasion from cell migration. Preliminary work in this area strongly supports a role for cysteine cathepsin proteases in the Na_v channel-dependent invasion of epithelial cancer cells (Gillet et al. 2009). It has been shown that ~75% of H460 cell invasion is regulated by secreted cysteine cathepsin enzymes, and that some of these enzymes (CatB, CatS and CatL) are, at least partially, regulated by Na_v channel activity. Moreover, the enzymes are more active in more acidic extracellular environments, brought about via Na_v channel activity. These observations provide interesting insights into how Na_v1.7 might ultimately promote invasion of non-excitable NSCLC cells and should clearly constitute an area of focus for any planned future work.
7.6 A model of EGFR-driven NSCLC cell invasion

In light of the findings of the experimental work performed in this project, together with the published data, a model of EGFR-driven Na,1.7-dependent invasion of H460 NSCLC cells can be proposed (Figure 7.2). It should be noted that other Na+-dependent mechanisms might also impinge on this pathway and that Ca²⁺ signalling might make a greater contribution to the progression of lung cancer to the metastatic mode than the model implies. Moreover, the work focussing on the downstream effects of elevated [Na⁺]ᵢ on cell invasion is very much preliminary and so conclusions drawn from it are speculative and open to interpretation. Nevertheless, the high consistency of the data in this study would suggest that the basic model described is robust, albeit incomplete.
Figure 7.2: Model of EGFR-driven H460 cell invasion. (1) A high concentration of EGFR ligand in the tumour microenvironment ensures stimulation of the EGFR. Stimulation of the EGFR may well be self-directed (Fujimoto et al. 2005; Volante et al. 2007; Wu et al. 2007). Intracellular signalling via the ERK1/2 signalling cascade promotes expression of the Na\(_{v1.7}\) gene (SCN9A). The mRNA transcript is translated into Na\(_{v1.7}\) protein which is trafficked to the plasma membrane. Stimulation of the EGFR also promotes NHE1 expression and post-translational upregulation of NHE1 at the leading edge of the cell (Chiang et al. 2008). (2) Na\(_{v1.7}\) channels are partially activated and not fully inactivated at the \(E_m\) of the cell, allowing a basal influx of extracellular Na\(^+\) into the cell, resulting in elevated [Na\(^+\)]\(_i\). Expression of the CAM-like \(\beta1\)-subunit is low, further promoting cell invasion (Patino and Isom 2010). The rise in [Na\(^+\)]\(_i\) has no effect on [Ca\(^{2+}\)]\(_i\), either via the reverse function of NCX1 (3) or any other conduit, or on PKA activation (4), thought to be involved in the activity-dependent regulation of Na\(_v\) channel expression in invasive cancer cells (Brackenbury and Djamgoz 2006; Chioni et al. 2010) and activation of V-ATPase (Alzamora et al. 2010). (5) Rather, elevated [Na\(^+\)]\(_i\) promotes extracellular acidification of the cell, either via NHE1-dependent H\(^+\) efflux (Brisson et al. 2011), or more likely, via Na\(^+\)-dependent downstream effectors activated by the combined influx of Na\(^+\) via Na\(_{v1.7}\) and NHE1, driving cell invasion through the generation of a favourable environment for cysteine cathepsin activity (Gillet et al. 2009). Extracellular acidification has also been reported to facilitate the anterograde transport of lysosome vesicles to the plasma membrane, allowing secretion of cathepsin enzymes into the perimembrane space (Steffan et al. 2009). Na\(_{v1.7}\) and NHE1 reside together in caveolae. However, a mechanism linking Na\(_{v1.7}\) and NHE1 activity with NSCLC cell invasion remains unclear.
7.7 Therapeutic potential

