Investigating Engrailed-2 (EN2) as a therapeutic target in prostate cancer

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The thesis is submitted in accordance with the requirements of the University of Surrey for the degree of Doctor of Philosophy

Supervisor: Prof Richard Morgan
Co-Supervisor: Prof Hardev Pandha

January 2017
DECLARATION OF ORIGINALITY

The work detailed in this thesis are the result of my own efforts unless otherwise stated - these are fully acknowledged and referenced. Nor has the thesis been adapted or submitted in short for any other qualification. I agree that the University of Surrey has the right to submit my work to the plagiarism detection service - Turnitin. The University also has the right to require an electronic version of the final thesis submitted here.

Natasha Punia
January 2017
Abstract

Engrailed-2 (EN2) is a transcription factor involved in development, where it is has multiple functions and is expressed in a caudal-to-rostral gradient in the midbrain. Its mRNA and protein expression are absent from most adult cells, but are switched back on in cancer cells. Although transcription factors are usually located in the nucleus, a number of previous reports have indicated that EN2 protein could be in the cell membrane and on the cell surface on tumour samples, from where some at least might be released, as EN2 has been found in the urine of prostate cancer patients - a more readily obtainable clinical sample than tumour biopsies.

In this study EN2 protein is definitively shown to be on the membrane of prostate cancer cells and in the tumour microenvironment. The commercial anti-EN2 antibody was found to be non-specific and we therefore used a tagged version of EN2 to study its cellular distribution and behaviour. This revealed different modes of EN2 protein transport and secretory mechanisms in different cancer cell lines. Live cell imaging further revealed the generation of secretory vesicles from PC3 cells, which are derived from metastatic prostate cancer, but not WPMY-1 cells that are derived from normal prostate fibroblasts. The findings further suggest that EN2 protein switches roles during tumour progression, from a transcriptional regulator to a regulator of protein translation in localised regions of the cytoplasm. The latter mechanism is especially significant as EN2 cellular localisation becomes dysregulated in cancer, becoming widely cytoplasmic and available for packaging into luminal vesicles. The findings also indicate that the translation factor EIF4E is a potential binding partner of EN2, in prostate cancer.

The study findings also indicate that a monoclonal antibody-drug conjugate targeting EN2 may not be the most effective method of targeting EN2-expressing cells. A blocking peptide or antibody would be more appropriate to prevent its secretion and transfer, both of which have been shown to be possible mechanisms of tumour progression. Alternatively, because less EN2 is secreted (and hence more is retained) in prostate cancer cell lines with low metastatic potential, such as LnCaP, T cells could be employed to target early-stage prostate cancer.

To conclude, cancer cells have seemingly retained the ability to tightly regulate the expression of EN2 protein in a spatial and temporal manner, unlike
normal adult cells. EN2 is secreted in large vesicles by cells from more advanced prostate tumours and thus monoclonal antibodies may not be the most effective approach to therapy.
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<td>eIF4E</td>
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<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
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<td>MHC</td>
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<td>Matrix metalloproteinase</td>
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<td>Small/short Interfering Ribonucleic Acid</td>
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<td>Western Blot</td>
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<td>Wheat germ agglutinin</td>
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Chapter 1: Introduction
1. Introduction

The chapter is arranged as follows. First, the thesis question is stated and the literature and theoretical framework on which the thesis was formed is presented. The motivation for the work to be carried out and the methodological approach are justified. The thesis aims, objectives and hypotheses are listed. Lastly, the overall structure of the individual chapters are outlined.

1.1 Thesis question

• Is Engrailed-2 (EN2) a suitable prostate cancer target for an antibody drug-conjugate?

1.2 Scope of research

EN2 is a developmental protein required for brain development, principally patterning the brain (for more detail see Section 2.3.1), and is subsequently downregulated. It is then re-expressed in cancer and thus can be used as a means of differentiating between the normal healthy tissue and tumour. However, its unique ability to be secreted and internalised means that it is able to be detected in bio-fluid, such as urine, and in tissue away from the tumour (expanded upon in Section 2.3.4). Aside from being used as a biomarker, if EN2 resides stably on the membrane of cancer cells only, it can also be used as a target for monoclonal antibody therapy (see Section 2.2.2).

EN2 could be involved in initiating tumorigenesis. In 2005 Martin et al. identified En2 as an oncogene in breast cancer (Martin et al. 2005). In addition to showing the overexpression of EN2 at mRNA and protein levels, they also suggest that EN2 was necessary for the loss of contact inhibition and an increased proliferation rate in breast cancer cell lines. EN2 forced expression was able to
generate adenocarcinomas after 14 weeks and with PBX co-expression there was accelerated development of adenocarcinomas.

It is possible that the expression of any potential binding partner of EN2 could also be regulated by EN2 in a similar feedback mechanism to PAX2. In 2008, Bose et al. looked at PAX2 as a potential downstream effector due to its vital role in prostate cancer cell survival (Bose et al. 2008). They concluded that PAX2 may be an activating transcription factor for En2 and proposed that there was a feedback mechanism in which EN2 then affects its own expression by regulating PAX2’s expression and so on.

In 2011 Morgan et al. showed that EN2 was a potential diagnostic biomarker for prostate cancer. Secreted EN2 was detected by an antibody-based test (ELISA) in the urine of prostate cancer patients with better accuracy than PSA (R. Morgan et al. 2011). Subsequently, EN2 was also found to be a protein biomarker in non-muscle invasive bladder cancer. High grade tumours had progressively less EN2 expression but, conversely, urinary EN2 detection was sensitive to increasing tumour grades.

More recently, there have been several reports of EN2 down regulation as a marker of poor prognosis for renal cancer, whilst higher amounts of EN2 protein was found in adjacent normal tissue (Lai et al. 2014) (Lai et al. 2016). Furthermore, Lai et al. showed that forced En2 expression (in vitro) slowed proliferation, increased apoptosis and reduced the invasive ability of the cancer cells.

1.3 Study rationale

The research into EN2 in cancer has been sporadic and has given contradicting results. EN2 has been reported to be both an oncogene and tumour suppressor and the size of EN2 has been reported at different sizes with no explanation (see Section 2.3.3).

The lack of knowledge in the function of EN2, compared to other homeoproteins, during fetal development and especially cancer has left a considerable gap in the research and hindered its application in the clinic. The structure of the homeodomain (depicted in Section 2.3.2, Figure 2.13), located within EN2 (and other homeoproteins), responsible for DNA binding, has been
studied extensively and found to be responsible for secretion. Penetratin, a cell-penetrating peptide that drives internalisation, has been largely studied for its potential to cargo molecules in and out of the cell (Dupont et al. 2015). However, a recent study shows that the whole homeodomain is fundamental to the transfer process, in and out of the cell (Carlier et al. 2013). Despite these advances, downstream effectors of EN2 and their physiological (and pathological) effects are still largely unknown. Nevertheless, EN2’s unique property of intercellular transfer makes it an attractive biomarker and target.

The specificity of the widely used PSA biomarker for prostate cancer is sub-standard and often results in unnecessary biopsies and over-treatment. PSA remains as a tool in the clinic for two reasons firstly, there is sufficient knowledge in the literature to guide its application in the clinic and secondly, there is no better biomarker available (for prostate cancer) to replace it, see Section 2.1.4 for further details.

EN2 could replace PSA or supplement PSA (in making a panel of biomarkers) to improve the diagnosis of prostate cancer, and has yet to be explored as a therapeutic target - this study was designed to explore these possibilities.

1.4 Methodological approach

The chosen therapy to investigate was an antibody-drug conjugate (ADC) against EN2. An antibody-drug conjugate is a type of targeted therapy, which falls under personalised medicine as it relies on the cell surface profile of the individuals cancer (as further described in Section 2.1.6.1). A good example of a successful treatment using this approach is Herceptin and its target, HER2, in breast cancer therapy (also mentioned in Section 2.2.3.1). An ADC has a low satisfaction criteria: EN2 must be accessible via the circulation and preferably reside on or tether the cell surface of cancer cells. In this way the drug (attached to the antibody) can be easily administered - injected into the bloodstream - and is targeted to the cancer cells, not the surrounding normal cells; this should lessen unwanted side effects in the clinic.

As this is, to my knowledge, the first time EN2 has been investigated in the context of a therapeutic target, the most logical type of investigation was an in vitro one. In vitro experiments are a cost-effective approach when investigating under an
exploratory nature; a necessity in this case as very little is known about EN2 and its role in cancer. In order to model prostate cancer \textit{in vitro}, the following cell lines were used: high grade (PC3) and low grade (LnCaP) prostate cancer cell lines and WPMY-1 normal cells that have been immortalised for laboratory use. For more information regarding their origin see \textbf{Chapter 3}.

Antibodies are commonly raised against a protein of interest and the antibodies produced are either monoclonal or polyclonal. Monoclonal antibodies all bind to the same peptide of a protein, this can be extremely limiting when dealing with an unstable protein which can, for example, drastically change its structure depending on its environment. Polyclonal antibodies bind to different peptides of a protein and thus, allow for a general overview of protein expression as it is not restricted to one peptide. Generally speaking antibodies are robust, inexpensive and amenable but they do require validation - polyclonal antibodies, especially, can bind to non-specific proteins (further expanded upon in \textbf{Section 2.2.4}). The validation experiments carried out in this study are:

- protein overexpression - where we’d expect the antibody to detect higher levels of protein
- protein knockdown - where we’d expect the antibody to detect lower levels of protein
- No primary antibody controls - this is to show if and to what extent the secondary antibody, which is raised against the primary antibody and provides the quantifiable marker, is binding non-specifically and falsely increasing the signal detected

The other part of an antibody, beside from its antigen binding properties, is the effector. The effector can also be engineered to a) indirectly kill the tumour by eliciting an internal immune response or b) directly kill the tumour by attaching a drug. For the latter - the chosen approach in this study - the whole structure has to be taken up by the cell otherwise the drug is overly exposed on the outside and is likely to cause harm to the surrounding healthy tissue in the clinic. Thus, it is imperative that the chosen antibody is shown \textit{in vitro} to be internalised preferentially by the cancer cell and not ‘normal’ healthy cells.
1.5 Thesis aims, objectives and hypotheses

The overarching aims of this study were to validate and explore EN2 as a cancer specific cell surface protein target and increase our understanding of the role and behaviour of EN2 in cancer.

The overarching hypotheses of the thesis was that a) there would be a significant level of EN2 on the cell surface of prostate cancer cells relative to ‘healthy’ cells \((in\ vitro)\), and b) EN2 localisation to the cytoplasm is promoted as prostate tumour grade increases due to its potential translational role on the cell surface of cancer cells.

In order to achieve this, the following objectives (1-5) were formulated:

1. determine EN2’s expression and localisation within cancer cells
2. further define EN2’s secretion and internalisation mechanisms
3. identify a suitable EN2 antibody for development into an antibody-drug conjugate
4. carry out preliminary \((in\ vitro)\) experiments on the candidate antibody-drug conjugate to direct the next set of experiments
5. further investigate EN2’s cell autonomous and cell non-autonomous role

1.6 Thesis structure

The thesis is organised as follows:

- **Chapter 2** collates the relevant literature in order to provide the necessary background knowledge to follow the thesis. It outlines the current state of prostate cancer management and highlights the gaps in the area of EN2 research, particularly in cancer. It also reviews prostate
cancer biomarkers and the potential role of EN2 in this respect. It also provides the underlying evidence required to formulate the objectives and hypotheses in Section 1.5.

- **Chapter 3** firstly outlines the general materials and methods used and then the specific and more novel methodology created for each of the three results sections that follow.

- **Chapter 4** is one of three self-contained results section and provides only the required background knowledge for the chapter, and a specific subset of objectives and hypotheses. It addresses the expression of EN2 in prostate cancer and seeks to validate it further as a biomarker before exploring its potential as a therapeutic target. Therefore, this Chapter addresses **Objective 1** (detailed in Section 1.5).

- **Chapter 5** is the second results section that further assesses EN2 on the cell surface of prostate cancer cells. It also explores the secretory and internalisation mechanism of EN2. The candidate antibody for developing an antibody-drug conjugate against EN2 is further investigated *in vitro*. This Chapter has dealt with **Objectives 1 - 4**.

- **Chapter 6** is the third and final results chapter which aims to further define the cell autonomous and non-autonomous functions of EN2; the Chapter focuses on **Objective 5**.

- **Chapter 7** concludes the thesis by drawing together the achievements from all three results chapters, and addresses future research aims.

This chapter has provided a guide and breakdown of the thesis. The next chapter will expand on this to provide the background knowledge and further highlight the gap in the literature.
Chapter 2: Literature review
2. Literature Review

The review outlines the characteristics of prostate cancer and its demography in the UK, and highlights the lack of biomarkers for early stage screening and for distinguishing indolent from aggressive prostate tumours. Thus, prognosis varies widely as does the subsequent treatment administered. For EN2 as a potential biomarker and therapeutic target, there is a significant gap in the basic etiology concerning EN2 and cancer and EN2’s secretory and internalisation mechanisms.

2.1 Prostate Cancer

The prostate is an exocrine gland of the male reproductive system, which can be found below the bladder, surrounding the urethra. It is about the size of a walnut and produces the milky-white fluid for semen. Its growth heavily relies on the androgenic hormone: testosterone (Aumüller 1983). Prostate cancer is the most common cancer in men and the second most common cause of cancer-related deaths in men in the UK (Cancer Research UK). Though most prostate cancer cases remain indolent, men who go on to progress to have an aggressive form have very few treatment options available to them and survival is poor.

2.1.1 Incidence and survival

The estimated prostate cancer incidence world-wide, in 2012, was more than 1.1 million (World Health Organisation), of which 417,000 were European (Office for National Statistics). In 2013, in the UK alone, prostate cancer was the most common cancer in men with over 40,000 cases registered (Cancer Research UK). The following year, there were 11,287 prostate cancer deaths in the UK (Office for National Statistics); accounting for 13% of all cancer deaths in males that year in the UK (Office for National Statistics).

Below Figure 2.1 highlights the percentage of men surviving prostate cancer even after ten years, falling to approximately 84% only.
The high survival percentage depicted in Figure 2.1 is due to the earlier detection of prostate tumours after the widespread use of transurethral resection of the prostate (TURP) and the diagnostic prostate specific antigen (PSA) blood test (Potosky et al. 1990) (Levy et al. 1993). This however, is accompanied by higher incidence. Figure 2.2, which highlights the number of new cases (per year) by age, it is clear that men aged over 50 years routinely undergo PSA testing and TURP when prostate cancer is suspected. However, 25% of the 40,000 diagnosed in 2013 would have presented with metastases, for which the 5-year survival rate is only 30% compared to 84% in Figure 2.1; only 10% will survive for at least 10 years (Office for National Statistics).
There are several reasons for the increasing number of cases, including an aging population, but the predominant reason is the unspecific nature of the PSA test. The PSA test has directly resulted in frequent over-diagnosis and over-treatment (Cary & Cooperberg 2013). Studies have been carried out to further determine whether it actually reduces mortality when there is a) an increase in incidence (Figure 2.2) and b) an increase in both the over-diagnoses and over-treatment of prostate cancer happening at the same time (further discussed in Section 2.1.4.1). The unnecessary tests and subsequent treatments can affect a patient’s quality of life and are not reflected in the national statistics (Figure 2.1 and 2.2). The general lack of clinical biomarkers and testing means that the usual next step (after measuring serum PSA) is to biopsy the patient’s tumour - a much more invasive test that can cause long-term damage (see Section 2.1.4.2 for further details). Equally, no good biomarker exists to distinguish (early-on especially) the subpopulation that will progress to have a more aggressive disease subtype.

2.1.3 Characteristic features
Only a limited number of common mutations and chromosomal alterations have been identified between prostate cancers that could potentially be used as biomarkers - forming the basis of a diagnostic test or be used as a therapeutic target. Prostate cancer, compared to other solid cancers, have: a) high heterogeneity, which means that the genetic mutations within the tumour itself are highly varied, and b) high multifocality - the cancer originates at multiple locations, each going on to consist of different mutational landscapes and inevitably forming a number of tumours within a prostate. Androgens, as biomarkers, are an exception - they play a wide and considerable role in promoting the progression of prostate cancer - but their use as a therapeutic target in androgen therapy has been exhausted. Furthermore, androgen therapy is redundant for those tumours that are or become androgen-independent (also known as hormone-resistant); and due to hormone deprivation therapy, most tumours eventually switch to become androgen-independent, for which the therapeutic options are few.

2.1.3.1 Pathophysiology

Research that aims to understand the fundamental aetiology of the disease such as the key pathways involved in the progression of cells down the tumorigenic path, are key to producing effective and reliable clinical biomarkers and targets for therapy. Unfortunately, the pathways are numerous in prostate cancer and the information cannot be translated in a sufficiently useful manner in the clinic, further details are provided below.

Prostate cancer can be broken down into three categories depending on its spatial relationship with the prostate gland: localised, where the cancer remains confined within the prostate and has not spread elsewhere in the body; locally advanced, cancer that has started to extend out of the prostate or has spread to the area just outside the prostate but is not yet metastatic; and metastatic cancer, like most other solid tumours, can spread to distant areas such as the brain. Prostate cancer tends to metastasise to the bone (Rucci & Angelucci 2014) (Jin et al. 2011); this is the most frequent cause of death in metastatic prostate cancer patients and, as such, the 5-year survival drops significantly to 31% (Figure 2.1) (Jin et al. 2011). Figure 2.3 below outlines a more common pathogenesis pathway of prostate cancer. Examples of mutational events that have been shown to be significant at
those stages are highlighted in red (the molecular mechanisms will be further discussed in Sections 2.1.3.2 and 2.1.3.3).

![Diagram of prostate cancer pathogenesis](image)

**Figure 2.3. The pathogenesis of prostate cancer.** A flow-diagram following the development and progression of prostate cancer (adapted from (Shen & Abate-Shen 2010)). Highlighted in red are events shown to be important at those steps and the genes associated.

As stated above, prostate cancer is difficult to manage because of its highly heterogeneous nature (Schoenborn et al. 2013). In order to further explain this characteristic, it is important to have a clear image of the different types of cells the prostate tissue is made up of - and the type of cells that the cancer can originate from - as depicted below:
When determining the origin of prostate cancer, there are some patterns or commonalities to the type of cell the cancer initiates; research has uncovered that these cells are namely basal and/or luminal cells - the cancer originating cells are also known as ‘cells-of-origin’. Basal and luminal cells in the prostate are two major epithelial cell types that strongly depend on androgen receptor (AR) signaling. The neuroendocrine cells, which are rare and interspersed through the prostate, act on the surrounding cells to stimulate growth (Terry & H. Beltran 2014). Neuroendocrine cells do not express androgen receptors and research that show neuroendocrine differentiation (NED) in prostate cancer have suggested this as a mechanism of resistance to androgen therapy (J. Huang et al. 2007; Parimi et al. 2014). It is important to distinguish cells-of-origin from cancer stem cells (CSCs), CSCs are largely seen as those that sustain and regenerate or replenish tumours and not cells from which the cancer initiates from (Rycaj & Tang 2015). Research that explores the mechanisms of tumour initiation have revealed a more detailed picture, such as the one below (Figure 2.5).

**Figure 2.4. Structure of the prostate tissue.** A schematic diagram that highlights the different cell types that make up the prostate. There are three types of epithelial cells: basal, luminal and neuroendocrine cells. Taken from Prostate Cancer UK.
Figure 2.5. Cells-of-origin in prostate cancer. a) During development stem cells commit to generate basal and luminal cells. b) Both the basal and luminal cells may serve as the cell of origin for (primary and metastatic) prostate cancer (Xin 2013).

Figure 2.5 depicts the common cell types involved at broad stages of tumourigenesis: initiation, progression, and metastasis. The research shows that the basal cells and luminal cells are independently self-sustained. Furthermore, a basal-luminal transition plays a significant role to the cancer cells - during tumour progression - that originate from basal cells; basal cells are seemingly more resistant to oncogenic signals and this infers a characteristic of stem-like plasticity (Xin 2013). In addition to this, prostate cancer tends to progress to form multiple tumours (multifocal prostate cancer) and this is problematic because each has its own cells-of-origin and tumourigenic pathways. There is currently investigations into how best to identify the index lesion (or the driving lesion), which is supposedly the best representative of the overall behaviour of the disease. Currently, the larger lesion is taken as the index lesion and tumour grading (Section 2.1.4.3) is typically based on the index lesion alone (Walz et al. 2011).
The resulting intratumour and intertumour (within the prostate and between patients) heterogeneity makes it difficult to: a) develop robust biomarkers that are successful and cost-effective in the clinic; b) to interpret these in a useful manner and c) predict how the disease will progress (i.e. the prognosis) (Wei et al. 2017). Thus, without sufficient studies that further delineate and categorise these markers - to certain traits such as responsive to a type of treatment or indicative of metastasis - it is difficult for the markers to be informative and aid in clinical decision-making. As an alternative approach to standard diagnosis that is based on its spatial localisation alone (please see Section 2.1.4 for current clinic diagnostic practices including the Gleason grading system), tumours could be further sub-typed according to the pattern of molecular markers or mutational events they harbour and patients assigned treatment on an individual basis (for more details see Section 2.1.6), however this could be argued to be less cost-effective.

2.1.3.2 Molecular basis of initiation and progression

Section 2.1.3.1 highlighted the highly varied nature of prostate cancer between patients particularly at the genetic level, compared to other cancers (Spahn et al. 2015).

Figure 2.2 shows that one of the early, more common events is the loss of chromosomal regions at 8p that leads to the loss of NKX3.1 (homeobox gene). This loss has been further shown to be restricted to cells within the prostate (and is not found in metastases) (Abate-Shen et al. 2008). Subsequently, the cancer commonly progresses with the loss of chromosome 10q and this most likely results in the loss of PTEN, a lipid phosphatase that inhibits PI3K/Akt signaling. This signaling pathway plays a key role in cell survival, proliferation and differentiation (P. Liu et al. 2009) and is the focus of current targeted therapy (Section 2.1.6.1).

Prostate tumours can be either androgen dependent (also known as androgen-sensitive) or androgen-independent (also known as castrate-resistant). The acquisition of castrate-resistance is defined as disease progression that is difficult to treat. After initial ADT response, resistant cells arise and ADT eventually fails; castrate-resistant tumours then develop in these patients with a much more aggressive phenotype discussed further in Section 2.1.3.3. Androgens (usually a steroid hormone such as testosterone) work by binding to androgen receptors
(ARs); these then translocate to the nucleus where they bind to androgen responsive elements and affect the expression of downstream effectors such as PSA (Richter et al. 2007). The AR has been found to be amplified, mutated and have several splice variants, post-translational modifications, aberrant transcriptional activity. Furthermore, associated factors or binding partners have been found to be dysregulated - all towards the same goal of progressing tumour growth (Karantanos et al. 2013). Androgen sensitive tumours can be targeted via androgen depletion and androgen receptor inhibition (Section 2.1.5.2.2) and thus are easier to treat as this is not an option for castrate-resistant tumours.

In addition, by identifying the molecular mutations that drive individual tumours it is possible to distinguish between indolent tumours (early-stage, androgen-sensitive) and aggressive tumours (late-stage, castrate-resistant). From this, a signature profile can be created for each tumour type. However, this is difficult when the tumour is highly heterogeneic by nature with so much variation between individual tumours and within the cells of the tumour itself. A recent study has focused on further defining the mechanisms that underlie indolent prostate tumours and a prominent marker here is the TMPRSS2-ERG fusion (Irshad et al. 2014) (Shen & Abate-Shen 2010). The role of this fusion remains undefined but recent evidence suggests that it functions in invasion (Hägglöf et al. 2014). The TMPRSS2-ERG fusion is actually the most common event throughout all cancers as it can be found in up to 70% of prostate tumours and is seemingly exclusive to prostate tumours (L. Huang et al. 2011). This means that it has the potential to be a highly specific prostate cancer biomarker (Sanguedolce et al. 2016).

Despite this, prostate cancer generally has high heterogeneity and individual management (that includes early PSA screening and monitoring) would be more cost-effective (Section 2.1.4.1). A collaborative molecular screening platform could be a possibility, suggested by Spahn et al, in which patients with specific genotypes are quickly included in a sub-group biomarker driven clinical trial (Spahn et al. 2015).

2.1.3.3 Molecular basis of metastases and beyond

The features of invasion and metastasis are well-defined in tumour biology and these are (in this order): angiogenesis; migration; invasion; intravasation;
circulation, and extravasation of tumor cells and further angiogenesis and colonization at the new site (Hanahan & Weinberg 2011).

The acquisition of androgen-independence plays a huge part in the progression of prostate cancer, switching to a much more mobile and aggressive form that is usually fundamental to metastasis (Arnold & Isaacs 2002). Besides androgens, another key molecule in the castrate-resistant transition is interleukin-6 (IL-6). IL-6 is a glycoprotein that has been shown (when overexpressed) to activate AR-mediated gene expression and therefore increase PSA mRNA levels (Lin et al. 2001) and induce the growth of neuroendocrine and neuroendocrine-like cells (Deeble et al. 2001). These cells can secrete a cocktail of growth stimulating factors and are associated with drug resistance (Hu et al. 2015). This neuroendocrine phenotype is a result of transdifferentiation, which typically occurs at advanced stages (beyond castration-resistance) and is therefore usually present in the metastatic sites rather than the primary site. Thus, these tumours are not treated with ADT, but with platinum-based chemotherapy (Aparicio et al. 2013), and consequently treatment options are even fewer at this stage. It was recently shown that there is bias towards neuronal features during transdifferentiation, as opposed to endocrine features (Grigore et al. 2015). Unfortunately, the neuronal phenotype is associated with higher proliferation rates and thus, a poor prognosis (Grigore et al. 2015).

Alternatively, ADT therapy produces a selective pressure towards androgen independence and instead drives alternative pathways that need to be further defined and characterised, in order to provide more appropriate targets for therapy (Karantanos et al. 2013; Karantanos et al. 2015). Even though AR - to some degree - stays active, these alternative pathways drive the tumours regrowth (such as the neuroendocrine differentiation just described) but other more common pathways become dominant, for example: C-MYC overexpression; PI3K/AKT/mammalian target of rapamycin (mTOR) pathway and survival pathways such as RAS/MAPK pathway (Karantanos et al. 2013). Thus, once a tumour becomes independent of androgen, it become harder to treat as the treatment options available are currently limited.

2.1.3.4 Risk factors
Although risk factors, such as smoking, exposure to UV and family history, sometimes indicate the likelihood of individuals developing cancer, prostate cancer is not significantly linked to such risk factors; instead it is strongly related to endogenous risk factors such as age and ethnicity (Cancer Research UK).

Men aged 65 and over are at higher risk of getting prostate cancer (Cancer Research UK). The same is true for African-American men compared to white men, and amongst the former the disease tends to progress at an earlier age and be more aggressive (Shenoy et al. 2016). There is a clear association between a history of prostate cancer in the family and the risk to that individual of developing the disease. Furthermore, the closer the relatedness, the greater number of family members affected and the earlier the onset (Madersbacher et al. 2011). Therefore, prostate cancer risk is linked to genetic markers such as the (BRCA2) mutation that has a role in DNA repair; a man is at higher risk of developing prostate cancer if he has then BRCA2 mutation (Cavanagh & Rogers 2015).

Discrepancies in copy number variations (CNVs) has recently come to the forefront of cancer research; it has been shown that there is greater diversity in the number of gene copies amongst the human population than previously thought (Zarrei et al. 2015). These have been studied in hereditary and familial prostate cancer especially (Shlien et al. 2008). Similarly, single nucleotide polymorphisms (SNPs), which describes a single nucleotide change within a significant proportion of the population (Erichsen & Chanock 2004). SNPs have been strongly associated with prostate cancer and are already under investigation for use as clinical biomarkers (Williams et al. 2014; Van den Broeck et al. 2014). Both CNVs and SNPs could be useful indicators of risk.

For hormones, insulin-like growth factor 1 (IGF-1) has been implicated as a cause of prostate cancer (Borugian et al. 2008);(Chokkalingam et al. 2001). For androgens (such as testosterone), which play a vital role in prostate tumour progression, there is surprisingly limited evidence to show that higher levels of androgen increases an individuals prostate cancer risk (Michaud et al. 2015). In addition, there is no compelling evidence that prostatitis (infection or inflammation of the prostate) is linked to prostate cancer (Roberts et al. 2004).

2.1.4 Clinical screening, diagnosis and staging
It is widely accepted that the earlier the tumour can be detected and treated, the better the survival outcome (Singal et al. 2014) (Pande et al. 2013). A national screening programme aims to detect cancer early when symptoms are not necessarily present. This could be, for example, screening the population for a genetic biomarker. In addition, the more information obtained at diagnosis, the easier it is to make clinical decisions regarding treatment.

Staging is a hierarchical system against which all prostate cancers are described; this ensures standardisation, allows changes to be easily adopted and more information to be obtained. Tumour biopsies are then further assessed by the Gleason grading system, which provides more information regarding the behaviour of the cells that is reflected by the architecture of the tissue. Further details about the ways in which prostate cancer is currently managed, specifically in the UK, can be found in the following subsections.

2.1.4.1 National screening programme

There are currently no prostate cancer screening systems in the UK as there are no suitable biomarkers or non-invasive tests. Though, men aged 50 and over can request a PSA test (Hodgson et al. 2012). PSA has been tried as a screening tool and the results are inconclusive as to its suitability as a number of studies have indicated that it is not specific and produces a number of false-positive results (Nna 2013). Thus, if PSA were to be used as a screening tool with a larger population it would result in more over-diagnosis and over-treatment. Several studies have shown that PSA screening actually reduces prostate cancer deaths. However, whether PSA screening is beneficial, when taking into consideration the amount of over-treatment and thus harm caused, is still to be determined (Nna 2013). One of the more established and successful screening programmes is the mammogram for women in their 40s, although is it non-invasive it is difficult to standardise and, similar to prostate cancer, breast cancer is often over-diagnosed (Brennan & Houssami 2016).

The big problem in prostate cancer management is distinguishing the slow-growing indolent tumours from aggressive ones at an early stage in the process. Many men with prostate cancer actually live a long time without being treated at all because no symptoms are apparent and in some cases the cancer is never diagnosed and only discovered at autopsy (K. J. L. Bell et al. 2015). Therefore, the
early detection of aggressive tumours can allow more radical treatment early whilst slow-growing ones can be monitored, treated much later or not treated at all. In addition, some believe that hormone therapy (even when a patient is asymptomatic) works better when used as early as possible and studies have shown this to be true after radical prostatectomy (Moul et al. 2008).

In summary, an effective screening programme requires a reliable marker or panel of markers (see section 2.1.4.1), and further work is needed to identify these. More recent advances in this area include discovery of the urinary biomarkers prostate cancer antigen (PCA3) mRNA and EN2, EN2 is further discussed in Section 2.3. The PCA3 gene expresses a non-coding RNA that is restricted to human prostate tissue; overexpression of the PCA3 mRNA in prostate cancer cells has led to the development of a PCA3 mRNA urinary test (Schmid et al. 2015).

2.1.4.2 Route to diagnosis

There are a number of ways men can be diagnosed with prostate cancer and these are highlighted in Figure 2.6 below.

![Figure 2.6. Percentage of Cases by Route to Diagnosis, Males Aged 15-99, England. Prostate cancer 2012-2013. Taken from Cancer Research UK.](image)

The initial indicators, for example, are urinary symptoms such as passing urine more often and erection problems (Jefferies et al. 2016). The primary test is a digital rectal examination (DRE). However, even with an experienced examiner, this
will only have fair reproducibility and these are likely to be diagnosed as advanced stages (Borley & Feneley 2008). The advantage of carrying out a DRE is that it can detect non-PSA-secreting tumours that might be missed, shown to be possible in Figure 2.6.

The PSA test is a relatively cheap and minimally invasive blood test that can be monitored continuously over time. PSA is a serine protease that is produced by the prostate gland to turn semen liquid. Only during prostate cancer are unusual (though still minute) amounts of PSA detected in the serum (blood) (Stamey et al. 1987). PSA is also commonly used as a monitoring tool, further discussed in Section 2.1.5.

There are numerous ways in which PSA has been tested as a diagnostic and monitoring tool. These include the quantification of PSA as free, complexed to serum proteins and a ratio of the two with age-specific delineation or biopsy (Sharma et al. 2016). Active surveillance, for example, heavily relies on PSA measurements that are then used to calculate PSA kinetics such as PSA velocity and doubling time (Vickers & Brewster 2012). Unfortunately, best practice and standardisation of these individual PSA tests has been difficult. The limitations of PSA are that PSA levels can rise due to factors such as prostate infection and hypertrophy, which means it is not specific to prostate tumours (Nadler et al. 1995). Furthermore, the PSA test cannot be a standalone diagnostic test. PSA is unspecific, and further tests must be carried out such as a DRE, Transrectal ultrasound (TRUS) and occasionally magnetic resonance imaging (MRI). In addition, PSA levels cannot distinguish between indolent and aggressive tumours. Despite this, continued improvements on the PSA test management are being explore. For example, the PRIAS (Prostate Cancer Research International: Active Surveillance), which is an online protocol, is aimed towards helping to standardise the PSA interpretation for men with early stage prostate cancer during active surveillance (Punnen et al. 2015). For more information about current and potential prostate cancer biomarkers see Section 2.2.2.

There is a need for additional prognostic biomarkers at the diagnostic stage to supplement PSA and provide more information regarding the disease state before advancing to biopsy. Despite this, PSA is currently used as a diagnostic and monitoring tool (see Section 2.1.5). Unfortunately, there are no better clinical biomarkers available to further delineate the disease state. Thus, the clinician must
make a decision to whether to go ahead with a biopsy or not currently based on the PSA test, risk factors and perhaps other (less-invasive) tests such as an MRI.

A biopsy is necessary to reconfirm the PSA findings - only 30% of those that test “positive” for PSA will actually go on to have a “positive” biopsy (Troyer et al. 2004). A TRUS-guided biopsy is carried out that takes 10 to 12 cores of prostatic tissue to help account for the high heterogeneity and multifocality that are associated with prostate cancer (Boutros et al. 2015); both of which make accurate staging, diagnosis and prognosis difficult. Furthermore, biopsies are unpleasant and can cause complications such as infection (Efesoy et al. 2013). Ideally, there could be biomarkers that slot in here to help decipher whether a biopsy is actually required; currently under investigation are further detailed in Section 2.2.1.1.

2.1.4.3 Histological staging and the Gleason grading system

TMN staging is used world-wide and separately assesses the tumour (T), lymph node involvement (N) and secondary metastases (M). The stages define the size of the tumour and how far it has grown (Borley & Feneley 2008). The T stage is split into four (T1-T4) where T1 tumours are too small to see on the scan, T2 tumours are completely localised within the prostate (T1 and T2 are classed as localised tumours), T3 tumours have broken through the prostate covering but have not spread into other organs, whereas T4 tumours have invaded neighboring organs (T3 and T4 are classed as locally advanced prostate cancer) (Borley & Feneley 2008). T2 and T3 are further sub-categorised according to the extent of tissue coverage.

The Gleason grading system is based on morphology using haematoxylin and eosin (H&E) staining of the tumour (under a microscope) (Humphrey 2004). This allows the assessment of the extent of cell differentiation relative to normal cells. Cancer cells tend to be poorly differentiated as they become less specialised and more proliferative and thus unpredictable or out of control. More than one biopsy sample is taken in order to take into account the heterogeneity within the tumour itself (Section 2.1.3.1), multiple sections are assessed from each sample and the two most common grades are added together. Grades 1 and 2 are normal prostate cells and 3-5 are cancer cells, increasing in abnormality (Humphrey 2004).
2.1.5 Clinical management

Guidelines (provided by NICE) or recommendations are closely followed by clinicians to treat their patients and are compiled of evidence-based data, which are continually evaluated (https://www.nice.org.uk/guidance).

2.1.5.1 Assessment

Men (typically over 65 years), diagnosed with prostate cancer, will be managed during their lifetime as the cancer progresses (or does not progress) through the following stages: risk at diagnosis, active surveillance, active treatment, recurrence, recurrence free, metastatic castrate-resistant prostate cancer, and death.

The risk is assessed using the PSA result with the inclusion of exogenous factors such as weight, as clinical evidence exists linking it to advanced prostate cancer (Cao & Ma 2011) and other factors such as prostate size and DRE, as described in Section 2.1.3.3, and a decision is made as to whether to carry out a biopsy. As mentioned in Section 2.1.4.2, it is at this stage where prognostic markers are lacking, prognosis varies widely and so does the treatment. Ideally, there should be molecular stratification that could allow for a better prognosis and a tailored treatment stream instead of the very general ‘one size fits all’ approach (Sedelaar & Schalken 2015), this is known as personalised medicine (see Section 2.1.6 for more details).

Next, the tumour will then be assigned a stage and a grade. Localised prostate cancer are then further assigned to one of the following risk groups: Low risk - less than 10ng/ml PSA, a Gleason score lower than 6 and Stages T1- T2a; Intermediate risk - PSA levels are between 10-20 ng/ml, Gleason score of 7 and Stage T2b; and High risk - PSA is more than 20 ng/ml, Gleason score between 8-10, Stage T2c, T3 or T4 (Borley & Feneley 2008). These classifications help assign patients to the most appropriate treatment streams currently available.

2.1.5.2 Treatment

Figure 2.7 is a summary of the most common treatments administered for different stages of the disease.
Figure 2.7. Summary of the standard treatment available as the prostate cancer develops. For localised and low-risk the patient is monitored closely, at high-risk immediate action is taken to remove the prostate with adjuvant radiation or hormone therapy and if recurrent (due to hormone therapy) then hormone therapy is stopped and chemotherapy and targeted therapy are used (taken from (J. Zhang et al. 2015)).

The routine treatments for most localised prostate cancer are active surveillance or active monitoring, surgery (such as radical prostatectomy), external beam radiotherapy and brachytherapy - depending on the stage at which the cancer presents or progresses to. Other treatment options, that are less common, include cryotherapy, high intensity focused ultrasound (HIFU) and watchful waiting (see Section 2.1.5.2.1 for more details). Targeted therapy are specifically designed drugs that target specific molecules that are largely found in or on cancer cells compared to normal cells (Fisher et al. 2013; Begley et al. 2008). As more targeted therapy comes through the clinical pipeline these will replace current treatment as they tend to be more effective and cause less side effects (see Section 2.1.6.1).

2.1.5.2.1 Localised and locally advanced prostate cancer

The majority of prostate cancers are slow-growing and asymptomatic and are therefore considered low-risk, and these men will normally be offered active
surveillance or watchful waiting to closely monitor the tumour (Loeb et al. 2013). Active surveillance is favoured, especially for young men, as an alternative to therapeutic intervention that could cause long term side effects. This usually involves serial PSA tests to monitor and calculate PSA kinetics such as velocity and doubling time (Section 2.1.4.2) and repeat biopsies. Alternatively, there is active monitoring that involves serial PSA tests only or watchful waiting that relies on a change in symptoms and so generally less tests are involved (Hayes et al. 2010) (Albertsen 2011). The latter methods avoid unwanted complications or side effects from biopsy or therapy, this is also useful for those who are asymptomatic. The problem is recognising those few that will go on to produce much more aggressive prostate cancer where treatment may be administered too late. Another issue is the anxiety of waiting and having no treatment that is often experienced by patients (Anderson et al. 2014).

For intermediate-risk and high-risk it is usually radical treatment such as radical prostatectomy or radiation therapy with adjuvant hormone therapy (Section 2.1.5.2.2). For high-risk individuals, the following factors must influence the overall decision: age, life expectancy and potential damage to their quality of life. A radical prostatectomy involves the complete removal of the prostate gland, although there is an approximately 30% chance of recurrence within 10 years (Roehl et al. 2004). PSA is used to detect this recurrence (see Section 2.1.5.3). Due to the large effects on quality of life, such as sexual dysfunction and incontinence (Alivizatos & Skolarikos 2005), this is generally an option only for men aged 70 and over.

It is possible that the cancer could be missed even though it showed a ‘normal’ PSA result. Conversely, PSA is can be elevated in asymptomatic patients with slow-growing, localised prostate cancer (Hugosson & Carlsson 2014). Thus, in the latter case it is the opposite problem and active surveillance or monitoring is a better option, preventing unnecessary treatment.

2.1.5.2.2 Hormone therapy

Excess androgens provide a growth advantage to cancer cells (Section 2.1.3.2) and this makes them a good therapeutic target. Hormone therapy or androgen-deprivation therapy (ADT) are a relatively simplistic type of targeted therapy, more sophisticated ways to target androgens and androgen receptors have been developed and are detailed in Section 2.1.6.1. ADT are either agonistic or
antagonistic to testosterone, as such they work by different mechanism but lead to the same result: to slowly decrease the amount of testosterone available in the serum. An agonist works similarly to androgen by binding to the androgen receptor and eventually they out-compete the androgens and saturate the androgen receptors, however this does cause an initial short surge in testosterone. The most common types of agonist are called LHRH agonists or luteinizing hormone releasing hormone agonists (Hellerstedt & Pienta 2002). Alternatively, and the option for more advanced disease, there are the antagonists that target the receptor directly - blocking the androgens from binding to the receptor. The antagonists commonly used are known as gonadotrophin releasing hormone antagonists or GnRH (Cook & Sheridan 2000). In addition, both LHRH and GnRH can be used as an agonist and antagonist (Hellerstedt & Pienta 2002). Thus, most clinicians struggle to understand, select and optimise these treatment for individual patients (Harris et al. 2009).

More research is required to uncover the exact mechanisms by which the cancer becomes resistant and thus which pathways to target subsequently. As hormone therapy is the most widespread treatment for prostate cancer, even after disease recurrence, the disease (as mentioned before, Section 2.1.3.2) almost always progresses to hormone-resistant status and the therapy becomes redundant (Dutt & A. C. Gao 2009). For advanced and metastatic disease hormone therapy is not normally enough but the progression rate varies so extensively that decisions on additional treatment are made on a patient-by-patient basis (as quality of life at late-stages become more pressing). Usually hormone therapy will be used as adjuvant therapy to surgery or radiotherapy (Gomella et al. 2010).

Controversy surrounds when exactly to administer this treatment, some believe that when it is used early on localised or locally advanced stages it could improve outcomes (Moul et al. 2008). However, this is only true for those with localised cancer that will go on to develop a much more aggressive form and until there is a better method of distinguishing this small proportion of patients it is difficult to know who to treat aggressively at an early stage.

