Meseanchymal stem cell mediated sodium iodide symporter gene therapy of breast cancer

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2014-05-07

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Chapter 1

Introduction
1.1 Breast Cancer Overview

Breast cancer was the most commonly diagnosed invasive cancer in Irish women between 2007-2009 with an average annual incidence of 2673 new cases [1]. In 2008, 736 women died from breast cancer in Ireland making it the second biggest cause (16 % relative frequency) of all cancer related deaths in Irish women. Breast cancer also affects men with an average of 20 new cases per year diagnosed between 2007-2009 and with 6 deaths reported in 2008 [1]. On a global scale an estimated 1.4 million females were diagnosed with breast cancer in 2008 with over 458,000 deaths [2]. This represents a huge financial cost to global health services but more importantly an even larger human cost. With a 2 % average annual increase in the incidence of female breast cancer in Ireland between 1994-2009 it is now urgent that our understanding of, and ability to treat this disease is significantly improved [1].

The structure of the breast is complex, consisting of milk producing alveoli housed within lobules and the ducts that carry this milk to the areole and onto the nipples (Figure 1.1). Between these are adipose tissue, connective tissue, and a network of ligaments and lymph ducts. Breast cancer comprises of abnormal cells growing initially in breast tissue but with the potential to invade other parts of the body. These cells have acquired mutations in genes responsible for proliferation and survival with the majority of breast cancers arising from epithelial cells. In the breast, cancer may initially arise as an in situ carcinoma, either in lobules or ducts. These accounted for 383 cases in Irish females in 2009 representing less than 14 % of the total number of breast cancer cases diagnosed that year [1]. In situ carcinomas have not yet shown evidence of invasion through the epithelial basement membrane. When this happens the tumour is known as an invasive carcinoma. These are more common than in situ types and accounted for the remaining 2974 (86 %) of cases diagnosed in 2009 [1]. Of the invasive carcinomas approximately 75-80 % are invasive ductal carcinomas [3]. The remainder is made up of less common, mixed ductal-lobular, inflammatory, colloid, tubular, medullary, papillary, metaplastic and micropapillary breast cancer.
Once a carcinoma has become invasive, it breaks through to the stromal tissues and has the capacity to metastasise either locally into the chest wall or skin, or distally through intravasion of lymphatic or blood vessels, followed by circulation, extravasion and subsequent seeding into sites such as the lungs, liver, nodes or vertebrae as shown in Figure 1.2. Depending on their inherent tumour initiating potential and the suitability of the new microenvironment to interact and support their growth, the newly located cells either die, become dormant or form a new tumour [5, 6].
1.1.1 Diagnosis

Breast cancer is diagnosed through an extensive process typically referred to as standard triple assessment [8, 9]. Patients can present with changes in shape or size of the breast, changes in the appearance of nipple or skin, the presence of a lump or pain in the breast or swelling of the axilla. These patients are assessed for general health and familial risk of cancer through compilation of a thorough medical history. This is followed by physical examination of suspected breast cancer patients which includes X-ray, ultrasound and mammogram or in special cases MRI scan. Further confirmation is provided by needle (core), stereotactic, excision or wire localisation biopsy with subsequent analysis by a pathologist whereby abnormalities if present can be identified and defined. Using this methodology sensitivity as high as 99% can be achieved [9].

Figure 1.2 Distal metastasis of an invasive ductal carcinoma [7].
1.1.2 Staging

Following confirmation of the presence of breast cancer in a patient, clinicopathological data is compiled and used to classify the cancer in order to better inform the treatment strategy. This is done using TNM staging [10]. This approach looks at size of the tumour in centimetres (cm) (T), the number of nodes affected by metastasis (N), and the presence of distant metastasis (M) to organs such as the liver, lungs, brain, etc (Table 1.1). 5-year survival rates are much higher at earlier stages (Stage I, 92 %) compared to later Stage breast cancer (Stage IV, 26 %). Staging allows for selection of more appropriate treatment strategies for individual cancer cases. More aggressive treatment regimes can be used to target more aggressive or advanced breast cancers such as Stage III or IV while less aggressive strategies may be used for Stage I or II.
<table>
<thead>
<tr>
<th>Stage</th>
<th>T, N, M attributes</th>
<th>5-year Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis, N0, M0</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>T1, N0, M0</td>
<td>92 %</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>(T0/T1, N1, M0) or (T2, N0, M0)</td>
<td></td>
</tr>
<tr>
<td>Stage IIB</td>
<td>(T2, N1, M0) or (T3, N1/N2, M0)</td>
<td>84 %</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>(T0-T2, N2, M0) or (T3, N1/N2, M0)</td>
<td></td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T4, N0-N2,M0</td>
<td>62 %</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any T, N3, M0</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T, any N, M1</td>
<td>26 %</td>
</tr>
</tbody>
</table>

**Tumour grading**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour ≤2cm</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour &gt;2cm, ≤5cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour ≥5cm</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour any size growing into chest wall or skin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nodal involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>N2</td>
</tr>
<tr>
<td>N3</td>
</tr>
</tbody>
</table>

**Distant Metastasis Grading**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX</td>
<td>Presence of distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis found by imaging methods or physical examination</td>
</tr>
<tr>
<td>MI</td>
<td>Distant metastasis present (typical sites include lungs, liver, brain, bone etc).</td>
</tr>
</tbody>
</table>

Table 1.1 TNM staging of breast cancer with 5-year survival rate [10]. 5-year survival data based on patients diagnosed in Ireland between 2004-2008 [1].
1.1.3 Grading

Another method of tumour classification involves the microscopic examination of cancer cells and tissue structure in order to determine how abnormal the tissue appears and how quickly it might proliferate and metastasise. Grading looks at the level of differentiation in a tumour. Less differentiated cells have more stem cell-like qualities and are likely to contribute to more aggressive cancers. Differentiation of breast cancer tissue is currently determined using the Nottingham modification (Elston-Ellis) of the Bloom-Richardson system established in 1957 [11, 12]. This is based on the capacity of the tissue to form ducts, nuclear pleomorphisms and mitotic count. Categories of grade are defined as follows:

GX  The grade cannot be determined
G1  Well differentiated
G2  Moderately differentiated
G3  Poorly differentiated

By itself, grading can be used to inform on the potential survival and treatment required for a breast cancer, however this data is usually represented as a constituent of a broader index known as the Nottingham prognostic index (NPI) [13]. This takes tumour size, grade and nodal involvement into consideration to determine a prognosis following surgery in a patient. It also provides an indication of the need for adjuvant chemotherapy. However, the NPI fails to provide any indication of the potential effectiveness of a treatment in individual cases. This limitation is a direct result of the heterogeneity of breast cancer, but a more recent system of molecular classification in breast cancer, known as epithelial subtype, has offered a substantial alternative to this failing.
1.1.4 Epithelial Subtype

Using microarrays, Sorlie et al. [14] examined a limited gene expression profile in 78 breast carcinomas and 4 normal breast tissue samples. Based on these expression profiles, the breast tissues were subdivided into 5 main subtypes of which normal breast tissue is one [14, 15]. The malignant subtypes can be distinguished in terms of their expression of estrogen, progesterone and human epidermal growth factor 2/neu (HER2/neu) receptors (Table 1.2) [14-17]. The luminal A subtype strongly expresses estrogen and/or progesterone but are negative for HER2/neu. Luminal B breast cancers (triple positive) are positive, while Basal (triple negative) are negative for all 3 receptors. HER2 carcinomas strongly express HER2/neu, and are estrogen and progesterone negative.

<table>
<thead>
<tr>
<th>Epithelial Subtype</th>
<th>Estrogen receptor</th>
<th>Progesterone receptor</th>
<th>HER2/neu receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HER2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Basal</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.2 Breast cancer epithelial subtypes as defined by gene expression profiling [14-19].

There is a strong association between prognosis and each subtype [14]. Luminal A is by far the most successfully treated subtype of breast cancer while the basal or triple negative subtype is the most difficult [14, 20]. Sensitivity to treatments varies by subtype. As a result, treatment regimes are often determined on the basis of epithelial subtype. Estrogen drives proliferation of estrogen receptor positive cancer cells. Hormone therapy works by blocking this effect. This is achieved using drugs such as Tamoxifen which
is an estrogen receptor antagonist. In postmenopausal patients, estrogen is produced by the enzyme aromatase. Aromatase inhibitors, such as Letrozole, Anastrozole or Exemestane block the action of aromatase and stop the supply of estrogen to cancer cells. While hormone therapy is frequently very successful in estrogen receptor positive breast cancer, it is ineffective in receptor negative subtypes. Cheang et al. [21] showed that the 10-year relapse-free survival in Luminal A cancers was 70 % after adjuvant therapy with the ER antagonist, Tamoxifen. This was significantly lower in Luminal B breast cancer (64 % complete response). However, addition of the HER2-neu targeting antibody, Trastuzumab, significantly improved outcomes in Luminal B cancers treated with aromatase inhibitors [22, 23]. While strong HER2/neu expression in the HER2 subtype offers an excellent target for Trastuzumab treatment it also diminishes sensitivity to radiotherapy [24]. However, combining Trastuzumab or other HER2/neu targeting treatments such as tyrosine kinase inhibitors has a radiosensitising effect on HER2/neu positive cancers and may prove to be beneficial in future studies [25-27]. Basal breast cancer also responds poorly to radiotherapy [28]. However, a similar monoclonal antibody: radiotherapy combination, shown to be successful in sensitising head and neck cancer to radiotherapy by increasing overall 5-year survival rate from 36.4 % to 45.4 %, may prove to be suitable for this subtype [29]. In contrast, luminal subtypes, particularly luminal A, respond well to radiotherapy [28, 30]. Using a cohort of 82 patients, Rouzier et al. [31] demonstrated that Luminal subtypes were relatively insensitive to a combination of paclitaxel, fluorouracil, doxorubicin and cyclophosphamide adjuvant chemotherapy with a pathological complete response (pCR) in only 7 % of cancers. In contrast a pCR was achieved in 45 % of HER2 and Basal breast cancers.
1.1.5 Breast Cancer Markers

A number of protein markers are available to help with identifying and defining the parameters of a breast cancer. These markers can be present on the tumour itself, or can be found circulating in the blood.

Tumour markers are usually assessed using immunohistochemistry following a biopsy or complete excision of the tumour. They offer less information in terms of definitively distinguishing between normal and cancer tissues and more as prognostic indicators. Tumour markers include estrogen, progesterone and HER2/neu receptors which, as outlined previously, are valuable in determining subtype and appropriate treatments [32-34]. Ki67 is an indicator of the rate of proliferation in cells and is used to predict a chemotherapeutic response in tumours [35]. High levels of Cyclin D in ER positive tumours tend to indicate a poor response to hormone treatment [36, 37]. Cyclin E is also associated with less successful treatment response [36, 38]. ER beta has been associated with a better Tamoxifen response [39]. Reduced levels of p27 indicate a higher histological grade of cancer [40]. Nuclear expression of mutated tumour suppressor gene p53 is associated with more tumour aggression and earlier metastases of inflammatory breast cancer [41]. The Sodium Iodide Symporter (NIS) has been identified as a potential cancer biomarker. Previous work has shown, using immunohistochemistry (IHC) techniques, elevated levels of NIS in malignant tissue compared to normal [42-47]. However, there was also an indication that NIS might fail to discriminate between benign and malignant tissues of the breast [47]. Further studies are needed to determine the true potential of NIS as a cancer biomarker since current data is either subject to small cohort size, or the subjective nature of IHC.