Ion channels are well recognised as important therapeutic targets in the treatment of a number of different pathophysiologies, and Na\textsubscript{v} channel-specific blockers are already in clinical use as local anaesthetics and epilepsy treatments (Wood and Boorman 2005). Currently, Na\textsubscript{v}1.7-specific inhibitors are being developed for the treatment of pain (Dib-Hajj et al. 2009; Clare 2010; Ghelardini et al. 2010; Chowdhury et al. 2011; Theile and Cummins 2011) and so Na\textsubscript{v}1.7 has already been identified as an attractive and tractable therapeutic target in a non-cancer disease model. Given its unequivocal functional significance in strongly metastatic cell lines of NSCLC and marked upregulation in tumour tissue, Na\textsubscript{v}1.7 potentially represents an important new target for therapeutic intervention and/or as a diagnostic/prognostic marker in NSCLC. Drug resistance remains a major cause of chemotherapy failure for current NSCLC treatments (Lynch et al. 2004; Paez et al. 2004; Domingo et al. 2010; Cataldo et al. 2011) and so there is a need for new therapies. If it could be shown that Na\textsubscript{v}1.7 is a convergence point in multiple growth factor signalling pathways, its appeal as a novel late-stage NSCLC therapeutic target would be considerably increased and may pave the way for its use alongside existing therapies.

As mentioned, Na\textsubscript{v}1.7-specific blockers are already in development for the treatment of pain. I have shown that two Na\textsubscript{v}1.7-specific inhibitors (AZ compound 1 and AZ compound 2, AstraZeneca) dose-dependently reduce the invasive capabilities of H460 cells (Figure 7.3). This not only supports all of the previous work which shows that Na\textsubscript{v}1.7 is the Na\textsubscript{v} channel isoform that promotes H460 cell invasion, but also demonstrates the potential of targeting Na\textsubscript{v}1.7 in the treatment of metastatic NSCLC. At the very least, this work shows that Na\textsubscript{v}1.7 can be considered a marker of progression of NSCLC to the metastatic mode.
Figure 7.3: Na\textsubscript{v}1.7-specific inhibitors dose-dependently reduce H460 cell invasion. Relative Matrigel™ invasion, recorded after 48 hours, for H460 cells treated with different concentrations (0, 5, 10, 25, 50 and 100 µM) of two Na\textsubscript{v}1.7-specific blockers, AZ compound 1 (A) and AZ compound 2 (B). Also shown for comparison is the effect of 1 µM TTX on H460 invasion (n=3 for all treatments, \(P<0.05\) (*), \(P<0.001\) (***) ; one-way ANOVA; SNK correction).
7.8 Future work

Whilst the high consistency of the data in this project allows a robust model of Na\textsubscript{v}1.7-dependent NSCLC cell invasion to be proposed, this is by no means an exhaustive study and many questions remain unanswered. For a more complete study, the following future work should be considered:

1. The bulk of the work conducted in this project demonstrates unequivocally that the functional expression of Na\textsubscript{v}1.7 is associated with the enhanced invasive potential of strongly metastatic NSCLC cells and that transcriptional regulation of Na\textsubscript{v}1.7 is regulated by EGFR signalling via the ERK1/2 signalling cascade. This conclusion is based on work carried out on just one model cell line, however. For a more detailed analysis of Na\textsubscript{v} channel-driven NSCLC invasion and its regulation by EGFR signalling, it would be desirable to perform the same experiments on multiple NSCLC cell lines. The initial IonWorks® electrophysiology screen identified four more NSCLC cell lines with detectible Na\textsubscript{v} channel currents (H23, H1703, H358 and HX147). It would therefore be logical to perform the same experiments on these cell lines to see if the same model of cell invasion applies to them.

2. Similarly, only one growth factor receptor and one growth factor receptor ligand was studied. As has been discussed, it may be the case that functional expression of Na\textsubscript{v}1.7 is a convergence point in several growth factor signalling pathways and so an investigation into the transcriptional regulation of Na\textsubscript{v}1.7-driven NSCLC invasion by multiple growth factors could prove extremely fruitful.

3. Whilst I successfully demonstrate the clinical relevance of Na\textsubscript{v}1.7 in human lung cancer through IHC analysis of patient lung samples, access to these samples was a limiting factor in this investigation. Moreover, access to staged lung cancer samples was not possible at all, thereby justifying the use of staged prostate cancer samples. A much broader IHC analysis of staged lung cancer samples from multiple patients is therefore needed to confirm a clinical role of Na\textsubscript{v}1.7 in late-stage/metastatic NSCLC.
4. Much of the required future work concerns events downstream of Na\textsubscript{v} channel activity in strongly metastatic NSCLC cells. This project addresses the likely pathways/mechanisms that elevated [Na\textsuperscript{+}]\textsubscript{i} might be expected to trigger to ultimately result in enhanced cell invasion, although a great deal more work is required in this area. The experimental evidence did not support a clear role for Ca\textsuperscript{2+} signalling as a downstream consequence of elevated [Na\textsuperscript{+}]\textsubscript{i} in H460 cells. Rather, its effect on pH\textsubscript{i} was much more robust and as this provided a direct link to an enhanced invasive cell phenotype, became the focus of much of the later work in the project. This is not to say that altered [Ca\textsuperscript{2+}]\textsubscript{i} in response to elevated [Na\textsuperscript{+}]\textsubscript{i} does not play any role at all in NSCLC invasion. In reality, only a very small subset of cells in a tumour need to respond in an invasive manner for it to pose a clinical problem and so there may be some aspects of NSCLC metastases dependent on this invasive mechanism, necessitating the need for further research in this area.