2.1.5.2.3 Metastatic prostate cancer

The main treatment for metastatic or advanced prostate cancer is chemotherapy and ADT (see Section 2.1.5.2.2). If the metastatic disease is a result
of biochemical relapse after ADT and is hormone-resistant, then Docetaxel - an anti-
mitotic chemotherapy agent that interferes with cell division - is usually administered
(Yagoda & Petrylak 1993; Lorenzo et al. 2007). After this, treatment options are
limited and so more chemotherapy is usually administered.

Palliative type treatment would usually be made available as soon as possible. Similarly, watchful waiting would be appropriate if the patient was elderly (70 or older) and had only a short-survival time; therapy would severely and unnecessarily affect the patients quality of life. Thus, treatment is often tailored to the patients needs at this stage.

Unfortunately, it is clear that not many options are available to patients that go on to have metastatic disease and thus, more research is desperately needed here. **Section 2.1.6** outlines some examples of ongoing research that aims to develop more targeted and effective therapy for those with more advanced prostate cancer.

### 2.1.5.3 Monitoring after treatment

In addition to active monitoring and surveillance discussed in **Section 2.1.5.2.1**, monitoring is also necessary for two different stages that follow one after the other. These are: a) monitoring effectiveness of the treatment and b) monitoring cancer recurrence after treatment is complete. The PSA levels are measured to monitor the success of radical prostatectomy where any detectable PSA is treated as suspicious and a sign of biochemical recurrence (Paller & Antonarakis 2013). No other (non-invasive) biomarkers for monitoring are clinically available, though some are currently under investigation such as PCA3 (Jamaspishvili et al. 2010).

### 2.1.6 Current advances in therapy

**Figure 2.7** shows that the standard medicine for localised tumours today is radical prostatectomy - a risky procedure for many of these men who then instead choose active surveillance and avoid unwanted side effects, such as sexual dysfunction. Thus, a wider range of treatment options are urgently needed at the early stages of the disease as this will have a huge impact on the patient’s quality of life. In addition, there is a bottleneck at the flow of molecular information coming from research, such as high-throughput microarrays, to an effective tool in the clinic.
- an issue that a more translational approach and push can only solve. For prostate cancer, it could be argued that there is a higher demand for personalised medicine due to its particularly high heterogeneic nature relative to cancers that originate from other areas of the body. The current model in which prostate cancer treatment is decided by stratification (using parameters such as PSA and the Gleason score) is therefore far too generalised and ineffective (Lazzeri 2015). Encouragingly, there have been recent developments in targeted therapy (Section 2.1.6.1), immunotherapy (Section 2.1.6.2) and a combination of the two - all of which are more personalised than current conventional therapy - and are being tested in clinical trials (Table 2.1) and are outlined in more detail below.

2.1.6.1 Prostate cancer targeted therapy

Targeted therapies are based on drugs that act on specific molecules found largely in cancer cells, for example proteins necessary for continued survival and progression, and its high specificity means that toxicity and side effects are minimal. Targeted therapy differs from conventional therapy such as chemotherapy, which instead acts more generally on rapidly dividing cells and is associated with higher levels of toxicity. Several targeted treatments for solid cancers exist in the clinic already, such as HER2 (over-expressed in breast cancer cells to enhance its growth) with the drug Herceptin, which has been highly successful (Ahmed et al. 2015). Hormone therapy in prostate cancer is an example of targeted therapy, where androgens and androgen receptors are targeted (Wong et al. 2014). There are two main types of hormone therapy, ones which work to either: a) block androgens (ADT) such as the agonists and antagonists detailed in Section 2.1.5.2.2 or b) target the androgen receptor with a drug, for example to block its translocation to the nucleus and preventing its association with heat shock protein 90 (De Leon et al. 2011). Unfortunately, androgen therapies have been exhausted and are not always effective, especially when androgen is no longer required for growth by the cancer cells (as is the case for hormone-resistant tumours). Thus, new therapeutic targets are urgently required to develop new targeted therapies or immunotherapy agents (Section 2.1.6.2) against it; the criteria for an ideal target to be used in therapy is detailed in Section 2.2.2. Currently in development are targeted therapies for advanced and metastatic disease that focus on blocking the PI3K/Akt/mTOR
pathway, which is upregulated in 30-50% of prostate cancers (T. M. Morgan et al. 2009).

Molecular analysis such as genomics, transcriptomics, and metabolomics have allowed for the stratification of prostate cancer into much more relevant and useful subclasses that are associated with distinct patterns of mutations or abnormalities (that could be used as biomarkers and/or targets for targeted therapy). High-throughput sequencing can now identify CNVs.

2.1.6.2 Immunotherapy for prostate cancer

Immunotherapy is a relatively new branch of treatment, which harnesses, guides and strengthens the immune system through engineering components of the immune system such as monoclonal antibodies and T cells. In addition, immunotherapy can evoke a whole systemic immune response, with the use of cancer vaccines (Noguchi et al. 2016).

The use of immune cells in a therapeutic context can be active or passive; the key difference being the mechanism of action. Active immunotherapy acts to stimulate a host immune response, for example through the use of a vaccine, and passive immunotherapy enhances an already existing immune response and acts immediately. An example of the latter is an antibody-drug conjugate (ADC) carrying a toxin; additional examples are depicted in Figure 2.8.

![Figure 2.8. Schematic representation of the different mechanism of actions performed by monoclonal antibodies to directly kill cancer cells. Antibodies targeting a cell surface antigen on tumour cells can directly cause apoptosis to the cancer cell it binds to. The antibodies act by different mechanisms and these include being used as a vehicle for a drug or toxin, inhibiting receptors and](image-url)
blocking downstream signaling or alternatively activating receptors that then leads to apoptosis (Neves & Kwok 2015).

These monoclonal antibodies are initially engineered or humanised, which means that the mouse Fc (or CH and CL) and Fv (or VH and VL) framework regions (see Figure 2.9) of the mouse antibodies are replaced by human germ-line amino acids, this minimises its immunogenecity and avoids rejection by the host immune system (Gonzales et al. 2005).

![Figure 2.9. Schematic representation of the humanisation of murine monoclonal antibodies.](image)

The variant unit gives the antibody its specificity to an antigen, if the minimum amount required to still recognise and bind that antigen the rest can be changed to be made human. (Carter 2001).

These humanised, chimeric or fully human monoclonal antibodies can then be further engineered to increase their anti-tumour activity (A. M. Scott et al. 2012). The Fv regions at the top of the antibody provide the specificity that can be modified to increase the affinity (strength of binding to one binding site) and valency (number of antigen binding sites), which both, in turn, would increase the overall strength of binding to the antigen (also known as avidity). Innovative approaches include single chain variant fragments (scFv) that have much better and more even tissue
penetration and distribution and a shorter serum half life (Ahmad et al. 2012). Another example are bispecific antibodies that target two cancer specific antigens that have the ability to block two pathways and limit the number of ways in which cancer cells can become resistant (Weidle et al. 2014). Knowing which approach is best heavily depends on the target antigen. Fortunately, monoclonal antibodies have been very successful with over 12 being approved by the FDA for use in the clinic so far (A. M. Scott et al. 2012).

Both targeted therapy and immunotherapy treatment strategies have shown to be more potent when used together, most drugs or toxins will induce an immune response to some degree anyway. Some examples are shown in Table 2.1.

A Phase III clinical trial has just ended, which sought to test the monoclonal antibody Ipilimumab (brand name is Yervoy) against metastatic castrate-resistant tumours (already in the clinic to treat advanced melanoma) with radiotherapy. The monoclonal antibody binds the receptor CTLA-4 on the surface of T cells, essentially activating it. This prevents the T cells from switching off and instead keeps them active (also known as checkpoint blockade). Unfortunately, the results indicated no significant overall survival (OS) difference (Kwon et al. 2014). However, the study indicated that it may work with those who had lower disease burden and thus, will be tested in early prostate cancer stages instead (Reese et al. 2015).

Adoptive cell transfer is a highly personalised form of immunotherapy. Here, T cells are taken from the patient (ex vivo), activated and expanded before being transferred back to the patient or another recipient (Neves & Kwok 2015). Tumour-specific T cells can be generated through the use of antigen-presenting cells (APCs). APCs, such as dendritic cells, are the cells which present the antigen to the T cells bound to the major histocompatibility complex (MHC), those T cells that recognise the MHC-antigen complex will go on to mature into CD8+ cytotoxic T cells (O’Hagan & Valiante 2003).

Sipuleucel-T (Provenge) is a cancer vaccine that is made by isolating dendritic cells and stimulating them ex vivo. These ‘primed’ cells (against the cancer) are then re-injected back into the patient. This was approved by the FDA but subsequently rejected NICE for use by the NHS, due to cost.

It is clear that different types of immunotherapy are at different stages, especially as so much is still unknown in immunology and good biomarkers (to be targeted) are also required. For prostate cancer, the immunotherapies in development are intended for patients who have metastatic and hormone-resistant
tumours. Key and recent clinical trials, for prostate cancer immunotherapy, are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of action</th>
<th>Patient group and number</th>
<th>Phase</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sipuleucel-T</td>
<td>DC-based vaccine, primed with PAP and GM-CSF</td>
<td>Asymptomatic metastatic CRPC 512 patients</td>
<td>III</td>
<td>Improved OS by sipuleucel-T compared to placebo (25.8 versus 21.7 months)</td>
<td>Kantoff et al. 2010</td>
</tr>
<tr>
<td>Ipilimumab</td>
<td>Targets CTLA-4 and keeps cytotoxic T lymphocytes active</td>
<td>Metastatic CRPC after docetaxel 799 patients</td>
<td>III</td>
<td>No significant difference in OS</td>
<td>Kwon et al. 2014</td>
</tr>
<tr>
<td>Prostvac-VF</td>
<td>Viral vaccine that infects dendritic cells and leads to the activation of PSA-specific CD8+ T lymphocytes</td>
<td>Metastatic CRPC 125 patients</td>
<td>II</td>
<td>Improved OS compared to placebo control (25.1 versus 16.6 months)</td>
<td>Kantoff et al. 2010</td>
</tr>
<tr>
<td>GVAX with docetaxel</td>
<td>Whole tumour cells genetically modified to secrete granulocyte-macrophage colony-stimulating factor</td>
<td>Taxane-naive patients with asymptomatic CRPC</td>
<td>III</td>
<td>Trial terminated</td>
<td>Petrylak 2006</td>
</tr>
</tbody>
</table>

Table 2.1. Four key clinical trials in prostate cancer immunotherapy. Immunotherapy is becoming the first-line treatment for metastatic castration-resistant prostate cancer (CRPC). Sipuleucel and Prostvac-VF are cancer vaccines that have shown improved overall survival (adapted from (Noguchi et al. 2016)).

Listed in Table 2.1 are just some examples of biomarkers that have been tested as a target for therapy and have come through the clinical pipeline, such as PAP and CTLA-4. With the emergence of high-throughput data analysis bringing us into the ‘-omics’ era (such as the increasing sophistication of genomic microarrays and mass spectrometry), there has been a rise in the number of potential biomarkers and targets that are currently being investigated. Deciphering how to best to streamline the biomarkers path to the clinic and ensure that it is more fruitful is still ongoing. For more information see Section 2.2.
2.2 Cancer biomarkers

The definition of a biomarker, according to the National Cancer Institute (NCI), is “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease” (NCI). Biomarkers are now widely used in the clinic and increasingly relied upon for vital clinical decisions. Biomarkers can emerge from a number of places such as transcriptional changes or post-translational modifications, which can include one or a combination of microRNAs, proteins, and antibodies, and more. Exosomes, other extracellular vesicles and circulating tumour cells are another rich source of biomarkers.

Unfortunately, not enough biomarkers are reaching the clinic. Some of the areas that need improvement are basic research and preclinical validation studies, both are integral and evolving parts of the biomarker pipeline. The most successful and informative biomarkers are proteins that are extracellular and/or located on the cell surface - importantly, some cell surface biomarkers are exploited for therapy.

2.2.1 The biomarker discovery and preclinical pipeline

The standard procedure for the clinical integration of biomarkers and therapeutic targets often follows a similar pathway involving: discovery (e.g. genomics, proteomics), in vitro pre-clinical validation (e.g. 2D cell culture), in vivo pre-clinical validation (e.g. mouse models), human clinical trials (phases I-III). If a biomarker is successful it then awaits approval by the European Medical Association (EMA) or MHRA (in the UK) (Goossens et al. 2015). Each stage is still a working process; it has to be adaptable to keep up with increasingly sophisticated technology and the size of the data that is being produced. The key issues and challenges that have to be considered are summarised in Figure 2.10.
Figure 2.10. A schematic representation of the biomarker pipeline from discovery through to clinical implementation. The four stages are outlined to the left and key issues and challenges to be considered and improved upon are listed on the right, at each stage (taken from (Goossens et al. 2015)).

The pipeline is essentially a funnel where the experimentation becomes more sophisticated, expensive and accurate from one stage to the next. Each stage requires a model that is balanced between cost-effectiveness and accuracy to test either a large number of potential biomarkers (cell lines) or one (human clinical trials). Usually, during biomarker validation these tests are specific to a cancer type (i.e. prostate, breast or ovarian cancer) and progress within this framework through to clinical trials. Through clinical trials a specific subset of patients are tested, such as those bearing metastatic castrate-resistant tumours.

A major problem of the pipeline is finding an ideal model (that is both cost-effective and accurate), which becomes exponentially difficult as the cohort to be tested becomes larger (Makawita & Diamandis 2010). It is unsurprising then that biomarkers fail to make it to the clinic when the validation stages lack rigour. **In vitro** preclinical validation testing commonly involves the use of cell lines but under non-physiological conditions. **In vivo** testing usually involves mouse models - the stage just before human clinical trials - and are crucial in helping to decide the therapeutic
strategy to be adopted, such as the optimal drug dosage. The features of the two most common mouse models used are shown in Figure 2.11.

**Figure 2.11. The standard mouse models used today in the preclinical stage for biomarkers.**
The two models outlined either side of the cancer patient are the best representative cancer models outside of humans to test new therapies, these were chosen due to a balance of cost and accuracy. Highlighted in green are the advantages and in red are the disadvantages. On the right, genetically engineered mice (GEM) focuses on mimicking the tumour microenvironment, whereas the patient-derived xenograft (PDX) mice on the left are explanted fragments of tumor tissue that have been directly transferred into immuno-suppressed mice that preserve the intratumour genetic diversity (taken from (Herter-Sprie et al. 2013)).

The two models in **Figure 2.11** fail to truly mimic the tumour microenvironment, which usually undergoes a constant and complex interplay with, for example, the immune system and human vasculature. The model fails to recapture the genetic diversity within a tumour and thus also fails to recapitulate de novo human tumour development, which can be responsible for drug resistance. Mouse models only represent a snapshot of what occurs in humans (Herter-Sprie et al. 2013).

The first *in vitro* phase is still necessary and should be retained; it is cost-effective when starting with a large cohort of potential biomarkers. This is a growing issue in the era of ‘-omics’ technology - such as genomics and proteomics - that have become much more cost-effective and widely used and are producing a large amount of data at a faster rate. Furthermore, it is especially important considering there is less than 100% concordance between mRNA and protein levels (Carter 2004). The pre-clinical *in vitro* phase is also a means by which the initial hypothesis can be tested and the study design re-evaluated before proceeding. The large amount of potential biomarkers fail to be adequately scrutinized and selected for a number of reasons such as the lack of rigorous pre-clinical *in vitro* testing (Makawita...
& Diamandis 2010). Thus, many ineffectual biomarkers are retained in the pipeline and moved into the preclinical stages. Consequently, very few new cancer biomarkers have been implemented into the clinic over the last 20 years (Diamandis 2012). Ideally, an additional in vitro pre-clinical validation step is required in order to shift the focus onto accuracy much earlier on in the pipeline. This could involve the use of in vitro 3D cell culture or tumour models that are evolving into cost-effective tools (Nyga et al. 2011), further discussed in Section 2.2.4.

2.2.2 Cancer biomarkers in the clinic

Section 2.1 shows how biomarkers are currently integrated into the management of prostate cancer, such as helping to decide whether treatment is needed and which treatment to administer to patients. Biomarkers can be used in the clinic in numerous ways: inherited genetic variation (or disposition) allow the clinician to assess the patient’s risk of cancer development in the future; population screening tools allow earlier detection of the cancer in order to reduce mortality (usually not very invasive, robust and fast); diagnostic tool, in order to confirm cancer and provide information on disease state (such as the grade); prognostic tool, which provide information about the likely clinical outcome; as a predictor in order to aid the clinician in choosing the most appropriate therapy; as a monitor to establish how well the current therapy is working or not in order to adjust the therapy appropriately and to determine (post-treatment) whether there is disease recurrence; or as a pharmacogenomic tool in order to determine suitable dosage and assess the risk for adverse effects at that dose.

Some biomarkers currently used in the clinic are listed in Table 2.2, alongside their application/s.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Biomarker type</th>
<th>Cancer type</th>
<th>Sample type</th>
<th>Clinical application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate-specific antigen (PSA)</td>
<td>Protein</td>
<td>Prostate</td>
<td>Blood</td>
<td>Diagnosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Treatment response</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Disease recurrence</td>
</tr>
<tr>
<td>Alpha-fetoprotein (AFP)</td>
<td>Protein</td>
<td>Hepatocellular carcinoma (HCC)</td>
<td>Blood</td>
<td>Diagnosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Germ cell tumours (GCT)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

59
Table 2.2. Cancer biomarkers with current clinical utility. A list of the more common biomarkers used in the clinic today with the cancer type, the type of detection and its specific application (NCI, Tumor markers http://www.cancer.gov/cancertopics/factsheet/Detection/tumor-markers).

The more established, accurate and reliable the biomarkers, the more information they will provide to clinicians, which then aids in clinical decision-making. Furthermore, patients with prostate cancer usually live for a long time and thus, there is limited patient history to strengthen the interpretation of predictive biomarkers. In addition, none of the currently available biomarkers provide optimal clinical utility and often fail to fulfill one or more of the following criteria: time, cost, and patient conscious. Commonly, the issues are that these biomarkers are not easily attainable (but invasive), do not have adequate sensitivity and specificity, and do not lead to patient benefit (Kohn et al. 2007). There is therefore an urgent unmet need for clinically robust biomarkers.

### 2.2.2.1 Extracellular protein biomarkers

Extracellular biomarkers, for example proteins found in ascites for ovarian cancer or urine for prostate cancer (see Section 2.3.4), are generally easier and less invasive to measure.
An example of an extracellular protein biomarker that has successfully reached the clinic, despite being limited by a low specificity, is PSA (Section 2.1.4.2). PSA is measured in the blood and satisfies three out of four of the criteria. It has multiple purposes in the clinical management of prostate cancer (Prensner et al. 2012). PSA's utility ranges from a screening tool to an indicator of disease recurrence (Table 2.2). There are a number of specific reasons for its widespread use in prostate cancer: it is inexpensive and easy to measure; it can be monitored over time and therefore tailored to each individual; it is a very good indicator of disease progression (Fleming et al. 2006) and there is currently no better biomarker to take its place despite the fact that its use is associated with the over-diagnosis of prostate cancer (Troyer et al. 2004). It may be impossible to completely replace PSA due to its widespread use.

The PSA test is a good first-line screening test as you can narrow down the population to those that are at higher risk. In doing so, it would reduce the number of men that are over-treated. However, PSA cannot distinguish between indolent and aggressive tumours and it is not a very established prognostic marker (Ballentine Carter 2012; Saini 2016). Unfortunately, no other biomarker has successfully reached the clinical pipeline that is able to do this. There are a number of biomarkers still under investigation that could make up a panel of biomarkers to supplement PSA, including PCA3 and TMPRSS2-ERG (Cary & Cooperberg 2013). Together, this panel of biomarkers would provide more prognostic information and reduce the number of men that are over-diagnosed.

As a therapeutic target (for monoclonal antibodies) PSA is not ideal because it is not located or stable on the cell surface, it is released into circulation (away from the tumour) and it provides no growth advantage to the tumour. Examples of cell surface biomarkers that could be targeted for therapy are discussed in Section 2.2.3.

2.2.3 Cancer Specific Cell Surface Proteins (CSCSPs)

Cancer specific cell surface proteins (CSCSPs) are generally those that are located in the plasma membrane, exposed to the tumor micro-environment and largely absent from the surface of normal adult tissue.
The cell surface location of CSCSPs means that they could be used to direct therapies to the cancer cells only and spare normal healthy tissue (that do not express the CSCSP on its cell surface). Therapies such as antibody drug-conjugates, which are made to target the CSCSP and engineered to carry a cytotoxic payload that becomes active once the antibody is internalised (Ducry & Stump 2010). Another advantage of using cell surface proteins is that major histocompatibility complex (MHC) presentation is not needed; peptides are constantly being broken down and complexed to the MHC to be presented on the cell surface. The advantage is that the MHC pathway allows intracellular proteins to become targets for monoclonal antibodies or peptide-based vaccine (Roche & Furuta 2015). However, cell surface proteins are generally more accessible on the cell surface as they are not complexed to MHC or fragments but the whole, or at least a substantial part of the protein.

Most of the biomarkers in Table 2.2 are proteins found on the cell surface, and these could be candidates for therapy. These candidates can be used solely as molecular flags on the cancer cell that do not directly contribute to tumour growth itself. These could then be targeted by an ADC, for example. PMSA is a transmembrane protein that has been investigated in clinical trials as a therapeutic (and imaging) target for prostate cancer because it is highly expressed at all stages of the disease, being an integral membrane protein that is not released into the circulation and which facilitates the internalisation of bound antibodies (by receptor-mediated endocytosis) (Bouchelouche et al. 2010). Alternatively, the cell surface biomarker is the target itself as it contributes directly to tumour growth and, for example, can cause apoptosis by interfering or blocking receptor dimerization or its downstream signaling with a peptide (A. M. Scott et al. 2012). Therefore, it is important to understand the functional significance of such markers to further validate their potential as a therapeutic target and to establish the best therapeutic strategy, such as deciding between the approaches just outlined.

### 2.2.3.1 Current CSCSPs in the clinic

In Section 2.1.6.2 it was described how monoclonal antibodies, in particular, have been very successful in the clinic. The protein targets of these monoclonal antibodies and other types of therapy are usually those found on the cell surface of
cancer cells.

There is an unmet need for clinically effective cancer therapies that can be tailored for personalised treatment (Section 2.1.6). The most efficient way to identify new targets (and biomarkers) is through a proteomic screen of cell surface proteins, a practice which tends to be faster, more accurate and informative than preceding techniques such as genomic profiling (Workman & Johnston 2005).

One of the most important CSCSPs to emerge, HER2, is a cell surface growth receptor over-expressed in only 15-20% of invasive breast carcinomas (Witton et al. 2003) and is currently the most successful target in breast cancer therapy. HER2 is utilised not only as a therapeutic target but also as a prognostic and predictive factor in breast cancer (Cooke et al. 2001) and other HER2 positive cancers. The success achieved by this antibody-receptor pairing is rare and is due to several factors: a) the lack of extensive heterogeneity with one common mutation (constitutive activation) that does not affect antibody binding, instead its overexpression increases the chances of the antibody finding its target on the cancer cell b) HER2 is stable with enough of it on the cell surface at one time and c) is fully exposed for antibody binding, which makes it a reliable target for antibody therapy.

2.2.3.2 Functional significance of cell surface proteins

It is now well established that phenotypic changes to the surface of cancer cells contribute to tumourigenesis (Yarden 2001). These changes can be seen clearly during invasion and metastasis (Karhemo et al. 2012) where the cell adopts a more rounded shape (Yin et al. 2013). Another common deregulation is aberrant signaling through the over-expression of a growth factor receptor or ion transporter that leads to cellular changes such as sustained cell growth or an increased metabolic turnover, respectively, two broad and defining hallmarks of cancer (Hanahan & Weinberg 2011). Therefore, the cell surface localisation of these proteins serves a functional role in maintaining normal physiological activity.

CSCSPs can be classified to the following functional taxonomies: nuclear receptors; growth factor receptors; G protein coupled receptors; ion channels and transporters; cell-adhesion molecules; proteases; stem cell-like markers, and others (Figure 2.12). From this, we can summarise the foremost changes that arise on the cell surface of cancer cells: loss of signaling feedback between the cell surface and
nucleus; increased solute transfer of essential nutrients; loss of cell polarity and cell-cell adhesion; acquisition of migratory and invasive properties with fibroblast-like shape (epithelial-mesenchymal transition), and increased cell surface proteolytic activity (metastatic properties).

Figure 2.12. A spider diagram stratifying CSCSPs into functional and phenotypic groups. The functional groups are coloured green, and these are further sub-grouped into the labeled blue and pink boxes. The CSCSPs that are being investigated within the subgroups are named in purple boxes. These include cell proliferation and survival. A number of these are candidate therapeutic
Figure 2.12 summarises the vast amount of cancer specific proteomic data available in the literature and highlights potential functional therapeutic targets. Despite this potential, only a small number of CSCSPs are used effectively in therapy (Brooks & Brooks 2012). The low success rate at clinical trials could be due to the following: heterogeneic populations that exist between tumours and within the cells of the tumour, (Meacham & Morrison 2013) such as cancer stem cells; a lack of robust clinical validation before entering clinical trials, as previously mentioned in Section 2.2.2 and a lack of understanding of the function of the markers that would help create the most effective treatment strategy.

2.2.4 Limitations of current techniques and approaches

Cell surface biomarkers that have been approved for clinical use are comprised of aberrantly expressed proteins, such as those in Figure 2.12. The proteome rather than the genome more accurately portrays the disease state and progression, as proteins directly mediate signaling. Unfortunately, the early promise of this approach was dampened and not many of these biomarkers have made it to the clinic over the past 20 years (Diamandis 2014). There has been difficulty in establishing the level of change that is functionally significant for a) therapeutic targeting with minimal toxicity b) identifying the subset of patients who are most likely to benefit from treatment and c) diagnosis and prognosis. Currently the over-expression of a protein receptor on the cell surface is assessed on the basis of a differential change between a cancer cell and its normal counterpart; for example, a tumor with a HER2 immunohistochemistry (IHC) score of 3+ is generally classified as a high HER2 expressing tumor (Gustavson et al. 2009).

To rely on the cell surface phenotype (as described in Section 2.2.3.2) as a diagnostic tool alone would be to assume that it accurately represents the complexity that exists inside the cell; this is not the case and especially true for prostate cancer that is highly heterogeneic. The cell surface phenotype is one way of uncovering part of this hidden heterogeneity and should be integrated into a panel of markers. The panel of markers for now could include genomic profiling (Lorico & Rappa 2011) and easy to attain extracellular protein in biofluid, as described in Section 2.2.2.1.
Inter-tumour heterogeneity is a problem for the development of robust biomarkers and targeted therapy, and intra-tumour heterogeneity is just as problematic for personalised medicine. Personalised medicine could help solve the first issue and for the second there is now technology such as next-generation sequencing. Next-generation sequencing is able to simultaneously sequence whole genomes and proteins at the single cell level, which then allows the detection of mutation clusters within the tumour (Gawad et al. 2016).

Antibodies are typically used to measure this level of change but are not accurate or reliable enough to be solely used as a tool for measurement, especially without appropriate controls and chances of cross-reactivity. Most commercially bought antibodies can be polyclonal or monoclonal, monoclonal antibodies are generally more expensive. A polyclonal antibody is produced by a number of B lymphocytes and consist of a mixed population of antibodies that recognise the same antigen but not necessarily the same epitope. Or, in the case of monoclonal antibodies, they are produced by a single B lymphocyte clone and so recognise the same epitope on the same antigen. Both these types are susceptible to cross-reactivity as they recognise a small component of an antigen and the chances of recognising a similar epitope on another antigen is relatively high (Lipman et al. 2005).

Furthermore, when working with biofluid samples such as blood and urine there is often a low signal-to-noise ratio that makes it more difficult to detect a single marker. However, monoclonal antibodies are more cost-effective and have the potential (through antibody engineering) to have a high affinity and avidity against the target protein and potentially be used as an antibody-drug conjugate instead (Section 2.1.6.2).

2.3 Engrailed-2 (EN2)

EN2 is a member of the homeobox-containing transcription factor family and is essential for the normal development and organisation of the nervous system. It is largely down-regulated after embryonic development and its expression is restricted to the purkinje cells in the cerebellum. However, its expression is upregulated again
in several different types of cancer and, thus far, EN2 has been detected by antibody assays in cancer cell lines, malignant tissue and urine samples.

EN2 has the ability to regulate gene transcription and protein translation, as well as participate in an extracellular signaling pathway. The protein is unusual for a transcription factor as it can be secreted from cells and taken up by other cells. This secretion results in the presence of EN2 in the urine of patients with prostate cancer, which in turn, has great potential as a biomarker. EN2 is currently being investigated as a biomarker in prostate, bladder, kidney and ovarian cancers. However, the potential therapeutic role of EN2 has yet to be explored and the role it plays in cancer remains unclear.

2.3.1 Homeoboxes

The homeobox genes are a superfamily of regulatory genes that all contain a signature homeobox domain. They were initially discovered by observing homeotic mutations in *Drosophila* that caused embryonic segments to be switched, indicating that they have a role in providing positional information (Lawrence et al. 1983).

Homeobox genes are found in all multicellular organisms and the homeobox sequence is highly conserved (Santini et al. 2003). The homeobox is encoded by a 183 base pair DNA segment that encodes a 61 amino acid (aa) homeodomain (McGrath, Michael, et al. 2013). In humans 235 functional homeobox genes (those that will translate to proteins) and 65 pseudogenes (structurally similar genes that do not translate to proteins) have been identified (Holland et al. 2007). Homeobox genes often occur in clusters on chromosomes and on this basis are broadly grouped into clustered and non-clustered genes. These are further grouped into subfamilies such as HOX, PAX and the Engrailed gene families, though the groupings are inconsistent (Holland et al. 2007).

The National Human Genome Research Institute has been collating the homeobox superfamily data online (found at https://research.nhgri.nih.gov/homeodomain) such as the number of DNA binding sequences, protein coding genes and organisms, known protein-protein interactions, and the number of proteins implicated in disease. This is an extensive resource that will help to create a bigger picture of the role of homeobox genes in cancer, which is currently highly varied and limited.
2.3.1.1 Homeobox gene expression and function

The homeogenes encode proteins that primarily act as nuclear transcription factors, known as homeoproteins (used hereafter). Homeoproteins are conserved both structurally and functionally through evolution (Akam 1989). They play a fundamental role in vertebrate development (Manak & M. P. Scott 1994), including the formation of limbs and organs along the anterior-posterior axis (Addison & Wilkinson 2016). Homeoproteins have multiple functions although they often regulate cell characteristics such as shape, size, cell movement, proliferation and differentiation (Stettler & Moya 2014). The majority of homeoproteins continue to be expressed in the adult, although their function in this context is less well understood.

The homeodomain enables the homeoproteins to bind to specific DNA sequences in the regulatory regions of target genes, resulting in transcriptional activation or repression (McGinnis & Krumlauf 1992). EN2-DNA binding specificity is increased through interactions with specific cofactors (Chan et al. 1997). An example of three homeoproteins that often interact during development are HOX, MEIS and PREP. Mutations that deregulate these interactions have been reported to be associated with disease (Section 2.3.1.3), an example is HOXA9 and its cofactors MEIS1, MEIS2, and PBX1 that promotes prostate tumour progression (J. L. Chen et al. 2012). The combination of cofactors for each homeoprotein is not strict, such that HOX proteins can act alone or with other homeoproteins and both MEIS and PREP can act as cofactors to other homeoproteins (Moens & Selleri 2006).

The majority of homeoproteins have different functions depending on its localisation and level of expression such as EN2 and OTX2 (Prochiantz & Di Nardo 2015). EN2 is initially expressed in the midbrain-hindbrain boundary an is largely switched off after embryonic development. However, EN2 continues to be present at low levels in adult cerebellum but its expression become restricted to cells such as purkinje neurons and granule cells (Table 4) (Choi et al. 2011). EN2 most likely shifts from a transcriptional role in the nucleus to a translational role as its moves into the cytoplasm and outside the cell (see Section 2.3.2.1 for more details). Both OXT2 and EN2 homeoproteins are seemingly required by the adult nervous system for the continued survival of mesencephalic dopaminergic (mDA) neurons (Joshi, Torero Ibad, et al. 2011) in a non-autonomous manner (expanded upon in Section 68).
2.3.2.1). A further role of transferred OTX2 has been identified in the visual cortex where it was found to regulate cortical plasticity (Sugiyama et al. 2009).

2.3.1.2 Homeoproteins and disease

Homeoproteins have been linked to many genetic and developmental diseases due to their vital role in development. For example HOXA13 and hand-foot-genital syndrome (Goodman et al. 2000; Goodman 2002). Many cancers exhibit expression or alteration in homeobox genes (Spatazza et al. 2013). Normal development and cancer progression have a lot in common; both processes involve shifts between cell proliferation and differentiation and therefore the deregulation of homeogenes found in many cancer types is unsurprising (Nunes et al. 2003).

HOX proteins have been extensively investigated in cancer. Fortunately, HOX expression and functions in development are well-characterised. Their role in development has been identified as fundamental to normal limb and organ development along the posterior-anterior axis, blood vessel formation and prostate gland development (Moens & Selleri 2006). HOX protein expression remains in the adult where they, for example, regulate normal hematopoietic differentiation (Magli et al. 1991). Consequently, Hox gene deregulation are often found in leukemia (Rice & Licht 2007). EN2 has been linked to autism (expanded upon in Section 2.3.2) and prostate cancer (detailed in Section 2.3.3).

2.3.2 The biology of EN2

EN2, like most other homeoproteins, is multifunctional and the defined functions have largely been discovered through its distinctive protein domains, highlighted in Figure 2.13 and its expression pattern during different developmental stages (Table 2.3). A key mechanism of action by homeoproteins is tight spatio-temporal expression that define boundaries during development, such as a caudal-to-rostral gradient in the developing tectum that can act as axon guidance cues for topographic map formation in the vertebrate visual system. This tight spatio-temporal expression of homeoproteins likely explains how EN2 was able to rescue...
the EN1 mutant phenotype (Hanks et al. 1995) and compensate for the depletion of PAX2/5 (Koenig et al. 2010). Therefore, it is possible that EN2 is able to carry out many of the functions identified in other homeoproteins and homeoproteins identified in other species. The C-terminal homeodomain of EN2, known as EH4 (EN homology region 4), is a highly conserved 61aa sequence (as described in Section 2.3.1.1) (Logan et al. 1992), which adopts an alpha helical protein fold allowing EN2 to bind to A/T-rich DNA sequences (Laughon & M. P. Scott 1984). Upstream of the homeodomain are PBX interacting domains, another family of homeodomain transcription factors, that confer DNA binding specificity to EN2 (Peltenburg & Murre 1996), represented in Figure 2.13 below.

![Figure 2.13. Schematic representation of EN2 protein structure with known functional domains.](image)

EN2 has the ability to carry out all three major functions assigned to homeoproteins, thus far, which are the following: regulating gene transcription and protein translation, as well as participating in an extracellular signaling pathway. EN2 has been reported to predominantly act as a transcriptional repressor (Choi et al. 2011). In order to do so EN2 uses two distinct mechanisms: a) EH1 confers direct transcriptional repression b) EH1 and EH5 recruit the non-DNA binding co-repressor, Groucho (Tolkunova et al. 1998), please see Figure 2.13 for reference. The translational regulation by EN2 in the cytoplasm may involve elf4E (Nédélec et al. 2004) (Choi et al. 2011). The eukaryotic translation initiation factor (eIF4E)
binding domain can be found at the N-terminal (Figure 2.13). A previous study, in adult mice, found a significant amount of Emx2 and eIF4E in high-density fractions outside the nucleus, enriched in vesicles, where RNase treatment failed to dissociate the two proteins; in order to locally control protein translation. This study also confirmed EN2 and OTX2 direct binding to eIF4E; in fact, there are approximately 200 homeodomains that contain eIF4E binding sites, which could dysregulate eIF4E activity (Topisirovic et al. 2005). EN2's association with vesicles has been reported in a number of studies; Section 2.3.2.1 focuses on EN2's ability to be translocated out of the nucleus, out of cells and internalised by other cells. The phosphorylation site, also found at the N-terminal, could be involved in regulating DNA binding as this was found to be the case in Drosophila (Bourbon et al. 1995).

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>E8.0-E12.5</td>
<td>Mid-hindbrain junction</td>
<td>A-P patterning, Neurotransmitter development</td>
</tr>
<tr>
<td>E12.5-E15.5</td>
<td>Developing cerebellum, colliculi, ventral mid-hindbrain nuclei including LC and RN, periaqueductal gray</td>
<td>Retinal-tectal mapping, Neurotransmitter development</td>
</tr>
<tr>
<td>E15.5-P0</td>
<td>Developing cerebellum, colliculi,</td>
<td>Retinal-tectal mapping, cerebellar connectivity</td>
</tr>
<tr>
<td>P0-P12</td>
<td>Cerebellum (differentiating Granule cells)</td>
<td>Cell cycle and differentiation</td>
</tr>
<tr>
<td>Adult</td>
<td>Mature granule cells</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 2.3. List of EN2 expression patterns and functions at different developmental stages in mice. Taken from Choi et al. 2011.

At early developmental stages the EN genes (EN1 and EN2) participate in neural development, determining the midbrain/hindbrain (MH) border and continue to be expressed at high levels in the alar (dorsal) cells of this region. Interestingly, when these alar (dorsal) cells were transplanted to other areas they then promoted EN expression in new neighbouring cells (Martinez et al. 1991). In addition, fibroblast growth factor 8 (FGF8) was identified as a transcriptional target of the EN genes; it is a secreted peptide that was found to induce the expression of EN genes (Gemel et al. 1999; A. Liu & Joyner 2001). Subsequently, neuronal subtypes arise from the MH region such as the mesencephalic dopaminergic (mesDA) neurons and
these have been shown to require *EN* expression for their continued survival (Simon et al. 2001; Albéri et al. 2004). *EN* is also involved in retinal-tectal mapping such as axonal guidance, it does so by two mechanisms: a) outside the cell where it is expressed in a caudal-to-rostral gradient and generally enhances the sensitivity of the cells to the action of other growth factors, such as EphrinA whereby EN2 promotes its transcription (Logan et al. 1996) and b) directly by being internalised by axons and modifying local protein synthesis (Brunet et al. 2005; Wizenmann et al. 2009; Nédélec et al. 2004), further discussed in Section 2.3.2.1.

There has been considerably more interest in other members of this superfamily, such as the *HOX* genes (Section 2.3.1.2), with respect to their developmental (Izpisúa-Belmonte et al. 1991) or etiological role (Boncinelli 1997). There are three main reasons for this: (i) EN2 expression and regulation have been difficult to understand both during development and in adult cells because of their complex and diverse nature (ii) EN2 has low and extremely restricted expression in the adult with unclear function, and (iii) its developmental function, mode of action and localisation are yet to be fully characterised, leaving an incomplete EN2 expression profile and difficulty in establishing its role in disease, such as cancer (expanded upon in Section 2.3.3). The only known sites of normal adult EN2 expression are in the nervous system, particularly the Purkinje neurones (Sillitoe et al. 2008), and in the tubular epithelial cells of the kidney (Lai et al. 2014; Guan et al. 2014).

Due to EN2's role in the continued survival of mesDA neurons, *EN2* was investigated in individuals with young-onset Parkinson's disease and a SNP was found in EN2's promoter region (Rissling et al. 2009). EN2 has also been linked to autism; EN2 is found at the Chromosomal region 7q36.3, which is an autism susceptibility locus. Subsequent investigations into EN2's elevated expression in autism revealed no *de novo* mutational event but strong indications of single nucleotide polymorphism (P. Yang et al. 2010) and/or an epigenetic change (James et al. 2013).

2.3.2.1 EN2's localisation and translocation properties
Although homeoproteins primarily reside in the nucleus and act as transcriptional regulators they do have the ability to translocate outside the nucleus. A conserved nuclear export sequence (NES) has been identified as the mechanism by which EN2 guides axons during retinal-tectal mapping (Stettler et al. 2012). The NES is an 11aa sequence that exists within the homeodomain and is essential to the intercellular transfer of EN2 (Maizel et al. 1999). Furthermore, the NES and lack of a classical secretory sequence suggests that EN2 has to initially translocate out of the nucleus and into the cytoplasm before being secreted out of the cell (Arnoys & J. L. Wang 2007).

The homeodomain consists of three helices that make a helix-turn-helix structure, and the third helix is required for translocation (Derossi et al. 1994). Therefore, due to conservation of homeodomain between homeoproteins, it is likely that this property is universal amongst all homeoproteins. The third helix of the Antennapedia homeodomain has been further exploited as a means to transfer molecules across membranes and between cells, these peptides are collectively known as cell penetrating peptides (Dupont et al. 2015).

The exact mechanism by which these proteins are secreted and internalised is yet to be fully characterised. The conventional route would normally involve receptor-mediated exocytosis and endocytosis (Baines & B. Zhang 2007). However, homeoprotein intercellular transfer occurs both at 37°C and 4°C degrees (Chatelin et al. 1996). Thus, the mechanism is likely to be receptor independent and an unknown or ‘unconventional’ pathway that has yet to be discovered. Cofactors and post-translational modification could play an important role in EN2’s localisation; EN2 has a number of phosphorylation sites and more recent advances revealed that phosphorylation by CK2 - that targets a serine rich site on the C-terminal end of the En protein - prevents EN2’s intercellular transfer (Maizel et al. 2002).