Blood borne markers such as cancer antigen 15-3 (CA15-3) and 27-29 (CA27-29), are expressed on the surface of tumour cells and shed into the bloodstream, and can be used to monitor progression or recurrence of breast cancer [48, 49]. The carcinoembryonic antigen (CEA) test is primarily used to determine recurrence in a treated patient due to its limited sensitivity [50].
These tests are severely limited in their potential to diagnose the presence of a tumour, however, there have been reports that blood borne microRNA may be successfully used for this function. Heneghan et al. [51] demonstrated that increased systemic levels of miR-195 was reflected in cancer tissue and reverted back to normal levels postoperatively. A similar trend was seen with Let-7a. Further work demonstrated that elevated miR-195 levels were specific to breast cancer and could differentiate breast cancer from controls and other cancers with a sensitivity of 88 % and a specificity of 91 % [52].

1.1.6 Treatment

Following a breast cancer diagnosis, classifications such as stage, grade and subtype are used to determine what treatment strategy is most suited to the presented cancer. These strategies often involve combinations of the following therapies:

Surgery: Usually the first option is to remove the tumour and surrounding tissue from the breast [53]. This can be done by lumpectomy which has a smaller margin of tumour-associated normal tissue or mastectomy in which the entire breast is removed. Further strategies include the removal of nearby lymph nodes to ensure containment of the cancer. The major drawback of surgical therapy is that the site of malignancy has to be identified and the tumour has to be operable. This limits its value in metastatic cases.

Chemotherapy: Cancer cells typically have a higher proliferative index than normal cells. As such, they are more vulnerable to damage by cytotoxic and mutagenic agents which impair mitosis and result in cell death. This can be achieved through different classes of chemotherapeutics. Alkylating agents attach an alkyl group to guanine in DNA interfering with replication and transcription of genes. These include cyclophosphamide which is used as part of a combination chemotherapy with methotrexate and fluorouracil [54, 55]. Anthracyclines intercalate with DNA or RNA and interfere with replication [55, 56]. Enzyme inhibitors interfere with key DNA enzymes such as
topoisomerase (doxirubicin) which is involved in DNA replication leading to DNA damage [57]. Chemotherapy is far from perfect. Some normal cells also proliferate quickly and are subject to the same cytotoxic effects intended for cancer cells. These cells include those of the gastrointestinal tract, bone marrow, testicles and ovaries, which if damaged can result in gastric side effects, reduced haematopoiesis, and sterility. Additionally, limited blood supplies in larger tumours can reduce distribution of chemotherapeutics to these sites leaving them unharmed [58].

Targeted Therapy: Although technically a type of chemotherapy, targeted therapy is starting to evolve into a separate category of treatment typified by improved specificity for cancer over normal cells, with reduced side effects. To date they include a selection of drugs (Trastuzumab, Lepatinib) concerned with interrupting the proliferative and anti-apoptotic effects of HER2/neu or inhibition of vascular endothelial growth factor A which is involved in tumour angiogenesis [59, 60].

Hormone Therapy: As previously described, this approach works by blocking the positive proliferative effect estrogen causes in ER-positive tumours [21].

Radiotherapy: High energy ionising radiation is used to damage DNA. This is achieved by direct ionisation of atoms in the DNA strand or indirectly through ionisation of water resulting in free radicals which have a damaging effect on DNA. Methods of radiotherapy used for breast cancer include external, internal (brachytherapy) and intraoperative radiotherapy [61-64]. External beam radiation is the most common type of radiotherapy and involves the targeting of a high energy beam of radiation, guided by a linear accelerator, to a point of focus within the breast. Internal radiotherapy involves the temporary insertion of radioactive seeds within the space created by a lumpectomy in order to prevent a local recurrence. Once the required radiation dose is delivered the seed is then removed. Intraoperative radiotherapy is performed during a lumpectomy whereby a single dose of radiation is delivered to the remaining high risk border tissue following excision of the tumour.
Radiotherapy, particularly external beam radiation has been long established as an effective tool in improving surgical outcomes. However in most cases it is necessary to know the location of the cancer in order to treat it, so like surgery, radiotherapy has limited effectiveness in treating metastatic breast cancer.

1.1.7 Treatment inadequacies

The success of breast cancer treatment in Ireland has improved significantly in the last 20 years with the relative 5-year survival rate increasing from approximately 72% (1994-98) to 81% (diagnosed 2004-08) in females [1]. This improvement can be explained by better treatments and diagnosis of breast cancer at an earlier stage in disease progression. Despite the relative success rates it remains the case that once breast cancer has spread to distant sites of the body such as the brain, lungs or bone, treatment becomes palliative. Radiotherapy, chemotherapy, hormonal therapy, targeted therapy and surgery only serve to reduce symptoms and slow progress of the disease. It is thought that some metastases may lie dormant and cease proliferating. These quiescent cells are usually insensitive to chemotherapy since it targets rapidly growing cells. There is a need for a treatment regime that can effectively eliminate all sites of metastases to ensure there is no recurrence of disease.

In 2003, Wicha et al. [65] demonstrated that as little as a 100 breast cancer derived cells which shared similar cell surface markers, were capable of seeding a tumour in immunocompromised mice. These cells, described as breast cancer stem cells (BCSC) have the ability to self renew and possibly regulate the tumour microenvironment [66, 67] and may also have the potential to be resistant to chemotherapy and radiotherapy [68, 69]. It is likely that where recurrences occur it is the cancer stem cell population that reinitiates the tumour. [70, 71]. For therapeutic efficiency and to prevent recurrence, it is critical that BCSCs are completely eliminated with whatever treatment is applied.
In addition, there is currently a plethora of potential adverse reactions associated with different treatment strategies [72]. These range from mild issues such as dry mouth or itchiness to more serious problems such as hepatotoxicity or infertility. While these differ significantly in severity, each side effect has the potential to deal a body blow to patient wellbeing and quality of life. Even targeted therapies, the newest category of cancer treatments, have the potential to cause discomfort and malaise. The monoclonal antibody Herceptin is associated with cardio toxicity leading to reductions in left ventricular ejection fractions, necessitating regular monitoring through echocardiographs [73]. It is critical that newer generations of cancer treatment eliminate the malignancy effectively but also that they do so in a smarter fashion with less collateral damage.

Looking past the personal cost of the disease, there is a huge financial cost to breast cancer. This affects both individual and national or global economics. Using a traditional capital approach Hanley et al. [74] estimated productivity costs of breast cancer at € 193,425 per person in Ireland in 2008. More alarmingly, the Irish Cancer Registry has estimated, based on trends from 2002-2006, that cases of breast cancer may increase by as much as 53 % by 2030 [75]. Kelley et al. [76] reported 3 cases where Imatinib therapy was discontinued in metastatic cancer patients owing to financial difficulties leading to a worsening of their disease. Ireland may be particularly vulnerable to this trend given that in 2010, 55 % of patients had to use personal savings as government support and private insurance was inadequate to cover the costs of cancer treatments [77]. It is hugely important that newer therapies not only be medically-effective but also cost-effective.

1.1.8 Ideal breast cancer therapy

Looking to the future there are a number of challenges to consider in developing the ideal breast cancer therapy. These are as follows:
Efficacy: New treatments must be more effective in achieving long term cancer free-survival.

Target cancer stem cells: To be effective and to prevent recurrence of breast cancer newer treatments must target and eliminate cancer stem cells as well as the remaining bulk of the tumour.

Target metastases: Currently, metastasised breast cancer treatment is palliative. In an ideal situation, newer treatments should be capable of destroying sites of metastasis or at the very least be capable of mapping out there location for surgical purposes.

Minimal side effects: The ability to distinguish between normal and cancerous tissue will be crucial in reducing the occurrence of adverse events. This would have a positive effect on compliance and improve outcomes.

Cost-effective: The difficulty of designing the perfect breast cancer treatment will undoubtedly result in a more expensive treatment at first. However, new therapies must have the potential to be cost-effective in the long term in order to address inequalities in survival outcome due to deprivation and reduce overall cancer costs.

Convenient to administer: More easily administered treatments have the potential to increase compliance and with it survival outcome as well as reducing costs, both personal and institutional, and reducing patient stress.

Potential to target other cancers: The ideal breast cancer should not only have the above traits but should also have potential applicability in other cancer types or at the very least provide a model of instruction for development of more effective treatments for other cancers.

1.2 Cancer Gene Therapy

In the last 2 decades huge strides have been made in advancing the potential of gene therapy. The fundamentals of gene therapy involve the delivery of a
therapeutic gene(s), to damaged or diseased tissue in order to rectify the abnormality. Cancerous tissue is a perfect candidate for this. The huge potential of gene therapy is reflected in the multitude of therapeutic approaches being investigated. These approaches often overlap but typically include:

Gene replacement: Where mutated oncogenes are responsible for driving a cancer it is believed that reintroducing normal wild type genes could restore normality to the effected tissue. Replacement treatment with miR-143 and mirR-145, both of whom are downregulated in bladder cancer, lead to synergistic inhibition human bladder cancer cell proliferation by regulating PI3K/Akt and MAPK signalling pathways [78].

Sensitisation to chemotherapy: In some cases, cancer subtypes are highly resistant to chemotherapy. Introduction of a gene(s) that interfere with the mechanism of this resistance can help sensitise cancerous tissues to the chemotherapeutics making them easier to treat. Adenovirus-mediated introduction of wildtype P53 was shown to increase chemosensitivity 18-fold in adriamycin resistant MCF-7 cells [79].

Anti-angiogenesis: In order for a cancer to grow it requires sustenance which is delivered through the vascular system. If a proliferating cancer doesn’t generate new vasculature it will starve. Anti-angiogenic approaches attempt to block the formation of new vasculature in cancer tissue. Adenoviral mediated ectopic expression of p16/INK4A in the breast cancer cell line MDA-MB231 demonstrated inhibition of in vivo angiogenesis, as well as in vitro proliferation, and induction of senescence and apoptosis [80].

Gene-directed enzyme prodrug therapy: Prodrugs are inactive forms of drugs which can be activated enzymatically. If these activating enzymes are localised to one place in the body then the prodrug can be administered systemically but only becomes active where the enzymes are present. Tissue targeted delivery of genes expressing prodrug enzymes ensure that those tissues get the primary dose of active pharmaceutical.
Suicide genes: Normal apoptotic programming is often lost in cancer cells. Targeted delivery of apoptotic genes can bypass these glitches and restore controlled cell death in aberrant cells. An example of this can be seen where adenovirus carrying the pro-apoptotic Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) gene was transfected in to Mesenchymal Stem Cells (MSCs). When co-cultured with A459 lung carcinoma cells this viral DNA was transferred from MSCs leading to TRAIL expression and TRAIL-induced apoptosis in A459 cells [81].

Gene silencing: Because of the complex nature of cancer, numerous genes play roles in the development and progression of a tumour. However, some are more culpable than others. By interfering with their post transcriptional expression it is possible to stop translation of their protein product. Small interfering RNA strands of 20-25 base pairs in length have the potential to do this [82]. Aliabadi et al. [83] targeted expression of anti-apoptotic protein myeloid cell leukaemia 1 and the efflux pump P-glycoprotein in drug resistant breast cancer cells introducing genes encoding short interfering RNA. This blocked expression of both proteins and lead to significantly increased doxorubicin cytotoxicity in resistance-induced cells.