5. Similarly, based on the experimental evidence, a role for V-ATPase in H460 cell invasion was quickly dismissed. Again, much more follow-up work is needed to either confirm or undermine this conclusion, particularly as a mechanism involving V-ATPase seems to be a prime candidate for the downstream effect of elevated [Na\textsuperscript{+}]\textsubscript{i} in the favoured model of Na\textsubscript{v}1.7-dependent H460 cell invasion. As a starting point, simply using a different V-ATPase inhibitor to test the effect of blocking V-ATPase on pH\textsubscript{i} would prove very informative. Supino and colleagues, for example, have demonstrated the antimetastatic effects of a small molecule V-ATPase inhibitor on H460 cells (Supino et al. 2008), thus highlighting the need for further investigation in this area.

6. To add greater support to the proposed models of Na\textsubscript{v}1.7-dependent H460 cell invasion, additional pH\textsubscript{i} experiments can be performed. Primarily, an analysis of the effect of EGF/gefitinib treatment on the resting pH\textsubscript{i} of H460 cells would, if shown to have an effect, reinforce the idea that EGFR-driven expression of functional Na\textsubscript{v}1.7 affects pH homeostasis in these cells. Furthermore, one would expect that the pharmacological manipulation of Na\textsubscript{v}1.7 would have a reduced effect on pH\textsubscript{i} in H460
cells that had been treated with gefitinib, owing to the fact that gefitinib treatment has been shown to significantly reduce Na\(_v\)1.7 expression in these cells.

7. Preliminary evidence suggests that Na\(_v\)1.7 and NHE1 might co-localise in invadopodia-like structures. NHE1 is recognised as an important component of invadopodia (Busco et al. 2010) and I would like to see if Na\(_v\) channels are too.

8. Time-permitting, I would have liked to investigate the putative role of pH-sensitive cysteine cathepsins as the likely endpoint in the proposed Na\(_v\)1.7-dependent metastatic cascade (Gillet et al. 2009).

9. Finally, having demonstrated the clinical relevance of this work via IHC experiments, future work in the field of Na\(_v\) channel-dependent NSCLC metastasis should focus on \textit{in vivo} experiments in which the development of targeted therapies against Na\(_v\)1.7 in the treatment of this disease is the ultimate goal. One possible experiment that could be performed relatively quickly and could provide very exciting results would be to obtain lung cancer tissue samples from patients treated with gefitinib and to compare Na\(_v\)1.7 expression in these samples versus untreated patient samples.

The field of Na\(_v\) channel-dependent invasion of non-excitable epithelial cancers is a relatively new one and the model presented here is based on the pharmacological and physiological findings of this project. However, as has been highlighted, further experimentation is needed to substantiate this model and to address the unanswered questions.

7.9 Conclusion

In conclusion, the data presented in this thesis demonstrate a potential role for the functional expression of Na\(_v\)1.7 in the progression of human NSCLC to the metastatic mode. Evidence suggests that the functional expression of Na\(_v\)1.7 in strongly metastatic cell lines of NSCLC is under the transcriptional regulation of EGFR signalling, working via the ERK1/2 signalling cascade, and that this has a direct effect on the invasive properties of the cells.
without affecting cell proliferation or migration. Na\(_v\)1.7 is strongly expressed in cancerous human lung tissue versus normal-matched human lung tissue and preliminary evidence suggests that elevated [Na\(^+\)]\(_i\), as a result of functional Na\(_v\)1.7 expression, promotes cell invasion in an uncharacterised mechanism involving NHE1, resulting in extracellular acidification of the cell, possibly at specific, caveolin-rich membrane regions.
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