Due to the ability to be transferred between cells, homeoproteins are likely to have distinct cell autonomous roles and non-autonomous roles (Joshi, Torero Ibad, et al. 2011; Prin et al. 2014; Rampon et al. 2015; Albéri et al. 2004). Interestingly, EN2 may have a role in cell-cell communication due to its ability to be secreted (Joliot et al. 1998) and internalized by other cells (Cosgaya et al. 1998; Rampon et al. 2015). The mechanism by which it does this has been investigated and concluded that it occurs through an unconventional route that is shared by other members of the homoebox family; further investigation into the mechanism is needed. However, Brunet et al showed that internalised EN2 was able to trigger...
rapid phosphorylation of proteins involved in translation initiation and thus, trigger local protein synthesis (Brunet et al. 2005). Furthermore, EN2’s ability to control the responses of both nasal and temporal growth cones occurred through its accumulation inside these cones within minutes, which would suggest a change to protein synthesis in such as short time, was stopped both when EN2 could not be internalised and when protein synthesis was blocked (in the recipient cells) (Brunet et al. 2005). An example of a protein involved in translation initiation is EIF4E that has also been shown to bind to EN2 and other homeoproteins (Nédélec et al. 2004), also mentioned in Section 2.3.2. EN2 has been shown to create extracellular caudal-to-rostral gradients (in the developing mid-brain) outside of the cell and be internalised to act directly on protein synthesis (Brunet et al. 2005), as discussed in Section 2.3.2; PBX is internalised by neighbouring cells to control brain patterning (Rampon et al. 2015) and OTX2 intercellular transfer regulates the plasticity of postnatal neurons in the visual cortex (Rebsam & Mason 2008). Other homeoproteins have also been detected outside of the cell and shown to be able to translocate between cells, such as VAX1 (Kim et al. 2014).

2.3.3 EN2 in cancer

EN2 is down-regulated after embryonic development with the exception of brain tissue where it is confined to Purkinje cells (Albéri et al. 2004). The differential expression of EN2 in cancer and a normal adult cells indicated a possible role for EN2 in tumorigenesis; much less progress has been made with the latter. EN2 was put forward as a candidate oncogene by Martin et al showed that aberrant EN2 expression in breast cancer (Martin et al. 2005), with ectopic expression of EN2 in ductal carcinoma and breast cancer cell lines and no detectable EN2 expression in normal tissue and cell lines. They showed that ectopic EN2 expression readily transforms mammary epithelial cells and enhances metastatic adenocarcinoma that involved a reduction in cell cycling time, a loss of cell to cell contact and a failure to differentiate in response to lactogenic hormones, and that siRNA knockdown of EN2 inhibits human breast cancer cell proliferation. SAGE libraries comprised of human brain glioblastoma, colon and ovarian carcinomas also revealed EN2 expression (Martin et al. 2005). Furthermore, there was no evidence of gene rearrangement or amplification. The study provided a rationale for further exploration of EN2 in
targeted therapy as it is expressed in only a subset of breast cancers and toxicity
was likely to be minimal as there was no EN2 expression in normal breast epithelial
cells. However, the lack of a large cohort of ductal carcinomas makes it difficult to
draw a definitive conclusion. EN2 expression was subsequently investigated in
prostate cancer (Bose et al. 2008), with findings similar to those made in breast
cancer (Martin et al. 2005). The study showed that the knockdown of EN2 in cell
lines resulted in a decrease in PAX-2 expression, which lead to a decrease in cell
proliferation. Further studies have been carried out in various cancer types that
show elevated EN2 expression and these are summarised in Table 2.4 below.

<table>
<thead>
<tr>
<th>EN2 involvement in cancer</th>
<th>Cancer type</th>
<th>Technique</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promotes malignant</td>
<td>Breast (mouse)</td>
<td>Forced overexpression</td>
<td>Martin et al. 2005</td>
</tr>
<tr>
<td>characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Required for cancer cell</td>
<td>Breast</td>
<td>RNAi knockdown</td>
<td>Martin et al. 2005</td>
</tr>
<tr>
<td>proliferation</td>
<td>Prostate</td>
<td>RNAi knockdown</td>
<td>Bose et al. 2008</td>
</tr>
<tr>
<td>Overexpression</td>
<td>Breast</td>
<td>RT-qPCR IHC</td>
<td>Martin et al. 2005</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>RT-qPCR IHC</td>
<td>Bose et al. 2008</td>
</tr>
<tr>
<td></td>
<td>Ovary</td>
<td>RT-qPCR IHC</td>
<td>Michael et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>RT-qPCR IHC</td>
<td>Morgan et al. 2013</td>
</tr>
<tr>
<td>Secretion in urine</td>
<td>Prostate</td>
<td>ELISA</td>
<td>Morgan et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>ELISA</td>
<td>Pandha et al. 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Morgan et al. 2013</td>
</tr>
<tr>
<td>Hypermethylation</td>
<td>Lung</td>
<td>Methylated-CpG island</td>
<td>Rauch et al. 2007</td>
</tr>
<tr>
<td></td>
<td>Follicular lymphoma</td>
<td>recovery</td>
<td>Bennett et al. 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methylation-specific PCR</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. A summary of the current evidence linking EN2 gene and protein expression to
cancer development. Adapted from (McGrath et al. 2013).
Immunohistochemistry (IHC) staining revealed the presence of EN2 in the cytoplasm and the membrane, exclusively in prostate cancer tissue and not normal prostatic epithelium (R. Morgan et al. 2011; R. Morgan et al. 2013; Killick et al. 2013). There was no detection of EN2 in the nucleus in prostate cancer tissue. Interestingly, high levels of EN2 were detected in ductal structures of tumours, which created ‘blebs’ in prostatic acini and ducts and provided further evidence and insight into EN2’s secretory properties. Morgan et al went on to describe a single EN2 ELISA test that detected EN2 in the urine of biopsy-proven prostate cancer patients, without a prior DRE test, see Section 2.3.3.1 for more details.

More recent investigations in renal cancer have shown a higher level of EN2 expression in the stroma surrounding the tumor than in the tumor itself (Lai et al. 2014). EN2 is best characterised as a transcriptional repressor during development (Choi et al. 2011), the activity of which is again located at the C-terminus (Figure 2.13). If EN2 was identified as a transcriptional repressor in cancer, it could suggest that EN2 is switched on as a consequence of tumorigenesis and not as a precursor. Unfortunately with no obvious pattern of expression it has been difficult to assign a definitive role to EN2 in cancer, which is likely to be multifunctional.

An EN1 study demonstrated, for the first time in 2011, increased EN1 protein expression in adenoid cystic carcinoma after identifying significant hypermethylation at EN1’s transcriptional start site (D. Bell et al. 2011). A more recent EN1 study showed the exclusive overexpression of EN1 in the breast cancer subtype: basal-like breast tumours. It was found to act as a pro-survival factor when short hairpin RNA (shRNA)-mediated knockdown of EN1 led to immediate cell death to breast cancer cells; overexpression of EN1 resulted in the increased resistance to drugs. The study went on to target EN1 with blocking peptides that mediated a strong apoptotic response in breast cancer cells and increased their sensitivity to drugs with no toxicity to normal cells (A. S. Beltran et al. 2014). The hypermethylation of En genes has also been demonstrated in lung cancer (Rauch et al. 2007), colorectal cancer (Mayor et al. 2009), astrocytomas (Wu et al. 2010) and prostate cancer (Devaney et al. 2011). The significance of the hypermethylation is not known but they could be useful DNA methylation markers for early diagnosis of cancer (further discussed in Section 2.3.3.1).

2.3.3.1 EN2 as a clinical biomarker
There have been very limited investigations in the use of homeoproteins as diagnostic and prognostic biomarkers, and those that exist are mainly investigating HOX proteins (Barba-de la Rosa et al. 2012; Javed & Langley 2013; R. Morgan & El-Tanani 2016). However, EN2’s presence in the urine of prostate cancer patients make it an attractive, non-invasive target and recent publications have continued to show EN2’s potential as a clinical biomarker (R. Morgan et al. 2013; McGrath, McGrath, et al. 2013).

Morgan et al in 2011 investigated EN2 as a diagnostic biomarker in prostate cancer (R. Morgan et al. 2011). In this study EN2 protein was shown to be secreted in the urine by prostate cancer cells, and enzyme-linked immunosorbent assay (ELISA) measurements of urinary EN2 in cancer patients and aged-matched controls revealed that this marker had a 66% sensitivity and 88.2% specificity (R. Morgan et al. 2011). Another study in 2012 showed that pre-treatment urinary EN2 levels were positively correlated with prostate tumor volume of men who subsequently underwent a radical prostatectomy (Pandha et al. 2012). Consequently, EN2 is being investigated in other urothelial cancers. An almost equivalent study was carried out recently in bladder cancer where EN2 was detected in the urine at slightly reduced levels compared to patients with prostate cancer (R. Morgan et al. 2013). Additionally, a weak correlation has been found between tumour grade and the level of EN2 expression (R. Morgan et al. 2013).

Studies that show the secretion and internalisation of EN2, together with the sequence of the EN2 homeodomain, suggest that some EN2 could be localised to the cell membrane (Joliot et al. 1997; Joliot et al. 1998; Maizel et al. 1999; Brunet et al. 2005; R. Morgan et al. 2011). In 1997 Joliot’s team demonstrated the association of EN2 with membrane fractions or caveolae-like structures (Joliot et al. 1997). In addition, EN2 was identified in the basal membrane of prostatic tissue by IHC analyses (R. Morgan et al. 2011). Intriguingly, there has been controversy surrounding a reliable, commercial anti-EN2 antibody for its detection in western blots and IHC (Guan et al. 2014). The goat anti-EN2 antibody, used in this project, was confirmed by Guan et al to detect EN2. The study also suggests that a ‘non-EN2’ protein is detected by other commercial EN2 antibodies. Additionally, EN1 shares 90% homology with EN2 and it is a possibility that some commercial antibodies could pick up this protein and, coincidentally, these commercial antibodies to EN1 also pick up the presence of a unspecific band of the same size. Thus far, no study has tried to identify this protein.
EN2 expression has already been investigated in epithelial cancers such as prostate, ovarian, bladder, breast, and kidney though knowledge regarding its role in cancer remains limited. EN2 could potentially be a useful biomarker for identifying the switch from indolent to aggressive phenotype when EN2 measurement in the urine is no longer correlative with the tumour volume. In the long term, this could help reduce the number of men that are over-diagnosed and over-treated.

### 2.3.3.2 EN2 as a therapeutic target

EN2 has yet to be investigated as a target for therapy. A recent publication provides further rationale for the use of EN2 as a therapeutic target by demonstrating its immunogenicity. The study showed that the number of EN2 auto-antibodies in prostate cancer patient sera were significantly higher compared to healthy controls (Annels et al. 2014).

EN2’s secretory and internalisation mechanisms are unconventional and undefined, though it is likely to be receptor independent (Section 2.3.2) without a classical secretion signal sequence (Joliot et al. 1998). EN2’s association with vesicles (Joliot et al. 1997; Maizel et al. 2002; R. Morgan et al. 2011) may prevent EN2 from being highly accessible to an antibody. However, a study using micelles to mimic the membrane environment sought to characterize the conformational transition of the full-length homedomain using circular dichroism (Carlier et al. 2013). Carlier et al concluded that EN2, placed in a lipid environment, loses its overall 3D shape but keeps its helical secondary structures of the homoedomain intact. Consequently, this conformation allows previously buried hydrophobic amino acids, such as Trp-48 (Le Roux et al. 1993), to become available to insert into the membrane. They also suggest that the initial electrostatic interactions with the membrane may be enough to trigger a conformational change and enable Trp-48 to insert into the membrane. Consequently, there is a possibility that EN2 is tethered onto the cell membrane for a prolonged period of time. This window could be enough to use EN2 to directly internalise an antibody such as an antibody-drug conjugate (Section 2.1.6.2).
2.4 Summary

The literature acknowledges that there is a problem in the biomarker pipeline: large numbers of potential biomarkers from genomic or proteomic analysis that do not equally translate to large amounts of biomarkers in the clinic. For prostate cancer biomarkers that initially show promise, they do not significantly fair better than PSA to warrant a change in practice and so PSA remains. It is possible to create a panel of biomarkers with PSA and EN2, which would make the information gained early on in the process more informative; this could lead to more appropriate treatment being administered.

There is a growing body of evidence for the role of EN2 in prostate cancer as a clinical biomarker and perhaps a therapeutic target. EN2 is a homeobox-containing transcription factor secreted specifically by prostate cancers into urine that is detectable by an ELISA assay. The functional significance of the overexpression of EN2 in cancer, and especially in prostate cancer, is not known. Thus, this study aims to evaluate EN2’s gene expression, protein expression and localisation in prostate cancer; and its translocation between cells. These experiments will be carried out in vitro in a handful of prostate cancer cell lines that range from low to high metastatic potential (or aggressiveness). In addition, this study will seek to confirm its presence on the cell surface of cancer cells; and further investigate its accessibility and stability on the cell surface, in order to provide further rationale towards its use as a therapeutic target. EN2’s role in cancer is further evaluated by looking at the cellular responses as a result of EN2 messenger RNA (mRNA) silencing and over-expression. Ultimately, if EN2 protein is confirmed to be stable on the cell surface and shown to have functional significance (such as being a vital component to tumour progression), it would provide information on how best to design the therapeutic antibody against EN2.

The next chapter outlines the specific materials and methodology utilised and designed for this study to address the specific objectives, restated below from Section 1.5:

1. determine EN2’s expression and localisation within cancer cells.

2. further define EN2’s secretion and internalisation mechanisms.
3. identify a suitable EN2 antibody for development into an antibody-drug conjugate.

4. carry out preliminary *in vitro* experiments on the candidate antibody-drug conjugate to direct the next set of experiments.

5. further investigate EN2’s cell autonomous and cell non-autonomous role
Chapter 3: Materials and Methods
3. Materials and Methods

All materials and methodology are described in the order of experimentation displayed in the results section from Chapter 4 to Chapter 6.

3.1 Tissue culture

3.1.1 Culture media

All culture media used for the \textit{in vitro} cell line experiments carried out in this study are listed below in Table 3.1 as well as the supplier.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>MEM</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>F-12K</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>IMDM</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>HBSS (Hanks Balanced Salt Solution)</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>Opti-MEM I Reduced Serum</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>FluoroBrite\textsuperscript{TM} DMEM</td>
<td>Life Technologies, UK</td>
</tr>
</tbody>
</table>

Table 3.1. A list of the culture media required for all cell lines and their source.

3.1.2 Cell lines

All cell lines (Table 3.2) were purchased from the American Type Culture Collection (ATCC, USA); except for the Fibroblasts, which were obtained from the University of Birmingham and Peripheral blood mononuclear cells (PBMCs) that were isolated from blood samples obtained from healthy donors on the premises (see Section 3.1.3).
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue type</th>
<th>Origin</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60</td>
<td>Human</td>
<td>AML Peripheral blood</td>
<td>ATCC, USA</td>
<td>Gallagher et al. 1979</td>
</tr>
<tr>
<td>SK-MEL-5</td>
<td>Human</td>
<td>Malignant melanoma Skin, metastatic axillary node</td>
<td>ATCC, USA</td>
<td>Fogh et al. 1977</td>
</tr>
<tr>
<td>PC3</td>
<td>Human prostate adenocarcinoma</td>
<td>Grade IV, adenocarcinoma Prostate, metastatic site: bone</td>
<td>ATCC, USA</td>
<td>Kaighn et al. 1979</td>
</tr>
<tr>
<td>Du145</td>
<td>Human prostate carcinoma</td>
<td>Prostate; derived from metastatic site: brain</td>
<td>ATCC, USA</td>
<td>Stone et al. 1978</td>
</tr>
<tr>
<td>LnCaP</td>
<td>Human prostate carcinoma</td>
<td>Prostate; derived from metastatic site: left supraclavicular</td>
<td>ATCC, USA</td>
<td>Gibas et al. 1984</td>
</tr>
<tr>
<td>WPMY-1</td>
<td>Human normal prostate stroma/ fibroblasts</td>
<td>-</td>
<td>ATCC, USA</td>
<td>(Bello et al. 1997)</td>
</tr>
<tr>
<td>T47D</td>
<td>Human ductal carcinoma</td>
<td>Mammary gland; derived from metastatic site: pleural effusion</td>
<td>ATCC, USA</td>
<td>Judge &amp; Chatterton 1983</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>Mouse embryo fibroblast</td>
<td>-</td>
<td>ATCC, USA</td>
<td>Jainchill et al. 1969</td>
</tr>
<tr>
<td>Dermal Fibroblasts</td>
<td>Human adult skin (epidermis)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2. A summary of all cell lines used in this study, including their source and derivation.

All cell lines that were purchased prior to the start of the study have a valid authentication certification. Other cell lines, including the clones that were created as part of the study, were sent for reauthentication using short tandem repeat (STR) profiling against the original STR profile (LGC, USA). All cell lines that were sent were confirmed to be an exact match.

The atmospheric conditions and working cell culture media required to maintain each cell line in cell culture are listed in Table 3.3, with the exception of the stable clones that were also maintained under selection pressure using an appropriate antibiotic.
Table 3.3. A list of recommended media, supplements and environmental conditions for optimal growth for each cell line. Working media for *in vitro* cell culture usually contain the following additional supplements: Fetal bovine serum (FBS) (Life Technologies, UK), Penicillin and Streptomycin (P/S) (Sigma, UK) and glutamine (glu) (Sigma, UK).

All cell lines are adherent lines except HL-60, which are a suspension cell line. Cells were generally kept at 5% CO$_2$ and at 37°C, only WPMY-1 required 10% CO$_2$ (Table 3.3). All tissue culture work was performed in a sterile class II biosafety cabinet (Kendro, UK); these were cleaned and maintained on a regular basis. The working media was prepared in 500ml bottles and supplements added (outlined in Table 3.3) in sterile conditions and this was kept at 4°C. Penicillin-Streptomycin (P/S) were used to prevent bacterial contamination, due to the combined gram-positive and gram-negative bacteria action. In addition, mycoplasma testing was carried out regularly using a MycoAlert™ Mycoplasma Detection Kit (Lonza, UK).

3.1.3 Isolation of PBMCs from healthy donors

A venesection of 10ml or 20ml of blood was taken from a healthy volunteer and collected in ‘green top’ heparin blood tubes (BD Vacutainer System, UK). These were diluted in HBSS and carefully layered on top of 15ml Ficoll-Paque (Invitrogen,
UK). This was spun at 690g for 25 minutes at room temperature after which, the ‘cloudy’ ring at the interface was carefully collected. This was topped up with HBSS to 50 ml and spun at 690g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 20ml red blood cell lysis buffer (Pierce, UK) for 10 minutes before pelleting (690g, 10 minutes). The final pellet were carefully resuspended in 1ml PBS to be counted.

3.1.4 Passaging adherent cells

All cells were cultured in appropriate media specified in the suppliers instructions (found on the ATCC website). Cells were generally grown in T-75 (75 cm$^3$) flasks. If cells were less than 60% confluent, half of the culture medium was removed and fresh medium added. If cells were more than 60% confluent (usually 3 days after previous passage) then they were harvested by removing the culture medium, washing cells with 10ml HBSS and subsequently 10ml (10X) trypsin ethylenediaminetetraacetic (0.5% trypsin, 0.2% EDTA), leaving the residual trypsin-EDTA on the cells. After a few minutes 10 ml culture medium was used to neutralize the trypsin, and cells were collected and centrifuged at 1500rpm for 3 minutes. Supernatant was decanted and the pellet was re-suspended in 1-10ml complete culture medium for either a 1:2 to 1:8 cell split depending on the experiment to be set up shortly after. The cell suspension was transferred to a new flask, which was subsequently topped up with complete culture medium. Cells were then incubated according to the conditions stated in Table 3.3.

3.1.5 Passaging suspension cells

A percentage of the culture media was simply removed, discarded and fresh culture media replaced what was taken.

3.1.6 Cell viability count
Cell viability was assessed by trypan blue (Sigma, UK) exclusion. Cell suspension was diluted 1:10 with trypan blue. 10µL of the mixture was loaded onto the grid of a Neubauer haemocytometer counting chamber. Trypan blue is membrane impermeable and so cells that had taken up trypan blue, observed by light microscopy, were considered non-viable. Once all four grids had been counted, the following calculation was then carried out:

\[
\text{Mean number of cells per quadrant} \times \text{dilution factor} \times 10^4 = \text{number of cells/mL}
\]

3.1.7 Cell cryopreservation and storage

Cells were harvested by centrifugation at 1500rpm for 3 minutes and resuspended in culture media to 1x10^6 cells/ml supplemented with 5% dimethyl sulfoxide (DMSO) (Sigma, UK). 1 ml aliquots were distributed into sterile cryovials Nunc® Cryo Tubes®, placed into Mr. Frosty™ freezing containers (ThermoScientific, UK) and frozen at -80°C overnight, to allow a steady decline in temperature, before being transferred to liquid nitrogen storage tanks.

3.1.8 Cell recovery from cryopreservation

Cryovials were removed from liquid nitrogen, kept on ice and quickly thawed in a water bath at 37°C. After approximately 2 minutes, the 1ml cell solution was pipetted into a to universal tube (pre-filled with 9ml of warm culture media) and centrifuged at 1500rpm for 3 minutes. The supernatant was discanted to remove traces of DMSO and the cell pellet was resuspended in approximately 6mL cell culture media and transferred to a T-25 flask and incubated at 37°C. The media was changed to fresh media the next day once cells had adhered.

3.1.9 General cell seeding densities for downstream applications

Table 3.4 provides the optimal culturing conditions for each cell line when carrying out an experiment using different sized culture-ware.
<table>
<thead>
<tr>
<th>Culture-ware</th>
<th>cells/ ml</th>
<th>Total volume</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>1x10^4</td>
<td>200µl</td>
<td>MTS assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cloning</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>siRNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>pH-Ab internalisation</td>
</tr>
<tr>
<td>24-well plate</td>
<td>1x10^4</td>
<td>500µl</td>
<td>Cloning</td>
</tr>
<tr>
<td>8-chambered slide</td>
<td>1x10^4</td>
<td>500µl</td>
<td>ICC</td>
</tr>
<tr>
<td>35mm glass-bottom dish</td>
<td>4x10^5</td>
<td>1.5ml</td>
<td>Live cell imaging/ time-lapse microscopy</td>
</tr>
<tr>
<td>6-well plate</td>
<td>4x10^5</td>
<td>1.5ml</td>
<td>RNA extraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Western blot lysates</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lipid plasmid transfections</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cloning</td>
</tr>
</tbody>
</table>

Table 3.4. A list of culture-ware used in this study and the optimal seeding densities, volume and their common applications.

3.2 Total RNA extraction

Total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen, UK). Cells were washed with HBSS and trypsinised before being counted. Cell pellets were fully resuspended and lysed using a suitable volume of RLT plus β-mercaptoethanol buffer recommended by Qiagen. For homogenization lysates were pipetted directly into a QIAshredder spin column (Qiagen, UK) placed in a 2ml collection tube and centrifuged for 2 minutes at 13,000rpm. Lysates were then treated according to manufacturer’s instructions. In summary, lysates were first passed through a gDNA eliminator spin column in order to remove genomic DNA. Ethanol was then added to ensure enable RNA to bind to the silica membrane of the RNAeasy spin column, which was then washed a number of times. The RNA was then eluted from the membranes with 14µL RNase-free water. The RNA concentration (ng/µl) was measured as well as the ratio of absorbances at 260nm and 280nm using a NanoDrop® ND-1000 Spectrophotometer (Labtech International, UK). A 260/280 ratio less than 1.7 was discarded as the sample was likely to be contaminated with
proteins, for example. Samples were immediately stored at -80°C or used immediately for cDNA synthesis (Section 3.3)

3.3 Complementary DNA (cDNA) synthesis from total RNA

A two-step cDNA Synthesis Reaction using nanoScript Reverse Transcription kit (Primer design Ltd, UK) was performed. First, the annealing step, samples were made up to equal concentrations with RNase/DNase free water in a total of 9µL. 1µL of Oligo(dT) primers was added to each RNA sample. The RNA template-primer solution was denatured by heating to 65°C for five minutes and immediately placed on ice to cool. Second, the extension step, a master mix was made up for all samples, which was made up of the following: 5µL of nanoScript2 4X reaction buffer; 1µL dNTP mix (10mM); 3µL RNase/DNase free water and 1µL nanoScript 2 enzyme. Subsequently, 10µL of the master mix was added to each sample on ice to make a total of 20µL reaction volume. These were transferred to a preheated PCR thermal cycler (Applied Biosystems, UK) and incubated at 42°C for 20 minutes, the reaction was terminated by incubating at 75°C for 10 minutes. Assuming the cDNA synthesis was 100% efficient the cDNA synthesised was equal to the amount of RNA initially added to the reaction; this was then diluted to 5ng/µL accordingly. The cDNA were stored as at -20°C or used immediately for RT-qPCR.

3.4 Real time-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR reaction was carried out using a Stratagene Mx3005P qPCR system (Agilent Technologies, USA) and SYBR Green fluorescence to quantify the amount of target cDNA in the sample. The MxPro software (Agilent Technologies, USA) measured the cycle threshold (Ct) for each sample, which is the number of PCR cycles required to amplify the target cDNA past the background level (reference dye) and up to a fixed concentration of amplicon (threshold). The amount of SYBR green fluorescence captured is equal to the amount of cDNA as it only emits light when bound to double stranded DNA. Therefore, Ct values are inversely proportional to the amount of target cDNA in the sample. House keeping genes are required as endogenous controls to normalise Ct values between samples, beta-
actin was initially used (see Table 3.5 for primer sequences). All qPCR reactions were carried out in duplicates on a 96-well and BrightWhite plate (Primer Design Ltd, UK).

A qPCR reaction master mix was prepared for all reactions, which consisted of the following components: 10µL PrecisionPlus Master Mix (Primer Design Ltd, UK); 1µL Primers (forward + reverse) and 4µL DNase/RNase free water. 5µL of cDNA sample (25ng total) obtained through the protocols outlined in Sections 3.2 and 3.3 was then added to the appropriate well, a total of 20µL per reaction. The primers used are listed in Table 3.5. The thermal cycling conditions were set as follows: enzyme activation at 95°C for 2 minutes (x1 cycle), denaturation at 95°C for 15 seconds and data collection at 60°C for 1 minute (x40 cycles). In order to check that only a single product (target cDNA) was amplified a melt curve was performed at 95°C for 30 seconds, 72°C for 1 minute and 95°C for 30 seconds (see Section 3.4.1).

The ΔCt relative quantification method was applied because it calculates the fold difference in EN2 expression between a treated sample and a non-treated control sample (calibrator), absolute quantitation was not necessary (Livak & Schmittgen 2001). Next, assuming the PCR reaction is 100% efficient, every cycle of PCR resulted in a 2-fold increase or doubling in PCR product, therefore the relative ‘fold change’ in gene expression was expressed as ‘2^(ΔCt)’. However, in order to determine the expression of EN2 normalised to β-actin expression the following formula was applied: 2^(ΔΔCt) where ΔΔCT = (Ct_{EN2} - Ct_{HKG})_{treated} - (Ct_{EN2} - Ct_{HKG})_{control}, which is expressed as a fold change in gene expression relative to the control cells (and scaled up as required).

3.4.1 Primer sequences and validation

All primers used in this study have been detailed in Table 3.5. Primers (3.2µM stock) obtained from Primer Design Ltd. were designed and validated before receiving and the working concentration of these primers was 300nM in a 20µl reaction (Section 3.4). All other primers (10µM stock) were designed using the NCBI Primer3 and BLAST tool (Ye et al. 2012), purchased from Sigma, UK (stored at -20°C), the working concentration was 1µM in a 20µl reaction.
Table 3.5. List of all qPCR primers in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Accession number</th>
<th>Sequence (5’-3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>EN2 (8)</td>
<td>NM_001427</td>
<td>Forward: GTGAGCTCGGACTCGGACAGCTCGCA&lt;br&gt;Reverse: GGCCCGCTTGCTCTTTGGTCGGTTC</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_001101.3</td>
<td>Forward: ATGTACCCTGGCATTGCCGCAGAC&lt;br&gt;Reverse: GACTCGTCATACCTGCTTGG</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>EN2 Exon1</td>
<td>NM_001427</td>
<td>Forward: GAAACCGAACAAGAGAGGAAC&lt;br&gt;Reverse: CGCTTGTCTTGGACACAAAT</td>
<td>Primer Design, UK</td>
</tr>
<tr>
<td>EN2 Exon2</td>
<td>NM_001427</td>
<td>Forward: GAAACCGAACAAGAGAGGAAC&lt;br&gt;Reverse: CGCTTGTCTTGGACACAAAT</td>
<td>Primer Design Ltd, UK</td>
</tr>
<tr>
<td>EN2 (IS)</td>
<td>NM_001427</td>
<td>Forward: GAAACCGAACAAGAGAGGAAC&lt;br&gt;Reverse: CGCTTGTCTTGGACACAAAT</td>
<td>Primer Design, UK</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046.5</td>
<td>Forward: GAAACCGAACAAGAGAGGAAC&lt;br&gt;Reverse: CGCTTGTCTTGGACACAAAT</td>
<td>Primer Design, UK</td>
</tr>
<tr>
<td>ATP5B</td>
<td>NM_001686</td>
<td>Forward: GAAACCGAACAAGAGAGGAAC&lt;br&gt;Reverse: CGCTTGTCTTGGACACAAAT</td>
<td>Primer Design, UK</td>
</tr>
<tr>
<td>qPCR EN2 (pGenScript)</td>
<td>NM_001427</td>
<td>Forward: AGACAGAACCCTCCCATGTGC&lt;br&gt;Reverse: CCCAGTCTCCTCTTGCCTTCAG</td>
<td>GenScript, USA</td>
</tr>
<tr>
<td>MAP1B</td>
<td>NM_005909.4</td>
<td>Forward: GCCGGAGCGGAGACACTTC&lt;br&gt;Reverse: ACTTGCTGTCAAGGAAGCGG</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>COL8A1</td>
<td>NM_001850.4</td>
<td>Forward: CTGGGTCAGCAAGATCCTCA&lt;br&gt;Reverse: GGACCTTGTCCCTCCTGTAA</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>INHBA</td>
<td>NM_002192.3</td>
<td>Forward: TTTCTGGGCAAGTTTTAGCG&lt;br&gt;Reverse: CGGGTCTCCTCTTGATGTC</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>EIF4E</td>
<td>NM_001968.4</td>
<td>Forward: GCAAGCTCGCATACACTCCT&lt;br&gt;Reverse: GCTGCTCTTCTGAGAGCGT</td>
<td>Sigma-Aldrich, UK</td>
</tr>
<tr>
<td>TMEM204</td>
<td>NM_024600.5</td>
<td>Forward: CCCATACCAACCTCCT&lt;br&gt;Reverse: GTTGATGGAGCGGCTGAGAAG</td>
<td>Sigma-Aldrich, UK</td>
</tr>
</tbody>
</table>

As outlined in Section 3.4 a melt curve was performed to ensure that the RT-qPCR assay had resulted in a single, specific product. Furthermore, a ‘no template (cDNA) control’ well was always carried out for every set of primers used in that assay and this revealed whether they could create a false positive signal. For the EN2 primers these were further tested by serial dilution of EN2 plasmid DNA in
order to create a standard curve. After applying a line of best fit to the standard curve, the amplification efficiency (E) was calculated using the following formula: $E = 10^{(-1/\text{slope} \ (m))}$, where m is derived from $y = mx + c$. An efficiency of more than 105% indicated that another product was likely to be co-amplified and less than 90% indicated that the primers were poorly designed and thus, the reaction was not efficient (Rutledge & Côté 2003).

### 3.4.2 Selecting house keeping gene/s for normalising RT-qPCR data

For accurate gene quantification, it is essential to normalise real-time PCR data to a fixed reference; one that is not affected by the experimental conditions. The geNORM kit measured the expression of 12 reference (house-keeping) genes in a range of representative samples. The geNorm software provided with the kit ranked the reference (house-keeping) genes in order of stability of expression. The panel of 12 validated HKGs were tested (included with the geNORM kit (Primerdesign Ltd., UK): SDHA; B2M; 18S; YWHAZ; EIF4A2; CYC1; ACTB; RPL13A; UBC; TOP1; GAPDH and AP5B. cDNA were a representative set of samples (1-10) that included cDNA from PC3 and WPMY-1 cell lines after EN2 forced expression, GFP-EN2 stable expression and EN2 knockdown. The plate layout is depicted in Table 3.6.

<table>
<thead>
<tr>
<th>Samples</th>
<th>HKG1</th>
<th>HKG2</th>
<th>HKG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
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</tr>
<tr>
<td>9</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3.6
Table 3.6. geNORM RT-qPCR plate setup. Cell line cDNA samples were tested for each housekeeping gene (HKG) 1 to 12 in order to calculate the most stable throughout these samples.

A qPCR reaction master mix was prepared for each HKG primers to be used, which consisted of the following components: 10µL PrecisionPlus Master Mix (Primer Design Ltd, UK); 1µL Primers (forward and reverse) and 4µL DNase/RNase free water. Enough was made for duplicate samples, and aliquoted to the appropriate wells in a BrightWhite 96well plate. Subsequently, 5µl of cDNA sample was added to each assigned well. The same qPCR thermal profile was used as outlined in Section 3.4. For the analysis the software provided, Biogazelle qBasePLUS, created two graphs: a) M - the average expression stability value of each reference gene and b) V - the optimum number of reference genes required. The protocol was followed in accordance with the instructions provided.

The chosen HKGs were GAPDH and ATP5B, the sequences of which are listed in Table 3.5.

3.4.3 EN2 primer design

Each EN2 primer pair was verified by generating standard curves and calculating the amplification efficiency (E) as detailed in Section 3.4.1. For EN2 the results verified that E was more than 105%, which meant that there was co-amplification. The qPCR products were also run on a 2% agarose gel that further confirmed the amplification of unspecific products. Therefore, new EN2 primers were designed, created and validated by Primer Design Ltd., UK to anneal as shown in Figure 3.4; the sequences are listed in Table 3.5.
**Figure 3.4. Schematic representation of EN2 mRNA and EN2 specific primer pairs.** Green blocks highlight the protein coding mRNA exon regions, of which there is only two, and the thin black line depicts the intronic region. The diagram shows the boundaries that Exon 1 (1), Exon 2 (2) and Intron-Spanning primer pairs with the black bars. The intron-spanning primer anneals to part of Exon 1 and Exon 2 and therefore the intron sequence must be fully excised in order for the RT-qPCR reaction to occur.

EN2 genomic DNA, depicted in **Figure 3.4**, is made up of two exons that are separated by a large intron sequence. EN2's mRNA would be made up on the two exons only, as the intron sequence is excised. Usually, qPCR primers are made to anneal over an exon-exon junction to avoid genomic DNA contamination as they could only anneal if these two exons sites had come together, which could then produce a single product (EN2 mRNA). Furthermore, mRNA splice variants could exist that contain, for example, only Exon 1 or only Exon 2 sequences.

### 3.4.4 Peptide sequences

Listed in **Table 3.7** are the translated sequences of the EN2 plasmids that are transfected into cell lines in this study, for reference.
<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Peptide sequence (N- to C- terminus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EN2</td>
<td>Origene, USA</td>
<td>MEENDPKPGEAAAAVEGQRQPESSPGGGSGGGGGS                                      GEDTWGRRRALMLPAPLQAPNGHQPHTSCRPINFFIDNLRIPE FGRRKDAGTCCAGAGGGGAGGAGGEGGASGAEGGGGA GEGSEQLLGSGSRPRQNPACAGGGLPAAGSPGD GEGGKTLHSLHGAGKKGDPGDGLDKARGLGGGDL SVSSDSDDSSQAGANLGAQPMLWPAWVYCTRTYSRDPSSG PRSRKPKKKNPNKECRPRRTAFTAELQRKAEQTFNRNYL TEQRQOSIAQLSLNESQIKIFQNKRAKIKKATGNKNTLAVHLMAQGLYNHSTTAEGKSDSE</td>
</tr>
<tr>
<td>EN2 (pGenScript)</td>
<td>GenScript, USA</td>
<td>Same sequence as EN2 (above).</td>
</tr>
<tr>
<td>EN2-GFP (tagged)</td>
<td>Life Technologies, UK</td>
<td>IEEMENDPKPGEAAAAVEGQRQPESSPGGGSGGGGGS                                      SPGEADTWGRRRALMLPAPLQAPNGHQPHTSCRPINFFIDNLRIPE RPEFGRKRDAGTCCAGAGGGGAGGAGGEGGASGAEGG GGGAGGSEQLLGSGSRPRQNPACAGGGLPAAGSPGD DSPQGDGEQGSKTLSLHGAGKKGDPGDGLDKARGL GGGDLSVSSDSDDSSQAGANLGAQPMLWPAWVYCTRTYSRDPSSG PRSRKPKKKNPNKECRPRRTAFTAELQRKAE QTNRYLTEQRQOSIAQLSLNESQIKIFQNKRAKIKKATGNKNTLAVHLMAQGLYNHSTTAEGKSDSEXKGNSAD IQHSGGRSSLXMASKGEELFTGVVPIELVLDGDVNGHKF VSSEGEGDANTGKTLKFICTTGKLVPVPWPTVTFSYGV QCFSRYPDHMKRDFFKSAMPEGYVQERTISFKDDGNY KTRAEVKFEDTENRILKGIDFKEDGNIALLHELYNYS HNVIYATDKQKNGIKANFKIRHNIEDGSVQLADHYQQQNPI GDGPVLLPDNYLSTQALSNDPNEKRDHVMLLEFVTAA GITHGMDELYK</td>
</tr>
<tr>
<td>Name</td>
<td>Source</td>
<td>Peptide sequence (N- to C- terminus)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GFP-EN2 (tagged)</td>
<td>Origene, USA</td>
<td>MSGGEELFAGIVPVIELDGDVHGKFSVRGEGEGDADYGKLEIKFICTTGKLI1PVWPWLVTTLCYGQICFHAPAYPMKMNDFKSAEMPEGYIQERTIQFDGGYKTRGEVKFEGDTLVNRIELKGKDFKEDGNIILGHKLEYSFN S VWYIIRPDKANGGLEANFKTRHNEIEGGVQLH DHYQTNVPLGDGPVLIPINHYLSTQTKISKDRNEARDHMVLLESFSACCHTHGMDLERYRSGLAIA MEENDPKPGEAAAVEGQRQPESSPGGGS GGGGSPGEADTGRRALMLPAVLQAPGNNHHQHPHRITNNFIDNLRPEGRRRAGTCCAGAGGGGARGGAGGEGGA SGAEGGGGAGGSEQLLGSREPRQNPPCPAGGAGPLPAAGSDDSPGDEGGSKTLSLHGAKKGGDPPGGLDGLSKA RGLGGDLSVSSDSSAQANLGAQPMWLPAWYCTRYSDRPSSGPRSRRKPKKKNPKEDKRPRTAFTAEQLQRLKAEFQTNRYLTEQRRQSLAESLSNESQIKWQNFNKR AKIKKATGNKNTLAVHLMAQGLYNHSTTAKEGKSDSETR</td>
</tr>
</tbody>
</table>

**Table 3.7. Peptide sequences.** The peptide sequences of the EN2 protein variants that were forcibly over-expressed in cell lines, in order to validate antibody detection and observe cell behaviour - specific experiments are described in the following sections. Green amino acids make up the GFP peptide sequence, the blue amino acids make a linker sequence, in red are the restriction sites and black the EN2 peptide sequences.

### 3.5 Immunocytochemistry (ICC) for the detection of EN2 protein in cell lines

This is carried out in order to demonstrate both the presence and localisation of total EN2 in cells. It is performed on intact cells, ensured by ‘fixing’ the cells. This method relies on the high specificity of the primary antibody against the known target protein. The secondary antibody is conjugated to a fluorophore to allow visualisation under fluorescence with a Nikon A1M confocal microscope and NIS elements acquisition software (Nikon, UK). Images were processed with ImageJ. Subcellular localisation of the antigen can also be determined when additional markers are used to demarcate the subcellular compartments. When the membrane is not forcibly permeated the antibodies can only bind to the cell surface providing a method to distinguish between cell surface and intracellular expression.
3.5.1 Cell fixation and staining

Adherent cell lines were initially grown overnight in an 8-chambered polystyrene culture treated glass slides (BD Biosciences, UK), in order to create a monolayer that is more than 70% confluent. Suspension cells and PBMCs were dropped onto microscope slides and left to dry for an hour before removing culture media. The cells were ‘fixed’ with 300µl/well of freshly prepared 4% paraformaldehyde solution (PFA): 0.8g paraformaldehyde (Sigma, UK) was dissolved in 20mL PBS (×1) (Fisher Scientific, UK) on a magnetic stirrer at 60°C until dissolved for 10 minutes. PFA was removed and cells were washed with 500µl PBS (x1) three times. Cells that were to be permeabilised were incubated with 300µl/well 0.2% Triton X-100 (Sigma-Aldrich, UK) for 10 minutes, after which the cells were washed three times with 500µl PBS (x1). Next, the cells were incubated with an appropriate blocking serum (see Table 3.9) for 20 minutes. The serum carries antibodies that bind to reactive sites and thus, blocks non-specific binding sites from the secondary antibody. The blocking solution was removed and cells were incubated with 100µl/well primary antibody diluted at 1:100 in freshly made PBS/1% BSA at room temperature for 2 hours or overnight at 4°C. A ‘no primary antibody’ sample was also included containing 300µL PBS/1% BSA for use as a negative control. All primary antibodies used in this study for ICC are listed in Table 3.8. In order to reduce unspecific binding and allow the detection of the protein of interest only, each primary antibody was optimised in order to get a clear signal with minimal unspecific binding. This meant the antibodies were serially diluted usually from 1:50 to 1:400 and tested with the positive and negative cell lines HL-60 and WPMY-1 respectively.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-EN2</td>
<td>Goat polyclonal, IgG</td>
<td>Abcam, UK</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-EN2</td>
<td>All sheep polyclonal, IgG</td>
<td>Bioventix, UK</td>
<td>1:50</td>
</tr>
</tbody>
</table>

Table 3.8. Primary antibodies used for ICC and their optimal dilution.
Table 3.9. Secondary antibodies used for ICC and their optimal dilution.

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Serum block</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-goat AF488</td>
<td>Abcam, UK</td>
<td>1:200</td>
<td>10% horse serum (Jackson ImmunoResearch, USA)</td>
</tr>
<tr>
<td>Donkey anti-sheep AF488</td>
<td>Abcam, UK</td>
<td>1:10,000</td>
<td>5% horse serum</td>
</tr>
</tbody>
</table>

After primary antibody incubation, the cells were washed three times with 500µl PBS (x1) and incubated with secondary antibody (see Table 3.9) in the dark for 45 minutes. After incubation cells were washed three times, chambers removed carefully using the white comb supplied (BD BioSciences, UK). The slides were mounted using 2-3 drops of propidium iodide (PI) Vectashield® mounting medium that stains the nucleus (Vector Laboratories, USA) and a cover slip was carefully placed over the top. The slides were imaged at X40 magnification using the confocal microscope.