Immunotherapy: There are two types of gene therapy which exploit the immune response. The first involves the targeted delivery of genes overexpressing an immune-stimulating cytokine. The potential for immunomodulatory cytokines in cancer therapy has been highlighted by Chikanna-Gowda et al. [84] using implanted K-BALB tumours in BALB/c mice. Intratumoral administration of Semliki forest virus expressing Il-12 which plays a key role in the induction of T-helper1 cell response, resulted in tumour regression compared to controls and inhibition of lung metastasis. Histological analysis of tumours revealed substantial necrosis as well as aggressive influx of CD4+ and CD8+ cells. The second approach involves a gene therapy-mediated immunisation against cancer antigens. One such antigen is polymorphic epithelial mucin, encoded by the MUC1 gene [85]. Upon administration of a live recombinant vaccinia virus expressing MUC1 and Il-2
(serves as an adjuvant chemokine) in 9 patients with advanced inoperable breast cancer recurrences in the chest wall, showed evidence of MUC1-specific cytotoxic T lymphocytes in two patients. One of these had a concomitant decrease in CEA serum levels for 10 weeks. No adverse reactions were observed.

Virotherapy: Although not technically a gene therapy, virotherapy employs many of the viral vectors used in gene therapy. Whereas these vectors are rendered non virulent for gene therapy purposes, when used in virotherapy they are manipulated to be virulent only in cancerous tissues. The potential of HER2 redirected oncolytic Herpes Simplex Virus therapy was previously demonstrated by Nanni et al. [86] in subcutaneous tumours induced by human HER-2 +ve breast cancer cells MDA-MB-453 and BT-474 in mice. Tumour growth was strongly inhibited by repeated doses of the oncovirus. Systemic administration of MDA-MB453 cells resulted in a metastatic model of breast cancer wherein all mice developed ovarian metastasis. All mice in receipt of IP oncovirus treatment appeared to be free from macroscopic ovarian metastases at necropsy. Reduced brain metastasis was also reported in treated mice.

1.2.1 Adenovirus as gene therapy vector

There are a number of methods of delivering gene expression to cells. Reagent-based methods include the use of lipids, cationic polymers, calcium phosphate, activated dendrimers, and magnetic beads [87, 88]. Instrument-based methods include electroporation, biolistic particle delivery, microinjection and laserfection [87, 88].

Viral candidates include but are not limited to retroviruses, lentiviruses, adenovirus, adeno-associated virus, herpes simplex virus, and vaccinia virus [89]. All these viruses possess excellent transfection efficiency when used with appropriate cells lines but they also have drawbacks. Retro and lentiviral infection can lead to long term transgene expression, however random chromosomal insertion can sometimes result in insertional mutagenesis [89].
Adeno-associated virus requires a helper virus for amplification. Herpes simplex virus infections can become latent leading to a cessation of transgene expression and vaccinia virus can be cytopathic [89].

Despite the many options available for delivering gene expression, adenovirus is the vector of choice in 23.5 percent of the gene therapy clinical trials worldwide [90]. Adenovirus consists of double stranded linear DNA, non-enveloped virus surrounded by an icosahedral nucleocapsid (Figure 1.3). The nucleocapsid is composed of hexon proteins and penton base proteins to which a fiber spike is attached. These fibers are critical for virus entry into cells as they initiate viral contact through the Coxsackie adenovirus receptor (CAR). This enables interaction between penton base proteins and cellular integrins such as alphavbeta3 and alphavbeta5 which in turn leads to internalisation (endocytosis) through clathrin-coated pits [91]. The efficiency of adenoviral infectivity in cells with low CAR levels of expression can be improved by exploiting this secondary interaction. Protocols involving centrifugation increase contact between integrins and the RGD motif in penton bases [92].

**Figure 1.3** Adenovirus structure. Consists of a double-stranded DNA genome surrounded by an icosahedral viral capsid composed of hexon proteins and penton base proteins to which a fiber spike is attached [93]. Permission granted to use image.
The genome is composed of genes expressed at early and late stages of infection. E1A, E1B, E2A, E2B, E3, and E4 transcription units are expressed early in the replication cycle and are involved in either regulation of viral transcription (E1A), replication of viral DNA (E2A, E2B), or suppression of the host response to infection (E1B, E3). Structural proteins such as those required for capsid formation are encoded by transcription units expressed later in the reproductive cycle (L1-5).

By deleting certain genes of the adenovirus it can be rendered replication-deficient but still maintains its ability to infect cells. Because of their importance to adenoviral reproduction, E1 genes are typically deleted to eliminate the capacity for genetic transcription. Amplification of adenovirus is then achieved by producing those packaging or helper cell lines which express these E1 genes.

As a viral vector for cancer gene therapy adenovirus has a number of advantages:

1. High transduction efficiency with leads to better penetration of the therapeutic gene.

2. Transduces a large range of cells both replicating and nonreplicating.

3. DNA insert sizes of up to 8 kb potentially accommodating a large variety of therapeutic cassettes. Helper-dependent adenovirus vectors can accommodate up to 37 kb [94].

4. Adenoviral vectors can be propagated at high viral titres facilitating relative ease of production.

5. Adenovirus does not integrate into the host genome so the risk from insertional mutagenesis is nil. This nonintegration leads to transient expression of therapeutic genes, which if sufficient for cancer therapy is much more preferably to long term expression.
For these reasons adenovirus is the leading vehicle of choice in gene therapy approaches with almost a quarter of clinical trials involving an adenovirus vector [90]. Several adenoviral-mediated gene therapies of cancer have already been investigated in a clinical setting.

A Phase II study targeting breast cancer was shown to deliver wildtype p53 transgene expression in a number of patients with locally advanced breast cancer using an adenoviral vector (AdCMV-p53) [95]. As well as p53 messenger RNA expression which persisted up to 21 days post injection, p21^{WAF1/Cip1} mRNA expression was also observed in biopsies of all but 2 patients analysed. The authors suggested that administration of the adenoviral vector produces local activation of the innate immune response followed by activation of adaptive immunity. Extensive tumour infiltration of CD8+ T-lymphocytes was also observed which the authors attributed to AdCMV-p53 and not the associated chemotherapy. Despite serum antibody levels revealing an adenoviral-specific immune response in all patients, the combination of AdCMV-p53 treatment with chemotherapy was deemed safe with no increase in systemic toxicity. All patients achieved a partial response which accounts for a >50 % reduction in tumour volume. Critically however, this trial failed to reach a complete pathological response in any patients.

1.2.2 Limitations of Adenoviral Vectors

Despite the obvious potential of adenoviral-mediated gene therapy of cancer, to date, this potential has not been realised in a clinical setting. Partial clinical responses have been observed in patients [95-98], and if optimised these treatments may have value in prolonging life or reducing symptoms in patients with advanced disease. However, adenoviral mediated gene therapy is rarely if never accountable for a complete pathological response in patients. This disappointing outcome is attributable to two major disadvantages of adenovirus vector:
1. It is unable to specifically target cancer tissue due to the ubiquity of CAR in a multitude of cell types and a relatively low level on primary tumour cells [99]. Dwyer et al. [100] demonstrated that systemic administration of adenoviral vector in a mouse pancreatic cancer model lead to high levels of transgene expression in the liver and no transgene expression in tumours. Use of a tumour-specific promoter MUC1 eliminated expression in the liver but also failed to deliver transgene expression to tumours. In a separate study using a mouse ovarian cancer model, Dwyer et al. [101] also highlighted weaker tumour expression following intratumoral injection of an adenovirus carrying a MUC1 versus CMV promoter controlled transgene. While exploiting promoters which selectively target tumours over normal tissue is valuable, it is just as important that these promoters do not undermine optimal expression of the therapeutic gene. Successful attempts to improve breast cancer cell transduction by modifying adenoviral fibres have been described by Stoff-Khalili et al [102]. However, where this approach was applied in a metastatic breast cancer mouse model only 8% of metastases were infected and expressed the adenoviral transgene after systemic injection. The authors also suggested that in order to overcome this limitation tumour-specific expression strategies need to be supplemented by strategies to increase tumour accessibility [103].

2. Adenovirus is highly immunogenic. Initial doses can induce a rapid innate response [104, 105], and post-infection clearance through the adaptive immune system [106]. Adverse immunological reactions are typically mild when they occur. However, higher systemic doses of Adenovirus vector may result in a serious and potentially fatal condition known as cytokine storm [107].

These hurdles must be overcome if such an approach is to be successful. Ideally, the adenoviral vector would be engineered to find and infect only cancerous cells without stimulating an immune response. This is a technically
challenging approach and may take many years to achieve. An alternative
approach would be to load adenoviral vectors into cellular vehicles in order to
protect and deliver therapeutic genes to tumour sites. The use of cell-based
therapies is already being explored. Leukaemia has been treated successfully
for years using bone marrow transplants, which is in itself a form of cell
therapy. Treatment of solid tumours has been more difficult. Some success has
been achieved with neural stem cells which were shown to migrate not only to
the main tumour burden but also to small intercranial microsatellite deposits
typical of most gliomas [108]. Treatment of mice with neural stem cell grafts
expressing cytosine deaminase with the prodrug flucytosine, resulted in an 80%
reduction in tumour volume relative to untreated tumours [108]. A more
recent study has demonstrated a neural stem cell tropism to breast cancer
metastases in a mouse model indicating potential in breast cancer therapy
[109]. Systemically administered endothelial stem cells have been shown to
home specifically to hypoxic pulmonary metastases in mice. Furthermore,
implanted endothelial stem cells expressing cytosine deaminase produced an
anti-tumour effect following systemic administration of flucytosine which
prolonged murine survival [110]. Engineered T-cells are currently being
investigated as a means of targeting and destroying tumours [111, 112]. This
involves isolation and expansion ex vivo of T-cells. They are subsequently
engineered to express a tumour targeting receptor, and reintroduced to the
patient in high numbers. Human trials are currently underway using T-cells
expressing anti-CEA in patients with CEA-positive tumours [112]. Another
study uses anti-p53 engineered lymphocytes to target p53 overexpressing
metastatic cancer [111]. The difficulty with both approaches is the potential for
unintended targeting of healthy tissue. Not all cells expressing CEA will be
part of a tumour. Low levels can still be expressed in adults, especially
smokers [113]. Cross reactivity between over expressed tumour-associated p53
and normal functional p53 of healthy cells may also occur. In both cases,
unwanted side effects may occur. In fact preliminary results from the anti-p53
T-lymphocyte study indicate a large number of side effects although none were
categorised as severe. Efficacy was poor with only 1 in 9 patients achieving
tumour regression. The anti-CEA trial was terminated due to safety concerns and a lack of efficacy. An alternative approach using MSCs may have significant potential. Due to their immune privileged status and ability to migrate and engraft at tumours sites, MSCs may represent a means of delivering adenoviral directed expression of a large variety of therapeutic genes to tumours [114-117].

1.3 Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs) are multipotent stem cells found predominantly in the bone marrow but also in trabecular bone, teeth, adipose tissue, muscle and the umbilical cord or placenta [118]. As stromal cells they have a fibroblastic morphology and play a role in providing a structural framework for other cells. When cultured in specific growth media they may be induced to differentiate into multiple lineages of connective tissue such as fat, bone, cartilage, muscle and tendons. For example, exposure to dexamethasone, β glycerophosphate and ascorbic acid-2-phosphate can lead to osteoblast formation [119]. Similarly adipogenesis can be achieved by cell culture in media containing reduced FBS (5 %), insulin, dexamethasone, indomethacin, and 3-isobutyl-1-methylxanthine. MSC culture in the presence of transforming growth factor beta leads to a chondrogenic lineage [120]. Additionally, neural and cardiac lineages can also be generated from MSCs indicating their huge potential in tissue engineering applications addressing issues such as osteoarthritis, bone fractures, and nerve and cardiac damage [121-123].