3.5.1.1 Wheat-germ agglutinin (WGA) membrane staining for EN2 co-localisation analysis

In order to demarcate the cell membrane the WGA stain (Alexa Fluor® 647, Molecular Probes®, Life Technologies, UK) was added before fixation in Section 3.5.1. A fresh working solution (1:200) of the 1mg/ml WGA stain in warm HBSS was made up. Media was removed from the 8-chambered slide and replaced with the WGA stain solution and incubated for 10 minutes at 37°C. The protocol outlined in Section 3.5.1 was followed with the addition of a 1:400 dilution of TO-PRO®-3 (Molecular Probes®, Life Technologies, UK) with the secondary antibody incubation for a blue nuclear stain and thus, an empty Vectashield® (hard set) Mounting Medium (Vector Laboratories, USA) was used instead.

3.6 Western blot analysis for EN2 detection in cell lysates

Similarly to Section 3.5.1 a western blot (WB) uses an antibody for its high specificity to detect a known protein of interest, however, the secondary antibody is
tagged to a chemiluminescent molecule instead of a fluorophore. Proteins are separated according to their size on a membrane. The antibody will only ‘stick’ to the membrane if the protein of interest is present. The chemiluminescent molecule attached to the secondary antibody undergoes a chemical reaction when a substrate is added, and bands appear that can be captured. The size of the band on a blot is dependent on the amount of protein available and thus, it can be semi-quantified relative to a house keeping protein (such as GAPDH).

3.6.1 Whole cell lysate preparation

Cells were washed twice with ice-cold PBS, harvested in PBS using a cell scraper and centrifuged at 1500rpm for 3 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in ice-cold RIPA buffer (Life Technologies, UK) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (100X; Sigma-Aldrich, UK) and EDTA, usually used at a 1X final concentration. Cells were kept on ice, vortexed and sheared using 21G needles before being centrifuged at 13,000rpm for 15 minutes at 4°C, after which the supernatant was collected and immediately stored at -80°C. The total amount of protein in the lysates were calculated using the Pierce™ BCA Protein Assay Kit (Life Technologies, UK) in accordance with the manufacturer’s instructions. To summarise, 25µl of lysate or albumin standard was pipetted into a 96-well plate and 200µl of the green working reagent was added to each well and incubated for 30 minutes at 37°C. The green working reagent turns purple when protein is present and this change was measured at 562nm on the Variskan® Flash plate reader (Thermo Scientific, UK). The albumin standards create a standard curve from which the unknown protein concentrations can be interpolated.

3.6.2 Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were diluted in RIPA buffer to the same concentration in 13µl and supplemented with 5µl NuPAGE lithium dodecyl sulfate (LDS) Sample Buffer (4X; Life Technologies, UK) and 2µl NuPAGE Sample Reducing Agent (10X; Life Technologies, UK). The XCell Surelock™ Mini-Cell Electrophoresis apparatus (Life
Technologies, UK) was assembled with a pre-cast polyacrylamide NuPAGE® Novex 4-12% Bis-Tris Protein Gel (Life Technologies, UK) and NuPAGE® MOPS SDS running buffer (Life Technologies, UK), diluted 1:20 in water. The samples were denatured by heating for 10 minutes at 95°C before being loaded into the pre-cast polyacrylamide 4-12% Bis-Tris protein gel (Life Technologies, UK). 10µL Novex® Sharp prestained markers (Life Technologies, UK) and 20µL lysate samples were loaded onto the lanes of the gels. Gel electrophoresis was performed at 200V for approximately an hour using a PowerPac (Bio-Rad, UK).

The recombinant EN2 (rEN2), purified from E.Coli, was used as a positive control and runs at 45kDa. The rEN2 was produced within the Oncology department with the following protocol: EN2 cDNA with optimized codon usage (GenScript, USA) was re-cloned into the pQE31 plasmid (Qiagen, UK), placing it under a T7 promoter and tagged the protein with histidine. This plasmid was transformed into E. coli host strain (M15; Qiagen, UK), the histidine tags allowed purification of rEN2 by affinity chromatography on Ni-NTA resin (Qiagen, UK) under denatured conditions (Annels et al. 2014).

3.6.3 Protein transfer, blocking and detection

Following SDS-PAGE, the separated proteins were transferred onto a nitrocellulose membrane by electroblotting at 20 volts for 7 minutes using the iBlot® gel transfer system (Life Technologies, UK). The membrane was then blocked with blocking buffer (5% milk powder in PBS, 0.1% Tween-20 (Sigma-Aldrich, UK)), overnight at 4°C with gentle shaking. It was then probed with primary antibody (diluted in blocking buffer) for 2 hours at room temperature, with gentle shaking. All optimal antibody dilutions were initially determined by serial dilution (Table 3.10). After three 10-minute washes with PBS/ 0.1% Tween-20, the membrane was incubated with an appropriate secondary Horseradish peroxidase (HRP)-conjugated antibody (listed in Table 3.11), diluted in blocking buffer.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Molecular Weight (kDa)</th>
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<tbody>
<tr>
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<td>Goat polyclonal, IgG</td>
<td>Abcam, UK</td>
<td>1:1667</td>
<td>33kDa</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>Bioventrix, UK</td>
<td>1:250</td>
<td>33kDa</td>
</tr>
<tr>
<td>Name</td>
<td>Type</td>
<td>Supplier</td>
<td>Dilution</td>
<td>Molecular Weight (kDa)</td>
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<td>----------</td>
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<td>anti-α-tubulin</td>
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<td>50kDa</td>
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<td>Abcam, UK</td>
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<td>27kDa</td>
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<td>Anti-HaloTag®</td>
<td>Mouse monoclonal, IgG</td>
<td>Promega, UK</td>
<td>1:1000</td>
<td>34kDa</td>
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<tr>
<td>Anti-NanoLuc®</td>
<td>Rabbit polyclonal, IgG</td>
<td>Promega, UK</td>
<td>1:5000</td>
<td>19kDa</td>
</tr>
<tr>
<td>Anti-GAPDH antibody, clone 2D9</td>
<td>Mouse monoclonal IgG</td>
<td>Origene, USA</td>
<td>1:2000</td>
<td>35.9kDa</td>
</tr>
</tbody>
</table>

Table 3.10. Primary antibodies used for WB analysis and their optimal dilution.

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey anti-goat, HRP-conjugated</td>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit anti-mouse-HRP-conjugated</td>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Donkey anti-sheep, HRP-conjugated</td>
<td>Jackson ImmunoResearch Laboratories, Inc.</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Goat anti-Mouse IgG, HRP-conjugated</td>
<td>Origene, USA</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG-HRP</td>
<td>Abcam, UK</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 3.11. Secondary antibodies used for WB analysis and their optimal dilution.

Lastly, three 10-minute washes in PBS/0.1% Tween-20 were carried out and the blot was covered in West Pico chemiluminescence substrate (Life Technologies, UK) for 5 minutes. The resultant protein bands were imaged using the ChemiDoc-It² imager (UVP, UK). The weight of the protein bands were identified by comparing them to the standards from the Novex® Sharp pre-stained marker. The blot was
carefully stored in PBS/0.1% Tween-20 and stored at 4°C for stripping at a later time (Section 3.6.5).

3.6.4 Membrane stripping for loading control detection

The membrane was washed three times for 10 minutes before decanting 15 ml of Restore™ PLUS stripping buffer (Fisher Scientific, UK) onto the membrane, rotating slowly for 20 minutes. The membrane was then quickly rinsed before being washed a further three times for 5 minutes. The detection process (outlined in Section 3.6.3) was then repeated with a mouse monoclonal anti-GAPDH antibody, clone 2D9 (Origene, USA). GAPDH was used as the loading control thereafter and membranes were not stripped more than three times.

3.6.5 Densitometry analysis

Blot images taken by the ChemiDoc-It² imager (UVP, UK) were further analysed by Image Studio™ Lite Version 4.0 software (LI-COR Biotechnology, UK). The individual bands were outlined, the band density quantified and then normalised to the band density of the loading control in order to directly compare the protein expression level between samples. The relative protein density in each sample was then plotted graphically.

3.7 Lipid-mediated transfection of EN2 plasmid in cell lines

Cell lines were transfected with a DNA plasmid created using the full-length human EN2 cDNA (accession number: NM_001427) and pcDNA CMV6 vector (TrueClone® OriGene, USA). Lipofectamine LTX (Life Technologies, UK), Lipofectamine® 2000 (Life Technologies, UK) and ViaFect™ (Promega, UK) are cationic lipid transfection reagent that mediates the interaction of the positively charged nucleic acid with the negatively charged cell surface membrane and the DNA is then endocytosed. The lipid-mediated transfection is highly dependent on the amount of transfection reagent as this can be toxic to the cells. Therefore, each
cell line was first tested with each transfection reagent and optimised, the results are listed in Table 3.12.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transfection reagent</th>
<th>Ratio of Transfection reagent (µl) :DNA (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>ViaFect™</td>
<td>3:1</td>
</tr>
<tr>
<td>LnCaP</td>
<td>ViaFect™</td>
<td>5:1</td>
</tr>
<tr>
<td>WPMY-1</td>
<td>ViaFect™</td>
<td>6:1</td>
</tr>
<tr>
<td></td>
<td>Lipofectamine® LTX</td>
<td>1.4: 0.1 + with 0.6µl of PLUS reagent (Life Technologies, UK)</td>
</tr>
<tr>
<td>Du145</td>
<td>Lipofectamine® 2000</td>
<td>6:1</td>
</tr>
<tr>
<td>SKMEL5</td>
<td>ViaFect™</td>
<td>5:1</td>
</tr>
</tbody>
</table>

Table 3.12. Optimal transfection reagent and transfection reagent: DNA ratio for each cell line.

Cells were seeded either in a 8-chambered slide at 1x10^4 cells/well (if the cells were to be analysed by ICC) at or in a 6-well plate at 1x10^5 cells/well (for all other analysis) and incubated with working media overnight to ensure at least 70% confluency. For ViaFect™, 1µg of DNA was typically diluted in 100µl of Gibco™ Opti-MEM™ I Reduced Serum Media (Opti-MEM I, Life Technologies, UK), mixed and incubated for 5 minutes. After 5 minutes, ViaFect™ was added straight to the diluted DNA and gently mixed. Lipofectamine® 2000 reagent was first diluted in an equal volume of Opti-MEM I and then mixed with the diluted DNA. For Lipofectamine LTX the protocol is depicted in Figure 3.1. A ‘media only’ control and transfection reagent only control (Life Technologies, UK) were prepared, which had no plasmid DNA.
The lipid:DNA complexes were then incubated for up to 30 minutes before being pipetted directly into the cell culture media and gently mixed. The transfections were typically left for 48 hours. The visual appearance of the cells was checked under a light microscope for signs of cytotoxicity before RNA was extracted from the cells (Section 3.2) in order to ensure forced EN2 mRNA had been achieved. cDNA was synthesised from the RNA and an RT-qPCR performed as described in Sections 3.3 and 3.4 respectively. Furthermore, the protein overexpression was clarified either by ICC and WB, as described in Sections 3.5 and 3.6 respectively.

3.8 Lipid and siRNA-mediated EN2 knockdown in cell lines

Short interfering RNA (siRNA) is a double stranded RNA molecule that operates within the RNA interference pathway (RNAi) and directs the RISC complex to a particular mRNA to be degraded, which then induces short-term silencing.
Optimisation was necessary as the siRNA transfection efficiency is highly influenced by cell density and the concentration of the transfection reagent. Therefore, the KDalert™ GAPDH Assay kit (Life Technologies, UK) was employed in order to obtain optimal conditions for an EN2 knockdown of more than 80%.

3.8.1 GAPDH siRNA knockdown for optimisation of transfection conditions

The KDalert™ GAPDH Assay kit silences glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cultured cells, which is ubiquitously and constantly expressed in all cells, and measures GAPDH siRNA efficiency. The negative control siRNA (Life Technologies, UK) included is a good indicator of cellular toxicity when varying the assay conditions. The KDalert™ bypasses the need for RT-qPCR and instead uses the GAPDH’s endogenous reaction during which NAD+ is converted to NADH. Under these assay conditions the rate of NADH production is proportional to the amount of GAPDH enzyme present.

Cells were first trypsinised and resuspended in working media at 1.5x10⁵ cells/well and further diluted to make 4x10³, 8x10³, 12x10³ cells per well (80µl per well) and stored at 37°C and 5% CO₂ until required. The siPORT™ NeoFX™ transfection reagent (Invitrogen, UK), another lipid-based reagent (similar to those in Section 3.7), was diluted in Opti-MEM I medium (Life Technologies, UK) to a total volume of 10µl/well at three different concentrations (0.2, 0.5 or 0.8 µl/well). Both the GAPDH siRNA and negative control siRNA were resuspended to 2µM in nuclease-free water (Life Technologies, UK). For each siRNA 1.5µl was then mixed with 8.5µl Opti-MEM I (Life Technologies, UK) to give a total volume of 10µl/well, and incubated for 10 minutes at room temperature. The diluted siPORT™ NeoFX™ reagents were mixed with the diluted siRNA samples in a 1:1 volume ratio and incubated for 10 minutes at room temperature. Therefore, a total of 20µl siPORT NeoFX:siRNA complexes were made per well. In addition to the negative control siRNA, which essentially has no specific target, there was also a media only control (no siRNA). The transfection was carried out by a reverse transfection method whereby the transfection reagents are added to the cells before the cells have adhered to the bottom of the well, as depicted in Figure 3.2.
The plate was then incubated at 37°C for 24 hours before being replaced with 100µl fresh working media for another 24 hours. A KDalert™ master mix was then prepared on ice as per manufacturer’s instructions. In summary, the visual appearance of the cells was first viewed by a light microscope to check for any signs of toxicity. The wells were emptied of media and replaced with lysis buffer (100µl/well) and incubated for 20 minutes at 4°C. The lysates were then transferred to a new 96-well plate and additional water only control wells were made; 90µl of the master mix were added to these and incubated for 15 minutes at room temperature. The absorbance was then measured at 620nm using the Variskan® Flash plate reader (Thermo Scientific, UK). The GAPDH activity was calculated by subtracting the average absorbance at 620nm of the water only control well from the average absorbance at 620nm of the sample wells. These values were then used to calculate the percentage of GAPDH remaining by dividing the GAPDH activity in the GAPDH siRNA sample by the GAPDH activity in the negative control siRNA sample and multiplying by 100.
The conditions that resulted in the maximum GAPDH knock-down whilst lessening transfection-associated toxicity were used thereafter (see Section 3.8.2)

3.8.2 EN2 siRNA knockdown (siPORT NeoFX) and western blot analysis

For PC3 cells the optimal conditions were, for a 96-well plate, 8x10^3 cells/well (80µl/well) in normal culture media, 0.8µl/well of EN2 siRNAs (listed in Table 3.13) and negative control siRNA at 2µM and 0.8µl/well of transfection reagent. The cells were resuspended in media, siRNAs and siPORT NeoFX solution were each diluted in Opti-MEM media to make an 1:1 volume ratio with a total overall volume of 20µl/well. The protocol outlined in Figure 3.2 was carried out and cells were analysed by RT-qPCR as described in Section 3.4, 48-96 hours after transfection. Simultaneously, cells were lysed for western blot analysis (Section 3.6) in order to evaluate EN2 protein levels using the goat anti-EN2 antibody and GAPDH antibody as the loading control.

<table>
<thead>
<tr>
<th>mRNA sequence accession number</th>
<th>EN2 siRNA</th>
<th>Targeted Exon</th>
<th>siRNA location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_001427.3</td>
<td>4674</td>
<td>2</td>
<td>946</td>
</tr>
<tr>
<td></td>
<td>4675</td>
<td>2</td>
<td>967</td>
</tr>
<tr>
<td></td>
<td>4676</td>
<td>2</td>
<td>1110</td>
</tr>
</tbody>
</table>

Table 3.13. EN2 siRNAs and their target location on EN2 mRNA.

3.8.3 EN2 siRNA knockdown for ICC analysis using Lipofectamine 2000 in a 8-chambered slide

The transfection volumes per well were increased to make a total of 50µl for efficient knockdown in 8-chambered slides. Therefore, 2µl siPORT NeoFX diluted in 25µl per well and 3µl siRNA diluted in 25µl per well. ICC staining was carried out as described in Section 3.5.1 with the goat anti-EN2 antibody. These were visualized under a confocal microscope (X40 magnification). RT-qPCR was carried out on a duplicate 8-chambered slide as described in Section 3.4.
3.9 Protein analysis: Kyte-Doolittle Hydropathy plot

The Kyte-Doolittle hydropathy plot (or hydrophobicity/ hydrophilicity plot) is essentially a method to display the hydrophobic and hydrophilic regions of a protein sequence. The software, found at http://web.expasy.org/protscale/, scans through sections along the protein sequence to evaluate the average hydrophobicity or hydrophilicity of the amino acids. Each amino acid has been assigned a score between -4.5 and 4.5 (Kyte & Doolittle 1982). The EN2 protein sequence (accession number: P19622) was run through the program and its hydropathy scores were graphically displayed.

3.10 Generating EN2-GFP using pcDNA3.1/CT-GFP-TOPO vector

pcDNA3.1/CT-GFP-TOPO expression vector kit (TOPO® vector, Life Technologies, UK) was used for cloning EN2. EN2 could then be expressed tagged with green fluorescent protein (GFP) to the C-terminus. Therefore, EN2 could be directly visualised under fluorescence using the confocal microscope.

3.10.1 EN2 PCR amplification

Primers (Sigma Aldrich, UK) were designed to amplify the EN2 sequence within the synthetic EN2 plasmid (GenScript, USA) using MacVector primer design software. EcoRI (5’) and XbaI (3’) restriction sites were inserted into the PCR product. The T at the 5’ of the reverse primer is added so that the product has ‘A’ overhangs. The sequences designed are as follows: forward 5’-ATCGAATTCGCCACCATGGAGGAAAACGACCCCAAG-3’ and reverse 5’-TTCTAGACTCGCTATCGGACTTGCCCT-3’ (Sigma-Aldrich, UK), collectively labeled EN2 TOPO primers. The primers are designed so that Taq polymerase (within the PCR mix) adds a single deoxyadenosine (A) to the 3’ end of the PCR products which is required because the vector has overhanging thymidine residues. The binding of these two residues is catalysed by an activated topoisomerase enzyme covalently attached to the vector.
The PCR reaction was made with 1µl DNA template (synthetic EN2 plasmid), 4µl of EN2 TOPO primers, 7.5µl distilled water and 12.5µl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, UK). The PCR product was then purified using QIAquick PCR Purification Kit (Qiagen, UK) and the manufacturer’s instructions were followed. The purified product was then evaluated by agarose gel electrophoresis with a 1.2% pre-cast E-gel (Invitrogen, UK) and the samples were diluted in E-Gel® Sample Loading Buffer, (1X; Invitrogen, UK) and also run alongside a 1 Kb Plus DNA ladder (Invitrogen, UK) to check that the PCR product was the correct size for EN2 (1 Kb). The gels were visualized and imaged under UV light by a BioDoc-It™ Imaging System (UVP, UK).

3.10.2 Plasmid transformation and amplification

The following cloning reaction was made up: 2µl fresh PCR product (from Section 3.10.1); 1µl salt solution; 2µl sterile water and 1µl TOPO® vector. As a negative control, no PCR product was included. The reaction was mixed gently and incubated for 5 minutes at room temperature and then placed on ice. 2µl of this reaction was placed into a vial of 50µl One Shot® TOP10 Chemically Competent Escherichia coli (E. coli (Life Technologies, UK)), mixed gently and incubated for 10 minutes on ice. Cells were heat-shocked for 30 seconds at 42°C after which they were immediately placed back on ice. 2µl of the transformed bacteria was then added to 250µl of warm Super Optimal Broth (SOC) medium (Sigma-Aldrich, UK). This was placed on a shaker at 200rpm at 37°C for 1 hour. Approximately 140µl of this was then spread onto a pre-set and pre-warmed Luria Broth (LB, Sigma-Aldrich, UK) agar plate and incubated overnight at 37°C.

The overall cloning procedure is depicted in Figure 3.3 below, DNA was extracted and purified from the overnight cultures of individually picked colonies.
Figure 3.3. Molecular cloning protocol with *E. coli*. The next day, after plating, approximately 8 colonies were picked and placed into separate vials of 5ml LB broth (plus ampicillin at 1:1000 dilution). These were then cultured overnight on a shaker at 200rpm at 37°C. To extract and purify the DNA the cultures were spun down at 2500rpm for 20 minutes at 4°C before using the Wizard® Genomic DNA Purification Kit (Promega, UK), the protocol was followed according to the manufacturer’s instructions.

To make sure that the insert was the correct size for EN2 (approximately 1Kb) the following digest reaction was set up for 2 hours at 37°C: 1µl of EcoRI restriction enzyme; 1µl of XbaI restriction enzyme; 2µl Buffer H; 14µl of distilled water and 2µl of the PCR product. The 5µl loading buffer was added to each 20µl digests and run on a 1.2% agarose gel. To make sure the PCR product was cloned into the vector in the correct reading frame and without substitutions or deletions the product was also sent for sequencing along with the appropriate primers. These were sequenced by DNA Sequencing & Services (Scotland). A glycerol stock was made for each colony and stored at -80°C.

3.10.3 *EN2-GFP* transfection into cell lines and protein analysis

0.5µg of EN2-GFP plasmid DNA, diluted in Opti-MEM I, was transfected into WPMY-1 or PC3 cells/well using the protocol outlined in Section 3.7, in a 8-chambered slide. The control vector with GFP alone and a sample with Opti-MEM I alone were used as positive and negative controls respectively. These complexes
were added straight to the working media to make a total of 500µl per well. After 24 hours the culture media was removed, cells were washed and stained as detailed in Section 3.5.1.1. However, antibodies were not necessary and in short: WGA stain was added to demarcate the membrane, the cells were then fixed with 4% PFA and incubated with TO-PRO®-3 in order to stain the nuclei and finally coverslipped with empty Vectashield® (hard set) Mounting Medium and imaged by confocal microscopy with X40 magnification. A duplicate assay was carried out with the same cell lines and transfection parameters in a 24-well plate (instead of an 8-chambered slide). After 24 hours, cell lysates were prepared for western blot analysis as outlined in Section 3.6 and detection was carried with the goat anti-EN2 antibody and anti-mGFP antibody.

3.11 GFP-EN2 (N-terminal tag) de novo expression and protein analysis in prostate cell lines

Cell lines were transfected with a DNA plasmid created using Myc-DDK-tagged ORF clone of Homo sapiens engrailed homeobox 2 (EN2) as transfection-ready DNA (accession number: NM_001427) and PrecisionShuttle mammalian vector with N-terminal mGFP (OriGene Technologies, USA), which enabled the expression of N-terminal GFP-tagged EN2. The transfection was carried out in both WPMY-1 and PC3 using ViaFect™ (Promega, UK) lipid-mediated transfection (the parameters for individual cell lines are found in Table 3.10) and evaluated by ICC and WB, all carried out as described in Section 3.10.3 using the goat anti-EN2 antibody and anti-mGFP antibody.

3.12 Stable expression of GFP-EN2 in PC3 cell line for siRNA evaluation

Unlike transient transfections that persist for a few days only, the DNA can persist for much longer by using antibiotic selective pressure. This pressure selects those that have incorporated the DNA into its genome and go on to pass it onto their
progeny; these cells express the vector that includes both the antibiotic resistant
gene and GFP-EN2. Therefore, under fluorescence microscopy all cells fluoresced
green. As a positive control a stable cell line expressing GFP only was created in the
same way using the PrecisionShuttle mammalian vector with N-terminal mGFP
(OriGene Technologies, USA). For this vector the antibiotic resistant gene was
Neomycin, which confers resistance to G418 (Geneticin disulfate salt) antibiotic
(Sigma-Aldrich, UK). In order to select the correct concentration of G418 a dose-
response experiment (kill curve) was carried out. G418 was serially diluted in cell
culture medium (replaced every 2 days) and each dilution was tested over time by
examining the cells under a light microscope for signs of a 'large kill'. For PC3 the
lowest concentration of G418 that was able to kill all cells within a week was 700µg/
ml.

In order to generate a stable cell line the transfection was carried out as
outlined in Section 3.11 but instead 1µg DNA was transfected into 5x10^5 cells in a
6-well plate. 24 hours later the cells were checked for GFP-EN2 and GFP
fluorescence. The cells were trypsinised and reseeded in a 10cm tissue culture petri
dish in normal culture working media and incubated at 37°C. Once the cells had
adhered, usually 24 hours later, the media was replaced with culture media
containing 700µg/ml G418. The media was replaced with fresh G418-containing
culture media every 2-3 days until a 'large kill' was achieved (of cells that had not
taken up the plasmid) and from which point, spherical colonies would start to
appear. The colonies were picked with a pipette tip and transferred to a v-shaped
bottom well that contained 50µl of trypsin before being transferred to a flat-bottom
96-well plate that contained 150µl of G418-containing culture media. Subsequently,
those that grew confluent and were seen to be expressing GFP under the
fluorescence microscope were transferred to culture wells of increasing surface
area, still under selection pressure through incubation with G418-containing media.
Once the expression levels had been established (after several passages) through
RT-qPCR (Section 3.4) and western blot with the goat anti-EN2 antibody and anti-
mGFP antibody (Section 3.6), early passages of the clones were stored in liquid
nitrogen (Section 3.1.7).

The GFP-EN2 stable clones were then used to test the efficiency of the EN2-
specific siRNAs by repeating the protocol outlined in Section 3.8.3 however, no
antibody staining was necessary and the cells were simply imaged (X20
magnification) under the fluorescence microscope as EN2 was tagged to GFP.
3.13 HaloTag®-EN2 expression in prostate cell lines for specific EN2 detection (with the anti-HaloTag® antibody)

HaloTag®-EN2 (N-terminal tag) plasmid was purchased from Promega (UK) and the sequence can be found here: http://www.kazusa.or.jp/kop/vd/pFN21AB8957/. HaloTag®-EN2 was transfected into cell lines as described in Section 3.7, and its expression was assessed by detecting the increased expression of EN2 mRNA by RT-qPCR (as described Section 3.4) and western blot analysis using the monoclonal antibody to HaloTag® (Promega, UK), in order to determine the size of the tagged protein. Western blots were carried out as described in Section 3.6 and according to manufacturers instructions, which meant that 5% BSA in PBS/0.1% Tween was used as the blocking buffer and antibody diluent instead of the 5% milk powder. In addition, non-denatured western blot analysis was performed where the samples were not boiled prior to being loaded onto the gel and thus, the proteins were kept in their native state.

3.13.1 Preparation of cell supernatants for western blot analysis

The flask of cells were initially washed with PBS twice, and then incubated with 15mls of serum-free culture media at 37°C. After 48 hours, the supernatant was transferred to a 10ml Universal tube and centrifuged at 1500rpm for 3 minutes to remove cellular debris. The supernatant, approximately 15ml starting volume, was transferred into a Amicon Ultra-15 tube (Merck Millipore, UK) with a 10 molecular weight cutoff (MWCO) and so anything larger than 10 kDa was retained. This was centrifuged at 4000 x g using a swinging bucket rotor at 25°C. The sample volume would reduce to approximately 200µl. The Halt™ Protease and Phosphatase Inhibitor Cocktail was immediately added at a 2X final concentration due to a higher presence of proteases in the supernatant and the samples were immediately stored at -80°C. A BCA assay and WB were performed as detailed in Section 3.6.
3.14 Identification of protease sites within EN2 protein

PeptideCutter software was used to assess the EN2 protein sequence (accession number P19622), found at: http://web.expasy.org/peptide_cutter/. The software predicts potential cleavage sites cleaved by proteases or chemicals in a given protein sequence. The results mapped and listed the potential proteases/chemicals and their corresponding cleavage sequence on the protein.

3.15 Membrane fraction preparation and western blot analysis

A flask of cells was initially washed in ice-cold PBS and harvested in 5ml ice-cold PBS using a cell scraper. These were then centrifuged at 1500rpm for 5 minutes at 4°C and resuspended with 1ml of TE buffer (10mM Tris, 1mM EDTA, pH 7.4). Cells were then disrupted by passing through a 25G fine needle twice and additionally homogenized using a hand-held Teflon-on-glass homogenizer. The samples were centrifuged at 1000rpm for 10 minutes and the resultant pellet (containing nuclei and debris) was discarded. The supernatant was subjected to centrifugation at 50,000rpm for 30 minutes using a Beckman Optima™ LE-80K Ultracentrifuge (Beckman Coulter, UK). The membrane pellet was resuspended in 0.2-1ml of TE buffer, depending on size, and a BCA assay was performed (Section 3.6.1).

A western blot was carried out as previously described (Section 3.6). Additionally, to confirm that the membrane fraction was not contaminated with cytosolic proteins and that it was a pure membrane fraction the western blots were carried out in triplicates. One was probed for EN2, the other pan-cadherin (membrane marker) and the other with α-tubulin (cytoplasmic marker), all primary and secondary antibodies and their dilutions can be found in Tables 3.8 and 3.9, respectively. Importantly, during protein transfer to the nitrocellulose membrane the gel that was to be probed by anti-pan-cadherin antibody (due to pan-cadherins high molecular weight) required an additional incubation step with transfer buffer, this was made up according to manufacturers instructions (Life Technologies, UK) and the gel was incubated for 20 minutes submerged in transfer buffer on a shaker. in
addition, a longer 9 minute transfer with the iBlot® gel transfer system (Life Technologies, UK) was required.

3.16 Fluorescent intensity line profile to evaluate co-localisation of GFP-EN2 and WGA membrane stain

Cells are stained as outlined in Section 3.13. Within the Z-stack function in the NIS elements acquisition software a line is drawn over the cell to be analysed, demarcating the diameter of the cell. In addition, a lower and upper boundary can be set (corresponding to the top and bottom of the cell) as well as the number of slices (images) to be taken as it moves through the cell (top to bottom). This was further assessed by plotting the fluorescence intensities, of both the GFP and WGA membrane stain, along the line profile.

3.17 Analysis of extracellular epitopes by confocal microscopy

Each sheep IgG antibody (Bioventrix, UK) listed in Table 3.14 binds to a different EN2 epitope across the length of the protein from the C- to the N-terminal, each epitope is 20 amino acids long and overlaps the next by 10 amino acids. These antibodies were generated in sheep using a nested peptide series conjugated to the metalloprotein, KLH.

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Epitope location (amino acid)</th>
<th>Peptide sequence (N’-C’ terminal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab33</td>
<td>330 - 310</td>
<td>AVHLMAQGLYHSTTAKEGK</td>
</tr>
<tr>
<td>Ab32</td>
<td>320 - 300</td>
<td>KKATGNKNTLAVHLMAQGLY</td>
</tr>
<tr>
<td>Ab31</td>
<td>310 - 290</td>
<td>IWFQNKRASSIKKATGNKNTL</td>
</tr>
<tr>
<td>Ab30</td>
<td>300 - 280</td>
<td>ELSLNESQIKWIFQNKRAKI</td>
</tr>
<tr>
<td>Ab29</td>
<td>290 - 270</td>
<td>EQRQQSLAQEELSLNESQIKI</td>
</tr>
<tr>
<td>Ab26</td>
<td>260 - 240</td>
<td>NKEDKRPRATAFTAEOQLQRLK</td>
</tr>
<tr>
<td>Ab25</td>
<td>250 - 230</td>
<td>RSRKPUNKNPKEDKRPRTA</td>
</tr>
<tr>
<td>Antibody name</td>
<td>Epitope location (amino acid)</td>
<td>Peptide sequence (N’-C’ terminal)</td>
</tr>
<tr>
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<tr>
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<td>240 - 220</td>
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<td>230 - 210</td>
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<td>Ab21</td>
<td>210 - 190</td>
<td>DSDSSQAGANLGAQPMMLWPA</td>
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<tr>
<td>Ab17</td>
<td>170 - 150</td>
<td>PGDGEGGSKTLSLHGGAKKG</td>
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<td>Ab16</td>
<td>160 - 140</td>
<td>GPLPAAGSDSPGDGEGGSKT</td>
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<tr>
<td>Ab12</td>
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<td>Ab8</td>
<td>80 - 60</td>
<td>QHPHRITNFFIDNILRPEFG</td>
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<tr>
<td>Ab6</td>
<td>60 - 40</td>
<td>DTGRRALMLPAVLQAPGNNH</td>
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<td>Ab4</td>
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<tr>
<td>Ab2</td>
<td>20 - 0</td>
<td>MEENDPKPGGEAAAVGQYRQ</td>
</tr>
</tbody>
</table>

Table 3.14. Sheep polyclonal IgG antibodies that bind to different epitopes along the EN2 protein.

HL-60 cells and NIH/3T3 were used as the positive and negative controls, respectively. For each sheep antibody the staining described in Section 3.5 was performed. An additional ‘no primary antibody’ control was carried out to highlight any unspecific binding from the secondary antibody.

3.18 Validation of sheep anti-EN2 antibodies by western blotting

The western blots were carried out as described in Section 3.6 to test a few of the anti-EN2 sheep antibodies: Ab2, Ab4, Ab8, Ab16, Ab24 and Ab32. These were compared to the Goat anti-EN2 antibody and APS2 anti-EN2 antibody, which has been used to detect EN2 in an ELISA assay (R. Morgan et al. 2011). The samples tested were the EN2 positive control whole cell lysate from: SKMEL-5, PC3 transiently overexpressing (untagged) EN2 and PC3. In addition, a EN2 fragment was used, which is a synthetically produced peptide consisting of 100 amino acids of EN2’s C-terminus (Biosynthesis Inc., USA) and used as the control in the EN2 ELISA (R. Morgan et al. 2011). WPMY-1 whole cell lysate was used as the negative control.
3.20 Time-lapse confocal microscopy

The cells were seeded in a 4-chamber (35mm) glass-bottom dish (MatTek, USA) and 24 hours later the cells were transfected with usually 0.5µg of GFP-EN2 and GFP (control) as described in Section 3.7. 24 hours after transfection the cells were observed under light microscopy and fluorescence to make sure cells were still viable and the transfection efficiency was substantial. In addition, stains were added to demarcate the subcellular compartments of the cell such as: NucBlue® Live ReadyProbes® Reagent fluorescence dye (ThermoFisher, UK) and the WGA membrane stain - these could both be used without fixing the cells. The media was also changed to Gibco® FluoroBrite™ DMEM (Life Technologies, UK) to enhance the fluorescent signal. The correct size adaptor was fitted in the confocal microscope in order to accommodate the 35mm dish. The confocal integrated incubator was then set to 37°C and 5% of CO2. The corresponding lasers were selected: 488nm, 595nm and 642 nm to excite GFP-EN2 or GFP (green), WGA (red) and TO-PRO®-3 stain (blue), respectively. The cells in the dish were focused from X10 magnification up to X40 magnification. The conditions were set automatically in accordance with the fluorescence dye in the scanning unit and the individual bandwidths optimised to detect fluorescence for each channel. The most suitable imaging conditions based on the fluorescent dye selection using the acquisition software. Time-lapse mode was selected and the areas to be imaged were chosen by focusing on the cells, labeling and setting the X and Y parameters. Images were set to be taken every 5 minutes over 24 hours.

3.21 Tracking EN2 in real-time using NanoGlo assay

NanoLuc® (Nluc) is a smaller tag, only 19kDa, highly stable, ATP independent, bioluminescent protein and thus, a highly sensitive screening system when used with the Nano-Glo® Luciferase Assay. The Nano-Glo® Luciferase Assay generates a glow-type signal in the presence of NanoLuc® luciferase by simply mixing substrate and buffer together. The reagent contains a lysis buffer, which
allowed the measurement of NanoLuc®EN2 directly in cells or the culture media when NanoLuc®EN2 was secreted.

3.21.1 Cloning EN2 into NanoLuc vector to create NanoLuc-EN2

The HaloTag®-EN2 FlexiVector (Section 3.14; pFN21A HaloTag® CMV-amp) and the native NLuc® FlexiVector® (pFN31K Nluc CMV-neo) from Promega (UK) both contain rare restrictions sites SgfI-Pmel as part of the FlexiVector system. This system provides an easy method for directional cloning of protein coding regions between N-terminus tagged (such as the HaloTag®-EN2 FlexiVector®) and native FlexiVectors (such as the NLuc® FlexiVector®), as depicted in Figure 3.5. The native vectors also contain a lethal barnase coding region that means there is a positive selection for those that have inserted the protein-coding sequence as expression of barnase is lethal to bacteria such as E.coli and thus, the cloning system is highly efficient. The donor vector HaloTag®-EN2 contains the resistant gene to ampicillin antibiotic and was amplified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, UK) and eluted at approximately 100ng/µl. The acceptor vector was native NLuc® (Promega, UK; 100ng/µl) contains the resistant gene to kanamycin antibiotic.

Figure 3.5. Transferring protein coding regions in the Flexi® Vector Systems. Protein-coding regions can be shuttled between vectors using two rare-cutting restriction endonucleases, Sgf I and
The Flexi® Vectors contain a lethal gene, barnase, for positive selection of the protein-coding sequence and an antibiotic resistance marker for selection of colonies containing the Flexi® Vector. Taken from the Flexi® Vectors Systems technical manual at www.promega.co.uk.

The protocol was followed according to the manufacturers instructions. To summarise: both the donor and acceptor vectors were digested and ligated in the same reaction, the vectors that retained the lethal barnase gene were eliminated once the reactions were transformed into E.coli. The E.coli transfected with the donor vectors were positively selected by plating the reactions onto kanamycin plates. A 'background' control was included where the acceptor vector only was digested, ligated and plated onto a kanamycin plate, the vector does not have the kanamycin resistant gene and so there should have been no growth. The ligation reaction was then transformed into One Shot® TOP10 Chemically Competent E. coli (Life Technologies, UK) and plated onto LB plates supplemented with 25µg/ml kanamycin antibiotic (Sigma-Aldrich, UK). At least 4 colonies were screened by digesting with Sgfl and PmeI and ran on a 1.2% pre-cast E-gel (Invitrogen, UK) to make sure the insert was at 1 Kb (EN2). Furthermore, sequencing primers, recommended by Promega (UK), were designed to anneal to the the promoter (Flexi R2) and NLuc tag to ensure the insert was fully sequenced and that it had the correct tag. These were sent to Cambridge Sanger Sequencing (UK) for DNA sequencing.

3.21.2 Nano-Glo® Luciferase Assay for tracking NLuc®-EN2

First, the NLuc®-EN2 construct was transfected into LnCaP cells and a WB was carried out in order to evaluate the size of the protein. The anti-NLuc® antibody was a kind gift from Promega (USA). The protocol outlined in Section 3.6 was loosely followed, except the membrane was blocked overnight with TBST + 5% BSA. After 3 washes, the membrane was incubated with Rabbit anti-Nluc IgG (1:5,000) overnight (in TBST + 5% BSA). After 3 washes (TBST) the blot was incubated with anti-rabbit IgG-HRP (1:2,500) for 60 min (TBST + 5% BSA), washed 3 times with TBST, and developed for 5 minutes with West Pico chemiluminescence substrate (Life Technologies, UK). Images were taken using ChemiDoc-It2 (UVP, UK). The size of the proteins were determined by comparing to the prestained markers.
The LnCaP cell line was then transfected with serially diluted NLuc®-EN2 DNA plasmid and the Nano-Glo® Luciferase Assay was performed on the cell culture media, 24 hours later, according to the manufacturers instructions. In summary: first, the Nano-Glo® Luciferase Assay Reagent was prepared by combining one volume of Nano-Glo® Luciferase Assay Substrate with 50 volumes of Nano-Glo® Luciferase Assay Buffer; cells were removed from the tissue culture incubator and gently shaken (100rpm for 2 minutes) to remove concentration gradients that may have existed in the culture medium; 10µl aliquots for each sample were dispensed in triplicate to a white 96-well plate and these were made up to a final volume of 100µl with distilled water; 100µl Nano-Glo® Luciferase Assay Reagent was added to each well (equal to the volume of the sample) and mixed for optimal consistency and after 3 minutes the luminescence was read by the Variskan® Flash plate reader.

3.22 Antibody internalisation assay

The pHAb amine reactive dye (Promega, UK) are pH sensor dyes that have very low fluorescence at pH more than 7 and undergo a dramatic increase in fluorescence as the pH of the solution becomes acidic (less than 7).

First, the antibodies to be tested for internalisation were conjugated to the pHAb amine reactive dye. All solutions were made and protocols carried out according to the manufacturer’s instructions via the on-bead conjugation method. Once completed the Dye-to-Antibody (DAR) ratio was calculated using the following formulae:

- Antibody Concentration (mg/ml) = $A_{280} - (A_{532} \times 0.256)/1.4$
- DAR = $(A_{532} \times 150,000)/(\text{Ab Concentration (mg/ml)} \times 75,000)$

Where the molecular weight of antibody is 150,000, the extinction coefficient of pHAb Reactive Dye is 75,000 and the correction factor for pHAb Reactive Dye is 0.256.
To show that the antibody was still intact, a small volume was denatured for 10 minutes at 95 °C with (LDS) Sample Buffer (4X; Life Technologies, UK) and 2µl NuPAGE Sample Reducing Agent (10X; Life Technologies, UK). The denatured antibody was then loaded onto a NuPAGE® Novex 4-12% Bis-Tris gel and run for 50 minutes at 120V. After which, SimplyBlue™ SafeStain (Invitrogen), to visualise protein bands, was then poured over the gel on a rotator and left to incubate for an hour or more. An image was then taken when the heavy and light chains of the antibody became visible. Once this was established, cells were seeded at 1x10⁴ cells/well (100μl) in normal culture media in a flat, clear bottom black 96-well black plate (Figure 3.6).

**Figure 3.6. The plate layout of the antibody internalisation assay.** A black plate was required for fluorescence reading of pHdye-Ab that fluoresces when the pH lowers such as an endosome after the antibody is internalised. The clear bottom of the wells allowed the cells to be imaged by a confocal microscope and the viability of the cells to be assessed by a light microscope.