Bone marrow MSCs are typically isolated through aspiration from the superior iliac crest of the pelvis in human donors. Aspirates are then subjected to fractionation on a density gradient such as Percoll and the mononuclear fraction is cultured in Dubecco’s Modified Eagles medium with 10 % FBS for 12-16 days. This allows for the nonadherent fraction such as haematopoietic cells to be depleted from the adherent MSCs. In order to optimise MSC
proliferation and limit cell loss through differentiation pre-selected FBS is used [124, 125]. To confirm the presence of MSCs it is important to test for certain defining characteristics. These include the ability to adhere to plastic, to differentiate into osteogenic, chondrogenic and adipogenic lineages as well as the presence or absence of surface markers [118, 126]. MSCs should be greater than 95 % positive for CD105 (endoglin), CD73 (ecto 5' nucleotidase), CD90 (Thy-1) while less than 2 % positive for CD45 (pan leukocyte marker) and CD34 (primitive hematopoietic progenitors and endothelial cell marker)[126]. Growth rates of bone marrow MSCs are reasonably fast but vary significantly depending on donor and characteristics of each clonal selection. Population doubling times typically range from 20-36hrs [127-130]. Furthermore, MSCs can be maintained in an undifferentiated state for a number of passages allowing for generation of high cell numbers and long term in vivo studies [131]. More recent studies have demonstrated MSC culture without FBS in a chemically defined medium, thus eliminating the risk of zoonosis where MSCs may be used therapeutically [132].

1.3.1 MSC tumour tropism

When introduced systemically into a healthy unconditioned model, MSCs have a natural, low level tendency to migrate to the lungs, liver and bone [133]. Allers et al. [134] describe a time-dependent localisation of human MSCs in a mouse model whereby MSCs were found in the bone marrow, spleen, lung, bone, skeletal muscle and teeth between 15-180 mins post-systemic infusion. MSCs could be found in the bone marrow and spleen between 1-14 days later and in the lungs 1-7 days post-infusion. Long term data (4-13 months) showed evidence of MSC localisation in the lungs, bone marrow, bone, liver, spleen, cardiac muscle, teeth skeletal muscle, tail, ear and liver. Using bioluminescent MSCs in a mouse model, Kidd et al. [116] demonstrated that donor MSCs initially localised in the lungs, egress to the spleen and liver and dissipate over time. The dynamic dissemination of MSCs in this model is in stark contrast to the highly focused migration of MSCs towards sites of injury. Upon
introduction of a wound in murine models, systemically-injected donor MSCs migrated and engrafted specifically at the sites of injury and remained detectable over time [116]. This targeted migration appeared to respond to the inflammatory signal originating from the wound and allowed MSCs to support healing processes [116, 133]. Historical and current comparisons drawn between the microenvironment of sites of injury and tumours highlight strong similarities, including the shared capacity for release of inflammatory signals such as cytokines, chemokines and other chemoattractant molecules [135, 136]. As such, it was not surprising when further studies tracking migration of bioluminescent donor MSCs revealed specific, persistent co-localisation with sites of breast carcinoma and ovarian tumours following systemic injection and intraperitoneal injection respectively [116]. ‘Dubernard et al. [137] reported migration of male foetal cells across the placenta into maternal breast carcinomas (9/10) during pregnancy. Using vimentin antibody they determined that in 2/9 carcinomas the male foetal cells displayed a mesenchymal phenotype.’ Interestingly, there have been no reports of donor derived MSC engraftment in any tumour type following bone marrow transplants. However, this may be due to the logistical difficulties in building a cohort of patients in receipt of a bone marrow transplant and concurrently or soon after being diagnosed with a solid tumour.

Other studies have examined in vitro and in vivo migration models of MSCs in response to tumours. Nakamura et al. [138] demonstrated an in vitro rat MSC tumour tropism which was confirmed in vivo following contralateral hemisphere inoculation into a rat glioma model. Stoff-Khalili et al. [139] have demonstrated migration of fluorescently labelled MSCs to macroscopic nodules of MDA-MB-231 cells 3 days after systemic administration in a pulmonary xenograft model. No fluorescent MSCs were observed in the lungs of metastasis-free mice. This absence of donor MSCs from the lungs of control mice conflicts with data from Allers and Kidd et al. [116, 134] which describes lung engraftment of donor MSCs 1-7 days after systemic injection. This highlights the need for further studies to give clarity to the actual itinerary of MSCs in tumour and control models. While investigating the effect of
inflammation on MSC migration, Zielske et al. [140] discovered that the efficiency of the MSC tumour tropism occurred independently of tumour volume. This leads to the assumption that MSC migration may extend beyond primary tumours to smaller sites of cancer dissemination such as metastases and micrometastases despite their size. This was confirmed by Dwyer et al. [115] who demonstrated migration of systemically administered fluorescently labelled MSCs to lymph node metastases in murine models.

Confirmation of the importance of inflammatory signal to MSC tumour migration can be seen in a study demonstrating a reduction of MSC-IFNβ tumour engraftment following the introduction of an anti-inflammatory agent [141].

There are a number of soluble factors arising from tumour inflammation which have been implicated in the MSC tumour tropism. These include, platelet derived growth factor (PDGF), epidermal growth factor (EGF), stromal derived growth factor 1α, interleukin-8 (IL-8), transforming growth factor-β1, neurotrophin-3, vascular endothelial growth factor, interleukin (IL-6), monocyte chemoattractant protein-1 (CCL2), urokinase plasminogen activator and Angiopoietin-1, [142-147]. Of these, CCL2 has been shown to recruit MSCs to breast tumours [115].

Because MSCs carry receptors similar to that of migratory cells of the immune system, it has been suggested that the mechanism of MSC migration may be similar to that of leukocytes [148]. A model for MSC migration shown in Figure 1.4 involves the mobilization of MSCs and movement towards the source of chemotactic signals. As the cells reach capillaries they decelerate due to their physical parameters relative to the very narrow capillaries. Alternatively they might slow down in a similar fashion to leucocytes which tether and roll through selectin and integrin protein interactions with the capillary endothelium. These proteins may include p-selectin, very late antigen-4 (VLA-4) or vascular cell adhesion protein 1 (VCAM-1). MSCs roll along a chemokine gradient until they come to a passive (maintain shape and may obstruct blood flow), or active arrest (flatten upon the endothelium) near
the sites of inflammation. Cells subject to active arrest then transmigrate, through diapedesis, across the vasculature which has been rendered leaky by inflammation [148, 149]. The fate of passively arrested cells is unclear.

Figure 1.4 Proposed model of MSC migration. It highlights the possible mechanism of MSC deceleration (A) facilitated by i) tethering and ii) rolling with subsequent arrest (B) at the source of inflammation by passive or iii) active means followed by extravasation (C) facilitated by (iv) transmigration (diapedesis) in the case of active arrest [149]. Permission granted to use image.

Hypoxia, which occurs in many tumours, arises from a lack of adequate vasculature. In response, hypoxia induced transcription factor (HIF) -1α activates transcription of vascular endothelial growth factor and proinflammatory cytokines such as nuclear factor κβ. Nuclear factor κβ has been shown to induce expression of several chemokines such as RANTES (CCL5), MIP-2 (CXCL2), MIP-1α (CCL3), MCP-1 (CCL2), IL-8 (CXCL8), which are implicated in leukocyte and possibly MSC migration [115, 150-154].
Hypoxia also plays an important role in perpetuating tumour inflammation recurring through cycles of DNA damage by reactive oxygen species, expansion and invasion of cells with virulent mutations leading to further oxygen demands, tissue damage and further inflammation [155].

1.3.2 Engineering MSCs to improve tumour tropism

Exploration of the MSC tumour tropism is only in its infancy. The natural ability of MSCs to migrate selectively to tumours is impressive in its own right. However, elucidating the mechanisms involved in MSC migration has provided insight into how this trait may be improved.

It is clear that inflammation plays an important role in beckoning MSCs to tumours. Since cytotoxic effects of breast cancer treatments may lead to further inflammatory signals, it is possible that these treatments could further encourage MSC localisation. In fact, low dose irradiation which increases inflammation in tumours has already been linked in this manner. Klopp et al. [156] experimented with bilateral 4T1 breast cancer cell xenografts in mice which were irradiated unilaterally followed 24 hrs later by systemic injection of MSCs. Luminescent MSCs were detected in irradiated tumours at a level 34% higher than nonirradiated controls. *In vitro* models demonstrated a 50-80% increase in MSC migration in response to pre conditioned medium from irradiated 4T1 cells which contained increased levels of CCL2, CCL5, TGFβ1, PDGF and VEGF, all of whom have been implicated in MSC migration [156]. A similar study demonstrated increased MSC tumour engraftment in irradiated versus non-irradiated tumours of an MDA-MB 231 breast cancer xenograft model with few MSCs found in normal tissues [140].

Since inflammation has also been implicated in the progression of tumours, irradiation for the sake of improved therapeutic MSC migration would be ill-advised [157]. However, the fact that between 2004-2008, over 60% of patients in Ireland received some form of radiotherapy for breast cancer
certainty presents the possibility of supporting MSC tumour targeting in combination with established radiotherapy regimes [1].

Other options for improving the MSC tumour tropism involve modification of the MSCs themselves. Considering the leukocyte model of MSC migration (Figure 1.4), it is clear that the process of rolling adhesion is critical to MSC deceleration which facilitates adhesion and transmigration towards a source of inflammation. Sarkar et al. [158] exploited this target by immobilizing sialyl Lewis X on MSC surfaces. This increased rolling adhesion and decreased MSC velocity from 61 µm/s to 8 µm/s on a P-selectin surface under dynamic flow conditions. MSC over expression of epidermal growth factor receptor (EGFR), which binds EGF and TGFα has been shown to enhance in vitro migration of MSCs towards glioma conditioned media [159]. These cells were also shown to be able to migrate to gliomas and melanomas in vivo. Klopp et al. [156] have previously indicated that chemokine receptor CCR2 is up-regulated in MSCs exposed to irradiated tumour cells. These cells showed enhanced tumour migration. However, inhibition of CCR2 led to a marked decrease of MSC migration in vitro. MSCs engineered to overexpress CCR2 may show a similarly enhanced migratory potential as observed with EGFR overexpressing MSCs. A study by Pulukuri et al. [160] demonstrated that epigenetic induction of urokinase plasminogen activator protein expression through treatment with histone deacetylase inhibitors trichostatin A and sodium butyrate, significantly enhances bone marrow MSC tumour migration. Another interesting development has been reported whereby MSCs subpopulations characterised as Stro-1 negative or positive displayed different migratory itineraries in nonobese diabetic severe combined immunodeficient (SCID) mouse models. Stro-1 positive cells were found in larger numbers than stro-1 negative cells in the kidneys, bone marrow, muscle and spleen while the opposite was reported in the lungs [161]. While not directly related to MSC tumour migration this study may provide clues as to how MSCs may be further characterised in order to select for cells with improved tumour tropism. It may also explain why some studies can report early MSC migration to lungs following systemic injection while others show none [116, 134].
As a gene therapy mediator this ability could facilitate more selective targeting of malignant over normal tissue resulting in a reduction of side effects. With selective targeting of tumours, the therapeutic index of a treatment strategy might be substantially lowered allowing increased and more effective dosing regimes. This tumour tropism not only targets primary tumours but also sites of metastasis [115, 162-164]. This is hugely important. To date, the majority of cancer gene therapies have depended on direct intratumoral injection. In the majority of breast cancer scenarios where a tumour has been identified in the breast it is usually operable rendering this approach redundant. However, the surgical approach is severely limited when metastasis of a tumour has occurred. Either widespread dissemination of the primary tumour or an inability to identify sites of metastasis can quickly render a cancer inoperable. MSC mediated targeting of metastases may provide a solution to a problem which cannot be addressed by currently approved treatment strategies.

1.3.3 MSC immune privilege

MSCs are immune privileged. This may be due to low expression of major histocompatibility class 1 protein (MHC1) and no expression of CD40, CD80 and CD86 [133]. MSCs have been shown to inhibit T-cell proliferation in vivo as well as inhibiting B cells, dendritic cells and natural killers cells [165]. Secretion of prostaglandin E2, transforming growth factors and hepatocyte growth factor which regulate T-cell responses to novel antigens may decrease the potential for cytotoxic T-cell responses to transplanted MSCs [114, 166]. Krampera et al. [167] demonstrated that bone marrow stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Further observations concerning MSC inhibition of T-cell proliferation and reduction of interferon (IFN)-gamma and TNFα production lead Zappia et al. [168] to test their therapeutic value in treating autoimmune encephomyelitis. They treated murine models systemically with 1 X 10^6 MSCs at various time points. MSC administration before disease onset, at disease onset and at the peak of disease effectively reduced inflammation, demyelination and inhibited
T-cell response to the autoantigen [168]. It is also believed that MSCs may exert an indirect influence on B-cells as they are largely T-cell dependent [169]. In addition, *in vitro* studies have demonstrated direct inhibition of B-cell proliferation by cell-to-cell contact as well as by MSC paracrine signalling [170, 171].

Other studies have implicated MSCs in the suppression of the innate immune response. *In vitro* studies have shown that they can limit maturation of monocytes to dendritic cells [172, 173]. MSCs can also reduce pro-inflammatory and increase anti-inflammatory cytokine secretion from macrophages and monocytes [114, 174, 175]. Natural killer cells have also been shown to be vulnerable to MSC modulation. MSCs may inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production such as IL-2 [176, 177]. They may also reduce the intensity of respiratory/oxidative bursts, produced by neutrophils as part of their pancreatic function [178].

In the context of an adenoviral-mediated gene therapy, a direct benefit of these features is the protection of the vector from exposure to the immune response and potential immune clearance. Previous studies have shown that MSCs loaded with an oncolytic adenovirus suppress the anti-adenoviral immune response in the cotton rat model [179]. Furthermore, as well as autologous transplantation of MSCs, this immune privileged status may facilitate syngeneic and allogeneic MSC transplantation, thereby eliminating any potential issues with sourcing MSC donors.
1.3.4 Safety of MSC transplantation

To justify applying an MSC-mediated gene therapy in human patients there needs to be strong preclinical evidence for potential effectiveness set against a strong safety profile. Luckily MSCs have a long and safe record of use as early components of bone marrow transplants. More recent studies using MSC populations which have been cultured and expanded and reintroduced to patients have shown little or no levels of adverse reactions to donor MSCs. Mohyeddin et al. [180] administered MSCs at the time of coronary artery bypass or percutaneous coronary intervention in 8 patients with previous myocardial infarctions and compared them with age-matched control patients over 6 months. They reported that the use of MSCs was safe with no serious adverse reactions. Le Blanc et al. [181] demonstrated that co-transplantation of MSCs with haematopoietic stem cell transplants resulted in improved outcomes in 7 patients and did not report any safety issues with MSCs. A further phase II trial involved 55 patients who received median doses of 1.4 million cells/kg bodyweight. Following a 60-month surveillance no patients had side-effects during or immediately after infusions of MSCs nor were any MSC-associated adverse reaction reported [181]. In a separate Phase I/II study the safety and effectiveness of MSCs expanded in vitro with human serum for the treatment of acute and chronic graft-versus-host disease (GVHD) was examined in 18 patients. Again no serious adverse reactions related to MSC treatment were reported. However, minor infectious events were seen in 4 of 10 patients with acute GVHD after MSC administration although the authors report that the incidence was not unusually high in this setting [182].

Despite the strong clinical safety profile developed over the years there are still some concerns associated with MSCs. One major concern has been that in a similar fashion to teratogenic potential of transplanted embryonic stem cells in vivo, MSCs may differentiate into lineages inappropriate for the sites to which they have engrafted. However, adult stem cells are much more stable than embryonic stem cells following in vivo transplantation. There has been no evidence of unintended, lineage-mismatched MSC derived tissue formation in
humans despite numerous clinical trials with expanded cultures and direct bone marrow transplants. However, one such outcome in an animal model has been described whereby autologous intracerebroventricular (ICV) transplantation of MSCs was performed in a mouse model of severe autoimmune encephalitis [183]. This resulted in the migration of MSCs to the brain parenchyma with the formation of MSC masses in 64% of cases, characterized by focal inflammation, demyelination, axonal loss and increased collagen-fibronectin deposition. The collagen deposition was indicative of osteo/chondrogenic differentiation. However, a study by Sasportas et al. [184] indicated that in a glioma model, tumour engrafted MSCs remained undifferentiated and did not report the formation of such masses. In the context of breast cancer treatment this concern is less problematic since systemic injection of MSCs is unlikely to result in significant numbers of cells crossing the blood brain barrier.

As important as the stability of engrafted MSC phenotype is the need for proper targeting of diseased tissue. Delivery of an MSC-mediated therapy to normal healthy tissue would be unacceptable. While the tumour tropism of MSCs may facilitate tumour targeting, options to identify aberrant itineraries of MSCs would be very useful. Where gene therapy strategies allow for activation of the therapeutic gene after administration of the MSCs, a number of in vivo imaging techniques are available to help determine the predominant sites of localisation informing the subsequent treatment strategy [185, 186]. Where MSCs localise to healthy tissues, methods involving constitutively expressed gene therapies run the risk of causing unintended damage which in the case of critical organs could cause severe adverse reactions.

Concerns about possible zoonotic transmission of disease originating from bovine serum are also no longer relevant. Significant progress has been made in the use of bovine serum free media for the growth of MSCs [187-189]. A chemically defined media was used to grow rabbit MSCs and significantly outperformed a serum containing media increasing the MSC population by 50-fold compared to 40-fold over a 10 day growth period while maintaining the characteristic properties of the MSCs [190].
1.3.5 Tumourigenic potential of MSCs

Expansion of isolated MSCs harbours another potential risk in the form of spontaneous cell transformation. Wang et al. [191] reported, changes in morphology, loss of contact inhibition, reduced CD90, loss of CD105, chromosomal aneuploidy and translocations in a bone marrow derived-transformed MSC line. They suggested that the transformed MSCs may have represented a pre-existing transformed population isolated and expanded from the donor or that they may have spontaneously developed in culture. In the case of the former, it is important to note that these cells would not qualify as MSCs on the basis of their low CD90 and low CD105 expression. The current minimal criteria for defining MSCs dictate that MSCs should be greater than 95% positive for both surface markers. The possibility of spontaneous development of this subpopulation in culture is more of a concern. However the opposite was reported by Bernardo et al. [192] who were able to demonstrate the absence of telomerase activity and hTERT transcripts in all of the examined cultures with telomeres shortening during the culture period, and that the MSCs tested were not susceptible to malignant transformation. In 2012, a carcinoma of donor origin was attributed to transfusion of allogeneic peripheral blood pluripotent cells, which included both mesenchymal and hematopoietic stem cells [193]. A similar study again suggested that donor derived mesenchymal and hematopoietic progenitors, possibly recruited to oral mucosa inflammation, was responsible for the formation of oral squamous cell carcinoma in 4 of 8 patients following allogeneic bone marrow transplantation [194]. While these reports may possibly implicate MSCs in tumour formation they do not represent conclusive proof that MSCs are tumourigenic since the MSCs represent only a very small portion of the administered cells. The majority of studies have shown that human MSCs are stable while murine MSCs are more vulnerable to in vitro transformation [195-198].

Provision of optimal culture conditions and maintenance of MSCs at low passage numbers may help avoid undue stress that could lead to senescence,
genetic variation or high passage associated MSC transformation. Additionally, numerous techniques already exist in the biopharmaceutical industry, which are used as quality control measures to characterise production cell lines and ensure consistency. Such techniques such as karyotyping, DNA fingerprinting, determination of copy number or restriction map analysis could also be performed on clinical batches of MSCs to determine potential genetic instability prior to treatment.

1.3.6 MSCs and cancer

Probably the most significant safety concern with MSC-mediated cell therapies is their potential role in the tumour microenvironment. While research has progressed in determining how MSCs migrate to tumours, it is not yet clear why they do so. It may simply be a case of misdirection, whereby MSCs responding to inflammation from a perceived site of injury, follow the chemokine trail to a cancerous tissue. Recent studies however suggest there is a darker side to MSCs.

1.3.6.1 Co-transplantation studies examining MSC effect on cancer cells

Following 1:1 co-transplantation of GFP-labelled breast cancer cells and bone marrow MSCs into immunocompromised mice, Karnoub et al. [199] tracked progression of tumour xenograft growth and metastasis. They showed that MSCs increased growth of MCF-7/RAS cell tumours. More alarmingly, there was a marked increase in lung micro and macro metastasis originating from MDA-MB231, MDA-MB345, MCF-7/RAS and HMLER tumour xenografts transplants in the presence of bone marrow MSCs compared to without. Furthermore in the case of MDA-MB231 tumours, the presence of MSCs led to metastasis to tissues other than the lungs including the mammary glands. Contralateral and nearby injections of MSCs did not have an effect on tumour progression indicating that the MSCs need to be a part of the tumour microenvironment in order to exert their influence [199]. Following creation of new tumour xenograft derived from MDA-MB231/MSC tumours (minus the
stromal component) these effects were no longer observed indicating that the MSCs exerted a reversible effect on the breast cancer cells. More recently Karnoub et al. [200] have shown that co-injection of human bone marrow MSCs promote de novo production of lysyl oxidase (LOX) from human breast carcinoma cells, thereby enhancing the metastasis of otherwise weakly metastatic cancer cells to the lungs and bones. LOX also appears to play a critical role in Epithelial to Mesenchymal Transition (EMT), a model which has been proposed as a mechanism of metastasis [200, 201]. *In vitro* studies by Martin et al. [202] support this implication. They showed that 1:1 co-culture of T47D, Sk-Br-3 and MDA-MB231 cells with MSCs resulted in significant upregulation of EMT specific markers (N-cadherin, Vimentin, Twist and Snail) accompanied by a downregulation in E-cadherin protein expression which plays an important role in the maintenance of tissue architecture [203]. Furthermore elevated levels of oncogenes (NCOA4, FOS), proto-oncogenes (FYN, JUN), genes associated with invasion (MMP11), angiogenesis (VEGF) and anti-apoptosis (IGF1R, BCL2) and downregulation of proliferation genes (Ki67, MYBL2) were also associated with MSC co-culture. Chaturvedi et al. [204] showed that tumour models created using 1:1 co-transplantation of MSCs with MDA-MB 231 cells in the mammary fat pad (MFP) promoted metastasis to the lung and lymph nodes in a Hypoxia-inducible-factor (HIF) dependent manner. They also showed that co-culture with MSCs increased HIF expression in BCCs as well as placental growth factor which in turn increased tumour metastasis and facilitated MSC tumour migration.