Once the cells had adhered, the antibody was initially vortexed and spun down to get rid of antibody aggregates and 15nM of it was added per well and directly to the culture media. Cells were incubated, 37°C, 5% CO2, inbetween reading the fluorescence at excitation wavelength: 532nm and emission wavelength: 560nm using the Variskan® Flash plate reader (Thermo Scientific, UK).
3.23 Exosome isolation and analysis

All cell culture media used in the following experiments had no FBS - FBS contain exosomes that would contaminate the experiment - and were incubated with cells in T-75 flasks for 48 hours prior to use.

3.23.1 Ultracentrifugation

The protocol was adapted from the methodology outlined by Thery et al. (Théry et al. 2006) and depicted in Figure 3.7 below.

Figure 3.7. Flow chart for the exosome purification procedure. Taken from (Théry et al. 2006).

The 10,000 x g for 30 minutes step was not carried out and instead the samples were concentrated using Amicon Ultra-15 tube (Merck Millipore, UK) with a
100 MWCO and filtered through a 0.2 μm filter to remove particles larger than 200 nm. The samples were imaged by electron microscopy (Section 3.23.4).

3.23.2 Norgen exosome isolation

The Norgen’s Exosome RNA Isolation Kit (Norgen, Canada) is based on spin column chromatography that employs Norgen’s proprietary resin. The protocol was followed according to the manufacturer’s instructions however, the kit does not leave the exosomes intact in order to obtain the RNA so it was at this point the protocol was stopped and so the exosomes remained intact.

3.23.3 Size exclusion chromatography (SEC)

qEV size exclusion columns (Izon, UK) contain a resin with an approximately 75 nm pore size. Proteins and other contaminating molecules smaller than EVs enter the pores of the resin and are delayed in their passage through the column, eluting in later fractions. The protocol was followed according to the manufacturer’s instructions. The cell culture media samples were overlaid on qEV size exclusion columns followed by elution with PBS. 500μl fractions were collected, and particle and protein concentrations determined by Nanoparticle Tracking Analysis (Section 3.23.5).

3.23.4 Electron Microscopy (EM)

This was carried out by David Jones within the MicroStructural Studies Unit at The University of Surrey using a scanning electron microscope (SEM). The samples were prepared as outlined in (Théry et al. 2006). In summary, the isolated pellets (either from Sections 3.23.1, 3.23.2 or 3.23.3) were resuspended in 50μl of 2% PFA, a drop (approximately 5μl) of each sample was placed onto clean Parafilm (Sigma-Aldrich, UK) and the Formvar-carbon coated EM grids (Agar Scientific Ltd, UK) were carefully placed on a drop using a sterile Dumont no. 5 forcep (Agar Scientific Ltd, UK), with the coated side facing the suspension, and left to dry for 20 minutes. The grids were then transferred to float on top of 100μl PBS drops to wash and then to a 50μl drop of 1% glutaraldehyde (Sigma-Aldrich, UK) for 5 minutes, which ‘fixed’ the samples.
3.23.5 Nanoparticle Tracking Analysis (NTA)

This was carried out by Dr Rebecca Townsend within the Department of Mechanical Engineering Sciences at The University of Surrey using a NanoSight machine (NanoSight LM10; Malvern, UK). The instrument is able to analyse the size distribution and concentration of all types of nanoparticles from 10nm to 2000nm in diameter. Samples were sonicated for 5 minutes in order to break up aggregated particles just before they were injected into the machine.

3.24 Apoptosis evaluation after forced EN2 expression

3.24.1 Caspase-3/7 detection

CellEvent® Caspase-3/7 Green ReadyProbes® Reagent (Life Technologies, UK) is a fluorogenic, no-wash indicator of activated caspase-3/7 for live- and fixed-cell applications. Activation of caspase-3 is an early indicator of apoptosis.

Time-lapse confocal microscopy was set up as outlined in Section 3.20, cells were seeded in the 35mm Glass-bottom dish, transfected with EN2 plasmid from Section 3.7. Two drops of the ReadyProbes® Reagent was added per ml of FluoroBrite™ DMEM media and this was incubated for up to 1 hour at 37°C before imaging the cells.

3.24.2 Cell titre 96® aqueous non-radioactive cell proliferation (MTS) assay

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega UK) is a colorimetric method for determining the number of viable cells in proliferation. The assay contains an MTS (3-(4,5- dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) compound and an electron coupling reagent phenazine ethosulfate (PES). PES combines with MTS to form a stable solution, MTS is bio-reduced by cellular oxidoreductase enzymes into a coloured formazan product, which can be measured by absorbance at 490nm and is directly proportional to the number of living cells in culture.
The cells were seeded in a 96-well plate and transfected with EN2 plasmid from Section 3.7 and using the same protocol. A duplicate plate was made in order to assess EN2 overexpression by RT-qPCR (Section 3.4). Each sample was carried out in triplicates, unless otherwise stated. The MTS reagent was made up in RPMI-1640 media with only 2% FBS (as it contains less phenol red that could interfere with the colorimetric assay) and hence there was a media only control with cells and a media only control without cells, the latter would be the ‘background’ control to be taken away from all readings.

3.25 Investigating the cell autonomous (transcriptional) effects of EN2

The relative expression of Inhibin Beta A (INHBA), Collagen Type VIII Alpha 1 (COL8A1) and Transmembrane protein 204 (TMEM204), in prostate cell lines both transiently and stably expressing EN2 was determined. These were just a few genes aberrantly expressed as part of a microarray carried out in the Oncology department. The microarray was carried out in order to investigate gene expression after the overexpression of EN2 in ovarian cancer cell lines (McGrath 2015). The microarray results suggested a role for EN2 in cell invasion and metastasis, particularly in the process of epithelial-mesenchymal transition (EMT). Furthermore, EN2 has been implicated in this study in cell fusion and cell-in-cell action and all three genes (INHBA, COL8A1 and TMEM204) have a role in cell adhesion. The RT-qPCR was carried out as detailed in Section 3.4.

3.26 Co-culture

3.26.1 WPMY-1 incubation with conditioned media incubation over time

To generate the conditioned media the cells were grown in T-25 flasks with 10ml cell culture working media without FBS for 48 hours. The media was collected and initially spun down to get rid of any cellular debris. The media was then transferred to an Amicon Ultra-15 tube (Merck Millipore, UK) with a 10 molecular weight cutoff (MWCO) and so anything larger than 10 kDa was retained. WPMY-1
were seeded in a 96-well plate and subsequently incubated with 100µl of conditioned media. After 24, 48 and 72 hours incubation the viability was measure using the MTS assay, as outlined in Section 3.24.2. Two technical repeats were performed for each conditioned media.

### 3.26.2 Direct co-culture using stable cell lines

All stable cell lines were produced as outlined in Section 3.13. The following stable clones were created for WPMY-1: HaloTag (native) and LifeAct® (Ibidi, Germany), which highlighted the cytoskeleton red as visualizes F-actin within fixed and living cells and does so without compromising cellular processes. For PC3, stable LifeAct® clones were created. All cells were seeded in 6-well plates at half the normal density (1x10^5 cells/well) in normal DMEM culture media. The TMR ligand (Promega, UK) was required to enable the imaging of the HaloTag protein under fluorescence and this was carried out according to manufacturer’s instructions. The images were taken using a fluorescence microscope at X20 magnification. NucBlue® Live ReadyProbes® Reagent was added to demarcate the nucleus. The media was subsequently changed to fresh media every two days.

### 3.26.3 Transwell and Nano-Glo® Luciferase Assay

Essentially this experiment mimicked the direct co-culture assay in Section 3.26.2, as the two cell lines shared the same medium however, the donor (Nluc-EN2 expressing) cells and the acceptor cells were separated by a membrane. The ThinCert™ Cell Culture Inserts (Greiner Bio-one, Germany) were employed and these had a 0.4µm pore-sized polycarbonate membrane to ensure that proteins could freely pass through the membrane but the cells could not. The donor cells were initially transfected with Nluc-EN2 and seeded in 6 well plates before being reseeded in the hanging insert (within a 24-well plate). An outline of the protocol is shown in Figure 3.8.
Figure 3.8. Evaluating intercellular transfer of EN2 in prostate cell lines by Transwell® (ThinCert™) assay. The setup separated the donor and the acceptor cells and so for EN2 to transfer between cells it would have had to do it without direct cell-cell contact. The transfer of Nluc®-EN2 was measured using the Nano-Glo® Luciferase Assay at different points in the experiments, outlined on the image. In order to know the amount of Nluc®-EN2 that was available in the media, a control was carried out in which there were no cells seeded at the bottom of the 24-well plate and would act as a reference. To normalise the difference in the rate of secretion and internalisation of proteins a positive control was employed, secNluc (Promega, UK) that was constitutively secreted and transfected into cell lines at the beginning of the experiment.

3.28 HXR9 treatment

The treatment was carried out as described by Morgan et al. where HXR9 and CXR9 (control peptide) were incubated with PC3 and WPMY-1 cells (in vitro) for 2 hours only (R. Morgan et al. 2014). In addition, stably expressing PC3 GFP-EN2
and GFP cell lines were used in this study. The MTS was carried out as described in Section 3.24.2. A dose-response curve was plotted, which indicated the percentage cell survival relative to increasing concentrations of HXR9 (and CXR9).

3.29 Data analysis

All values are carried out as duplicates for two technical repeats and two biological repeats to calculate the mean value and ± standard error (SEM) where possible. The GraphPad Prism software was used to draw the graphs and to apply a p value, usually by a Student’s t Test, where possible as follows p<0.05=*, p<0.01=**, p<0.001=***. Many of the biological repeats were analysed at the protein level in a qualitative form such as ICC and western blot, no quantifications were made and therefore no statistical model could be applied.
Chapter 4:
Investigating the expression of EN2 in prostate cancer
4. Investigating the expression of EN2 in prostate cancer

4.1 Introduction

This chapter presents the current understanding of EN2’s expression and localisation in development and cancer and, more importantly, the areas requiring further exploration. Following this, the objectives and hypothesis are stated. The experimental results are analysed and discussed. These emphasise that EN2’s expression and localisation in cancer cells are still not well-defined and this is partly due to the ambiguous nature of the antibody used against EN2. It is concluded that EN2’s potential as a therapeutic target remains undecided and a deeper understanding of its localisation and function is required before proceeding to develop an antibody-drug conjugate against EN2.

4.2 Background

EN2 in cancer falls under the unique umbrella of expression-restricted developmental proteins; reported to be both cell autonomous and non-cell-autonomous owing to its ability to be secreted and internalized (Joliot et al. 1998). EN2 expression is largely switched off after embryonic development though low levels remain in the adult nervous system, specifically in Purkinje neurons, for a purpose yet to be fully elucidated (for more details see Section 2.3.2). One hypothesis is that it inhibits the formation of neuronal dendrites through its ability to affect vesicle transport and cell morphology (Holst et al. 2008). Another report suggests a role in the continued survival of mesencephalic dopaminergic (mDA) neurons (Albéri et al. 2004). Elevated expression levels of EN2 at mRNA and protein levels have been reported in various cancers, which are listed in Table 5 (Section 2.3.3).

Potentially, there is huge scope for EN2 to be used as a cancer biomarker with the right detection assay. However, fundamental quantitation and definition has been difficult to obtain and interpret, largely due to the lack of understanding about its
function (for more detail see Section 2.3.2.1). Early cancer reports suggest that it is an oncogene and works to increase proliferation and aggressiveness of the disease (Martin et al. 2005; Bose et al. 2008). However, recent reports instead suggest that EN2 plays an anti-oncogenic role as more EN2 is detected in the surrounding tumour microenvironment than the tumour itself (Lai et al. 2014). Currently little evidence exists to show that EN2 expression correlates with increasing tumour grade but actually suggests the opposite: EN2 exogenous detection in biofluid surrounding the tumours increases with tumour volume and grade (Pandha et al. 2012; Guibinga et al. 2012). The extracellular detection is similar to PSA (see Section 2.3.2), detected instead in urine, and also provides rationale for further investigating EN2 as a diagnostic marker either alone or to supplement PSA.

Traditionally, polyclonal antibodies have been used as a generic means to detect and semi-quantify proteins, though in some cases these antibodies lack the necessary specificity (Lipman et al. 2005), further expanded upon in Section 2.2.4. Some of the EN2 commercial antibodies have been investigated and as a consequence an unspecific protein product, detected by a few but not identified, was dubbed ‘non-EN2’ (Lai et al. 2014), or a potential artifact that coincidentally matches the size of the IgG heavy chain. The goat anti-EN2 antibody used in this investigation was deemed to be specific to EN2. It is important to note, however, that it detects EN2 at 50kDa (results unpublished) instead of 40kDa, as previously reported in the literature (Martin et al. 2005). This study, foremost, sought to answer why there is apparent protein detection when there are no mRNA transcripts and to validate the goat anti-EN2 antibody.

If it is assumed that EN2 is the 50kDa protein band, instead of 40kDa, then EN2 must have tight regulation of expression, translationally or post-translationally, which differs from that in embryonic development. An inverse correlation between levels of EN2 promoter methylation (gene silencing) and levels of protein expression has been reported (James et al. 2013). This finding is unusual as gene silencing typically results in lower protein expression and further confirms EN2’s complex regulation. This inverse correlation is one of only a few published data available for EN2 promoter methylation status, the majority of which are in the context of neurological and developmental disorders. In addition, the research in this area has largely focused on EN1, where a similar pattern was found. The study revealed, in human salivary gland adenoid cystic carcinoma, EN1 high promoter methylation status with high protein detection (D. Bell et al. 2011). They hypothesised that this may be due
to a cyclical feedback mechanism. When a certain level of protein is obtained the promoter becomes hypermethylated; mRNA is no longer transcribed until the protein levels subsequently dip down to a certain threshold triggering promoter hypomethylation and mRNA transcripts are produced again. It is possible that EN2 is regulated under a similar feedback mechanism to EN1.

If EN2 is 50kDa then a possible explanation for the larger than predicted size is post-translational modification (PTM). PTM adds groups to proteins such as a phosphate group or a glycosyl group in order to control its localisation (Duan & Walther 2015). Investigations into post-translational modifications such as deglycosylation (that removes the glycosyl group) showed some shift in size but the results were not conclusive (McGrath 2015). It is possible that EN2 is regulated by microRNA's - another area of investigation left unexplored despite an EN2 targeting microRNA having been reported - miR181a (Guibinga et al. 2012). Another possibility is that EN2 has mRNA splice variants, though this is unlikely as EN2 has just two exons, but is yet to be investigated. Whilst most of these investigations are beyond the scope of this study, they will be addressed in the discussion as part of the results may support these.

Taking into consideration EN2’s ability to be secreted and internalised, it is then pertinent to ask: could protein isoforms exist? Is EN2 secreted in vesicles? Does EN2 undergo post-translational modification such as proteolytic cleavage and how much of what is detected at protein level is the result of internalisation or intercellular transfer?

De novo expression of fluorescently tagged EN2 allows EN2 to be tracked without using antibodies in order to further clarify its expression; subcellular localization; trafficking and regulation. These were mainly carried out through western blot and imaging experiments.

4.3 Chapter objective, hypothesis and approach

The following objective was made (taken from Section 1.5):

1. to determine EN2's expression and localisation within cancer cells.
In order to investigate this the following experiments were performed:

- Engrailed-2 mRNA expression by RT-qPCR was carried out in order to confirm the presence of higher EN2 mRNA levels in cancer cell lines compared to normal cells.

- Immunocytochemistry and western blot analysis of cell surface EN2 expression on cell lines was performed in order to confirm the RT-qPCR results and show higher levels of EN2 protein in cancer cell lines compared to normal cells.

- Goat anti-EN2 antibody validation was necessary to confirm the specificity of the antibody to EN2.

- Further investigation into the goat-anti-EN2 antibody specificity: Kyte-Doolittle Hydropathy plot.

- Further investigation into the goat-anti-EN2 antibody and the subcellular localisation of EN2 using GFP tagged EN2 de novo expression in prostate cell lines.

- House keeping gene optimisation as EN2 forced expression showed the creation of EN2-containing vesicles and so EN2 likely influences the cell’s cytoskeletal network of proteins including the house keeping gene beta-actin.

- Engrailed-2 primer design of exon-specific primers that are validated prior to RT-qPCR experiments and to explore the possibility of EN2 splice variants.

- GFP-EN2 stable expression for EN2 siRNA validation in order to ensure that the EN2 siRNA succesfully knock down EN2 at the protein level and confirm the detection of a unknown antigen at 50kDa.
- HaloTag® -EN2 expression in prostate cell lines for highly specific EN2 detection with the anti-HaloTag® antibody

- Identification of protease sites within EN2 protein to provide further rationale to the detection of cleaved fragments of EN2

- Goat anti-EN2 antibody re-evaluation as the 50kDa protein is not likely to be EN2

4.4 Results

It was imperative to reconfirm the findings that had been reported about EN2 in the literature in order to ensure that EN2 detection and expression patterns could be repeated in vitro, with prostate cancer cell lines. This began with qRT-PCR for EN2 mRNA detection and immunocytochemistry and western blot assays for EN2 protein detection. These included the necessary controls for quality assurance such as primer design, primer efficiency and further validation at protein levels with knockdown and overexpression experiments to determine antibody specificity.

4.4.1 Engrailed-2 mRNA expression by real-time quantitative PCR

EN2 mRNA expression was calculated from the extraction of total RNA and the synthesis of cDNA in order to quantify the amount of EN2 upregulated between cancer and normal (immortalised) cell lines (WPMY-1) and normal primary cells (fibroblasts) in Figure 4.1.
Comparing the cell lines in Figure 4.1, SKMEL-5 had considerably larger amounts of EN2 mRNA and WPMY-1 had no detectable EN2 mRNA. The fibroblasts had low amounts though some EN2 mRNA were still detectable compared to WPMY-1, which may have been a technical error as the primer pair was found to create primer-dimers and therefore this data is unreliable.

4.4.2 Immunocytochemistry and western blot analysis of cell surface EN2 expression on cell lines

The goat anti-EN2 antibody (Abcam) was used to develop the EN2 ELISA test (R. Morgan et al. 2011). In order to verify the EN2 RT-qPCR data in Section 4.4.1, EN2 protein expression in a number of cell lines was assessed by immunocytochemistry (ICC) using the goat anti-EN2 antibody. Subsequently, a western blot assay was performed to observe the size of the protein detected and verify the detection of EN2 (Figure 4.2).
Figure 4.2. EN2 detection in cancer cell lines and normal WPMY-1 cell line with goat anti-EN2 antibody. **a)** ICC cell surface staining (X40 magnification) of various cancer cell lines (labeled) and their ‘normal’ equivalent WPMY-1; derived from the same tissue or the periphery. **b)** Western blot analysis of EN2 in whole cell lysates, recombinant EN2 (rEN2) was the positive control and WPMY-1, PBMCs and fibroblasts (Fibros) were used as negative controls. α-tubulin housekeeping protein was chosen as the loading control.

In Figure 4.2, the negative controls (no primary antibody) gave no background or unspecific green staining produced by the secondary antibody. The secondary antibody is raised against the species of the primary antibody but it can sometimes detect different species or proteins (known as cross-reactivity) and
requires further dilution in order to prevent false-positive results. The top panel (a) showed the primary antibody detection of EN2 (c-terminus) on the cell surface of SKMEL5 and PC3 cancer cell lines. The normal (immortalised) cell line WPMY-1 revealed, subjectively, a lower amount of green staining compared to the cancer cell lines but nevertheless it was positive (Figure 4.2a). These observations were reproduced in the western blot analysis below (Figure 4.2b) - WPMY-1 was positive for the 50kDa band. A strong 50kDa band was detected in PC3 and LnCaP cell lysates only because a higher amount of protein was loaded onto the gel, revealed by the alpha-tubulin loading control. Healthy PBMCs were also positive for a band at 50kDa and much less was detected in fibroblasts.

4.4.3 Goat anti-EN2 antibody validation by EN2 plasmid expression and mRNA knockdown

These results (Section 4.4.2) indicate that the western blot data is spurious and the antibody may only be useful for immunocytochemistry, but required validation nonetheless. Consequently, the goat anti-EN2 antibody specificity was further tested. To carry this out EN2 was transiently overexpressed in WPMY-1 cell line (no EN2 mRNA detected). 0.1µg and 0.05µg of EN2 plasmid (Origene) was used for transfection before repeating the ICC and western blot assays with the goat anti-EN2.

![Graph showing EN2 overexpression](image)

![Images of transfection](image)
Transfection with an increasing amount of plasmid resulted in an increasing amount in EN2 mRNA expression (Figure 4.3a), which was reflected in the ICC (Figure 4.3b). To semi-quantify this and confirm the size of EN2, a western blot was performed showing that the over-expressed EN2 was detected at 41kDa - complementing the literature (Martin et al. 2005). However, the 50kDa band did not change and instead stayed uniform - this indicated the detection of a extremely stable isoform of EN2 (that was present in almost all cell lines) or unspecific binding by the antibody and was in need of further investigation.
To further confirm the specificity of the anti-EN2 antibody, siRNAs were employed as a negative control. The siRNA's were used to knock down EN2 mRNA in PC3 prostate cancer cell line (moderate EN2 mRNA expression).

a) 

![Graph showing EN2 knockdown in PC3 cells](image)

b) 

![Graph showing EN2 knockdown in PC3 cells](image)
Figure 4.4. siRNA mediated knockdown of EN2. a) with qPCR analysis of EN2 RNA expression (top) and subsequent protein analysis with goat anti-EN2 antibody (X40 magnification) (bottom). b) carried out in an 8 chambered slide with subsequent protein analysis with goat anti-EN2 antibody (X40 magnification); error bars represent the standard error of the mean (SEM) of two repeats. c) PC-3 cells underwent EN2 siRNA treatment with the 4676 EN2 siRNA for 48-96 hours, plotted both relative to B-Actin and GAPDH mRNA. The peaks on the graph are numbered 1-9 and correspond to the lanes on the western blot, found directly underneath; a negative (scramble) siRNA and media only repeats were carried out as controls.

Three different siRNAs were tried, and Figures 4.4a confirmed that an approximately 80% mRNA knockdown was achieved using the 4676 EN2 siRNA, and this was used for all subsequent knockdown experiments. An 8-chambered slide bypassing the need to trypsinise the cells showed a better reflection of the EN2 knockdown at the cell surface (Figure 4.4b) but was extremely subjective and far from conclusive. Subsequently, the experiment was repeated using a western blot
instead. The 50kDa band was not knocked down but again stayed uniform throughout all samples.

It is possible that mRNA regulation of EN2 (transcriptional control) is independent of protein regulation (translational control) or this particular protein isoform is highly stable. If it is the latter than either a longer knockdown assay or a stable knockdown should show a difference at the protein level. 96 hours after siRNA transfection again showed no reduction at 50kDa (Figure 4.4c). Only a 60% EN2 stable knockdown clone was obtained, which was not reflected at the protein level with the 50kDa band remaining (data not shown).

4.4.4 Further investigation into the goat-anti-EN2 antibody specificity: Kyte-Doolittle Hydropathy plot

Upon closer examination off the EN2 immunogen against which the goat anti-EN2 targets confirms that it binds close to the c-terminus. Analysis of the protein sequence by a Kyte-Doolittle Hydropathy plot reveals that, just before the c-terminus, EN2 is extremely sticky due to it being highly polarised, as demonstrated by the most negative hydropathy score (approximately -3.5) in Figure 4.5.

![Figure 4.5. Kyte-Doolittle Hydropathy plot (ExPAsy tool) of EN2 protein. Left - analysis of full-length EN2 protein. Right - analysis of the 100 amino acids at the EN2 C-terminus commonly used as the immunogen for commercial EN2 antibodies available.](image-url)
Occasionally, the size of the antigen targeted by an antibody raised against a specific target is too small and the specificity decreases. Thus, an antibody that targeted a larger antigen of EN2 (80 - 220 aa) that was centre of protein (away from the c-terminus) was tried on the positive control cell line: SKMEL-5 (Figure 4.6).

a) 

**EN2 siRNA knockdown in SKMEL5 cells**

![Graph showing EN2 siRNA knockdown in SKMEL5 cells.]

b) 

![Image showing media only, ViaFect™ only, Negative siRNA, and EN2 4676 siRNA with Gapdh loading control.]

Abnova mouse anti-EN2 (Randox) Gapdh loading control
Figure 4.6. EN2 siRNA knockdown in SKMEL5 cells for detection by mouse monoclonal antibody to EN2 (Abnova, UK). a) qRT-PCR confirming the mRNA knockdown of EN2 using a EN2 siRNA (4676) after 48 hours. b) A western blot using the protein lysate from cells treated with the EN2 siRNA after 48 hours. The controls were media only, ViaFect™ transfection reagent only and a negative (unspecific) siRNA.

However, three different band sizes were detected. The 40kDa band is the correct size for EN2, but no reduction was detected. Due to the lack of specificity to EN2 after the siRNA knockdown and the other two unspecified bands the antibody was not used any further.

4.4.5 Further investigation using GFP tagged EN2 de novo expression in prostate cell lines

At this point, an approach that did not rely on antibody detection was required and EN2 was studied by expressing it (de novo) in cell lines tagged to GFP. EN2 was initially cloned into a C-terminus GFP TOPO vector (Figure 4.7).
Figure 4.7. GFP-tagged EN2 expression in PC3 and WPMY-1 prostate cell lines. Both GFP-EN2 and EN2-GFP were transiently transfected into PC3 and WPMY-1 and a) were stained with WGA membrane dye, fixed and imaged by confocal microscopy at X20 magnification, b) the mRNA and protein lysate after 48hrs were collected for a RT-qPCR assay (top) and western blot assay (bottom). The peaks on the graph are numbered 1-8 and correspond to the lanes on the western blot, found directly underneath; rEN2 was used as a positive control. The western blot was probed with both the goat anti-EN2 antibody from Abcam and a GFP antibody.

Figure 4.7a shows that, for WPMY-1, EN2-GFP’s protein was localised at the nucleus in most cells, whereas, PC3 EN2-GFP expression was generally much less and vesicles could already be seen to be forming 24 hours post-transfection (Figure 4.7b and c). Unfortunately, the CT-GFP-EN2 expression was low and most commercial antibodies to EN2 bind to the c-terminus that would be obstructed by the bulky GFP - a limitation that should be taken into consideration at all times. Furthermore, the homeodomain and penetratin peptide is located at the c-terminus.
(Figure 2.13) where these are crucial to EN2’s ability to secrete and internalise - a GFP tag is likely to interfere with this.

Following N-terminal GFP-EN2 transient transfection, large amounts of EN2 mRNA were detected in WPMY-1 cells (Figure 4.8a), which subsequently underwent cell death (data not shown), addressed later in Chapter 5.

![GFP-EN2 overexpression graph](image)

![Western blot](image)
Figure 4.8. N-terminal GFP-tagged EN2 expression in prostate cell lines. GFP-EN2 were transiently transfected for 48 hours into a) LnCaP, PC3 and WPMY-1 cells and EN2 mRNA was detected by RT-qPCR and b) LnCaP and PC3 cells, the protein lysate was used for a western blot assay and probed with an anti-mGFP antibody. c) PC3, LnCaP, SKMEL5 and WPMY-1 cells and imaged by fluorescence microscopy at X20 magnification.

Similarly to the c-terminal GFP-tagged expression (Figure 4.7a), secretory vesicles, carrying EN2 were observed in PC3 cells, after N-terminal GFP-EN2 expression. These micro-vesicles are unique to PC3 and not detected in the other prostate cell lines. Prostasomes are large vesicles that have frequently been reported to be secreted from PC3 cells in vitro (Llorente et al. 2004; Sandvig & Llorente 2012).
Secretory vesicles containing EN2 in the micro-environment have not been observed in this way or reported in the literature and thus, will be further investigated in Chapter 5. In Figure 4.8b, the stable GFP-EN2 PC3 cell line shows a second band at 33kDa, as GFP is only 26kDa, as seen in the GFP lane, EN2 could be cleaved.

4.4.6 House keeping gene optimisation

Holst et al suggest a role for EN2 in cell morphology (Holst et al. 2008). A recent microarray (McGrath 2015) implicates a role in cytoskeletal re-organisation. Furthermore, the revelation that EN2 is secreted in vesicles meant that β-ACTIN, a cytoskeletal protein, may not be suitable as a RT-qPCR reference gene. Actin is fundamental to cytoskeletal rearrangement and vesicle formation (Sandvig & Llorente 2012).

Consequently, the geNORM kit (Primer Design Ltd) was employed to establish a more appropriate house keeping gene (reference) for normalisation.
12 reference genes were tested with a number of cell lines. The gene expression normalisation factor (M) is plotted against the set of reference genes.

The 12 house keeping genes were tested across a range of samples (Chapter 3, Section 3.4.2) and analysed by qbase+ software (Biogazelle, BE) which ranked the genes according to increasing stability (left to right). GAPDH and ATP5B showed the least variability between the samples tested. Therefore, these two HKGs were used thereafter for all RT-qPCR assays in order to accurately compare EN2 expression across all cell lines used in this study. GAPDH Ct values was used for all normalisation calculations unless it showed too much variability, at which point ATP5B was used instead.

4.4.7 Engrailed-2 primer design

To further investigate the discrepancies in EN2 protein size shown in Sections 4.4.3, 4.4.4 and 4.4.5 and to explore the possibility of different EN2 isoforms, exon-specific primer pairs were designed (Primer Design Ltd). This would also help to elucidate the possibility of EN2 mRNA alternative splicing.

Furthermore, ‘in-house’ intron-spanning (IS) primers produced an amplification efficiency (described in Section 3.4.1) of more than 105%, indicating
the co-amplification of an unspecific product (data not shown). The detection of low Ct values (or low mRNA detection) but high protein warranted the design of new EN2 primers pairs: exon-specific, in order to investigate splice variants, and intron-spanning (Primer Design Ltd), which are usually used to avoid detection of contaminating gDNA.

![Graph a) Standard curve - EN2 plasmid](image1)

![Graph b) Stable PC3 overexpression](image2)
Figure 4.11. Validation of exon-specific primers and intron-spanning primers. RT-qPCR were carried out in order to validate each primer by: a) generating a standard curve by EN2 plasmid serial dilution from 0ng to 10ng and plotting the raw Ct values against them; b) generating cDNA from EN2 overexpression assay using stably expressing PC3 GFP-EN2, PC3 GFP (control) and PC3 only (negative control), the Ct was normalised to GAPDH house keeping gene and the values were multiplied by 10000 and c) generating cDNA from EN2 siRNA assay, PC3 cells were treated with EN2 4674 siRNA, negative control siRNA (EN2 neg) and a media only (negative) control. The Ct values were normalised to GAPDH and multiplied by 100000.

The Exon 1 specific primers produced very high Ct values and this suggests that they detected few EN2 transcripts, Exon 2 specific primers produced the lowest Ct values and so detected lots of EN2 and intron-spanning detected EN2 at values in between these two primer pairs. Accumulatively, this would imply that the intron-spanning primer pair could not detect as many transcripts as the Exon 2 primers, perhaps due to improper splicing that would leave all or part of the intron sequence in the transcript and the primer pairs unable to anneal to it. Exon1 primers cannot pick up EN2 mRNA transcripts because the primers are unable to anneal to their target sequence; the standard curve revealed that the Exon 1 primers is inefficient as they could not pick up the EN2 plasmid and is most likely a sequence design issue (Figure 4.11b). The pattern of higher detection from Exon 2 over IS primers is unlikely to be gDNA contamination as DNase I (digests gDNA in sample) was used.
Next, all three primers were used to detect EN2 in a range of cell lines. SKMEL5 and HL-60 cancer cell lines were used as positive controls (previously shown in-house to have high EN2 protein). WPMY-1 and COS-1 are normal (immortalised) cell lines that were used as negative controls.
a) Exon1 specific primers

b) Exon2 specific primers

c) Intron spanning primers
Figure 4.12. EN2 mRNA expression in cell lines assessed by real-time quantitative PCR using validated exon-specific primers and validated intron-spanning primers. These primers have been validated using a standard curve and EN2 plasmid by Primer Design Ltd. They were designed to bind to either Exon 1 or Exon 2. Another intron-spanning primer pair were designed and validated, that would not detect any contaminating genomic DNA is detected.

SKMEL5 were shown to have the highest EN2 mRNA detection and PC3 (most aggressive) had the highest EN2 mRNA detection out of the prostate cancer cell lines. WPMY-1 and COS-1 were calculated to have no or very little EN2 mRNA, respectively. Using Exon 2 and IS primers (Primer Design Ltd) it was possible to detect increasing EN2 plasmid DNA, creating optimal binding efficiencies (Section 4.4.7). Exon 2 primers detected the highest expression of EN2 and were used thereafter.

It is still not clear whether mRNA splice variants exist for EN2 from Figure 4.12. It is interesting that the standard curve (Figure 4.11a) shows that IS primers detect the same amount of EN2 as the Exon 2 primer pair and notably, T24 showed no detection with IS primers but high detection with Exon 2 primers. The discrepancy between is inherent within the cell and is worth further investigating as another possible level of EN2 regulation. However, this study focused on EN2 as a protein biomarker and so progressed to look specifically at EN2 protein detection and regulation.

4.4.8 GFP-EN2 stable expression for EN2 siRNA validation

Now that a reliable RT-qPCR assay was established it could be used to reconfirm that a large percentage of EN2 mRNA was actually knocked down by the 4676 siRNA (Figure 4.13a). In addition, a stable GFPEN2 PC3 clone was employed as a positive control in order to verify that the 4676 siRNA resulted in a reduction of EN2 protein. Just 24 hours after siRNA transfection, there was considerable reduction in GFP fluorescence (Figure 4.13b). Importantly, the goat anti-EN2 antibody was able to detect this GFP-EN2 siRNA knockdown via western blot, at the correct size of 33 kDa (if the 33kDa of the GFP protein is taken away from the total
size of the band, which is approximately 66 kDa). However, it is further confirmation (to what was observed previously in Figure 4.2) of cross-reactivity by the goat anti-EN2 antibody and thus, it is likely that the antibody detects another antigen.

Notably, WPMY-1 failed to produce a stable GFP-EN2 cell line and this will be addressed in Chapter 6.
Figure 4.13. EN2 siRNA knockdown in PC3 GFP-EN2 stable cell line assessed by fluorescence and western blot. a) All three new primers designed by Primer Design Ltd were tried on the EN2 siRNA mRNA and all three show 80% or more knockdown of EN2 mRNA. b) This was repeated on PC3 GFP-EN2 stable cell line to test the siRNAs efficiency, after 48 hours these cells were imaged at X20 magnification using a fluorescence microscope. c) Protein lysates were obtained of each control and EN2 siRNA treated sample, twice for two biological repeats, and analysed by western blot assay (left) and probed for by the goat anti-EN2 antibody, the western blot was further analysed by densitometry, which measured the chemiluminescence of all bands that could then be plotted relative to its house keeping gene counterpart for accurate comparison. (1) EN2 4676 siRNA and the siRNA controls include (2) siRNA neg (scramble) and (3) media only, (4), (5) and (6) are biological repeats of (1), (2) and (3).

This result highlighted the efficacy of the siRNA, which can target and knockdown EN2 mRNA and subsequently knockdown the protein (that equates to 33kDa as part of GFP-EN2 and not 50kDa) at 66kDa (GFP-EN2) completely.
disappears when EN2 is expressed \textit{de novo} (Figure 4.13c, image on the left). However, the 50kDa does not decrease.

To make sure that no reduction occurred at 50kDa, the intensity was measured over the loading control. This time GAPDH was used as the loading control because EN2 has been implicated in cytoskeletal rearrangement and GAPDH had been shown to be one of the optimal house keeping genes in Section 4.4.6. Even still, no reduction was calculated (Figure 4.13b).

4.4.9 HaloTag\textsuperscript{®} -EN2 expression in prostate cell lines

To further investigate the potential cleavage of EN2 shown in Figure 4.8b, EN2 was N-terminally tagged to HaloTag\textsuperscript{®}, which has a high specificity monoclonal antibody for detection. This high specificity also meant that EN2 detection in the culture media was possible (Figure 4.14b and c).
HaloTag EN2 overexpression

EN2 Ct normalised to Gapdh (x100)

PC-3  LnCaP  Du145

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EN2 +</th>
<th>EN2 -</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>70</td>
<td>53</td>
</tr>
<tr>
<td>LnCaP</td>
<td>41</td>
<td>30</td>
</tr>
<tr>
<td>Du145</td>
<td>22</td>
<td>22</td>
</tr>
</tbody>
</table>
Figure 4.14. HaloTag®-EN2 expression in prostate cancer cell lines. 

a) PC3, LnCaP and Du145 were transfected with HaloTag®-EN2, HaloTag® control and media only control and analysed by RT-qPCR for EN2 mRNA detection (left), all values were normalised to Gapdh; the lysates were analysed with a western blot (right) and probed with an anti-HaloTag® antibody. 

b) The protein variants were further investigated by looking at their detection in both LnCaP cell lysate and culture media/ supernatant (super) as well a denatured and undenatured samples. 

c) To provide further confirmation that EN2 was being detected in lysate and culture media (super) from LnCaP (transfected with HaloTag®-EN2), antibodies against EN2 were used to detect the protein construct.

HaloTag®-EN2 was expressed in prostate cancer cell lines PC3, LnCaP and Du145 with RT-qPCR confirming expression. A western blot performed simultaneously revealed protein expression, HaloTag®-EN2 was detected at the correct size, approximately 70kDa, and HaloTag® alone at 35kDa. A similar pattern
to GFP-tagged EN2 expression emerges: high expression at the mRNA level results in relatively lower protein expression. Surprisingly, different sized bands are revealed, exclusive to HaloTag®-EN2, with LnCaP cells only. This time, EN2 is detected in the culture media although cleaved, as these bands are sized approximately 53, 45 and 41kDa and HaloTag® is only 35kDa.

To further investigate these variants, the western blot was repeated: denatured and non-denatured; with the HaloTag® to confirm EN2 specificity and was then further investigated with C-term and N-term specific anti-EN2 antibodies (Figure 4.14b and c). From denatured to non-denatured there was an increase in intensity of bands sized 70kDa and approximately 41kDa. The N-term specific EN2 antibody detects the cleaved bands in the lysate at 70, 53, 41k and 35kDa and supernatant at 41kDa only. This would suggest that the c-terminus of EN2 is being cleaved. However, it is clear that the EN2 antibodies are not exclusively specific to EN2 as Ab2 has detected the HaloTag® protein alone in the cell lysate only. Therefore, further confirmation is required by mass spectrometry.

4.4.10 Identification of protease sites within EN2 protein

To identify potential proteases and their cleavage sites within EN2 and to confirm, if any, the production of corresponding sized bands, EN2 protein sequence was ran through the PeptideCutter (ExPASy) program (Figure 4.15).

![PeptideCutter output](image-url)

- Cleaved by Aspartic protease after this residue (P1 position)
- Cleaved by Cysteine protease after this residue (P1 position)
- Cleaved by Metalloprotease after this residue (P1 position)
- Cleaved by Serine protease after this residue (P1 position)
- Cleaved by different multiple protease superfamilies after this position (P1 position)
Figure 4.15. Proteases cleavage sites within EN2 identified by PeptideCutter (ExPASy). a) EN2 protein sequence highlighted with enzyme cleavage sites, listed underneath. b) A list of positions and segments of EN2 to highlight where on EN2 MMP-9 could cleave it and the resulting fragment size.

The program confirmed that MMP-9 cleaves towards the N-terminus and would produce a peptide of the right size, 6.5kDa (Figure 4.15b). Other proteases cleave EN2 (Figure 4.15a) and so there are a few possibilities but this, at least, provides rationale for further investigation.

4.4.11 Goat anti-EN2 antibody re-evaluation

Knowing that EN2 detection should be approximately 33-40 kDa and is capable of being detected by the goat anti-EN2 antibody perhaps it was missed (Figure 4.16) because the band was ‘overshadowed’ by the 50kDa band thought to be EN2.
4.5 Discussion

The goals stated in Section 4.3 were to develop a more reliable assay for EN2 detection and quantitation in cell lines; to validate the anti-EN2 antibody; and to shed light on EN2’s regulation. In so doing, to gain a better understanding of its function and thus make better use of EN2 as a protein biomarker. It is not known how and when the cell starts secreting EN2 along the tumorigenesis timeline. Answers to these fundamental questions are undeniably pertinent to pursuing a more tailored and informative EN2 clinical test.

This study focused on looking at EN2 in vitro, limited by a 2D environment, and sought to answer whether endogenous EN2 protein is really being detected in
these cell lines. It also enquired as to why there is apparent protein detection when there are no mRNA transcripts; and ultimately intends to explain how EN2 could be tightly regulated.

ICC surface staining of cell lines with the goat anti-EN2 antibody revealed positive staining on all cell lines including a WPMY-1 normal cell line that had almost no EN2 or mRNA, and thus required further investigation. The western blot provided the same result: bands appeared at 50kDa with normal cell lines that were revealed to have almost no or very low levels of EN2 mRNA transcripts. Therefore, the antibody successfully detected EN2 but could not be used further to reliably discriminate EN2 regulation and quantitation between cell lines. For future studies, an EN2 CRISPR knockout kit could be employed, to knock the EN2 gene out completely (Sander & Joung 2014), this would then ideally be verified by mass spectrometry before using an EN2 antibody (Sections 7.4.2.1 and 7.4.2.2). Preferably, each antibody should be verified for its specificity this way otherwise it leads to questionable results, especially for EN2 antibodies that were recently investigated (Guan et al. 2014).