Tumour associated fibroblasts (TAF), also known as activated fibroblasts are critical for tumour progression. They provide a functional and structurally supportive environment to cancer stem cells by synthesising/degrading extracellular matrix, supporting angiogenesis, producing growth factors, cytokines, chemokines, and modulating the immune response. While the origin of TAFs is unclear Spaeth et al. [205] have indicated that MSC may transition to TAFs contributing to fibrovascular network expansion and tumour progression when co-transplanted in a 1:1 ratio with Sko-3 cancer cells in mice.
The immune privilege of MSCs has been implicated as an important attribute in supporting tumour progression [206]. It was shown that the subcutaneous injection of B16 melanoma cells could only lead to tumour growth in allogeneic recipients in the presence of MSCs. Once again, this data was based on a 1:1 co-transplantation ratio. While the data informs us of how MSCs may behave at very high concentration in the tumour microenvironment, this ratio of MSCs in tumours and at the point of tumour formation, is not representative of what occurs naturally. In fact the majority of studies which report MSC involvement in tumour growth or metastasis involved co-injection of MSCs with cancer cells in order to establish the tumour model [199, 200, 204-211]. A study by Wicha et al [66] may provide an additional insight into variable results observed with MSCs and tumour formation. They established a murine model where MSCs were inoculated into the tibia of irradiated mice 7 days prior to MFP injection of cancer stem cells. Monitoring of the subsequent tumour formation not only revealed MSC recruitment but also tumour promotion compared to MSC free irradiated mice. This study is different by the fact that cancer stem cells were used as opposed to cancer cell lines. A preceding in vivo study showed that 1:1 co-transplantation of Aldehyde Dehydrogenase (AD) positive MSCs greatly accelerated tumour growth, whereas AD negative MSCs had no effect on the growth of cancer stem cell tumours. This study suggests that subsets of MSCs population may be responsible for the negative tumour effects of MSCs and that their removal could improve their safety profile.

There is also a small number of studies involving 1:1 co-transplantation of MSCs with cancer cells which report reduced tumour size and metastasis in models of colon cancer [212], hepatoma [213] and melanoma [214].

1.3.6.2 Effect of MSCs on pre-established tumours

While some of the previous observations are clearly negative in terms of MSC therapeutic application there are some more positive reports. Nakamura et al. [215] have demonstrated MSC inhibition of 9L glioma cell proliferation in vitro. They subsequently demonstrated migration and inhibition of pre-established 9L glioma cell tumour models in vivo following injection into the contralateral hemisphere [215]. Otsu et al. [216] administered 1 X 10^6 rat MSCs directly into a 700 mm^3 subcutaneous melanomas in a mouse model. The MSCs induced apoptosis and abrogated tumour growth. Cousin et al. [217] performed an intra-tumoural injection of human adipose-derived stromal cells (ratio of 1 X 10^3 cells per mm^3) in a model of pancreatic adenocarcinoma which induced a strong and long-lasting inhibition of tumour growth. A similar outcome was seen when mouse MSCs were administered, 1-2 x 10^5 MSCs intraperitoneally to tumour-bearing mice resulting in inhibition of ascitogenous hepatoma cell growth [218]. Likewise Dasari et al. [219] demonstrated tumour inhibition using umbilical cord blood derived MSCs. These cells were inoculated at a 1:4 ratio of MSC to original tumour forming cancer cells used and induced apoptosis by down regulating X-linked inhibitor of apoptosis protein (XIAP) which was over expressed in the glioma cells used for the tumour model.

Interestingly, Klopp et al. [220] who did an extensive review of discrepancies between studies reporting conflicting roles for MSCs in cancer concluded that timing of MSC introduction into tumours may be a critical element. For the purposes of MSC-mediated gene therapy of cancer it is important to highlight that most studies in which MSCs were administered after establishment of tumours highlighted tumour and/or metastasis inhibition [216-219, 221].

While the possible negative influence of MSCs is an undesirable trait, it does seem natural that cells displaying a tumour tropism would play some role in the tumour microenvironment. In treating cancer all attempts should be made not to exacerbate the condition and given the examples above the long term tumour engraftment of transplanted MSCs may potentially pose a risk to patient health.
Luckily however, therapeutic approaches which use MSCs as a delivery method for cancer treatment only require MSC engraftment for as long as it takes to deliver the MSC mediated cytotoxic treatment which by default also destroys the therapeutic MSCs removing any further risk.

1.3.7 MSC delivery of therapeutic agents

Because of their relatively easy isolation, capacity for large expansion, potential for growth in chemically defined serum free media, tumour tropism, immune privilege, ability to target hypoxic tumours and suitability for transduction by a range of vectors, MSCs have been frequently selected as the cellular vehicle of choice for a number of cancer gene therapy strategies. Past studies have had varying degrees of success in exploiting these characteristics for delivery of therapeutic genes to tumours. In many of these studies impressive therapeutic effects were observed with inhibition or reduction of primary tumour growth or size [81, 222-225]. Even more impressive was the report by Chen et al. [162] who established a 4T1 cancer cell based metastasis model, which in untreated animals lead to significant invasion of lymph nodes and internal organs. They showed that upon systemic administration every 5 days over a period of 20 days MSCs overexpressing IL-12 not only inhibited the progress of metastasis into lymph nodes and internal organs, but also reversed established metastases [162]. Similar work involving a murine pulmonary metastasis model of breast cancer showed that upon systemic administration of MSCs transduced with tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) under the control of a tetracycline responsive promoter, metastasis was cleared in 38 % of mice compared to 0 % of controls [163, 164]. All mice treated with activated MSCs had a lower lung weight and lower metastases number per lung area than observed in control mice.

Other studies used MSCs to target tumours with interferon beta [141, 142, 226], IL-2 [138, 227], IL-12 [162, 223, 228], pigment-epithelium derived
factor [224], NK-4 [229], tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) [81, 163, 164], herpes simplex-thymidine kinase [230, 231], cytosine deaminase [222, 232, 233] and oncolytic viruses [234]. They were used to treat a variety of tumours including gliomas [138, 142, 222, 226, 235], breast carcinoma [162, 223], pancreatic [141], hepatocellular [162, 164, 224], lung [81, 163, 164, 229], prostate [230], gastric [232], ovarian [234], melanomas [162, 227, 233] and sarcomas [228].

However, compared to other therapeutic gene candidates, the Sodium Iodide Symporter (NIS) offers a unique benefit when combined with the tumour tropism of MSCs as it may potentially facilitate both therapy and imaging of breast cancer. By conferring the ability of iodide accumulation to MSCs, the NIS gene may facilitate imaging of tumour engrafted MSCs through gamma camera detection of nonablative I\textsuperscript{125} (or technetium-99m pertechnetate) accumulation. This could identify areas of MSC localisation within the patient potentially pinpointing sites of metastasis or unintended localisation to sites of injury or other inflammatory sites. Such data could inform clinicians on the best course of action by highlighting the extent of metastasis or avoiding radioablative damage to unintended tissues. Following confirmation of appropriate MSC localisation ablation of tumours could be attempted by administration of cytotoxic I\textsuperscript{131}.

1.4 Sodium Iodide Symporter (NIS)

The Sodium Iodide Symporter (NIS) is a transmembrane glycoprotein found predominantly in the basolateral membrane of thyroid follicular cells [236]. Also known as solute carrier family 5, member 5 (SLC5A5), NIS belongs to a family of more than 60 membrane transporters all with very similar sequences. Weighing 87 kDa, it is composed of 643 amino acids which fold into 13 transmembrane domains. The NH\textsubscript{2} terminus is positioned outside of the cell membrane while the COOH terminus is intracellular (Figure 1.5).
NIS facilitates iodide uptake into thyroid follicular cells for the synthesis of the iodide containing hormones Triiodothyronine (T₃) and Thyroxine (T₄). Mutations in NIS can lead to congenital iodide transport defects which manifest clinically as hypothyroidism [237]. The function of extrathyroidal NIS expression in the salivary glands and gastric mucosa is unknown but expression in the mammary glands is known to facilitate translocation of iodide into breast milk for neonatal nutrition [236].

Like all its relative transporters, NIS exploits an extracellular:intracellular Na⁺ cation concentration gradient (intracellular 12mM, extracellular 140mM) to drive the transport of iodide into cells against its own intracellular:extracellular concentration gradient. NIS requires 2 Na⁺ cations to carry one I⁻ anion through the membrane. In this way, under normal physiological conditions, the NIS protein can produce concentrations of I⁻ 20-40 times higher in thyroid tissue compared to blood plasma [236]. For NIS to function in this way it is critical that NIS is properly localised within the membrane. NIS is primarily localised at the basolateral membrane of thyroid follicular cells allowing uptake of iodide from the extracellular milieu into the cytosol (Figure 1.6). Reduced capacity for membrane localisation results in reduced iodide accumulation. NIS
function can be inhibited by thiocyanate or perchlorate which is a very useful feature as a control parameter in NIS experimentation.

**Figure 1.6** Basolateral localisation of NIS in thyroid follicular cells. A) Immunohistochemical staining of NIS in human thyroid tissue. B) Thyroid follicle map [238]. Permission granted to use image.

Once in the follicular thyroid cell cytoplasm, the iodide diffuses to the apical membrane where it is translocated to the colloid by the membrane protein Pendrin and also the apical I⁻ transporter (Figure 1.7). Upon the outer colloid membrane, thyroid peroxidase (TPO) catalyses the organification of I⁻. Here I⁻ is oxidised to iodonium (I⁺) and is incorporated into tyrosyl residues of thyroglobulin which is also secreted into the colloid by thyroid follicular cells. Iodinated thyroglobulin can either be stored in the colloid or taken back into the follicular cells. Thyroid stimulating hormone (TSH) released from the pituitary gland, binds its receptor on the basement membrane and stimulates endocytosis from the colloid. The resulting lysosomal protease digestion cleaves T₃ and T₄ from iodinated thyroglobulin [236].
Figure 1.7 Synthesis of thyroid hormones. NIS, located at the basolateral membrane of thyroid follicular cells is crucial for iodide uptake facilitating subsequent thyroid hormone synthesis [239]. Free licence image.

This ability to accumulate iodide in the thyroid has been crucial in the treatment of thyroid cancer. The partnership of radioiodide and NIS has been successfully used with high efficiency together with an excellent safety record which includes low toxicity [240].