When comparing GFP-tagged EN2 between PC3 and WPMY-1 cell lines, it became apparent that EN2 was differentially regulated. PC3s were visibly unaffected, found to be true of all cancer cell lines used here, but EN2 caused a large amount of cell death to WPMY-1 cells after expression of GFP-EN2 (further explored in Chapter 6). PC3 cells were able to quickly down-regulate EN2 (as there was a reduction in GFP fluorescence) whereas WPMY-1 cells could not and this resulted in large EN2 overexpression at mRNA and protein levels, which was only detected in the nucleus. This suggests that the PC3 cells have mechanisms in place to down-regulate EN2, with regards to mRNA and protein levels, and translocate EN2 out of the nucleus (further explored in Chapter 6). Furthermore, GFP-EN2 transfection in PC3 cells did not reveal high protein expression via western blot, which supports the above finding. This pattern of expression differed to LnCaP cells, a less aggressive prostate cancer cell line, where high EN2 protein was detected. However, a stable PC3 GFP-EN2 cell line was able to be produced, which resulted in higher protein detection, presumably because of EN2's constitutive expression. Furthermore, the stable cell line also revealed another possible level of EN2 regulation, post-translationally, as you find an additional shorter band that suggests that the protein was cleaved (Figure 4.8b).
The pattern of protein expression mentioned above is reproduced with HaloTag®-EN2 expression: high mRNA expression in PC3 and LnCaP that produces low protein in PC3 cells and high protein detection in LnCaP cells (Figure 4.14a, image on the right). It is interesting to compare EN2 de novo expression between the most aggressive cell line: PC3; and the least aggressive, LnCaP. This expression reveals that EN2 is regulated differently between cell lines. LnCaP reveals high protein expression, both lysate and supernatant (via western blot) but also highlights the large amount of proteolysis EN2 underwent - shown by the production of multiple bands (Figure 4.14a, right image). This supports earlier findings that EN2 is cleaved within these prostate cancer cell lines (Figure 4.8b) - as a post-translational means of regulating EN2. Exploring this, by revealing potential cleavage sites, further showed that EN2 is actually highly susceptible to a number of proteases. Bands depicting the correct size on the western blot can be produced this way, notably through MMP-9 cleavage. However, further confirmation is required by cutting out the bands for mass spectrometry due to EN2 antibody ambiguity. If this is true and EN2 is largely cleaved, it would be important to discover whether this is by a regulated or random process as it would have important implications to the ELISA test (or any detection test) whereby EN2 is detected in urine. Employing antibodies, especially monoclonal antibodies, to detect a cleaved target is not the most efficient method as the are made using only one B lymphocyte and all detect the same epitope in the native conformation (Section 2.2.4).

Exclusive to PC3, EN2 is secreted in microvesicles (further explored in Chapter 5). This secretion is perhaps another means of decreasing EN2 post-translationally. The other cell lines in figure, including LnCaP, showed no signs of vesicle formation. Overall, these results show that PC3 cells - the most aggressive cell line - and to a lesser extent, the other cancer cell lines, do not readily express and/or retain EN2 protein - they instead immediately dispel EN2. This would suggest that the protein is redundant in these cancer cell lines or play a role outside the cell. The large amount of proteolytic cleavage found with LnCaP cells suggests that it is likely to be the former, however, the encapsulation within vesicles formed by PC3 cells suggests the latter, and the vesicles could be used as a means of protection from proteolysis. Surprisingly, EN2 detection in the supernatant was only found with LnCaP, albeit cleaved, and not PC3. Perhaps, with PC3, EN2's protection by these
robust microvesicles made EN2 inaccessible or kept the protein in a native state that was simply unable to be detected by the antibody.

It was important to establish EN2 detection within culture media or supernatant (Figures 4.14b and c) as this formed the basis of the EN2 ELISA (R. Morgan et al. 2011). This confirmed that some EN2 must be released freely and without association to vesicles, although this is seemingly cell dependent. The antibodies used thus far may not have been sensitive or specific enough to detect what little of full-length (and free) EN2 is secreted into the environment with in vitro conditions and this is presumably a lot less than in vivo conditions.

The only way to be sure that these bands (detected with HaloTag®) are cleaved EN2, is to carry out mass spectrometry. In addition, future work would need to confirm the cleavage of EN2, without the tag, to rule out the chance that the tag itself causes an unusual confirmation of EN2, which then exposes it to proteolytic cleavage. Overall, this provides good evidence that EN2 cleavage could influence EN2’s translation into the clinic and is therefore worth further investigating.

Unfortunately, if the 50kDa protein band is confirmed to be unspecific binding, it is possible that endogenous EN2 has not been successfully detected here within these cell lines. EN2 protein is not expected to be present in normal cell lines and perhaps not in cancer cell lines if EN2 is secreted quickly and efficiently in late-stage tumours; and within high-density vesicles (Nédélec et al. 2004). EN2 seemingly becomes redundant or switches to a role outside the cell, such as cell-cell communication, and so more should be detected exogenously. However, without reliable antibodies, this is difficult to quantitate and tagging EN2 comes with its own set of limitations such as GFP interference with structure and function as a bulky protein and toxicity due to fluorescence (Jensen 2012). EN2’s tight regulation still seems to be keeping mRNA expression, perhaps low EN2 protein expression, within these cancer cell lines, perhaps too low or varied to be detected by methods that are not very sensitive (for example antibody-based detection assays). This is especially true when taking into consideration the finding here that EN2 is regulated post-translationally and likely to be cleaved. The shRNA clones made in this study underwent cell death after only a few passages (no data was able to be collected). Similar findings have been made with EN1 where blocking peptides against EN1 caused a rapid and strong apoptotic response in tumour cells (A. S. Beltran et al. 20163).
implicating EN1 as a pro-survival factor. This might be why 100% knockdown of EN2 has been difficult to achieve thus far.

**Figure 4.16** shows bands that may be endogenous EN2 (40kDa) within these cancer cell lines that have been shown to have high amounts of EN2 mRNA. Thus, one possibility about the lack of detection by the goat anti-EN2 antibody at the correct size is, the antibody preferential binds to the ‘unknown target’ over the small amount of EN2 protein within these cell lines and EN2 is consequently overlooked.

Ideally, to study EN2 protein dynamics (shown here to be highly complex), an *in vivo* or 3D cell culture environment is required as 2D *in vitro* cell culture is far too limited - especially when studying a protein that undergoes intercellular transfer - and thus prevents proper observation of EN2 regulation. Furthermore, due to the ambiguous nature of the antibodies available commercially (Guan et al. 2014), mass spectrometry should be employed for accurate and reliable quantitation; and antibody validation.

### 4.6 Conclusion

This preliminary investigation has indicated that EN2 protein is ectopically expressed in cancer and is subsequently downregulated at a later stage. The *in vitro* experiments carried out here suggest that this is mostly by post-translational mechanisms and extracellular transfer with increasing grade or aggressiveness (also referred to as metastatic potential). Consequently, only a low amount of full-length EN2 remains within these cancer cell lines, this however, has been overlooked due to unspecific binding by the goat-anti-EN2 antibody and has led to significant confusion. We show here that the goat anti-EN2 antibody binds to an unspecific protein product that needs to be verified by mass spectrometry. These findings here have serious implications for the use of EN2 as a protein biomarker, this is especially true if future work confirms that EN2 is cleaved and cleaved randomly.
Chapter 5:
Exploring the potential of EN2 a target in prostate cancer
5. Exploring the potential of EN2 a target in prostate cancer

5.1 Introduction

In this chapter the reasoning behind EN2 as a candidate target for monoclonal antibody therapy is explained. The following section sets out the objectives and hypotheses. Section three presents the results obtained, which overall reveals that more work is needed to ensure EN2 is suitable as a target for a monoclonal antibody drug-conjugate, particularly for high grade tumours. The discussion brings together the findings of this chapter and proposes other avenues of immunotherapy that could be explored and that the significant gap in the understanding of EN2’s function, particularly in cancer, is obstructing its utility.

5.2 Background

The rationale behind EN2’s potential utility as a cancer target for immunotherapy originated from an immunohistochemistry staining of prostate cancer tissue, where EN2 seemed to be located within microdomains at the membrane (R. Morgan et al. 2011). There have been several reports of EN2’s subcellular localisation within the cell membrane. These include an association of EN2 with caveolae-like domains (Joliot et al. 1997). Joliot et al. drew similarities between neuronal microdomains and the apical domain of epithelial cells to show a common pattern of localisation and a possible role of EN2 in axonal transport.

Joliot et al. also stated that the importance of the tissue and cellular distribution of EN2 had been overlooked due to a lack of specific EN2 antibody. Thus, cellular distribution is a focus of this chapter. However, the report also noted that only a small fraction of EN2 was associated with these domains, making it more difficult. Furthermore, an even smaller fraction of EN2 is known to be secreted and protected from proteinase K or trypsin proteolysis. In Chapter 4 (Figure 4.14) the proteolysis of EN2 in LnCaP cells (and PC3 stably expressing GFP-EN2) and the
encapsulation of EN2 in microvesicles within PC3 cells were reported. These results therefore agree with the conclusions of Joliot et al. that only a small percentage of full-length EN2 is secreted and accessible to the environment (Joliot et al. 1997). It also highlights the importance of defining the EN2 antigen that is accessible from the environment in order to effectively engineer a monoclonal antibody-drug conjugate. The antigen is likely to be at the C-terminus: L Carlier et al. placed the EN2 homeodomain into a membrane-mimetic environment and observed the tertiary structure unfold but not the secondary, which allowed the Trp-48 to insert directly into the membrane; Trp-48 is located towards the C-terminus (Carlier et al. 2013). However, it is possible that proteolysis is a mechanism that allows for the controlled release of EN2 into the environment:

‘A second possibility is that it reflects the capability of homeoprotein isoforms or fragments to be released from cells and passaged into other cells through a paracrine mechanism.’ (Derossi et al. 1994).

This could be a problem especially when targeting EN2 with a monoclonal antibody, which recognises only one epitope.

EN2, intriguingly, has a nuclear localisation signal (NLS) and a nuclear export sequence (NES) but no classical secretory signal though it is still capable of translocating out of the cell. EN2 most likely resides within the cytoplasm prior to being trafficked to the membrane, from which some is subsequently released into the environment (R. Morgan et al. 2011). However, there is little consensus as to how this final step is achieved, although it may involve association of EN2 with microdomains (R. Morgan et al. 2011), cavealoe-like domains or luminal vesicles (Prochiantz & Joliot 2003). The mechanism is still poorly characterised and this is mainly because research has focused on the utility of the penetrating peptide (third helix) as an intercellular carrier (Prochiantz 2010) rather than its function in development and disease.

The membrane penetrating part of EN2, mentioned above, has been pinpointed to the third helix of its homeodomain (Derossi et al. 1994). As this is the most conserved region of all the homeodomains, it was hypothesised that this property (of intercellular transfer) could be common to all homeodomain containing proteins, and subsequently additional homeodomain-containing proteins including OTX2, ISL-1 and VAX1 have indeed been shown to be secreted from cells (Kim et
More recently, it was shown that the whole of the homeodomain was required for efficient EN2 secretion (Carlier et al. 2013). Substantial evidence of EN2’s ability to be secreted exists, however, much less is known regarding its internalisation, mainly how it is taken up by the cell and why. The ambiguous nature of its translocation mechanism (and the unspecific nature by which it is subsequently internalised). It is conceivable that EN2 transport might involve exosomes or other microvesicles such as prostasomes that are thought to be mediators of intercellular communication (G. K. Ronquist et al. 2012). It would be advantageous as EN2 would no longer be susceptible to proteolysis, which bypasses the problem associated with EN2 ‘stickiness’. If EN2 were not encapsulated the paracrine function would be inefficient because the protein is too sticky and too susceptible to proteolysis. It has been reported that EN2 internalisation can happen at 4°C and 37°C though this it is not via the conventional endocytic pathway and is receptor-independent (Maizel et al. 2002). It could be a highly unspecific mechanism and therefore might be problematic when injecting an antibody-drug conjugate (ADC) to target EN2 that may end up in a normal cell. With this in mind, the next question is: Is EN2 internalisation targeted or non-targeted?

The rationale behind EN2 as a therapeutic target also comes from an investigation into its immunogenicity, which is the ability to induce a systemic immune response (Annels et al. 2014). This study was able to show that autoantibodies against EN2 were present in the sera of patients but not healthy volunteers. It was found that the quantitation of such was not useful as a diagnosis or prognosis tool. However, EN2 was naturally able to produce an immune response. If it were the case that the antibody-drug conjugate was not internalised and therefore could not kill the cancer cell than these findings would provide a rationale to explore other immunotherapy options. This could include using an anti-EN2 antibody for its binding specificity and modifying the other end, not with a drug, but with the aim of boosting an already existing immune response and homing T cells to the tumour site.

The paracrine activity of homeoproteins (including EN2) have been studied in detail (Joshi, Ibad, et al. 2011; Layalle et al. 2011). EN2 has been reported to bind to PBX, non-cell autonomously and during development, to pattern the brain (Rampon et al. 2015). This would suggest that EN2 secretion, in prostate cancer, is regulated and carries out a specific function. Regardless, an EN2 ELISA detection assay has
been developed to detect secreted EN2 in urine and by doing so detecting prostate cancer with a sensitivity and specificity of 66% and 88.2% respectively (R. Morgan et al. 2011). Without a defined role it is difficult to know how best to utilise EN2. EN2, for now, could fit into a panel of biomarkers to better facilitate the diagnosis of prostate cancer. To make advances in this area, this study sought to define EN2’s regulation and function. By knowing how EN2 relates to cancer (cell) progression makes EN2 much more useful by gaining more information than simply the presence or absence of cancer, such as prognosis.

Ultimately, this present study sought to confirm and quantify the presence of EN2 on the cell membrane to further validate it as a target for an antibody-drug conjugate; mainly through immunocytochemistry and live cell imaging experiments. In addition, it sought to identify a suitable antibody, which recognised a small antigen on EN2, to take forward (and engineer into an ADC).

5.3 Chapter objectives, hypotheses and approach

The objectives of this chapter (taken from Section 1.5) were to:

1. determine EN2’s expression and localisation within cancer cells.

2. further define EN2’s secretion and internalisation mechanisms.

3. identify a suitable EN2 antibody for development into an antibody-drug conjugate.

4. carry out preliminary in vitro experiments on the candidate antibody-drug conjugate to direct the next set of experiments.

In order to investigate these the following experiments were performed:

- Co-localisation analysis by immunocytochemistry using the goat anti-EN2 antibody and a membrane marker, the combined fluorescent signal would be confirmation that EN2 can be found on the membrane
Subcellular fractionation by ultracentrifugation and western blot analysis of PC3 and WPMY-1 cell lines using the goat anti-EN2 antibody, to semi-quantitatively measure EN2’s presence on the membrane

Confirmation of the subcellular localisation of EN2 using GFP-EN2 de novo expression, due to the unspecific nature of the goat anti-EN2 antibody, using confocal microscopy co-localisation analysis

Defining the extracellular epitopes of EN2 using polyclonal sheep anti-EN2 epitope-specific antibodies and immunocytochemistry, in order to identify the antibody to take forward and engineer into an ADC

Validation of sheep anti-EN2 antibodies by western blotting due to the unspecific nature of polyclonal antibodies and the chance of cross-reactivity

GFP-EN2 secretion and internalisation by PC3 cells to gain a better understanding of the limitations and challenges that may need to be overcome when using EN2 as a therapeutic target

pH-dye conjugated EN2 antibodies to determine EN2 antibody internalisation

Tracking EN2 secretion and internalisation from prostate cancer cell lines with NanoLuc® EN2 de novo expression and Nano-Glo® detection assay

Identifying GFP-EN2 containing microvesicles from PC3 cells

Tracking EN2 secretion and internalisation from prostate cancer cell lines with NanoLuc® EN2 de novo expression and Nano-Glo® detection assay
5.4 Results

5.4.1 Co-localisation analysis by immunocytochemistry using a membrane marker

Chapter 4 concluded that the subcellular localisation of EN2 was different between cancer and normal (immortalised) cell lines, and between the most aggressive and least aggressive prostate cancer cell lines. It was therefore important to observe its subcellular localisation in more detail, particularly as EN2 is a candidate target for therapy.

Figure 5.1. Investigating EN2’s subcellular localisation in vitro using immunocytochemistry and wheat germ agglutinin membrane marker. Immunocytochemistry was performed using the goat anti-EN2 antibody, WGA membrane stain (red) and TOPRO-3 nuclear staining (blue) with HL-60, PC3 cell lines and Fibroblast (Fibros) cells, this was carried out at X40 magnification.

The cells were initially co-stained with a red membrane marker, wheat-germ agglutinin (WGA), TOPRO-3 nuclear stain that fluoresced blue and an anti-EN2 antibody (green fluorescence) (Figure 5.1). In all the cancer cell lines, co-localisation of EN2 with the cell membrane was detected through the combined fluorescent signal. There was a high level of EN2 at the cell membrane. No EN2 was observed in normal fibroblast cells.
5.4.2 Subcellular fractionation by ultracentrifugation and western blot analysis of PC3 and WPMY-1 cell lines

To semi-quantify EN2 membrane localisation, PC3 and WPMY-1 cells were first ultracentrifuged in order to isolate the membrane fraction (as detailed in Chapter 3, Section 3.15) and analysed by western blot assay. To make sure a pure membrane fraction was obtained, pan-cadherin membrane marker was used as a positive control. Alpha-tubulin cytoplasmic marker was used as a negative control. This western blot was also compared to the whole cell lysate (Figure 5.2).

![Figure 5.2. Western blot of isolated membrane fractions of prostate cell lines PC3 and WPMY-1. PC3 and WPMY-1 membrane fractions compared to whole cell lysate, additional controls include pan-cadherin membrane marker (middle), α-tubulin cytoplasmic marker (bottom) and rEN2 in the last lane.](image)

The cytoplasmic marker was not detected in the preparation indicating that it mostly consisted of membrane components. There was a slight enrichment of the membrane marker compared to the whole cell lysate within both cell lines. EN2 was also detected in the membrane of both cell lines. However, taking into consideration previous western blot results from Chapter 4 (where the antibody likely detected an unspecific product) it cannot be stated that more EN2 is detected in the PC3 cancer cell line than the WPMY-1 normal (immortalised) cell line. Therefore, this western blot analysis is both limited and unreliable.
5.4.3 Confirmation of the subcellular localisation of EN2 using GFP-EN2 
*de novo* expression and confocal microscopy co-localisation analysis

An alternative method for assessing EN2 localisation GFP-EN2 was both transiently and stably expressed in PC3 cells and transiently expressed in WPMY-1 cells (due to apoptosis that will be explored in Chapter 6). In Figure 5.3 both cell lines were stained with WGA membrane marker.

a)

![PC3 (TRANSIENT) GFP-EN2](image)

![PC3 (TRANSIENT) GFP](image)
b) WPMY1 (TRANSIENT) GFPEN2

PC3 (STABLE) GFPEN2

WPMY1 (trans) GFP

PC3 (STABLE) GFP
Figure 5.3. Co-localisation analysis of wheat-germ agglutinin and GFP-EN2 using confocal microscopy. The fluorescence intensity was plotted against the distance in µm across the cell, which are highlighted with red arrows in the confocal images to the left of the plots. Cells were stained with a red WGA membrane marker to show that green GFP-EN2 fluorescence co-localised with the red fluorescence of the membrane, at the same site in the cell. This was carried out in a) PC3 cells transiently expressing GFP-EN2 (left) and GFP (right) and b) WPMY-1 cells transiently expressing GFP-EN2 (left) and GFP (right) and c) stable PC3 GFP-EN2 and GFP cell lines.

*De novo* expression of GFP-EN2 showed a large difference in EN2 expression and localisation between PC3 and WPMY-1 cell lines; despite the limitations associated with tagged proteins. PC3 cell fixation after 24 hours showed EN2 as located in the nucleus, cytoplasm and membrane within these cancer cell lines. The easy discrimination between the three subcellular localisation domains is due to the lower amount of EN2 detection within the PC3 cells as opposed to normal (immortalised) WPMY-1 cells. WPMY-1 expressed large amounts of GFP-EN2 (when compared to PC3 or GFP only control) that is seemingly ubiquitous (Figure 5.3b, left). The co-localisation analysis and imaging for PC3 showed that GFP-EN2 largely resided at the membrane and occupied discreet areas unlike GFP only and WPMY-1 GFP-EN2 expression, particularly after stable expression in Figure 5.3c (right). GFP was located universally within both cell types.

5.4.4 Defining the extracellular epitopes of EN2 using polyclonal sheep anti-EN2 epitope-specific antibodies and immunocytochemistry

When designing an efficient monoclonal antibody that can be used as an antibody-drug conjugate, it is important to define the immunogen to which it will bind. To do this, a panel of 19 polyclonal sheep antibodies, Ab2 - Ab33, were made to target short EN2 peptides along the whole of the protein from the N- to the C-terminus.
Figure 5.4. ICC for EN2 surface and intracellular staining using epitope-specific antibodies from N-terminal to C-terminal. (X40 magnification). Each number represents a 20 amino acid peptide that overlaps its neighboring peptides by 10 amino acids. The outside images depict intracellular staining and the innermost images are surface staining only. All slides were fixed with propidium iodide (red) nuclear stain.

In Figure 5.4 the PC3 (left) and WPMY-1 (right) showed the clearest epitope distinction with Ab32 corresponding to an epitope located between amino acids 310-330. Predominantly, a lower amount of EN2 was detected at the N-terminal half of the protein in both PC3 and WPMY-1 cells. This suggests that either: the N-terminus is not accessible from the outside of the cell, the N-terminus has been cleaved off (a possibility due to the results in Chapter 4, Section 4.4.5) or the antibodies that target the C-terminus are unspecific and cross-react to bind another antigen, enhancing the fluorescent signal.

5.4.5 Validation of sheep anti-EN2 antibodies by western blotting

In order to verify the specificity of these EN2 antibodies, those that detected EN2 in section 5.4.4 above were analysed further using western blotting (Figure 5.5).
Figure 5.5. Western blot analysis of cell lines using the Sheep anti-EN2 antibodies. First lane is the protein standard marker. The ELISA peptide (detects the last 100aa of EN2’s C-terminus) was used as a positive control for the c-terminus targeting antibodies and negative control for n-term targeting antibodies. All cell lines are labeled above the lanes and the antibodies used are labeled on each western blot.

These western blot results highlighted the lack of specificity for some of these EN2 sheep antibodies. In particular: Ab24; Ab4; Ab2; and the goat anti-EN2 antibody. However, Ab16, APS2 and Ab32 detected a single band at 40kDa, which corresponds to the size of EN2 that has been detected in the literature (Martin et al. 2005). Ab2, Ab4 and Ab16 detected much more of the EN2, at 40kDa, from EN2 plasmid transfected PC3 cells, but were not as specific. However, this may have been due to differences in antibody concentration and so individual optimisation would have been beneficial.

5.4.6 GFP-EN2 secretion and internalisation by PC3 cells

The observation that EN2 was contained within microvesicles indicated that it might be less accessible to a therapeutic antibody. EN2 secretion from PC3 was therefore further investigated in order to better understand the mechanisms by which it was internalized using live cell imaging.
Figure 5.6. Time-lapse confocal microscopy of PC3 cells transfected with GFP-EN2. 

a) Internalisation of the microvesicles was observed in GFP-EN2 expressing cells only (depicted by the white arrows)

b) Staining with WGA (red) membrane marker seemingly revealed that the direct intercellular transfer of GFP-EN2 was dependent on cell-cell contact and the subsequent migration and aggregation of cells. The arrows highlight the cells that are involved in the EN2 transfer when they are in contact. Live cell imaging was carried out with X40 magnification and up to 24 hours.

EN2 containing vesicles were again observed (Figure 5.6a) that were seemingly only taken up by other GFP-EN2 expressing cells. The images also revealed that EN2 was able to transfer inter-cellularly via multiple mechanisms, although it is unclear whether other mechanisms exist for PC3 cells and whether the direct transfer of EN2 (Figure 5.6b) is exclusive to PC3 cells.

Taken together, Figure 4.8c (Chapter 4) and Figure 5.6a showed that PC3 was the only cell line of those tested that produced EN2 containing vesicles.
5.4.7 pH-dye conjugated EN2 antibodies to determine EN2 antibody internalisation

Using the information gained in Sections 5.4.5 and 5.4.6 experiments the goat EN2 antibody, Ab32 and Ab2 were used for the internalisation assay. The goat anti-EN2 antibody was used to further investigate its specificity. The sheep antibodies were both shown to detect EN2 by ICC and western blot assays (Figure 5.5); and bind to opposite ends of the protein. The cell lines tested were LnCaP, PC3 and WPMY-1; all of which have shown in this study to regulate EN2 differently. These antibodies, along with the relevant isotype IgG control antibody, were conjugated to a pH amine reactive dye that fluoresced at low pH.

Figure 5.7. pH amine-reactive dye conjugation verification. a) The newly conjugated pH-antibodies were denatured and loaded onto a gel for coomassie staining, lane 1 is the protein...
standard marker, the blue arrow points to the repeat coomassie staining of the goat anti-EN2 antibody conjugated and unconjugated both native and denatured. b) Conjugated antibodies were then tested for their ability to fluoresce under low pH. c) Fluorescent image of antibody aggregates that can contaminate assays by producing a false signal.

The antibodies were found to still be intact after conjugation as both the heavy (50kDa) and light (23kDa) chains could be detected using coomassie staining (Figure 5.7a). However, the goat anti-EN2 antibody failed to denature correctly at the first attempt. After further repeating the assay, the conjugated goat antibody successfully denatured and the heavy and light chains were detected at the correct size. However, the control, unconjugated antibody (even without denaturation) had broken down and created a number of artefacts contaminating the blot. This suggests that the antibody is either not stable at -20°C or that it became unstable before it was loaded onto the gel.

The conjugated antibodies fluoresced after changing the media to pH 4 (Figure 5.7b). Under fluorescence microscopy, these antibodies aggregated and created artefacts. This problem was subsequently overcome by vortexing and spinning down the antibody to collect the aggregates at the bottom of the tube prior to use. In order to optimise the methodology a time-course assay was performed using the pH-conjugated anti-clathrin antibody as a positive control; and a range of cell lines were included (Figure 5.8a). The fluorescence emitted was measured as this equated to the amount of antibody internalisation.
a)

pHAb-conjugated Ab internalisation assay (18hrs)

Fluorescence fold increase vs WPMY-1

pHdye-conjugated Ab

pHdye-conjugated Ab internalisation assay (36hrs)

Fluorescence fold increase vs WPMY-1

pHdye-conjugated Ab

pHdye-conjugated Ab internalisation assay (42hrs)

Fluorescence fold increase vs WPMY-1

pHdye-conjugated Ab
Figure 5.8. pH internalisation assay optimisation. a) pHAb-conjugated antibody was incubated for 18, 36 and 42 hours to find the optimal time needed for the antibody to be internalised enough to emit a strong enough signal to be picked up. b) The cells were imaged at 36 hours at X20 magnification to capture the fluorescent signal emitted, alongside a blue (fluorescent) nuclear dye to identify the cells.

In Figures 5.8a and b the optimal detection time was shown to be between 18-36 hours as cells appeared to begin apoptosis at 42 hours (Figure 5.8a). Clathrin plays a major role in endocytosis and so the anti-clathrin antibody was included as a positive control (McMahon & Boucrot 2011). Fluorescent imaging and direct fluorescent measurements after 36 hours incubation revealed an increase in fluorescence, whilst there was decreased fluorescence with the EN2 antibodies compared to the anti-clathrin antibody. This is most likely due to the variability of dye-to-antibody ratio (DAR) and the quality of antibodies. These limitations made it difficult to optimise the assay for all antibodies in one experiment. Consequently, 24 hours was chosen as the optimal time for incubation.

In addition, a sheep IgG control was used under the same conditions to measure background (unspecific) internalisation, which differed markedly between cell lines (Figure 5.9a and b). This allowed the fluorescence to be normalised for a
more accurate comparison. The optimum time the conjugated antibodies was then normalised further by using the same concentration of each for each sample.

Figure 5.9. pH internalisation assay with prostate cancer cell lines. a) Each antibody was tested for their ability to fluoresce under low pH (pH4) and high pH (pH10) reflecting the dye-to-antibody

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**Figure 5.9. pH internalisation assay with prostate cancer cell lines.**

a) Each antibody was tested for their ability to fluoresce under low pH (pH4) and high pH (pH10) reflecting the dye-to-antibody interaction.

b) The RFU values were normalised to the control IgG concentration (x100).

c) Representative images showing the internalisation of pH-dye-conjugated antibodies in prostate cancer cell lines LnCaP and WPMY-1 under different pH conditions.
b) The fluorescence microscope captured red fluorescence emitted from Sheep (anti-EN2) Ab2 and Ab32 under artificial pH4. c) Cells were imaged under a fluorescence microscope at X20 magnification after 24 hours incubation with the EN2 pH-conjugated antibodies, Ab2 (top) and Ab32 (bottom). Ab2 showed a higher amount of red fluorescence with LnCaP cells than WPMY-1 cells and the Ab32 assay. To the right of both figures the fluorescent images for LnCaP were enhanced by increasing the exposure.

The results shown in **Figure 5.9c** revealed LnCaP had the highest antibody internalisation with Sheep Ab2, (anti-EN2) antibody. This was not unexpected as LnCaP produced the highest protein expression with HaloTag®-EN2 and revealed a high degree of proteolysis that could still be detected with Ab2. PC3 antibody internalisation was very low for both EN2 antibodies (Ab2 and Ab32), which might reflect the secretion of GFP-EN2 in microvesicles in this cell line that could prevent antibody binding. The PC3 GFP-EN2 stable cell line did not produce more fluorescence or antibody internalisation compared to non-transfected PC3 cells (data not shown).

### 5.4.8 Identifying GFP-EN2 containing microvesicles from PC3 cells

**Figure 4.9 (Chapter 4)** shows GFP-EN2 encapsulated within microvesicles from PC3 cells. No secretory vesicles were seen with LnCaP, which is derived from a less aggressive cell line. In order to further characterise these microvesicles, secretion was observed over time using live cell imaging.

Live cell imaging over 24 hours captured vesicle secretion from PC3 GFP-EN2 transiently-transfected cells (**Figure 5.10a**). **Figure 5.10b** is a plot of the ratio of fluorescent change over time. The spike at 60,000 seconds is consistent with the secretion captured in the image stills (**Figure 5.10a**). This rapid secretion of a large number of microvesicles would suggest that EN2 secretion is a regulated process, in agreement with the findings of Maizel et al (Maizel et al. 2002).
Figure 5.10. PC3 GFP-EN2 (transient) time-lapse confocal microscopy stills. a) Stills of PC3 after transient transfection with GFP-EN2 live cell imaging up to 24hrs with X40 magnification. b) GFP-EN2 fluorescent detection of PC3 after transient transfection with GFP-EN2 live cell imaging up to 24 hours. c) Images of the vesicles formed within the cell which contain GFP-EN2. Far right image: the scale bar is 50µm wide. DOI: https://dx.doi.org/10.6084/m9.figshare.4076337.

If these microvesicles were to be used as a source of EN2 detection, perhaps as an additional or alternative biomarker test, it is important to further define them. The scale bar suggests that these vesicles are approximately 1-2µm, which is 10 times the normal size of exosomes (Figure 5.10c). These microvesicles are also
seemingly created within the cell as opposed to forming at the cell surface (Figure 5.10c).

After isolating the microvesicles, electron microscopy was used to image them. Microvesicles isolated from PC3 cell culture media appeared to have been disrupted by ultracentrifugation. On the left (Figure 5.11a), the vesicles were obtained using the Norgen kit only (which used precipitation, as outlined in Chapter 3, Section 3.23.2) and have a distinct round shape whereas in the right picture (Figure 5.11a), the microvesicles were obtained through ultracentrifugation and appear flattened and unevenly shaped.

Figure 5.11. Electron microscopy of fixed microvesicles. a) Left image: microvesicles were obtained without ultracentrifugation and concentrated with 100MWCO concentrator only. Right image: microvesicles were obtained by ultracentrifugation. Both were imaged at 15kV, x30,000 magnification. b) Left image: commercially bought PC3 exosomes were used as a positive control. Right image: microvesicles were obtained from a serum sample obtained from a healthy volunteer. The Norgen (precipitation) kit was used to isolate the microvesicles and imaged at 15kV, x45,000 magnification.
The Norgen Exosome Purification Kit ensured that the microvesicles stayed intact (Figure 5.11b) and so the kit was used thereafter. The isolated microvesicles were further investigated to determine their exact size and to quantify their CD9 expression, which is an exosomal marker (Caby et al. 2005; Mizutani et al. 2014) in Figure 5.12 below.

**Figure 5.12.** Further analysis of PC3 isolated microvesicles. a) CD9 ELISA for CD9 detection in microvesicles isolated from a positive control (PC3 media spiked with 10µg of purified exosomes), PC3 media alone and patient serum. b) NanoSight analysis of serum isolated by the Norgen Exosome purification kit. c) NanoSight analysis of patient serum fraction 8 after isolation by size exclusion chromatography. d) NanoSight analysis of microvesicles from PC3 GFPEN2 stable cell media (fraction 8) after isolation by size exclusion chromatography. 1st, 2nd and 3rd data sets represent three repeats of the same sample and reveal particle aggregation over time.

A lower amount of CD9 was detected in the microvesicles from PC3 culture media and patient serum compared to the spiked (purified exosomes) control.
These isolated vesicles probably represent a mixed population, which is verified by nanosight analysis of the patient serum (Figure 5.12b). The nanosight revealed the population, isolated by the Norgen kit, to be made up largely of microvesicles sized between 200-800nm (Figure 5.12b). Thus the vesicles seen in live cell imaging were unlikely to be exosomes.

Size exclusion chromatography was used to separate the different sized vesicles. The nanosight showed that fraction 8 consisted mainly of vesicles sized approximately 55nm, for both serum and PC3 cell culture media (Figure 5.12c and d). Therefore, it is possible to analyse these different sized populations and to further define them if necessary. The isolated fractions were analysed by mass spectrometry, to confirm the presence of EN2 - as of writing we are still awaiting the results.

5.4.9 Tracking EN2 secretion and internalisation from prostate cancer cell lines with NanoLuc®-EN2 de novo expression and Nano-Glo® detection assay

To track EN2 secretion and to investigate the differences between cell lines regarding EN2 regulation EN2 was tagged to NanoLuc®. This allowed EN2 to be detected, in real-time, whereby the amount of luminescence given off could be normalised to calculate the relative amount of EN2 present (in the media or within cells).

EN2 was first cloned into the N-terminus NanoLuc® vector (Nluc®-EN2) and serially diluted for expression in LnCaP cells. The correct EN2 protein size was initially verified through a western blot (Figure 5.13a) that included the positive control secNluc, a constitutively-secreted protein. The mRNA and luminescent detection in media increased as the amount of EN2 plasmid (µg) increased (Figure 5.13b), indicating that the assay was specific for EN2. This was carried out using an anti-NanoLuc® antibody which detected a single band only. NanoLuc® is 19kDa and the fusion protein was detected around 55kDa, corresponding to the predicted mass of EN2 (approximately 36kDa). Figure 5.13c shows the bioluminescent signal captured by the NanoGlo® detection assay from LnCaP when transfected with secNluc, Nluc®-EN2 and media only (no transfection) after 48 hours.
Figure 5.13. NanoLuc®-EN2 expression in LnCaP. a) The size of EN2 was confirmed after cloning EN2 into the NanoLuc® plasmid (N-terminus tag) by a western blot assay using a rabbit anti-NanoLuc® antibody (Promega, UK) and goat anti-EN2 antibody (Abcam). b) The Nano-GLO® assay was tested for its specificity to NanoLuc®-EN2 detection (in LnCaP cell culture media) by serial dilution of the NanoLuc®-EN2 plasmid transfected into LnCaP cells that was confirmed by RT-qPCR and calculated as a fold increase from media only transfected LnCaP. c) LnCaP cells were transfected with the NanoLuc®-EN2 and secNluc and the protein expression was measured 48 hours later with the NanoGLO® assay.

The rabbit anti-NanoLuc® (Nluc) antibody was not commercially available and was kindly provided by Promega (US). However, this meant that the antibody had not been optimised, the detection signal was quite weak and would not be sensitive enough for additional downstream analysis such as detection after inter-cellular transfer.
In order to determine whether the uptake of EN2 varied between cell lines the experiment was designed to explore whether there was preference, in EN2 internalisation, between the most aggressive cell line (PC3) and least aggressive cell line (LnCaP); and also between a normal (immortalised) cell line (WPMY-1) and the two cancer cell lines.

**Figure 5.14. Nano-GLO® detection assay for regulated internalisation of Nluc-EN2.** The Nano-GLO® reagent measured the amount of luminescence emitted from the sample, secNluc was used as the positive control and a media only negative control was included. 

- **a)** Nluc-EN2 was detected in the media to ensure that similar amounts of EN2 were present for each cell line at the beginning of the experiment. This was plotted as a fold increase against the media only control and the average of three repeats.

- **b)** Nluc-EN2 was detected 36hrs later in the cells. This was plotted as a fold increase against the media only control and the average of three repeats.

- **c)** Regulated internalisation was determined by calculated the selective uptake of Nluc, which meant normalising values to the secNluc positive control in order to take into account differences in the rate of internalisation between the cell lines.
The results show that the same amount of secNluc and NLuc-EN2 were present in the conditioned media as the experiment began. EN2 is generally taken up by all cells as a luminescent signal is detected within the cells, which was made visible with NanoGlo® (Figure 5.14b).

In order to determine whether there was regulated uptake, the signal was normalised to the secNluc positive control, to account for the rate of internalisation between the cell lines. The results revealed that differential uptake of Nluc-EN2 as PC3 internalised the least and LnCaP took up the most Nluc-EN2. Importantly, this supports the observation that the more aggressive cancer cell line the more redundant EN2’s role. The results also revealed that WPMY-1 internalised EN2 and thus, this process may not be regulated. The unspecific nature of EN2 internalisation must be taken into consideration when using EN2 for therapy as EN2 targeted drugs may not exclusively be internalised by the cancer cells and thus, this requires further investigation.

In addition, the results of Figure 5.14 support those of the pH internalisation assay that also revealed LnCaP to have the highest rate of EN2 internalisation. It is yet to be defined whether this is due to intercellular transfer, cell surface localisation, or the accessibility of EN2.

5.5 Discussion

The aims of the work described in this chapter were to quantify EN2 on the cell membrane of prostate cancer cells, investigate the mechanism and regulation of EN2 secretion and internalisation, validate EN2 as a marker, and provide a rationale for utilising EN2 as a target in prostate cancer.

The initial experiments used to determine the membrane localisation of EN2 were carried out using the goat anti-EN2 antibody previously referred to in Chapter 4, which was shown to produce spurious western blot data. Nevertheless, the ICC experiments showed EN2 co-localisation with the WGA membrane marker, and GFP-EN2 was used to confirm the antibody finding. This was carried out with transient and stable expression of GFP-EN2 and a GFP only control in prostate cell lines. Although PC3 cells could stably integrate the GFP-EN2 plasmid, WPMY-1
cells underwent apoptosis when transfected with GFP-EN2 (this finding is explored further in **Chapter 6**). The co-localisation studies were able to show that GFP-EN2 protein occupied discreet areas of the membranes on PC3 cells transiently or stably expressing GFP-EN2, which was not the case for WPMY-1 cells or GFP only controls (transient and stable).

In order to follow up on this observation that EN2 was present in discreet areas of the membrane, the portion of EN2 on the cell surface was identified using a series of antibodies to establish whether it would be ultimately possible to generate an EN2-targeting antibody for therapy. In **Chapter 4** it was determined that EN2 was highly susceptible to proteolysis (**Figure 4.14**), in LnCaP cells (at the N-terminus). Furthermore, **Figure 4.5 (Chapter 4)**, showed that EN2 C-terminus was highly polarised, which means that it is ‘sticky’ and has an increased likelihood of binding to another membrane-bound protein; the C-terminus was hypothesised to be accessible on the membrane (**Section 5.3**). Correspondingly, it was found that the antibody that detected the most EN2 on the cell surface of the PC3 cells was sheep Ab32 that binds very close to the C-terminus. The panel of sheep antibodies were then further tested for EN2 specificity using a western blot analysis. This showed that the Ab32 was specific only to the EN2 41kDa band (in line with the literature, (Martin et al. 2005)) and did not detect any of the unspecific bands that the other antibodies also picked up. Interestingly, Ab32 was one of the few that did not pick up the 50kDa band. Ab2 was also used in the next assay as it recognised an epitope at the opposite end of the protein, this was especially important because EN2 was found to be cleaved at the N-terminus. The fact that some antibodies detected a greater number of non-specific bands than others (such as Ab2) could have been due to the differences in antibody concentration.

As the end-goal is to attach a drug to Ab32, it was imperative to know whether the antibody could be internalised by the cancer cell. Unfortunately, if Ab32 could not be internalised, then the drug would most likely concentrate itself outside the cancer cell or be taken up by normal cells close by, both of which could cause large cytotoxic effects at a later stage. If this is the case and the antibody is further determined to not be internalised, engineered T cells against EN2 might be a better option such as chimeric antigen receptor (CAR) T cells (Fesnak et al. 2016). To develop these, the antigen binding regions of the monoclonal antibody (anti-EN2) are fused to the T cells however, it would not rely on the antigen localising (stably) on the cell surface or on antibody internalisation, in order to be effective. Instead,
these CAR T cells detect the MHC-EN2 complex and not EN2 alone (Sadelain et al. 2013; Guo et al. 2016). Alternatively, if it could be proved that blocking EN2 secretion had the potential to cause large-scale cell apoptosis or significantly halt tumour progression alone (explored further in Chapter 6), than another option would be to use the antibody without modification to simply block EN2 secretion or a blocking peptide similar to EN1 (A. S. Beltran et al. 2014).

In order to determine the extent of antibody internalisation, Ab32, Ab2, and the goat anti-EN2 antibody were each conjugated to the pH amine-reactive dye along with their IgG control antibodies. This dye fluoresced at low pH (such as the environment of an endosome). The greatest fluorescent signal was observed in LnCaP cells after incubation with Ab2, which binds towards the N-terminus of the protein. As EN2 is secreted and internalised, it is difficult to know whether EN2 was stable on the cell surface and subsequently internalised with the antibody or whether the antibody was internalised during intercellular transfer. If the latter proved true, further investigation would be needed to make sure that the EN2 intercellular transfer was cancer cell specific a regulated process. Alternatively, the extracellular EN2 could have been a hindrance to antibody internalisation. In either case, it was important to establish whether EN2 was taken up through a regulated process by the cancer cells or normal cells in the tumour microenvironment.

To investigate whether EN2 was taken up by a regulated process once in the tumour microenvironment and to understand more about its regulation, Nanoluc® technology (Promega, UK) was employed. Tagging EN2 to Nanoluc® allowed EN2 to be detected by a simple luminescence assay. However, because EN2 is a ‘sticky’ protein (as shown in Chapter 4, Figure 4.5) EN2 could still have been present on the cell surface despite several washes because of strong protein-protein interactions with a membrane bound protein. This in turn would make it more difficult to determine whether it is truly membrane bound. Consequently, EN2 that was still on the membrane were unavoidably ‘cell-associated’ rather than free (in the culture media). The likely limitation here is not being able to factor in the differences in cell surface area, especially when EN2 extracellular detection has been shown to positively correlate with tumour volume (Pandha et al. 2012). WPMY-1 has a large surface area and could potentially have a larger amount of EN2 binding to the outside of the membrane without actively taking up EN2. This could also explain why more EN2 is detected within WPMY-1 over PC3 cells (a much smaller cell).
Therefore, is it not clear whether there is regulated uptake of EN2 between normal cells and cancer cells.