A huge advantage of NIS radioiodide accumulation is that there is a substantial bystander effect. In fact there are two bystander effects. The first is due to iodide accumulation in a cell expressing functional NIS. Accumulated radioiodide emits energy through radioactive decay. Approximately 90% of this decay is released in the form of beta decay or beta radiation. Depending on the quantity accumulated this beta radiation can be strong enough to penetrate cells up to 2 mm away from the source [241]. The energy from this beta radiation causes mutations and breakage of DNA leading to cell death. In a uniform tissue environment the beta decay could efficiently kill cells in a three dimensional sphere centred around the site of iodide accumulation. Given
enough sites of iodide accumulation a targeted tissue could potentially be ablated with only a small proportion of cells expressing functional NIS. The second bystander effect occurs indirectly whereby cells damaged by a radiation insult exert biological effects on non-irradiated adjacent cells. In a mouse model, Xue et al. [242] demonstrated this type of bystander effect by injecting a mixture of human colon LS174T adenocarcinoma cells and LS174T cells prelabelled with lethal doses of DNA-incorporated $^{125}$I. The approach was designed to minimise direct radiation exposure to non-labelled cells facilitating a clear determination of the effect of radiated cells on non-irradiated adjacent cells. Xue et al. [242] found a significant inhibitory effect on the growth of subcutaneous LS174T tumours providing strong evidence of the indirect bystander effect.

In addition, functional NIS expression in the thyroid facilitates imaging. This is possible since 10% of the energy released from decay in radioiodide occurs as gamma radiation. Gamma radiation has the ability to penetrate through significant amounts of tissue escaping outside of the body. Gamma cameras can detect this gamma radiation and the combination of sensitive instrumentation and computer software allows the generation of clear images of the source of iodide accumulation (Figure 1.8). By using less cytotoxic radiiodide doctors can visualise thyroid tissue and determine their surgical strategies and also the safety of a subsequent treatment with cytotoxic radiiodide.
1.4.1 Thyroidal NIS Regulation

Regulation of NIS expression in the thyroid is well understood. The primary thyroid hormone TSH plays a strong positive role in expression of NIS. It has been shown to increase transcription of the NIS gene in human primary thyroid cells via the cyclic adenosine monophosphate (cAMP) pathway. TSH stimulates the TSH receptor which activates adenylyl cyclase through the G-protein. The resulting accumulation of cAMP in thyroid cells stimulates NIS transcription through cis regulatory elements of the NIS promoter such as NIS upstream enhancer (NUE) [243-246]. This NUE also requires binding of Pax-8 and cAMP responsive element binding protein (CREB) for full activity [245, 246]. Similarly, binding of SP-1 to the GC box of the human NIS promoter is needed for full activity [247]. Negative feedback by thyroid hormones inhibit release of TSH which in turn leads to reduced NIS expression. Inhibition of Phosphoinositide-3-kinase (PI3K) induces NIS expression in rat thyroid cells and human papillary thyroid cancer cells [248, 249]. Spitzweg et al. [250] reported a 50% reduction in NIS mRNA in FRTL-7 cells grown under high I\(^\text{3}\) conditions indicating a strong inhibitive effect. This was corroborated by Eng et al. [251] in an in vivo rat model over 6 days of iodide administration. A number of cytokines have also been implicated in inhibition of NIS expression as well as NIS function [250]. These include TNF-\(\alpha\), TNF-\(\beta\), interferon-\(\gamma\), IL-
1α, IL-1β, IL-6 and TGF-β1. Kohn et al. [252] demonstrated that TSH-stimulated NIS expression could be inhibited by follicular Tg both in vitro and in vivo. This supported earlier studies showing Tg inhibition of NIS mRNA expression [253]. Furlanetto et al. have reported decreased NIS expression after estrogen stimulation between concentrations of 10^{-7} and 10^{-11} M both in the presence and absence of TSH [254]. Treatment of thyroid cells FTC-133 and FTC-238 with all trans retinoic acid for 24 hrs markedly increased NIS expression [255].

On a post-transcriptional level, TSH has been shown to increase the half-life of NIS protein, and stimulates trafficking of NIS to the plasma membrane [256]. It has also been suggested that phosphorylation, subcellular localisation and proteolytic degradation play a role in transporter activity [257-262].

1.4.2 NIS expression in breast tissue

NIS is also expressed in lactating mammary tissue to support iodide concentration into breast milk for neonatal nutrition [44]. It is not normally expressed at significant levels in nonlactating tissue. However, many studies have demonstrated NIS expression in malignant breast tissue [42, 43, 45-47, 263, 264]. Using immunohistochemistry, Tazebay et al. [44] demonstrated that more than 80% (n = 29) of the human breast cancer tissues expressed the NIS protein while none was detected in normal samples (n = 8). Kilbane et al. [47] showed increased NIS mRNA in 6/7 breast carcinomas and 2/2 breast fibroadenomas using RT-PCR. Further confirmation of NIS expression in human breast cancer tissue was provided by Upadhyay et al. [45] who demonstrated both NIS gene and protein expression throughout a small cohort of samples. Paraffin-embedded infiltrating ductal carcinomas were tested for NIS protein expression using a NIS monoclonal antibody and it was found that NIS was expressed in 90% of cases [43]. A larger study, which again targeted NIS protein expression by immunohistochemistry demonstrated 69/91 invasive carcinomas and 15/17 in situ carcinomas were positive for NIS [46]. Out of 23
triple negative breast cancers, Renier et al. [42] demonstrated that NIS was present in 15 with 11 exhibiting strong expression. Even functional NIS expression has been identified through scintigraphy [45, 265]. There is little doubt that NIS expression can be found in cancerous breast tissue but there are still major questions surrounding the clinical relevance and regulation of this expression.

1.4.3 Clinical relevance of mammary NIS expression

Following reports of much higher levels of NIS expression in malignant compared to normal breast tissue, there have been claims that NIS may well be valuable either as a biomarker, imaging or therapeutic tool in breast cancer diagnosis and treatment [42-45]. Demonstrations of functional NIS expression have supported these claims [45, 266]. As have iodide uptake identified in soft tissue breast cancer metastases through scintigraphy [267]. More recently, immunohistochemistry (IHC) detection of NIS protein in brain metastases derived from breast cancer indicated that this characteristic of abnormal NIS protein expression in breast tumours may be maintained in derivative metastases [268].

However, the majority of studies on NIS protein expression in human tissue have been based on IHC which is not a quantitative technique [42, 43, 45, 46, 263, 264]. Studies investigating NIS gene expression in malignant breast tissue provide a profile of expression which is limited due to small cohort sizes [45, 47]. Further complicating these issues have been reports of NIS expression in benign breast tissue (fibroadenoma) which casts doubt on its suitability as a biomarker [46, 47]. Also, while functional NIS expression has been demonstrated, doubts have been cast as to whether adequate iodide uptake can be achieved to support imaging or treatment. Interestingly, Berger et al [269], did highlight functional NIS expression in a fibroadenoma which was visible using a whole body gamma camera scan after administration of cytotoxic radioiodide for treatment of thyroid cancer. However, this study also
compounds the issue of NIS expression in benign breast tissue. It is critical that the issue of benign and malignant NIS expression is properly addressed using quantitative techniques in a large cohort of human samples to finally resolve the question of NIS potential as a biomarker of breast cancer. Some reports have suggested higher iodide uptake in benign compared to malignant breast tissue [47, 269], and the gamma camera scan of a fibroadenoma by Berger et al. [269] which, while clearly visible displayed a large amount of comparable or higher iodide accumulation in other parts of the body. This data suggests that imaging and therapeutic applications of native NIS expression in breast cancer are not feasible. However, if a clear understanding of the regulation of NIS expression in breast cancer could be established these possibilities may reopen.
1.4.4 Mammary NIS regulation

Regulation of NIS expression in breast tissue is not nearly as well understood as that of the thyroid. However, it is clear that the positive control of TSH seen in the thyroid doesn’t apply to the breast.

By treating nubile mice with various hormones and inhibitors of NIS, Tazebay et al. [44] have demonstrated that NIS expression is optimal in the presence of estrogen, prolactin and oxytocin. A number of potential regulators of NIS have also been identified. RARα has been shown to increase NIS expression in MCF-7 cells in vitro [270]. Willhauck et al. [271] demonstrated the induction of functional NIS expression in breast cancer xenografts after treatment with Retinoic acid and Dexamethasone. Further studies showed that carbamazepine stimulation of retinoic acid receptors led to increased functional NIS expression leading to $^{131}I$ cytotoxicity in cancer cells [272]. RARβ has been shown to promote a greater increase in NIS expression when similarly stimulated with RA in MCF-7 cells [273]. The unliganded ERα has also been shown to activate mammary NIS transcription in estrogen receptor-positive breast cancer cell lines [274]. This is supported by the presence of an estrogen-responsive element in the NIS gene promoter [274]. Two separate studies by Knostman et al. [275, 276] have suggested that PI3K activation leads to increased NIS expression in MCF-7 cells but decreases membrane localisation of the protein in these cells. Although no link has been demonstrated in mammary tissue, the importance of thyroid hormone receptors in thyroidal NIS expression [236], and their differential expression in breast cancer warrants their investigation as potential mammary NIS regulators [277-279]. Previous studies of potential regulators of NIS expression in breast cancer, although informative, have been performed using breast cancer cell lines and animal models. Expression of potential regulators and their pertinence to NIS expression in human breast tumour tissue must be assessed in order to confirm these in vitro and in vivo observations.
The lack of TSH involvement in mammary NIS expression is very interesting. While it leaves a significant gap in our understanding of how mammary NIS is regulated, the disparity between thyroid and mammary NIS regulation has opened up significant opportunities in terms of NIS therapy. In the event that NIS expression could be stimulated in the breast or exogenous NIS expression could be introduced for the purposes of radioiodide accumulation, thyroid NIS expression could be selectively suppressed using negative feedback loops involving TSH, without effecting expression in the breast. Using T3 to suppress thyroid NIS expression, Wapnir et al. [267] showed that thyroid iodide accumulation in patients could be reduced to negligible levels with little risk of radiation damage.