When exploring the transfer of EN2 between cells, the results indicate that the source of EN2 is important. The Nanoluc® results show that the mechanism of free EN2 transfer favours EN2 intake by LnCaP over PC3. In both chapters (Chapter 4 and 5) it was shown that PC3 cells secreted EN2 protein in vesicles, most likely in order to protect it from proteolysis (Figure 4.9 (Chapter 4) and Figure 5.6). Conversely, LnCaP cells (which was used to produce EN2 in this chapter) had detectable cleaved amounts of EN2 that was also secreted into the media (as proven in the western blot in Figures 4.14 (Chapter 4) and 5.13. Furthermore, EN2 housed within large secretory vesicles (approximately 2µm) would not have been detected by this assay because the pore size of the membrane was too small (only 0.4µm). It was therefore not surprising that EN2 was not efficiently taken up by PC3 cells from conditioned media. However, detectable levels of free EN2 are secreted from the LnCaP cell line only based on the western blot (Chapter 4, Figure 4.14) and NanoGLO® analysis of media after transfection (Figure 5.14a). In hindsight, an additional control that could have been carried out was to repeat the same NanoGLO® assay using PC3 as the source of EN2, which could have confirmed the inefficiency of EN2 transfer between PC3 cells when simply transferring media. Based on these considerations, PC3 cells (for EN2 transfer) may rely heavily on microvesicles as a form of regulated internalisation or intercellular transfer (to be investigated in Chapter 6); PC3 cells do not readily take up free EN2 from the media, when compared to LnCaP cells. It also again reflects the limitations of the 2D experimental setup, and provides a rationale for further investigation in a 3D setting.

Morgan et al showed that, in urinary bladder (R. Morgan et al. 2013) and prostate cancer (R. Morgan et al. 2011), higher grade tumours produced less EN2 protein. They hypothesised that this is due to the poor (and highly undifferentiated) structure of high grade tumours which consequently allows for a more effective release of EN2. The in vitro results in Chapters 4 and 5 also reflect this, as EN2 was more readily secreted from PC3, a highly metastatic cell line with an aggressive phenotype, compared to LnCaP (a less metastatic cell line). However, the artificial insertion of EN2 does not reflect true EN2 behaviour in vivo and the significance that tumour structure plays compared to the regulated secretion of EN2 (within microvesicles) in the total amount of EN2 that is secreted is unclear.
Based on all of these considerations, the following EN2 regulatory mechanism is proposed (depicted in Figure 5.15): low grade tumours cannot efficiently shut down or down-regulate EN2 protein expression and protein production is upregulated, which means that as EN2 is being secreted it is replenished within the cell. However, as the tumours progress to higher grades EN2 is no longer required and therefore EN2 protein production is shut down. Consequently, as EN2 is secreted it is not being replaced and so the levels of EN2 within the cell decreases and eventually EN2 detection in the urine (media) decreases. Thus, the ability to utilise EN2 as a biomarker in the urine of prostate cancer patients is likely restricted. EN2 detection in the urine positively correlates with tumour volume at early stages and is unlikely to correlate with tumour grade due to the promoted secretion of EN2 and protein down-regulation as tumours progress.

Figure 5.15. Proposed EN2 mechanism of regulation from low to high grade prostate tumours. EN2 mRNA stays fairly consistent whilst EN2 protein, along the tumorigenic pathway, is heavily downregulated and consequently the secretion of EN2 also declines later on. Blue line is the amount of EN2 secretion; the red line is the amount of EN2 protein being translated and the grey line is the amount of mRNA present.

The stage at which EN2 starts to be tightly regulated (pre- or post-transcription or translation) is not clear. As detectable levels of EN2 mRNA are still present in PC3 cells it is conceivable that it occurs at a post-transcriptional level and
involves a process such as alternative splicing, RNA editing and microRNA translational repression (Ryazansky et al. 2011) but further work is needed to confirm this.

As more aggressive tumours secrete more EN2 but retain correspondingly less of this protein (western blot data), increased EN2 should be correspondingly detected outside in the urine or in stromal cells in the microenvironment (Guan et al. 2014). Brunet et al showed that EN2 is released as a means to guide retinal axons extracellularly, and Joliot et al showed that EN2 was associated with caveolae-like domains (Brunet et al. 2005; Joliot et al. 1997). Therefore, EN2 is secreted from live cells for a purpose yet to be explained in cancer, but EN2 is highly likely to be involved in cell-cell communication (Prochiantz & Joliot 2003; Brunet et al. 2005). To take this forward it would be beneficial to determine whether: EN2 is more likely to be taken up by normal cells, once secreted, in order to aid the tumorigenesis process; or whether it is internalised by neighbouring cells, where it could have a secondary tumorigenic role most likely involving local protein synthesis (Nédélec et al. 2004). Unfortunately, due to the sticky properties of EN2 and the limitations of a 2D experimental setup, the results presented here do not allow a firm conclusion to be drawn, and further study is needed.

The findings of the experiments on microvesicle secreted from PC3 cells also failed to define them unambiguously. The electron microscopy and NanoSight analysis revealed a mixed population that mainly consisted of vesicles 10 times too big (approximately 2µm instead of 0.2µm) to be exosomes. It was not possible to confirm whether EN2 was present within them as there was no reliable or sensitive antibody to do so. Perhaps, after learning that the vesicles clumped together (as shown by the NanoSight analysis in Figure 5.12) and the 100MWCO spin concentrators were actually reducing the amount of exosomes in the sample, it would have been beneficial to repeat the experiments without concentrating the media.

The isolation of a specific population of vesicles was successful from serum and cell culture media (Figure 5.11 and Figure 5.12). The best methodology to do so was precipitation and size exclusion chromatography. Despite not defining the large vesicle population GFP-EN2 is still present within them (fluorescence detection) and this study provides both a protocol for isolating a specific population and a rationale for further investigating EN2 in these microvesicles.
The functions of microvesicles in cancer, predominantly exosomes, have been reported to be the transport of molecules between cells and the modification of the tumour microenvironment. It has also been reported that a large population of microRNA’s are present (J. Zhang et al. 2015) within them. It may be possible to use mass spectrometry to identify a signature panel of biomarkers (miRNA, mRNA and proteins) within a subset of these vesicles. This could make a much more sensitive, robust and non-invasive diagnostic and/or prognostic test. Due to the size of these microvesicles there is a possibility that they are ‘prostasomes’, which have recently been reported in the literature to be released from PC3 cell lines (G. K. Ronquist et al. 2012; Llorente et al. 2004) and internalised by them too.

5.6 Conclusion

Further work is needed before it can be satisfactorily demonstrated that EN2 can be used as a target for an antibody-drug conjugate. It is evident that EN2 has the ability to transfer between cells in culture, but the exact mechanism and the significance for this remain unclear. In Chapter 4, it was shown that the cell lines differed markedly in the way they regulated and secreted EN2, which is further confirmed in this chapter.

LnCaP cells that cleave EN2 were able to internalise Ab32 and Ab2, suggesting that free EN2 is transferred between the cells are easily accessible to these antibodies as opposed to vesicle-associated EN2. Alternatively, there is altogether a larger amount of EN2 present on LnCaP membranes. As LnCaP cells represent low grade prostate cancer it falls in line with the theory that the rate of EN2 secretion increases with increasing metastatic potential. Therefore, for PC3 cells that encapsulate EN2 within microvesicles, both Ab32 and Ab2 were unable to show internalisation. Furthermore, there is likely to be low amounts of EN2 on the cell surface as the PC3 cell line is highly metastatic (representing high grade prostate cancer).

When EN2 is secreted within microvesicles they are readily taken up by other GFP-EN2 PC3 cells (live cell imaging) and thus, providing further evidence for differential uptake of PC3 secreted microvesicles, EN2 transfer being a regulated process and hints at an involvement in cell-cell communication.
Chapter 6: Exploring the role of EN2 in cancer
6. Exploring the role of EN2 in cancer

6.1 Introduction

In order to further identify the pathophysiological effects of EN2 in cancer, this chapter focused on exploring both the cell autonomous and cell non-autonomous roles of EN2. The reports on EN2 in the literature, in regards to development and cancer, have been used to guide the experiments designed in this section. The findings of this study support a role for EN2 in cell-cell communication that relies on its regulation and localisation, with differences between normal, low and high grade tumours, and indicates that EIF4E is a candidate downstream effector.

6.2 Background

EN2 in development has, thus far, been assigned to or implicated in the following roles: segment-polarity (Millen et al. 1995), axon guidance (Brunet et al. 2005), vesicle formation and transport (Holst et al. 2008), transcriptional activation/repression (Gemel et al. 1999) and translational regulation (Nédélec et al. 2004). EN2 expression continues in the adult, although its expression is restricted to the Purkinje cells (Albéri et al. 2004) and it is expressed at low levels only, and is believed to be required for the continued survival of neurons (Fuchs et al. 2012), where they showed that low levels were retained. It could be that cancer cells require EN2 to promote and enhance cell survival, which was identified for EN1 (A. S. Beltran et al. 2014)). The function of EN2 is further detailed in Section 2.3.2.

During development EN2 functions primarily as a transcriptional repressor (Choi et al. 2011) and regulates cell identity and differentiation, functions that it may also have in cancer cells. As the purpose of EN2 expression in cancer cells is not yet known, a better understanding of its spatial and temporal expression patterns (along the tumorigenic pathway) and its localisation, could help further our understanding. Recent reports from (Lai et al. 2014) and (Guan et al. 2014) support the findings in Chapter 4 and 5, which suggests that larger amounts of EN2 are found in the tumour microenvironment and that lower expression in the tumour is indicative of poor prognosis (R. Morgan et al. 2013; Lai et al. 2014). This unusual and highly complex expression pattern, found in prostate cancer (McGrath, Michael, et al. 2014).
2013) and other cancers (Lai et al. 2016), which may reflect the tight special and temporal control during development.

In Chapter 4, we found that EN2 seemingly caused apoptosis in WPMY-1 cells and this will be further explored in this Chapter. The apparent pro-apoptotic function of EN2 may explain why a GFP-EN2 WPMY-1 stable cell line was unable to be created, though this was not the case for PC3. It was therefore imperative to look at the relationship between EN2 expression and apoptosis in these cell lines. Further evidence is provided by (Lai et al. 2014), which revealed that EN2 inhibition led to reduced apoptosis. This is an interesting finding as EN2 is required for neuron survival in the adult. Thus, EN2 could act as a switch between cell survival and apoptosis.

It was important to further uncover the mechanism by which EN2 is secreted and internalized; and to establish whether it is a regulated process or not. This would ensure minimal side effects are endured during therapy because if EN2 were to transfer unspecifically to any cell (including healthy cells) it could also take up the EN2-ADC complex. It is already well-known that there is a general increase in nuclear-cytoplasmic transport in cancer (Hill et al. 2014) and it is conceivable that this might also be true for protein secretion.

However, evidence in Chapter 5 reveals that EN2 is packaged into large vesicles (and are likely protected from proteolysis) and taken up by neighbouring cells. This is only seen in PC3 cells, which are highly metastatic and therefore representative of high grade tumours. The EN2 homeodomain (HD) has been recently studied in a membrane mimicking environment to observe its interaction and insertion into lipid micelles (representative of vesicles) (Carlier et al. 2013). This study revealed that EN2 HD forms a tight protein:micelle complex, deeply embedding each helical segment at the core (Carlier et al. 2013).

It is known that factors in the tumour microenvironment have an important role in regulating RNA and protein expression - the signals exchanged between the stroma and other neighbouring cells can dramatically alter cell signaling (Quail & Joyce 2013; M.-Q. Gao et al. 2010). EN2, in development, is able to manipulate the environment by building an external gradient that repels optic axons and attracts nasal axons (Brunet et al. 2005). In addition, it was found that extracellular EN2 was able to make growth cones more sensitive to Ephrin A5. This action is vital to the patterning of retinal axon terminals during development (Stettler et al. 2012). It is plausible that EN2 has a similar role in cancer (when in the microenvironment) but
the exact role is unclear, particularly in the context of prostate cancer. Similarly, the role of EN2 once it is internalised by surrounding cells - prostate epithelial cells or other cancer cells - is not completely understood.

The results of Chapter 4 and 5 confirmed that secretion was a regulated process, especially in highly metastatic or aggressive cell lines such as PC3 as EN2 was secreted within microvesicles. Chapter 5 explored regulated internalisation but the results are unclear. However, this could be because media conditioning and transferring does not allow for cell-cell communication. Time-lapse confocal microscopy in Chapter 5 revealed that the intercellular transfer of EN2 occurred through direct contact between cells or through vesicle transfer. Moreover, the antibody internalisation assay (Figure 5.8) was not highly successful with PC3 showing little or no antibody internalisation. Thus, in order to make an antibody-drug conjugate or any drug, further evidence is required to show selective uptake by cancer cells over normal cells; taking into consideration the experimental setup that should allow for cell-cell communication and contact.

Few reports exist that have explored the pathophysiological effects of EN2 in a cell autonomous manner and those identified thus far are: loss of contact inhibition; slowed proliferation; increased apoptosis and weakened invasive ability (Martin et al. 2005; Bose et al. 2008; Lai et al. 2014). Bose et al reported PAX2 as an activating transcription factor of EN2 that is potentially regulated by a feedback mechanism as a result of EN2 expression (Bose et al. 2008). In contrast, the non-autonomous effects of EN2 are largely unexplored in cancer (Section 2.3.3). EN2 has been shown to induce rapid phosphorylation of proteins involved in translation initiation (Brunet et al. 2005), which could include EIF4E (Nédélec et al. 2004).

Intriguingly, for cell lines such as PC3 that are derived from highly metastatic disease, EN2 is primarily cytoplasmic (Chapter 4 and 5), indicating that it might in fact regulate translational rather than transcription, especially as it can bind to the EIF4E translational initiation factor (Topisirovic & Borden 2005; Nédélec et al. 2004). EIF4E is usually the rate-limiting factor in protein translation and its increased availability and/or overexpression have been associated with tumour progression (Xu et al. 2016) and thus, can be considered as an oncogene (Carroll & Borden 2013; Konicek et al. 2008). There have been several reports of homeoproteins residing close to or on the cell surface and affecting local protein synthesis during development, such as VAX1 (Kim et al. 2014), EMX2, OTX2 and EN2 (Nédélec et al. 2004) all of which have been found in axons. Furthermore, EMX2 was shown to
bind to EIF4E in vesicles near the cell surface where it interacts with EIF4E. Both Emx2 and EIF4E proteins failed to detach from ‘high-density fractions enriched in vesicles and granular structures’ even after treatment with detergent and RNase (Nédélec et al. 2004). These large vesicles could be similar to the vesicles secreted by PC3 cells in Chapters 4 and 5 (Figures 4.6 and 5.8). It was suggested that Emx2 could be regulating mRNA localisation and local protein synthesis to aid axonal growth through its interaction with EIF4E (Nédélec et al. 2004). EN2 and EIF4E could carry out similar functions to Emx2 and EIF4E. Interestingly, some of the downstream targets of EIF4E include pro-survival proteins (BCL-2, Survivin) and tumour invasion and metastasis proteins (MMP-9, heparanase) (Konicek et al. 2008).

A recent report identified the PBX binding domain of EN2 as being integral to its ability to transfer between cells (in a zebrafish model), and that EN2’s secretion has both intracrine and paracrine activity that work towards the same goal, patterning the brain, but do so using distinct mechanisms (Rampon et al. 2015). Another downstream effector of interest is MAP1B due to its ability to regulate the cytoskeleton, and induce vesicle formation and membrane blebbing, all of which have featured in this study. More importantly, MAP1B is has been reported to be under the transcriptional control of homeoproteins (Montesinos et al. 2001).

6.3 Chapter objective, hypothesis and approach

The objective of this chapter (taken from Section 1.5) was to:

5. further investigate EN2’s cell autonomous and cell non-autonomous role

In order to test this, the following experiments were performed:

- Time-lapse confocal microscopy of EN2-GFP (C-terminus tag) transiently transfected into prostate-derived cell lines in order to observe the intercellular transfer of EN2 and cell behaviour
6.4 Results

6.4.1 Time-lapse confocal microscopy of EN2-GFP (C-terminus tag)
transiently transfected into prostate-derived cell lines

In order to explore the effects of over-expressing EN2 within a cancer cell line compared to a normal (immortalised) cell line (with no detectable EN2 mRNA) EN2-GFP was transfected into PC3 and WPMY-1, respectively. These transfected cells were imaged intermittently to create a time-lapse confocal microscopy video. The GFP fluorescence emitted was also monitored over time.
Figure 6.1. Time-lapse confocal microscopy of WPMY-1 and PC3 cells after EN2-GFP transfection. a) EN2-GFP and GFP control monitored in WPMY-1 cells over time and plotted as a ratio over time zero (F/F0). b) Snapshots of WPMY-1 undergoing apoptosis after GFP-EN2 transfection. c) GFP-EN2 and GFP control monitored in PC3 cancer cells and plotted as a ratio over time zero (F/F0).

The time-lapse video revealed that the WPMY-1 cells quickly underwent apoptosis as EN2-GFP began to express. The EN2-GFP fluorescent signal rapidly disappears (Figure 6.1a, right image). This was not the case with the GFP control, which had a strong and consistent GFP expression profile (Figure 6.1b, left image). EN2-GFP and GFP expression were both reasonably consistent in PC3 cells (Figure 6.1c), neither of which caused a significant degree of apoptosis.
6.4.3 Time-lapse confocal microscopy after GFP-EN2 (N-terminus tag)
 transient transfection in prostate cell line WPMY-1

The N-terminus of EN2 was tagged with GFP (GFP-EN2) and this was transfected into WPMY-1 as it was previously observed in Chapter 4 that this was more active than the C-terminal version. This was also monitored closely for EN2 localisation and transfer using the WGA membrane marker (red fluorescence).

Figure 6.2. Analysis of WPMY-1 transfected with GFP-EN2. a) Image stills of time-lapse confocal microscopy of WPMY-1 cells after transfection with GFP-EN2 and co-stained with a red fluorescing membrane marker to depict the boundary of the cell. The white arrow shows points out the formation of seemingly EN2-containing apoptotic blebs. b) WPMY-1 GFP-EN2 transiently transfected cells are again seen to traffic EN2 along membrane protrusions and furthermore, the cell expressing GFP-EN2 has fused with a non-transfected cell. The white arrows show the two cells that undergo major morphological changes.
The membrane blebbing in Figure 6.2 is clearly visible as the image stills depict the creation of red and green blebs that come off at the cell surface as the cell starts to disintegrate. In addition, other cells formed membrane protrusions between cells and the images show that GFP-EN2 is transported across them. Apoptotic blebbing may be a potential mechanism for removing EN2 from the cell. It is not clear from Figures 6.2a and b whether EN2 is taken up by an adjacent cell that is connected and would require further investigation with a much more sensitive assay than antibody detection. However, in figure b two cells have fused together with EN2 clearly visible close to or on the cell surface. This is an interesting observation as cell fusion events are rare and have been suggested as a mechanism of tumour initiation and progression (Lu & Kang 2009). Also, it is interesting to note that one of these cells (not expressing GFP-EN2) rounded first and then underwent fusion.

RT-qPCR was performed to detect MAP1B mRNA in WPMY-1 cells 48 hours after transfection. MAP1B protein has been reported to facilitate in cytoskeletal changes, vesicle formation, and membrane blebbing, and importantly it is under the transcriptional control of EN2 (Montesinos et al. 2001).

![MAP1B mRNA expression](image)

Figure 6.3. MAP1B mRNA detection by RT-qPCR in PC3 and WPMY-1 cells transfected with GFP-EN2. Detection of Map1b mRNA after EN2 transfection in PC3 and WPMY-1 cells, WPMY-1
shows a marked increase in expression relative to media only control. Two technical repeats were performed for each sample and these were plotted as an average.

**Figure 6.3** shows that *MAP1B* is up-regulated by approximately 4-fold in WPMY-1 cells expressing GFP-EN2 only and not in PC3 cells. Moreover, Chapter 5 shows PC3 cells after transfection with GFP-EN2, that do not undergo apoptosis (no apoptotic blebs) but instead transfer EN2 by multiple mechanisms.

### 6.4.3 Apoptosis evaluation after EN2 (untagged) transient transfection in WPMY-1

In order to confirm that EN2 was causing apoptosis in WPMY-1 cells and not GFP toxicity, WPMY-1 cells were transfected with untagged EN2. A marker of apoptosis was used this time, which was Caspase 3/7. This live stain reagent stained Caspase-3/7 with green fluorescence that was then imaged. In addition, an MTS assay was performed to determine cell viability.
EN2 induced apoptosis (Figure 6.4a) in WPMY-1 cells and, to a lesser extent, PC3 cells as there was an increase in green Caspase-3/7 signal and cell aggregation (highlighted by the blue fluorescence of the nuclei). The induced cell aggregation is prominent, but it is unclear whether EN2 caused this directly or it was a consequence of apoptosis.
In either case, WPMY-1 cells subsequently underwent extensive cell death, based on the MTS assay (Figure 6.4b) results that showed low percentage cell viability. However, PC3 cells did not undergo cell death and the percentage cell viability stayed consistently high under each transfection condition.

Figure 6.5. Time-lapse confocal microscopy with Caspase 3/7 detection. Cells were transfected with untagged EN2 plasmid and a negative control (or media only), after which the CellEvent™ Caspase-3/7 Green ReadyProbes® Reagent (Molecular Probes™, Life Technologies, UK) was added and the resultant green fluorescence was measured over time (in seconds) a) WPMY-1 cells captured by X40 magnification, bright-field confocal microscopy overlapped with FITC fluorescence detection after EN2 transfection and plotted as a ratio of fluorescence: fluorescence at time zero over
time. b) PC3 captured by X40 magnification, bright-field confocal microscopy overlapped with FITC fluorescence detection after EN2 transfection and plotted as a ratio of fluorescence: fluorescence at time zero over time.

This experiment highlighted the immediate activation of apoptosis in WPMY-1 by EN2 overexpression. It highlights the clear difference in EN2 regulation as PC3 had a steady expression (a fluorescence detection ratio (F/F0) of 2) that was much lower than WPMY-1 expression, which instead had a fluorescence ratio of 6 at time 0 which then reduced very quickly. Thus, reconfirming a previous finding that WPMY-1 is unable to down-regulate EN2 successfully, which leads to EN2 expression becoming too high leading ultimately to cell death.

6.4.4 Exploring the cell autonomous role of EN2 in prostate cancer cell lines

Time-lapse confocal microscopy was used to further elucidate the role of EN2 by observing its behaviour between cells.
Figure 6.6. Time-lapse confocal microscopy of cells after transfection with GFP tagged EN2 with cell fusion. a) PC3 cells transiently transfected with EN2-GFP seemingly show a large vesicle being internalised by a PC3 cell or a PC3 cell (with a more rounded morphology) has invaded another cell. b) PC3 cells transiently transfected with GFP-EN2 show a cell invading another and coming back out, which then becomes seemingly apoptotic.

After transiently transfecting PC3 cells with EN2-GFP, live cell imaging captured a large vesicle or cell that contained EN2-GFP that had invaded a non-EN2-GFP expressing PC3 cell. Below, Figure 6.6b, is the same experiment carried out but with GFP-EN2.

When looking further at the cell that internalised EN2-GFP from the invading cell or internalised vesicle (Figure 6.6a), the cell adopted a more rounded
phenotype as a consequence, which could be the cell seen in Figure 6.7. This was also seen by WPMY-1 that underwent cell fusion. Thus, this suggests that figure 6.6a could be showing cell-in-cell action by a cancer cell (Y. Wang & X.-N. Wang 2013).

Figure 6.7. Time-lapse confocal microscopy of cells after transfection with GFP tagged EN2 with cell invasion. A PC3 cell that was seemingly invaded by a PC3 EN2-GFP expressing cell evidently changed morphology to a more rounded shape.

Further live-cell imaging was carried out on PC3 cells after transient transfection with GFP-EN2 and images were captured over 24 hours.

Figure 6.8. Time-lapse confocal microscopy of cells after transfection with GFP tagged EN2 with unsuccessful cell-fusion. Two PC3 cells expressing EN2-GFP were imaged over time where they can be seen to attempt to fuse or invade one another, which seemingly leads to cell death. DOI: https://dx.doi.org/10.6084/m9.figshare.4057197.
The video shows that PC3 cells (Figure 6.8a) expressing GFP-EN2 attempted to fuse together after cell-cell contact. Alternatively, both cells have tried to invade each other. Both of these cells have a distinct round shape, which signifies the switch to a more invasive phenotype (an increase in cell-cell adhesion markers at the cell surface). Thus, similar to Figure 6.6 perhaps the switch to cell-cell adhesion markers on the cell surface has caused both to pull one into the other instead of just one (where there is a clear imbalance), and the last image shows the cells lifting off and most likely undergoing cell death.

With this in mind, it was important to search for other evidence of increased cell-cell interaction (such as cell adhesion and cytoskeletal molecules) to provide further evidence that cell adhesion molecules at the cell surface had in fact increased due to increased EN2 expression. These candidate downstream effectors were selected from a microarray, where EN2 was over-expressed and knocked-down, in ovarian cancer cell lines, carried out by Sophie McGrath in the Oncology department (McGrath 2015). The results showed a general increase of expression of mRNA that had a role associated with the cytoskeleton. Within this category the selected candidates were COL8A1, TMEM204 and INHBA as these had a role specific to cell surface interactions. To confirm this finding for prostate cancer cell lines, a RT-qPCR was performed with PC3 GFP-EN2 expressing cells (and GFP only and media only controls).
a) Investigating downstream effectors after GFP-EN2 transfection in PC3 cells

![Bar chart showing fold increase in expression of INHBA, COL8A1, and TMEM204 with GFP-EN2 transfection compared to GFP only and media only transfections.]

b) MTS assay of PC3 GFP EN2 transfected cells

![Bar chart showing viability of GFP-EN2, GFP, and media only transfected PC3 cells.]

Figure 6.9. Investigating the cell autonomous role of EN2. a) RT-qPCR of potential downstream effectors INHBA, COL8A1 and TMEM204 after GFP-EN2 transfection (and GFP only, media only transfections). b) MTS time-course assay to shows the difference between viability of GFP-EN2, GFP and media only transfected PC3 cells, measured by OD492nm. Two technical repeats were performed for each sample and a student t-test.

Figure 6.3a shows that all three downstream effectors increased in expression with GFP-EN2 transfected PC3 cells only compared to non-transfected PC3 cells. COL8A1 showed the highest increase in fold expression relative to the
media only control and has been implicated in cell-adhesion (A. Chen et al. 2014). **Figure 6.9b** shows that the cells with EN2 overexpression were still viable (and presumably proliferating) even after 120 hours when the cells were seemingly confluent. Together with the increase aggregation seen in **Figures 6.6 and 6.8**, it suggests that EN2 may be involved in the loss of cell contact inhibition - a property required at the early stages of tumorigenesis. However, when considering that there is likely an increase in cell-cell adhesion (as seen in **Figure 6.9b**), loss of cell contact inhibition would be a direct consequence of that - which one comes first would need further exploration (such as the detection of specific markers). Both of these (increase in cell-cell adhesion and loss of contact inhibition) would indirectly enhance cell survival.

These findings further suggest a role for EN2 is involved in cell-cell communication, although the exact mechanism remains unclear.

**6.4.5 Exploring the cell non-autonomous function of EN2 in prostate cell lines by co-culture assays**

A conditioned media transfer assay was first performed to assess whether conditioned media that contained secreted EN2 protein evoked a response to viability. From **Section 6.4.1** it is evident that EN2 expressed endogenously causes WPMY-1 cells to undergo cell death.
Figure 6.10. MTS assay for WPMY-1 after conditioned media incubation over time. WPMY-1 cells were incubated for 24, 48 and 72 hours with media that had been conditioned (no serum and concentrated with 10MWCO spin concentrator) and the viability was measured using an MTS assay. Two technical repeats were performed for each sample and a student t-test showed no significant difference.

No significant differences were found between the tested conditioned media with respect to cell survival (Figure 6.10) and the cells are still viable after 72 hours. However, the WPMY-1 cells incubated with media from stable GFP-EN2 expressing PC3 cell line are still increasingly proliferating at 72 hours compared to 24 hours and compared to the other cells incubated with different media sources. This is similar to the result from figure where EN2 expressing cells were able to keep proliferating for longer.

This implies that EN2 in the microenvironment (and once internalised) may work similarly to endogenous expressed EN2. However, it is not possible to know if EN2 was present or effectively internalised with this experimental setup. The lack of cell-cell communication may have been an obstruction, especially considering the results from Figure 6.2. By simply transferring conditioned media the following limitations arise: EN2 is a ‘sticky’ protein and it is likely that much of it was lost after handling the media; Chapter 5 revealed EN2 secretion occurred through different mechanisms that seemingly required cell-cell contact, which could not be achieved.
by this method and PC3 cells secreted EN2 within microvesicles that were most likely not efficiently transferred through media transfer.

Therefore, a co-culture assay was used instead to show the transfer of EN2 from PC3 cells to WPMY-1 cells and observe the influence of EN2 on WPMY-1 both in the environment and once it is internalised. The following fluorescent stable cell lines were used: PC3 expressing GFP-EN2 and GFP and WPMY-1 HaloTag® (with a red fluorescent ligand added in at a later stage). The co-cultures were monitored by time-lapse confocal microscopy.

![Figure 6.11](image-url)

**Figure 6.11. Investigating the cell non-autonomous role of EN2 by co-culture assay with PC3 and WPMY-1 cell lines.** PC3 GFP-EN2 and GFP stable cell lines co-cultured with WPMY-1 stable HaloTag® cell line (with red fluorescing ligand stain), imaged at X20 magnification with a fluorescence microscope. The dotted lines outline the aggregated cells.

After just 48 hours the GFP-EN2 stable PC3 cells (green) had seemingly caused WPMY-1 cells (red) to aggregate together (Figure 6.11), mimicking the effect that endogenous EN2 overexpression had on WPMY-1 in Figure 6.4a. This was not the case with GFP only and media only control transfections. No EN2
transfer was observed and this is based on the observation of no yellow signal which would be generated as a result of the merged GFP fluorescence and red fluorescent ligand. Unfortunately, no antibody was sensitive enough to detect small amounts of internalised EN2. Interestingly, no EN2-containing microvesicles were produced and there was seemingly no EN2 transfer from PC3 cells to WPMY-1 cells.

6.4.6 Investigating the intercellular transfer of EN2 using co-culture assays

To further investigate the intercellular transfer of EN2, a similar experimental design to Section 6.4.5 was used. The following stable cell lines were employed: PC3 GFP-EN2 (green fluorescence) and PC3 GFP and PC3 LifeAct (red fluorescence) and WPMY-1 LifeAct, as listed in Figure 6.12. These assays were carried out for longer than 48 hours due to the findings in Section 6.4.5 that seemingly showed no EN2 intercellular transfer at 48 hours.
Figure 6.12. Cell co-culture assay to show GFP-EN2 transfer with prostate cell lines. The stably expressing LifeAct (actin) highlighted the cytoskeleton (red fluorescence). a) PC3 LifeAct and PC3 GFP-EN2 stable cell lines. b) PC3 LifeAct and PC3 GFP stable cell lines. c) WPMY-1 LifeAct and PC3 GFP-EN2 stable cell lines. d) WPMY-1 LifeAct and PC3 GFP stable cell lines. All images were either taken at x20 and x40 as shown on the images using a fluorescent microscope.

These combinations were co-cultured for 72 hours. A combined fluorescent signal is only observed with the PC3 GFP-EN2 and PC3 LifeAct co-culture combination. This indicates that the effective transfer of EN2 only occurred between PC3 cells.

Figure 6.13 below highlights the images in Figure 6.12 (co-cultured PC3 LifeAct and PC3 GFP-EN2 or GFP stable cell lines), which were enhanced by
increasing the gain of the red cytoskeletal fluorescence and zooming in to compare the difference in morphology.

**Figure 6.13. Enhanced images of cell co-culture assay to show GFP-EN2 transfer between PC3 cells.** The stably expressing LifeAct (actin) highlighted the cytoskeleton red. 

- **a)** PC3 LifeAct and PC3 GFP-EN2 stable cell lines.
- **b)** PC3 LifeAct and PC3 GFP stable cell lines.
- **c)** WPMY-1 LifeAct and PC3 GFP-EN2 stable cell lines.
- **d)** WPMY-1 LifeAct and PC3 GFP stable cell lines. Images were taken at x40 using a fluorescence microscope.

The polarity of the cells that have taken up GFP-EN2 apparently changes relative to PC3 GFP co-culture (right) in which the polarity and boundaries of the cell are clear. Furthermore, it is not obvious where the internalised EN2 is localising to once internalised by the recipient cancer cell. Therefore, the assay was then repeated but imaged over time and with a blue fluorescent nuclear stain to further determine EN2’s localisation after internalisation by the recipient cells.
Figure 6.14. Cell co-culture assay to show GFP-EN2 transfer between PC3 cells imaged at 72 hours and 96 hours. The stably expressing LifeAct (actin) highlighted the cytoskeleton red and blue fluorescent highlighted the nucleus (recipient cell only). The PC3 GFP-EN2 and GFP stable cell lines were also the EN2 donor cell lines. The images were taken at X20 and X40 magnification (shown on the image) using a fluorescent microscope.

The resulting yellow signal (in the recipient cell) was seemingly located within discrete areas close to or on the membrane. Interestingly (and subjectively), no EN2 in the recipient cell was detected in the nucleus.

Overall, Figure 6.12 and 6.13 suggests that EN2 intercellular transfer is more efficient when cell-cell communication and contact is permitted. Furthermore, EN2 localises within discrete areas close to and/or on the membrane even after intercellular transfer, consistent with previously reported findings that EN2 has been reported acts locally to affect protein synthesis (Brunet et al. 2005). This would suggest that EN2’s cell non-autonomous function is predominantly through protein-protein interaction rather than protein-DNA interaction.
6.4.7 Evaluating intercellular transfer and mechanism of EN2 in prostate cell lines by Transwell® (ThinCert™) assay

Thus far, EN2 intercellular transfer has been carried out by two methods:

a) co-culture assays with PC3 GFP-EN2 as the donor cell and efficient EN2 intercellular transfer was observed between PC3 cells and not between PC3 and WPMY-1 cells.

b) NanoLuc®EN2/ NanoGLO® experiment whereby EN2 in the media (LnCaP conditioned media) were internalised by WPMY-1 more than PC3 cells.

The obvious differences between these assays were the EN2 donor cells (PC3 versus LnCaP) and the cell-cell contact permitted only by the co-culture assay. Having PC3 as the donor cell meant that EN2 would be transferred in vesicles whereas LnCaP would cleave EN2. The following additional experiments were therefore performed in order to:

a) investigate whether cell-cell contact and microvesicle transfer was necessary for EN2 intercellular transfer between PC3 cancer cells and

b) establish if free EN2 in a 2D experimental setup, rather than the 3D tumour microenvironment that is better represented by the co-culture assay, could have led to the misinterpretation of EN2 function and behaviour.

A Transwell® (Thincert™) co-culture experiment was designed so that the two cells (donor and acceptor) were physically separated. To directly compare this to the co-culture experiment in Section 6.4.6: PC3 cells were transfected and seeded in the insert as the EN2 donor cells and PC3 or WPMY-1 cells were the acceptor cells seeded at the bottom of the 24 well plate and co-cultured for 72 hours. NanoLuc®EN2 was detected at two stages in order to determine the following, respectively:
1) for media (in the control well) at the beginning of incubation, was the same amount of NanoLuc®EN2 available in the media?

2) for cells after 72 hours incubation, was NanoLuc®EN2 internalised, and if so was it selectively internalised?

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**Figure 6.15. Nano-GLO® bioluminescence detection assay for Transwell® (ThinCert™)**

**NanoLuc®EN2 experiment.** PC3 NanoLuc®EN2 transfected cells were donor cells and seeded in a ThinCert™ within a 24 well plate. secNluc® is a protein control that is constitutively secreted. Nano-GLO® assay was performed at two stages: 1) media before the experiment began to and 2) cells after co-culture for 72 hours. a) Two biological repeats of PC3 as acceptor cells b) Two biological repeats of WPMY-1 as acceptor cells. For each assay a duplicate control was performed in which no
cells were seeded at the bottom to allow the accurate quantitation of the total amount of EN2 in the media available throughout the experiment. For each biological repeat there were three technical repeats for the Nano-Glo® assay, these results are displayed as an average.

The results show that the same amount of EN2 was in the media at the start of the experiment. secNanoLuc® (that is constitutively secreted) was used as both the secretory control and background internalisation reference to account for the difference in rate of internalisation between the cell lines, which allowed the direct comparison of selective internalisation of the NanoLuc®EN2.

![Figure 6.16. Nano-GLO® assay investigating the differential internalisation of NLuc-EN2 between PC3 and WPMY-1 cell lines. a) Percentage internalisation and Nano-GLO® assay was performed to detect the presence of NanoLuc®EN2 after 72 hours of Transwell® ThinCert™ co-culture. A and B are biological repeats, three technical repeats were performed for each sample and the average has been plotted as a fold increase of the media only control (media Ct). b) Differential internalisation of NLuc®EN2 was calculated by normalised to the secNLuc secretory control that was used as the background internalisation reference. Calculated as an average of the two technical repeats and two biological repeats.](image)

The results now show that for WPMY-1 A repeat these cells selectively took up a lot more of the EN2 than any of the PC3 repeats, which were both markedly lower in comparison. The result for WPMY-1 A wasn’t repeated in WPMY-1 B and so
this would need extensively repeating to ensure that the experimental design is robust and accurate.

Furthermore, the cells were then seeded into a 96 well plate and allowed to grow for 48 hours before testing for viability using an MTS assay.

![Figure 6.17. Downstream analysis of NanoLuc®EN2 internalisation with MTS assay.](image)

Figure 6.17. Downstream analysis of NanoLuc®EN2 internalisation with MTS assay. The two biological repeats for each cell line were individually seeded into a 96 well plate and left for 48 hours before carrying out an MTS assay. **a)** The percentage cell viability was calculated for PC3 A and PC3 B cancer cells after 72 hours co-culture in the Transwell® ThinCert™ with PC3 NanoLuc®EN2 expressing cells. **b)** The percentage cell viability was calculated for WPMY-1 A and WPMY-1 B cells after 72 hours co-culture in the Transwell® ThinCert™ with PC3 NanoLuc®EN2 expressing cells. Each MTS assay consisted of three technical repeats.

No significant differences were observed. However, WPMY-1 A that selectively internalised the greatest amount of NanoLuc®EN2 showed a marked increase in cell proliferation compared to the other transfection controls.
As the results in Figure 6.17 are not consistent with those in Figure 6.12 it indicates that: a) PC3 cells require cell-cell contact for efficient intercellular transfer of EN2 or EN2-containing microvesicles or b) the use of conditioned media to study EN2 dynamics is a misrepresentation to actual events because cell-cell communication has been shown here to play a vital role in EN2 intercellular transfer, for PC3 cells at least.

Consequently, 3D cell culture methods must be considered as an alternative in vitro/ ex vivo model, the setup is more representative of the tumour microenvironment that includes cell-cell contact and communication to further study the behaviour of EN2.

This has also provided a novel method of tracking EN2 (in real-time) without the use of an antibody.

6.4.8 Preliminary experiments to study the effects of EN2 forced expression both cell autonomously and non-autonomously

The main objective was to explore the phenotypic downstream effects of EN2 particularly with cells that have taken up EN2 from the microenvironment.

Bose et al suggested that EN2 is able to regulate PAX2 (an activating transcription factor of EN2) by a feedback mechanism (Bose et al. 2008). Thus, EN2 could regulate the expression of potential protein binding partners. PBX and EIF4E protein binding domains within EN2 have already been defined (Chapter 2, Figure 2.13). Overexpression of EN2 could then affect the expression of PBX and EIF4E and to investigate this a RT-qPCR assay was performed using GFP-EN2 forced expression in prostate cell lines.
a) EIF4E mRNA expression

b) PBX mRNA expression
Figure 6.18. RT-qPCR of potential downstream effectors. The data for both graphs have been normalised to the GAPDH housekeeping gene, the cDNA from cell lines are labeled at the bottom. **a)** EIF4E mRNA expression was displayed as a fold increase relative to WPMY-1, included cDNA from PC3 GFPEN2 stable and transient (trans) cells, PC3 GFP stable and transient control cells. A 5-fold increase in EIF4E expression was detected in the GFP-EN2 stable cell line only. **b)** PBX1-4 mRNA were detected and displayed as normalized to GAPDH only (x100000) to highlight that higher amounts are detected in the WPMY-1 normal cell line. **c)** Meis 1-3 and Prep 1-2 mRNA detection after GFPEN2 stable and transient (trans) transfection, Ct values are displayed relative to GAPDH (x1000).

The RT-qPCR in **Figure 6.18a** reveals that EIF4E could be a potential downstream effector of EN2 as a 5-fold increase is calculated, interestingly this was only the case for the stable overexpression and not the transient overexpression of GFP-EN2 in PC3 cells. **Figure 6.18b** shows that PBX is not upregulated nor downregulated by EN2 and neither are PBX co-factors MEIS 1, MEIS2 and MEIS 3 (**Figure 6.18c**) and this fits with the literature (J. L. Chen et al. 2012).

The increased activity of EIF4E is typically attributed with enhanced survival (Konicek et al. 2008). In order to test this the peptide HXR9 was employed. HXR9
prevents PBX binding to HOX, which in turn triggers apoptosis in malignant cells only.

Figure 6.18. Cell viability of PC3 cell lines after HXR9 treatment with an MTS assay. HXR9 peptide and the control CXR9 peptide were serially diluted from 120µM to 0µM and cells were treated for two hours before carrying out an MTS assay to calculate cell viability. A and B are biological repeats and three technical repeats were performed for each sample.

The calculated IC50s were not significant (data not shown) but the MTS did show a slight increase in percentage cell viability between PC3 GFP and PC3
GFPEN2 expressing stable cells (Figure 6.18). This is consistent with the literature (Bose et al. 2008) however, the mechanism by which EN2 enhances cell survival remains unclear.

6.5 Discussion

There is a striking difference between PC3 and WPMY-1 expression of GFP-EN2 and the resulting downstream effects. EN2 elicits cell death in WPMY-1 shortly after expression of EN2. Untagged EN2 produced a similar cell death response to GFP-EN2 in WPMY-1 cells and therefore GFP cytotoxicity could be eliminated as the cause. MAP1B was found to be elevated in WPMY-1 cells only and not PC3 cells, after GFP-EN2 transfection. This is consistent with the observed vesicle formation and large amount of membrane blebbing - MAP1B has been linked to both of these physiological effects during autophagy (Harrison et al. 2008). Autophagy is a lysosomal degradation process, also known as a non-apoptotic cell death mechanism, and has also been reported to both inhibit apoptosis and enhance survival (Codogno & Meijer 2005). The observed increase in caspase-3/7, which is part of the apoptotic pathway, would indicate that apoptosis is activated by EN2. However, more quantitative evidence is required to define whether both these mechanisms are turned on as a direct result of EN2 expression.

The MTS cell viability assay (Figure 6.4b) confirms that WPMY-1 cells definitively undergo cell death but PC3 cells do not. Thus, the mechanism of cell survival in cancer cells were further explored by examining EIF4E expression as this protein can bind to EN2 and prominent downstream effectors of EIF4E include the anti-apoptotic protein BCL-2 and MMP-9, which enhance cell survival and increase metastatic potential, respectively. It was found that only the stably expressing PC3 GFP-EN2 cell line showed a marked increase level of EIF4E mRNA expression relative to WPMY-1 as opposed to transient expression and GFP expression. Thus, this provides rationale for further investigation of the role of EN2 in enhancing cell survival through its interaction with EIF4E (discussed further in Section 7.2.2). Interestingly MMP-9 was flagged as a candidate enzyme for the proteolysis of EN2 in Section 4.4.10 and this will be further expanded upon in Section 7.2.2 also.
Another experiment carried out to confirm this finding on enhanced cell survival was to challenge GFP-EN2 stably expressing PC3 cells with the HXR9 peptide. Although EN2 has a PBX binding domain and could hinder the mechanism of action of HXR9 through competitive binding, this experiment was carried out because: co-localisation by ICC of the two proteins was not observed (data not shown); EN2 did not directly affect the mRNA expression of PBX (Figure 6.18b); PBX is generally downregulated in advanced prostate cancer cells (Figure 6.18b); there have been no reports of cell autonomous binding of EN2 and PBX (in cancer cells) in the cytoplasm.

The mechanism by which EN2 enhances cell survival has been largely unexplored. A MTS assay was performed on GFP-EN2, GFP stably expressing PC3 cells after treatment with HXR9. This assay showed a slight increase in percentage cell viability between GFP-EN2 and GFP PC3 stable cell lines, for both repeats (Figure 6.18). Thus, EN2 did confer a survival advantage (as reported by Bose et al., (Bose et al. 2008)) but this may have involved EIF4E upregulation rather than direct competition with peptide binding. The other possibility was that EN2 causes loss of contact inhibition, as reported by Martin et al., where cells do not stop growing once they come in contact with each other (Martin et al. 2005). This could explain the increase in viability and proliferation with GFP-EN2 expressing cells, as reflected in the MTS assay results. However, it does not explain the increased resistance to HXR9.

It is possible that there was a link between the number of cell fusion events and enhanced cell survival and thus, the increased resistance to HXR9. Cell fusion has been reported to be involved in disease progression (particularly cancer) including an increased proliferation rate, a switch to a more invasive and metastatic behavior and an increased resistance towards drugs and apoptosis (Lu & Kang 2009; Y. Wang & X.-N. Wang 2013). Live cell imaging showed that the cell fusion events were between GFP-EN2 expressing and non-expressing cells, for both PC3 and WPMY-1; two GFP-EN2 expressing cells were not able to fuse (Figure 6.8). This points to the spreading or regulated dispersal of EN2 to perhaps allow the evasion of apoptosis and continued cell survival (Figure 6.2b), which have recently been reported in the literature (Noubissi et al. 2015; Noubissi & Ogle 2016). Membrane protrusions or nanotubes such as those made between WPMY-1 cells after GFP-EN2 transfection (Figure 6.2a) have also been implicated as a rescue
mechanism (Rustom 2016), and GFP-EN2 can be observed trafficking along these nanotubes.

A role for EN2 was also suggested by a general increase in all the candidate markers for cell-cell adhesion at the cell surface picked from the microarray (INHBA, COL8A1 and TMEM204). The elevated expression of these genes provides further evidence that EN2 is involved in cell-cell communication. However, the evidence in the literature and in this study suggests that EN2 is most likely to be affecting local protein synthesis due to its cytoplasmic localisation and perhaps, where the focused should be in future (Prochiantz & Joliot 2003; Brunet et al. 2005; McGrath, Michael, et al. 2013). Translational changes will affect the cell more quickly than transcriptional changes, and a role for EN2 in regulating translation is supported by the observations that: EN2 in highly metastatic cells resides on or close to the membrane (Chapter 5, Figure 5.3 and 5.4) and EN2 has the ability to bind to the translation initiation factor, EIF4E, with high affinity outside the nucleus (Nédélec et al. 2004). Furthermore, Figure 6.2b shows that EN2, even after intercellular transfer, remains close to the membrane.

This chapter has provided a protocol in which EN2 can be tracked in real-time using NanoLuc® tagging (just 17kDa) and the NanoGLO® detection assay. Its secretion and internalisation can be measured both in cells and in the media by simply detecting the luminescence. This assay doesn't have to be an end-point assay when detecting extracellular EN2 only and so can be performed on live cells. This is a flexible platform to test varying conditions and to investigate, for example, the differential secretion and uptake of EN2 in vitro, especially when no reliable antibody is available. Investigation into the non-autonomous effects of EN2 on the recipient cells (Figure 6.17) showed disparities between conditioned media and co-culture assays. This problem was predicted due to the findings in all three results chapters that showed cell contact to be a main mechanism of EN2 transport.

The NanoLuc®EN2 Transwell® (ThinCert™) system was used to investigate whether cell-cell contact was necessary for the intercellular transfer and paracrine activity of EN2, as it physically separated the cells. The results suggest that PC3 cells require cell-cell communication in order to transfer EN2. From the co-culture assay it was evident that EN2 transfer occurred much more frequently between PC3 GFP-EN2 and PC3 cells than PC3 GFP-EN2 and WPMY-1 cells, whilst this was not seen when the cells were physically separated. It was not possible to quantify the amount of intercellular transfer. One approach to quantification is to measure the
downstream effects to the EN2 recipient cells after co-culture with EN2 expressing cells. However, the non-autonomous effects of EN2 are yet to be defined. Despite this, the viability of the recipient cells were tested by an MTS assay because of the cell death caused by EN2 endogenous expression in WPMY-1 cells (Figure 6.4b). No significant difference was measured, which confirms that EN2 is not effective in this setup of no cell-cell communication and contact. Chapter 5, Figure 5.6 shows that EN2 was transferred to PC3 but it does not induce cell death, thus a viability assay would have been redundant. The results with WPMY-1 supports the notion that EN2 requires cell-cell contact in order to carry out its paracrine function. Other experimental approaches are explored in Chapter 7, Section 7.4.2.3.

Furthermore, when we add together the effects seemingly caused by EN2 when outside the nucleus (cell aggregation, cell-cell adhesion and cell-in-cell action) it supports the notion that EN2 switching from a predominant nuclear to non-nuclear localisation is critical to tumour progression.

6.6 Conclusion

The findings of this chapter revealed different modes of EN2 expression, regulation and affects between PC3 and WPMY-1 cells. PC3 cells are able to survive forced EN2 expression, unlike WPMY-1 cells. WPMY-1 cells try to evade cell death by activating different rescue mechanisms such as intercellular trafficking of EN2 via membrane protrusions, and membrane blebbing, as reflected by MAP1B expression. PC3 cells (after forced EN2 expression) show a slight increase in caspase 3/7 protein expression - indicative of apoptosis - but do not undergo cell death and do not show an increase in MAP1B expression. These cells can transfer EN2 through direct cell-cell contact, cell-in-cell action and in large vesicles. The increased EIF4E mRNA expression detected in these cells could also allow for their continued survival as it activates the expression of the anti-apoptotic protein BCL-2. Furthermore, EN2 is located at the periphery of these cells, even after intercellular transfer, unlike in WPMY-1 cells.

The increased amount of cell fusion events between the EN2 over-expressing cells and non-transfected cells also coincides with a general increase in cell-cell
adhesion markers. However, this is different from cell-in-cell action whereby cancer cells invade other cells. Thus, further studies are required to determine exactly which one of these mechanisms EN2 is inducing.

The mechanism of cell survival in cancer cells (as opposed to cell death in normal cells) through EN2 is unclear - this study presents two modes:

a) an increase in $EIF4E$ and

b) cell fusion or cell-in-cell events that have been reported as a rescue mechanism to evade cell death (W.-J. Yang et al. 2012; Noubissi et al. 2015).
Chapter 7: Final discussion
7. Final discussion

Firstly, for refreshment, the thesis question is restated. The three results chapters will be collated together to address the hypotheses made at the beginning of the thesis in Section 1.5 and to ultimately answer the thesis question. Furthermore, the non-envisaged limitations of this work and the research novelty are highlighted. Future work for EN2 are recommended, the future of cancer biomarkers is discussed as well as the wider implications of this study to the field of cancer research. Finally, the thesis work is concluded.

7.1 Thesis question

- Is Engrailed-2 (EN2) a suitable prostate cancer target for an antibody drug-conjugate?

7.2 Thesis overview

7.2.1 EN2 as a cancer specific cell surface target

At the beginning of the study, it was hypothesised that there would be a significant level of EN2 on the cell surface of prostate cancer cells relative to ‘healthy’ cells (in vitro). Immunofluorescence experiments showed that all cells expressed EN2, even WPMY-1 cells that had no detectable EN2 mRNA. In most cases, mRNA expression correlated with protein expression, and so this finding was unusual. Furthermore, for all cell lines, EN2 expression was confirmed to be outside the nucleus however, the apparent size of EN2 protein was unexpectedly large. The bands were analysed by mass spectrometry, which is likely to confirm that this band was not EN2 - as of writing we are still awaiting the results. In hindsight, contacting the authors of the reports that did detect EN2 at the correct size would have been beneficial (to ask for the antibody that was used). The higher mass of EN2 could have been a result of post-translational modifications, but this modification was not investigated here (see Section 7.4.3.2).
To carry on the study, EN2 was expressed *de novo* tagged to GFP, and live cell imaging showed that it could still be secreted (in vesicles). Moreover, confocal analysis revealed that the GFP-EN2 protein was close to or on the membrane only in cancer cells. For normal cells, GFP-EN2 resided mainly within the nucleus and caused cell death. Further analysis of the results suggest that in PC3 cells EN2 is downregulated and secreted by multiple mechanisms. PC3 cells represent highly metastatic tumours (high grade) in which EN2 has been previously reported to be packaged in secretory blebs at the periphery of the cell; only EN2 from the urine samples of low grade tumour patients positively correlated with tumour volume (R. Morgan et al. 2011). Taken together, the findings indicate that it is likely that only small amounts of endogenous EN2 protein remains in highly metastatic prostate cancer cells and therefore endogenous (and clinical) EN2 protein detection requires a more sensitive method (see Section 7.4.2.1).

If an antibody-based diagnostic test is to make it to the clinic it is important that it targets the right antigen; if an antigen is concealed, cleaved or shed at the cell surface - all of which has been shown in this study - in a random manner, an antibody-based test or an ADC may not be appropriate at all. The panel of antibodies - that targeted short peptides along the EN2 protein - showed the C-terminal detecting antibody Ab32 as producing the strongest cell surface signal. However, this antibody was seemingly not very well internalised. Previously published findings indicate that penetratin (the secretion and internalization motif of homeoproteins) internalisation is multi-mechanistic (Dinca et al. 2016) and the live cell imaging results in Chapters 4 and 5 reconfirm this as it shows that EN2 can, in addition to secretory vesicles, transfer directly between PC3 cells. As the antibody internalisation experiment (in Chapter 5) relied on endocytosis, it may not fully represent the extent of internalisation. These alternative mechanisms of internalisation and the possible ways that they can be measured are described in more detail in Section 7.4.2.3. The findings in Section 6.4.7 further suggest that EN2 intercellular transfer is not specific and/or not exclusive to cancer cells as it is internalised by normal cells in the microenvironment. This means there is a high risk of toxicity when using an ADC against EN2 in the clinic (even if the antibodies are delivered directly to the tumour site), especially if largely acting at a short distance outside the cell as reported by Layalle *et al.* (Layalle et al. 2011).

The diagram below depicts a possible mechanism by which an equilibrium is established for the distribution of EN2 between the nucleus and cytoplasm that
could account for differences between the normal (embryonic), low and high grade prostate cancer cell lines:

**Normal (embryonic) cell**

**Low metastatic cancer cell**

**High metastatic cancer cell**

**Figure 7.1. Nucleo-cytoplasmic shuttling of EN2.** The diagram depicts a theory to how EN2's localisation could be disrupted (in cancer) so that EN2 is mostly located in the cytoplasm and cell surface. Under normal physiology, EN2 is exported in and out of the nucleus because it has both a nuclear localisation sequence and a nuclear export sequence. However, when this shuttling is (somehow) disrupted it favours one location over the other and this is mostly cytoplasmic in cancer, which allows more EN2 to reach the environment presumably via luminal vesicles as EN2 lacks a classical secretion sequence.

EN2 contains both a nuclear localisation signal and a nuclear export signal, which means it can shuttle in and out of the nucleus, and the resulting nuclear-cytoplasmic
distribution may be disrupted in cancer cells. As a consequence, it could be that an increasing amount of EN2 protein localises to the cytoplasm, leading to a higher chance of EN2 trafficking into luminal vesicles and being secreted out of the cell. A possible modification - that could also be the cause of disruption in cancer - is SUMOylation, which is a post-translational modification that essentially adds sugar groups to the protein and has been reported to affect the nucleo-cytoplasmic shuttling of proteins (this will be discussed further in Section 7.2.2).

7.2.1.1 Summary

En2 mRNA expression was successfully shown to be higher in cancer cells compared to normal cells. For the use of ADC in cancer cells, in which EN2 is packaged into secretory blebs reported here and by Morgan et al. (R. Morgan et al. 2011), there would only be a narrow window of opportunity for the ADC to bind to EN2 on the cell surface. This study advocates that if ADC treatment was to be pursued, to restrict its use to tumours with a low metastatic potential that are unlikely to secrete EN2 in vesicles. However, further study is needed of the translational and post-translational regulation of EN2; EN2 protein expression is complex and generally, the area of translation regulation is often overlooked in cancer research.

7.2.2 Understanding the role and behaviour of EN2 in cancer

Before proceeding with the study, it was hypothesised that EN2 localisation to the cytoplasm is promoted as prostate tumour grade increases due to its potential translational role at the cell surface. To investigate EN2’s role in cancer more closely, an attempt was made to characterise the secretory vesicles containing EN2 - a protocol was successfully developed for isolating a mixed population from culture media. This population was then further separated by size-exclusion chromatography and the size of which was determined by NanoSight technology. Unfortunately, after separation, the presence of EN2 within the smaller exosomal population could not be confirmed without mass spectrometry. At the time of writing, these mass spectrometry results are still to be received. Regardless of whether EN2 is present in exosomes (or other microvesicles), the other constituents could be
used as biomarkers; these could be obtained from serum and urine that are easily accessible biofluids and far less invasive than a biopsy, which unfortunately is still the next step after PSA measurement. Exosomes house a rich source of microRNAs, which have been shown to be transferred between cells and facilitate metastasis (J. Zhang et al. 2015). Thus, this could be a promising avenue to uncover novel prostate cancer biomarkers.

For EN2 packaged into these larger (and yet to be defined) vesicles, an understanding about the destination and regulation of these vesicles as well as their function within the tumour microenvironment would be useful in determining the best approach to therapy. This is especially true if EN2 is still to be targeted with a monoclonal antibody, which requires high accessibility to an antigen on the cell surface of the cancer cell or an antigen destined to be taken up by a cancer cell. These vesicles were seemingly regulated because, on multiple occasions, the live cell imaging revealed EN2 secretion and furthermore, large amounts of secretion were detected at a specific time-point during the experiment. Unfortunately, whether the vesicles were destined to be taken up by other cancer cells, normal cells, or any cell type was not clear from the results of the live cell imaging; as opposed to secretion, internalisation was seemingly unregulated or unspecific.

MMP-9 was shown to be a candidate proteolytic enzyme for EN2 (in Chapter 4), producing a fragment size corresponding to that detected by western blot analysis - this requires further confirmation by mass spectrometry. In keeping with this, the results of Chapter 6 revealed that EIF4E (translation initiation factor) is a potential candidate downstream effector of EN2 in cancer and that it may (indirectly) increase the translation of MMP-9 protein (Hamdy et al. 1994). It has been previously reported that the knockdown of EN2 results in the down-regulation of MMP-9 protein (Li et al. 2015). Taken together, this study suggests the potential pathway outlined in Figure 7.2.

![Figure 7.2. A diagram to show the theory of EN2's indirect regulation of MMP-9 translation.](image)

The increased localisation of EN2 in the cytoplasm rapidly phosphorylates EIF4E, which then
increases local protein synthesis of MMP-9. MMP-9 is involved in cancer invasion and metastasis.

MMP-9 breaks down the extracellular matrix, driving invasion and metastasis in cancer (Plowright et al. 2009; Stewart et al. 2004). EIF4E can promote translation both directly and indirectly by selectively increasing mRNA trafficking to the cytoplasm (Topisirovic et al. 2005). The co-culture assays confirmed the presence of EN2 on or close to the membrane of the recipient cells that had internalised it; its absence from the nucleus would imply that its paracrine function is not transcriptional either. A previous study reported an autocrine function for EN2 during axon development. This provides further rationale that EN2 largely affects translation and local protein synthesis in cancer cells due to its predominant localisation outside the nucleus.

A closer examination of the live cell imaging videos revealed that GFP-EN2 expressing cells fuse with or invade non-GFP-EN2 expressing cells (Figure 6.6), but cannot do so with other GFP-EN2 expressing cells (Figure 6.8). This invasion by a cancer cell has been reported previously and is known as cell-in-cell action (Y. Wang & X.-N. Wang 2013). This process may allow the switching from a cell-substrate phenotype to a cell-cell phenotype (such as an increase in cell-cell adhesion markers at the cell surface). This in turn pulls cells towards each other and indeed into each other. This invasion requires cell-cell contact and so in order to further study this a 3D setup is required (please see Section 7.4.1 for more details). When considering whether EN2 is instrumental to orchestrating this switch in phenotype and promoting invasion or is itself a protein that participates in this process directly, when at the cell surface, the answer is unclear. There are three possible consequences of cancer cell invasion that has been reported thus far (and observed in this study), which are outlined in the Figure 7.3.
Figure 7.3. **Cell-in-cell action and three known consequences.** Adapted from (Y. Wang & X.-N. Wang 2013). Cancer cells have been observed to acquire invasive properties and invade cells. Consequently, there are three known fates (A) the cell survives and goes on to divide within the invaded cell (B) the cell manages to escape and survive or, most usually, (C) the cell dies.

When two cells express GFP-EN2 both cells try to invade the other, which triggers cell damage and death. This would mean that a tumour cell is more likely to invade the normal stroma (that still have the cell-substrate phenotype) than another tumour cell in that microenvironment, suggesting a role in the tumour microenvironment. This is an important avenue to explore as it would provide further rationale to EN2’s oncogenic role, and blocking its function and secretion could slow tumour progression.

Ideally, the experiments described above need to be looked at more closely with more cell lines in order to: a) further understand this difference in EN2 localisation between a cancer cell and a healthy cell, b) confirm this switch in phenotype from cell-substrate to cell-cell interactions and c) observe the invasion of the normal stroma in the tumour microenvironment. Preferably, this would be done using 3D cell
culture and co-culture models and progress to using primary cell lines (for more details please see Section 7.4.1 and Section 7.4.2.4).

On a another note, it was also important to reveal the function of EN2 because biomarkers can be useful in a number of ways, including as screening, diagnostic, and prognostic tools (outlined in Section 2.2.2). It is not always obvious how biomarkers should best be used in the clinic; the more that is known of their basic etiology such as expression, regulation, localisation and function can help to guide their use. For example, the stage at which these biomarkers are highly expressed (early or late) is important in order to know whether it can be used as a screening or a predictive tool.

7.2.2.1 Summary

The following can be concluded, but require further verification:

• EN2 acts through translation (outside the nucleus) and outside the cell to promote an invasive phenotype.

• EN2 in the nucleus prevents tumour progression by transcriptional activation of aggregation, autophagy and cell death.

• The equilibrium of EN2 movement in and out of the nucleus can change so that it becomes predominantly cytoplasmic. This, in turn, increases the amount of EN2 in luminal vesicles, at the cell surface and secreted into the microenvironment - where it then acts by translational regulation/ local protein synthesis (and in neighbouring cells that take them up).

This study has shown that EN2 can be secreted directly from one cell to another and within vesicles, and further study of this process will play an important part in deciding the best method of detection for diagnosis and therapy. These results strongly advocate the following: a) to look more closely at the local translational effect of EN2 at the cell surface and b) to use a more complex model such as 3D cell culture to continue deciphering the complex regulation and function of EN2.

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7.3 Research novelty

The novelty findings of this research are that:

- **the commercial goat antibody against EN2 does not specifically detect the EN2 protein and is unreliable.** This research provides an alternative method to accurately track EN2 using HaloTag® and NanoLuc® technology (Promega, UK), and further advocates the use of a more specific detection method for native EN2 in clinical samples. Antibody-based detection methods should be avoided, unless the antibody can been validated.

- **EN2 is secreted and transferred within large microvesicles between PC3 cells.** PC3 is a highly metastatic prostate cancer cell line, whilst cleaved EN2 is secreted from LnCaP cells. LnCaP cells have low metastatic potential and EN2 was not associated with vesicles. This suggests that PC3 cells are able to protect EN2 from proteolysis through its inclusion in large microvesicles, which are secreted. Isolating and purifying these vesicles could provide another means of using EN2 for clinical purposes. As this is a positive selection for the vesicles, EN2 levels would be higher and mass spectrometry or other less sensitive detection methods would become suitable. EN2's intercellular transfer, protected within vesicles, infers a role during disease progression possibly in the tumour microenvironment.

- **once internalised, EN2 does not localise to the nucleus but within discrete areas close to or on the membrane.** This was observed after the co-culture assay whereby EN2 remained within discreet areas close to the cell surface, after being internalised. Thus, EN2 could have a role in translation of local proteins at the cell surface. If EN2 was able to manipulate proteins at the cell surface it could play a role in cell morphology and migration; and manipulating the tumour microenvironment.
microvesicles of a defined size can be isolated. The protocol developed in this study involves an initial precipitation step followed by size exclusion, that was successful with media, urine and serum samples and allowed the isolation of vesicles of a defined size using NanoSight analysis. This is a method (in progress) for isolating large microvesicles that are seemingly abundant in prostate cancer.

EN2 is probably not suitable for targeting by a monoclonal antibody-drug conjugate, particularly in high grade tumours. EN2 is packaged within vesicles and is therefore less accessible to the antibody. Free EN2 is susceptible to proteolysis. The discovery that EN2 expressing cells can invade non-expressing cells is a further complication. Taken together, these findings suggest that EN2 is not stable on the cell surface and that an ADC against EN2 is unlikely to be internalised or prove therapeutically effective.

EN2 may have a role in the tumour microenvironment. PC3 GFP-EN2 expressing cells were found to be able invade non-GFP-EN2 expressing cells. This infers that prostate cancer cells that express EN2 are more likely to invade cells in their environment that do not express EN2, which are the surrounding normal stroma; and thus, promoting pro-tumour changes to the microenvironment. Both EN2 transference and invasion is also more likely to occur by the more metastatic cells, as this was only observed with PC3 cells and not LnCaP or Du145 cell lines.

The new hypotheses created because of these findings are outlined in Section 7.4.3. The wider implications of these findings are further discussed in Section 7.5.

7.4 Future work and recommendations

7.4.1 Practical limitations for future consideration
Due to the unspecific nature of the commercially available polyclonal antibodies, including the ones used in this study against EN2, additional in-house validation is vital; for example, using CRISPR/Cas9 knock-out of the protein of interest, if available, for each sample type to be used in the study (further detailed in Section 7.4.2.1).

The use of only in vitro experiments limits the interpretation of the study findings. The in vitro experiments were initially chosen because not many studies have been performed and so a more general, less expensive and easy to handle approach was necessary. However, 3D cell culture would be more suited in this case as EN2 is a dynamic protein, and to understand its behaviour requires a more complex 3D setting. It is clear now that the protein plays an important role extracellularly, most likely in the tumour microenvironment, including cell-cell communication that is also made more complex by temporal and spatial patterning (further confirmed with research into its function during development (Brunet et al. 2005; Rampon et al. 2015). “Most in vitro experimental systems use 2D cell culture and trans-well assays to study these interactions even though these paradigms poorly represent the tumor, in which direct cell-cell contacts in 3D spaces naturally occur.” (Majety et al. 2015). The advantages and potential of using such a model are further discussed in Sections 7.4.2.4 and 7.5.1.

In addition, it is estimated that only a small proportion of EN2 is actually secreted from cells (Joliot et al. 1997), and considering that GFP and HaloTag® are bulky proteins (approximately 30kDa) this could have altered the folding of the protein and hindered the movement of EN2 within the cells. The GFP especially may have been toxic to the cells and may well have contributed to EN2 degradation and cell death.

EN2 secretion is continuous and (for PC3 cells especially) a larger amount can be detected in culture media and within robust microvesicles, which may account for the difficulty in detecting EN2 within PC3 cells. In a number of experiments GFP-EN2 protein could not be detected in PC3 cell lysates by western blot even though it could clearly be seen by immunofluorescence, and was often cleaved. It is important, if EN2 continues to be explored as an ADC target, to establish whether the conformation of GFP-EN2 prevented the antibody from binding to its antigen or whether it was inaccessible to the antibody as a result of being encapsulated in vesicles.
7.4.2 Future experiments

The overarching aims continue to be: a) validate and explore EN2 as a cancer specific cell surface protein target and b) increase our understanding of the role and behaviour of EN2 in cancer. Furthermore, with reflection and critical analysis of this present study it has been proven to be fundamental to continue unveiling the intercellular transfer of EN2 in order to guide the therapy developed against it. These experiments listed below are the logical next steps that will need to be carried out before investigating the new hypotheses listed in Section 7.4.3.

7.4.2.1 Antibody target validation by EN2 knockout

This study did not achieve 100% knock down of En2 using siRNA in order to confirm the specificity of the antibodies used in this study. Another molecular tool is zinc finger nucleases (ZFN) that have two functional domains: a DNA-binding domain and a DNA cleaving domain (Gaj et al. 2012). When two ZFN's are paired they act like genomic scissors excising the unique target sequence. A new technology that is becoming widely adopted is CRISPR/Cas9 technology (Gaj et al. 2013). This technique has high precision and uses a lentiviral vector to deliver the machinery into cells and colonies are then selected for and thus, the methodology is simple and accessible (X. Liu et al. 2017). After knocking down EN2 completely and permanently, we would hope to see a much larger effect at the protein level via ICC and WB analysis with an EN2 antibody. This approach would validate the EN2 antibodies and enable them to be used with confidence for further functional assays, without the need to tag the protein to a fluorophore.

7.4.2.2 Mass spectrometry for protein size determination, protein isoform and post-translational modification verification

In order to verify that EN2 is cleaved in LnCaP cells the protein bands need to be analysed by mass spectrometry. Mass spectrometry can be used for protein identification in two ways: a) searching for a known peptide mass (EN2) or b) de novo sequencing that would assign amino acids depending on the mass of the
peptide fragments. Whether these peptides are isoforms is difficult to determine using mass spectrometry alone as they have very similar sizes. RNA sequencing could help address this. EN2 protein may undergo SUMOylation that in turn would help regulate nuclear import and export. The addition of a SUMO protein has a similar effect to ubiquitination, although it may also be involved in other cellular processes. EN2’s protein sequence was run through a predictive software, SUMOplot™, and the results are outlined in Figure 7.4 below. The results suggest there is a very high probability that EN2 is SUMOylated within the homeodomain.

![Figure 7.4. SUMOplot™ analysis for detecting SUMOylation sites.](image-url)

The commercially available kits to detect SUMOylation are unlikely to be sufficiently sensitive to detect a very small proportion of SUMOylated EN2, and additional techniques including ELISA and immunoprecipitation (using a antibody against the SUMO protein) may also be necessary.

### 7.4.2.3 Exploring EN2 protein-protein interactions and intercellular transfer using the NanoBRET assay.
An alternative approach to measuring the efficiency of EN2 intercellular transfer would be to use EN2 protein-protein interactions to detect internalised EN2 in a recipient cell, using NanoBRET. Antibodies are generally not sensitive enough to detect trace amounts of protein. NanoBRET® detects resonance energy transfer between two proteins denoted as the donator and acceptor, as shown below (Figure 7.5).

**Figure 7.5. NanoBRET® mechanism of action.** The donor cell line that expresses the donor protein with the NanoLuc® tag and the acceptor cell line expresses an acceptor protein tagged with HaloTag® with a ligand attached. When the substrate is added to the culture the NanoLuc® will luminescence and if the acceptor protein has interacted with the donor protein it will close enough for the ligand to accept the energy from the luminescence and fluoresce.

If a protein-protein interaction is identified cell non-autonomously, such as EN2 and EIF4E, the NanoBRET® assay could also be used to establish whether this interaction is more or less efficient in a co-culture assay compared to separated cells (Transwell®), outlined in Figure 7.6, and in a quantitative manner.
Figure 7.6. Measuring EN2 intercellular transfer using a Transwell® assay and NanoBRET®

system. The donor cell line (NanoLuc®-EN2 expressing cells) is seeded at the top inside the
ThinCert™ and the acceptor cell line (HaloTag®-POI expressing cells) at the bottom of the Transwell
plate. Once settled the ThinCert™ is transferred to the Transwell containing acceptor cells and
incubated. The negative controls are no acceptor cells and/or acceptor cells that have not been
transfected with the HaloTag-POI.

In this study, EIF4E has been shown to be a primary candidate for this assay,
though more evidence is required to show co-localisation before proceeding with the
experiment (such as the confocal line profile carried out in Section 5.4.3).

Another experiment that could be setup is, for example, to determine whether
EN2 dimerizes or not. In this instance, EN2 would be both the donor and acceptor
and if a fluorescent signal is produced it shows that they have come together. It is a
flexible system, especially when little is known about EN2’s protein function and binding partners. Furthermore, EN2’s ability to induce rapid phosphorylation of translation initiation proteins could be investigated with this method, ideally within a 3D cell culture model as discussed below in Figure 7.4.2.4.

7.4.2.4 Investigating EN2’s part during cell-in-cell action and in the tumour microenvironment using a 3D setup

The dynamic behavior and possible signaling function of EN2 (between cells) makes 3D cell culture particularly useful. In addition, Chapter 6 further implicated EN2 in the loss of cell polarity and the detachment from the natural basement membrane (adopting a more rounded shape, as shown in Figure 6.7), both of which are early events in carcinogenesis. If EN2 has a distinct role in cell-cell communication and interaction it could have been previously overlooked when studied with a 2D approach. In order to look at cell-in-cell action or cell fusion more closely and determine which one of these events is taking place and EN2’s role within it, the 3D cell culture would more accurately mimic the tumour microenvironment than previous studies (Nyga et al. 2011).

Figure 7.7. Schematic diagram of 2D and 3D cell culture models. The diagram highlights the difference between 2D cell culture where cells are relatively flat and detached on a hard plastic surface (a), 3D cell culture in which multiple cell types are embedded within a porous scaffold, as a spheroid, that could be multicellular (b) that then closely resembles cell-cell and cell-matrix
interactions *in vivo* such as (c) that is also grown supported by stromal cells (taken from (Nyga et al. 2011)).

3D model systems allow cells to delineate and mimic the tumour microenvironment. It allows cells to grow in all directions and retains the complex interplay between the cancer cells and the microenvironment that includes multiple cell types (such as immune cells and epithelial cells). For more details about 3D cell culture as a prerequisite to *ex vivo* and *in vivo* studies, and prostate cancer and the tumour microenvironment see **Section 7.5.1**.

**7.4.2.5 Isolation and characterisation of EN2-containing vesicles and further investigation into EN2’s intercellular transfer**

To continue investigating the best therapeutic approach, it would be useful to further characterise EN2’s secretory and internalisation mechanisms, particularly the EN2-containing vesicles seen in Figures 4.9, 5.6a and 5.12. **Section 7.4.2.4** describes 3D cell culture, which would be an ideal experimental setup to study the downstream effects of EN2 intercellular transfer and paracrine activities, between cancer cells, neighbouring cancer cells and the normal stromal epithelial cells. The seemingly regulated secretion and internalisation of EN2-containing vesicles require cell-cell contact and communication, which have been shown in this study to be fundamental to the process.

Due to the large size of the microvesicle they could be prostasomes. These have been investigated in a similar manner to this study by Ronquist *et al* where they sought to confirm whether there is differential uptake between prostasomes and PC3 exosomes by PC3 cells (K. G. Ronquist et al. 2016). The Norgen and SEC protocol worked well in this study for the isolation of microvesicles of a specific size-range, as shown in **Figure 5.14** (Chapter 5). After isolation and purification, mass spectrometry would still be required to confirm EN2’s presence. Furthermore, these vesicles could also be a source for other potential cancer biomarkers (Lance et al. 2011). For more details see **Section 7.5.1** below.
7.4.3 New hypotheses

The following hypotheses are suggestions for the continued exploration of EN2 as a clinical tool in prostate cancer, based on the discoveries and information acquired in this study.

- EN2 promotes cell-in-cell action.
- The function of EN2 changes during tumour progression from transcription to translation; EN2 close to the cell membrane can alter local protein synthesis.
- Genetically modified T cells against EN2 could be more effective therapy.

7.5 Wider implications of this study

7.5.1 Outlook: cancer biomarkers

From a clinical perspective, the field of cancer biomarkers is moving toward measuring molecules that require much less invasive testing such as EN2 - an extracellular protein in urine (Thomas et al. 2010; Minciacchi et al. 2017). These tests are usually less painful, costly and risky. In some cases, the biomarkers are able to be detected much earlier in the process (even when tumours are not yet visible and the patient is relatively healthy) than a tissue biopsy and act as a screening tool - this is known as a liquid biopsy (Karachaliou et al. 2015; Kaisaki et al. 2016). Examples of other non-invasive biomarkers include microvesicles such as exosomes, circulating tumour DNA (ctDNA), circulating tumour cells (CTCs) and prostasomes - commonly found in prostate cancer, see below for more details. For microvesicles, defining and isolating the right size population has been particularly difficult; the methodology to do so is still being developed, using techniques such as ultracentrifugation and size exclusion chromatography (Lobb et al. 2015). Subsequently, there is also difficulty in standardising the collection, storage and
analysis due to the variation between laboratories. However, ctDNAs and CTCs are able to be quickly identified, in blood, due to the development of next-generation sequencing (or high throughput sequencing) (Kaisaki et al. 2016).

The interplay between cancer cells and their local environment (known as the tumour microenvironment or TME) is a vital component of metastasis and resistance to therapy. Strong interactions between the tumor-stroma will help promote metastasis (Balkwill et al. 2012); a change in receptors on the surface of cancer cells can manipulate the microenvironment to strongly favour regulatory T cells and promote immunosuppression (Facciabene et al. 2012). These are just two examples of how the TME is a barrier to therapy, the mechanisms of which can vary widely depending of the stage and location of the disease. The non-malignant cells found within the TME, which are often harder to delineate, are another source of tumour-promoting signaling (Balkwill et al. 2012). Recently, there has been a focus on proteomic profiling of the heterogenous population of extracellular vesicles - particularly in prostate cancer - in order to find novel biomarkers and determine the role of microvesicle transfer between cancer cells and neighbouring non-malignant cells (Cesi et al. 2016; Minciacchi et al. 2017).

The search for biomarkers in the TME is vital as there is still a huge deficit in the number of biomarkers that make it to the clinic - such as those that can indicate the level of tumour aggressiveness and at early stages. Biomarkers that provide more information to the clinician can prevent unnecessary or inadequate treatment (due to poor stratification) and improve a patient’s quality of life. Those biomarkers that perhaps have failed as a standalone test could be part of a ‘panel of biomarkers’ that collectively are more informative. However, there is a more immediate issue of getting biomarkers into clinical trials in the first place, this is further discussed in Section 7.5.2 below.

7.5.2 Perspective: cancer research

It must become standard practice to validate all commercially bought products. A huge setback in this study was the over-reliance on a commercial antibody - one that had been used widely ‘in-house’ - against a protein that undergoes large changes in cell shape, folding and behaviour depending on its environment, such as cell type and cell culture media. Consequently, the levels of
detection observed and/or quantified becomes highly inaccurate. When a commercial product such as an antibody is validated by the company, it is important to check the method of validation and repeat it. Ideally, validation should be carried out in the environment that further in vitro experimentation are to be done. The actual validation methods usually consist of knocking down the protein of interest with a subsequent western blot analysis - this is semi-quantitative and can give an approximate size of the protein detected. There are several methods of knocking down a protein, the best approach (if possible) is to completely knock it out using new technologies such as CRISPR/ Cas9 (Section 7.4.2.1). If relatively little is known about the protein, such as EN2, it is pertinent to go on to immunoprecipitate the protein for further analysis by mass spectrometry (Persson et al. 2017). Mass spectrometry (MS) can identify and carry out high-throughput proteomic quantitation without prior knowledge of the protein of interest beforehand (unlike antibodies that are raised specifically against an antigen). Furthermore, the method/s and practices of antibody validation should be under continuous evaluation as techniques and technologies improve.

Mass spectrometry (MS) has been mentioned many times here, and as another means of protein detection it often goes head-to-head with antibody-based detection methods (Hale 2013). However, depending on the target and specimen to be analysed it may not be appropriate. MS is not as sensitive as antibody detection; for example, MS cannot replace the antibody detection of EN2 because EN2 is not found at high enough concentrations relative to the other proteins that are also present in the urine - urine typically has as high noise-to-signal ratio. The latter should not stop MS from being used in the laboratory as a standard practice for validating antibodies (Persson et al. 2017). However, mass spectrometry is yet to be made simple and cost-effective and improvements to it’s sensitivity are required before it becomes more widely adopted (Wasinger et al. 2013).

I have previously mentioned (in Section 2.6.1) that there is a bottleneck between biomarkers going from the lab to the clinic - despite the huge surge in information (also known as ‘big data’) such as metabolomics, genomics and proteomics. The biomarkers that do make it through often fail at clinical trials. This study further advocates the need for a more translational approach and rigorous selection process during preclinical evaluation. 2D in vitro cell culture loosely mimics the environment in which these biomarkers act and thus allows only very general
questions to be asked - the analysis can therefore only guide the next set of questions. This is especially true here and for many studies that look at secreted proteins where the environment hugely influences the way the protein behaves, making it very difficult to interpret the data. The questions asked at early stages could be more vigorous if the following becomes widely adopted: a) 3D *in vitro* models sit in between 2D *in vitro* cell culture and *in vivo* mouse models and b) there is a shift from 2D to 3D earlier along the pipeline. 3D *in vitro* tumour models would improve accuracy (as mentioned already in Section 7.4.2.4); for example, 3D spheroids enable the cells to adopt a normal physiology instead of being stretched out unnaturally on a flat surface - this majorly affects the expression and the spatial organisation of surface receptors. In addition, due to advances in tissue engineering, these 3D models can include multiple cell types and thus, will develop to better imitate the tumour microenvironment and enable further advances to be made in studies that look at metastasis in particular (Nyga et al. 2011) - see Section 7.5.1 for more information. However, wide adoption of 3D *in vitro* models can only happen when it becomes easy-to-use and cost-effective; the continued development of these models by 3D bioprinting will play a vital role here (Albritton & Miller 2017).

In cases where sparse research has been conducted - such as EN2 and prostate cancer - standardised methods and existing data to work from are not available. Thus, it is important that the information (such as that in this thesis) becomes openly available to those also working on similar areas. Knowing what had been tried before and the methods used to do so - not necessarily those that had been published - would have saved time and resources. Furthermore, a platform to share the novel data, such as the time-lapse videos that were fairly subjective, could have helped to provide a wider and inter-disciplinary perspective when designing the next set of experiments.

### 7.6 Thesis conclusions

This study has explored the potential of EN2 as a target for a monoclonal antibody drug-conjugate (ADC). The complex regulatory mechanism controlling EN2 expression means that mRNA expression and protein expression do not strongly
correlate with one another and that the protein expression needs further confirmation using alternative methodology.

The findings of this study indicate that there is regulated secretion of EN2 within vesicles (in high grade cell lines), which would restrict the use of the ADC to early stage prostate tumours. Furthermore, the unconventional and complex secretion and internalisation mechanisms of EN2 make it difficult to accurately quantify the total amount of antibody internalisation with a single assay and thus establish the most effective therapy using EN2.

An alternative therapeutic strategy are engineered T cells that do not rely on the native protein localising to and on the membrane, but instead recognise peptide fragments displayed in a complex with MHC. This study indicates that the EN2-MHC complex will be present on the cell surface of cancer cells at elevated levels. Furthermore, there is no drug attached to the antibody and hence toxicity is less likely to be a problem. Alternatively, small blocking peptides targeting the largely protected domain (at the N-terminus) of EN2 may prevent its secretion and subsequent paracrine function. Doing so would allow the significance of these events to be further elucidated.

In conclusion, Engrailed-2 (EN2) protein remains a potential therapeutic target and diagnostic marker in cancer. A more robust understanding is needed of its secretion and internalisation mechanism, not only because it could help us better understand alternative mechanisms used by other proteins - perhaps for cell-cell communication during tumorigenesis - but also to help tailor a detection assay and future therapy against it.
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