1.4.5 NIS for gene therapy

Based on its value in treating thyroid cancer using radioiodide, scientists began to speculate on the potential of NIS as a therapeutic gene in the treatment of other cancers. When the human NIS gene was first characterised in 1996 this became a distinct possibility [280]. This facilitated the generation of plasmid and viral vectors carrying the NIS gene. Infection of cells with NIS carrying vectors drives expression of NIS protein to levels which make the targeted cells susceptible to I131 treatment. In this context the NIS gene has been described as a suicide gene. This approach could potentially be used in any targeted tissue type. An early attempt to realise this potential was performed by Carlin et al. [281] who transfected UVW glioma cells with a plasmid carrying the NIS gene. They demonstrated a 41-fold increase in iodide uptake compared to control cells, and following exposure to I131 clonogenic survival in 2-dimensional glioma cell cultures was reduced to 21 %. Furthermore, just 2.5 % of cells survived exposure to I131 when cultured in a 3-dimensional format. The authors concluded that the decreased survival in 3d compared to 2d culture could be attributed to the radiological bystander effect. This potential was supported by Spitzweg et al. [282] who used an adenoviral vector to deliver the NIS gene under the control of the cytomegalovirus (CMV) promoter to prostate
cancer xenografts in a murine model. Functional NIS expression was observed through highly active iodide uptake, and tumour volumes were reduced on average by 84 +/- 12%. Dwyer et al. [283] advanced this application by demonstrating its safety in a large animal model. Following direct intraprostatic injection of adenovirus carrying the NIS gene, beagle dogs received intravenous injection of 3 mCi $^{123}$I and serial image acquisitions were performed. Dogs were also given a therapeutic dose of $^{131}$I (116 mCi/m2) and were observed for 7 days. Clear images of NIS transduced canine prostates were visible using SPECT/CT fusion imaging. In addition dosimetry calculations revealed that prostate adjacent organs absorbed acceptably low levels of radiation while the prostates on average received 23 +/- 42 cGy/mCi $^{131}$I. Despite this successful trial, it is clearly not ideal to completely destroy an organ or gland in order to eradicate cancerous tissue surrounded by otherwise healthy tissue. In the same year, Dwyer et al. [284] attempted to address this lack of selectivity by exploiting the tumour specific expression of MUC1. They infected MUC1 positive and MUC1 negative cells with adenovirus vector carrying the NIS gene under the control of the MUC1 promoter (Ad5/MUC1/NIS). Iodide uptake studies revealed a 58-fold increase in MUC1 positive T47D cells but none in MUC1 negative MDA-MB231 cells. Intratumoral injection of the vector in T47D xenograft models followed by iodide treatment revealed an 83% reduction in tumour volume while control tumours increased in size. This MUC1 dependent expression of NIS further highlighted the potential of NIS gene therapy. A similar approach in ovarian xenograft models successfully halted the growth of tumours while CMV controlled NIS expression reduced tumour size by an average of 53% [101]. With pancreatic xenografts, a 51% reduction in tumour volume followed an intratumoral injection of Ad5/MUC1/NIS and radioiodide treatment [100]. In both experiments successful imaging of iodide accumulation in vector infected tumours was achieved [100, 101]. In all of these animal models the thyroid tissue was effectively protected by suppression of functional NIS expression following administration of $T_4$ prior to radioiodide treatment [100, 101, 283, 284].
Along with the obvious advantages of using NIS as a gene therapy such as effective reduction of tumour volume and potential imaging of tumours, there is a built in self-destruct to NIS-radioiodide gene therapy whereby cells accumulating iodide are themselves destroyed in the treatment process. Accumulated radioiodide dissipates, losing its concentration-dependent deleterious effect on human tissue and is eventually excreted through the urinary system. However, in the event that radioiodide treatment may be deemed inappropriate following imaging of iodide accumulating cells, future gene therapy approaches involving NIS should consider the inclusion of a secondary suicide gene cassette as a failsafe which facilitates killing of NIS-transfected cells.

While these studies showed the huge potential of NIS as a gene therapy, it is issues with the vector carrying NIS that limits it application. In terms of adenovirus these disadvantages, such as the lack of a tumour tropism and immunogenic effects, have been previously described but they may be overcome using the valuable and unique characteristics of MSCs. MSCs infected with adenovirus expressing NIS may be capable of migrating to and engrafting onto tumours or sites of metastasis following systemic injection. Administration of $^{125}$I could facilitate tracking of these MSCs and mapping of tumour locations while $^{131}$I could be used to ablate MSC-adjacent tumour tissue. Given the cancer stem cell model of cancer treatment, it may not even be necessary for this approach to kill all cells within the tumour environment to be effective in halting progression of tumours [70, 285-287]

As a combined strategy MSCs expressing NIS could represent a highly effective targeted imaging and therapy approach for breast cancer or many types of cancers.
1.5 Aim of study

There were two overall objectives of this PhD project.

The first was to investigate the presence, relevance and regulation of NIS in breast cancer. In order to achieve this, the levels of native NIS gene expression in normal, benign and malignant breast tissue were determined and the relationship between NIS expression levels and tumour epithelial subtypes and patient clinicopathological were examined. In addition gene expression levels of putative regulators of NIS, such as RARα, RARβ, ERα, PI3K, THRα and THRβ, were determined in normal benign and malignant breast tissue. This data was subsequently analysed for potential relationships with NIS expression. The true role of retinoic acid, estrogen and thyroxine in the regulation of mammary NIS expression was determined using breast cancer cell lines. Similar models were used to determine the effect of these ligands in combination on NIS expression.

The second overall objective was to examine the potential for mesenchymal stem cell-mediated NIS gene therapy of breast cancer. This was achieved by determining the expression profile over time of the NIS transgene in MSCs infected with the vector Ad5/CMV/NIS. The effect of proliferation on this expression profile was also determined. Ad5/CMV/NIS was investigated for its transfection efficiency and impact on osteogenic potential and surface marker expression in infected MSCs. The cytotoxic effects of I131 on breast cancer epithelial cell lines co-cultured with MSC-NIS were also examined. In addition the migratory itinerary of MSCs was determined in two murine models of breast cancer. The distribution of systemically administered, Ad5/CMV/NIS infected MSCs was determined in a murine model of subcutaneous breast cancer using RQ-PCR, while the distribution of systemically administered, PKH26 labelled MSCs was determined in a murine model of metastatic breast cancer established in the mammary fat pad using IHC, Flow cytometry and RQ-PCR.
Chapter 2

Materials and Methods
2.1 Cell culture

2.1.1 Background

Cell culture is the technique by which mammalian cells are propagated and maintained under aseptic conditions. To do so, a suitable environment is required. This is provided by control of temperature, pH, humidity, culture medium, a growth vessel suited to cell adhesion and proliferation, and maintaining sterile conditions. Temperature is maintained at 37°C using incubators while the provision of 5% CO₂ and media buffers help regulate pH. The pH indicator Phenol Red is used as a constituent of most media and changes colour in response to changes in pH. Humidity is produced by placing stainless steel trays of sterile water at the bottom of each incubator. Basal media provide essential amino acids, sugars, vitamins and salt to support cell nutrition while hormones/growth factors are added as constituents of fetal bovine serum. Gamma-irradiated culture flasks with a hydrophilic surface are used for adherent cells facilitating optimal cell attachment and growth. Provision of adequate space is critical to allow cultured cells to grow unimpeded. If kept in a proliferative state the physiological and genetic profile of a cell population is more reproducible. Overcrowded or confluent cell populations tend to react in a less predictable fashion. Contact inhibition slows growth and may bring about senescence. Gene expression profiles may reflect stress responses to limited nutrients or increased pH. Passaging of cells alleviates space, nutritional, and pH pressures and encourages optimal growth and stable expression profiles in cells [288, 289]. Sterility is maintained by using sterile vessels, media, operating equipment, the use of sterilised biosafety cabinets and aseptic technique.

Cultured cells can be obtained commercially or through primary culture directly from digested tissue. In the case of commercial cell lines, they are usually homogenous, immortalised cells precisely defined in terms of growth parameters, suitable growth media and distinctive biological traits. They are also tested and certified as free from contaminants potentially hazardous to operator health and existing cell culture stocks. Cells obtained through primary
cell culture are typically developed locally through tissue digestion and a cell-
specific selection process yielding a relatively pure but not completely
homogenous cell line. Primary culture minimises the generational gap between
true biological and commercial cell line models leading to more biologically
relevant data, however disadvantages include the unknown biological risk
associated with primary cell culture (particularly that derived from human
tissue), variation in primary cell line traits, and limited value with progressive
generations.

Method

To perform cell culture techniques all cells were carefully handled using
aseptic technique. Asepsis was achieved by wiping all surfaces and equipment
with 70 % industrial methylated spirits (IMS), through the introduction of
antibiotics such as penicillin/streptomycin to media, and the manipulation of
cells in class 2 laminar airflow hoods which limits cell exposure to unfiltered
air. Hoods were sprayed with 70 % IMS and allowed to clear for 15 mins
before and after use. Two separate hoods were used. The first is dedicated for
manipulation of primary tissue cultures originating from human tissue and the
second was for commercially available cell lines. This segregation is intended
to protect expensive and certified contagion-free cell lines from potential cross
contamination (particularly viruses) from human tissue derived primary
cultures.

All liquid waste from cell culture was treated overnight with 1 % W/V Virkon
before disposal. Solid waste was placed into Biohazard autoclave bags and
incinerated. Waste contaminated with genetically-modified microorganisms
(GMM) was segregated and treated as follows. Liquid waste was treated
overnight with 1 %W/V Virkon solution and thick bleach. Deactivated waste
was disposed of post treatment down the sink with multiple volumes of water.
Solid waste was treated by autoclaving at 1230°C for 30 mins, and stored in
biohazard bin for removal and incineration by a registered waste contractor.
2.1.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a subset of non haematopoietic stem cells found in the bone marrow. They are multipotent cells and have the ability to differentiate into osteoblasts, adipocytes and chondrocytes [118, 126].

MSCs were supplied by the Regenerative Medicine Institute (REMedi), at NUI Galway. These cells were harvested from bone marrow aspirates of the iliac crests of healthy donors after ethical approval and informed consent [118, 124]. They were then isolated by direct plating to remove the non-adherent haematopoietic stem cell population and maintained in alpha minimal essential medium (α-MEM) supplemented 100 IU/ml Penicillin G- 100 mg/ml Streptomycin sulfate (P/S) and 10 % selected Fetal Bovine Serum (FBS) for 12-15 days. Adipogenic, chondrogenic and osteogenic potential was confirmed as well as characterisation of cell surface markers CD105, CD73, CD90 (positive) and CD34, CD45 (negative).

Where, possible more than one donor was used for replicates of each experiment.

MSCs were grown in α-MEM supplemented with 100 IU/ml Penicillin G- 100 mg/ml Streptomycin sulfate (P/S) and 10 % Fetal Bovine Serum (FBS). The FBS used was serotyped and specially selected based on maintenance of MSCs in their undifferentiated state.

2.1.3 Breast cancer cell lines

T47D and MCF-7 epithelial cell lines are representative of the Luminal A epithelial subtype of breast cancer being both estrogen and progesterone receptor (ER and PR) positive and Her2-neu negative. T47D cells originate from an infiltrating ductal carcinoma in the breast of a 54 year old female patient while MCF7 cells stem from a breast adenocarcinoma. T47D cells were grown in RPMI 1640 + GlutaMax™-I media and MCF-7 cells were grown in Eagle's Minimum Essential Medium. Sk-Br-3 cells are representative of the
HER2 epithelial subtype and originate from a breast adenocarcinoma. They overexpress Her2-neu and are ER and PR negative. Sk-Br-3 cells were maintained in McCoy’s 5A + GlutaMax™-I media. MDA-MB-231 epithelial cells originate from an adenocarcinoma of a 51 yr old Caucasian female. MDA-MB-231 cells are triple negative for ER, PR and Her2-neu and represent the basal epithelial subtype of breast cancer. These cells have the highest capacity for metastasis when used in \textit{in vivo} models and represent the most difficult subtype of breast cancer to treat. MDA-MB-231 cells were grown in Leibovitz’s L-15 Medium + GlutaMax™-I media.

All media types used for maintenance of breast cancer cell lines were supplemented with P/S and 10% FBS.

2.1.4 Recovery of frozen cells

To recover frozen stock, the appropriate media, warmed to 37°C was aliquoted into cell culture flasks. A vial of frozen cells was immersed in a 37°C water bath until thawed and the contents were immediately transferred to cell culture flasks. Media was changed 24 hrs later to remove traces of toxic DMSO as well as any non-adherent cells.

2.1.5 Cell line inspections

Thorough inspection of cell lines is crucial to identify problems with cell cultures at an early stage to avoid lost time, eliminate confounding factors for experimental results, and to identify morphological changes resulting from experimental treatments. Contamination of cell cultures is a chief concern. Familiarity with the normal morphology of the cell lines is informative as to the health of cells, monolayer confluence, or simply providing evidence of an experimental response. Cells were inspected on a daily basis on a macroscopic level for changes in media appearance such as colour or turbidity which are
indicative of cell stress or contamination, and at a microscopic level to
determine the health and confluency of the cell monolayer.

2.1.6 Feeding Cells

Cells were fed three times weekly and passaged every 7-10 days. Replacement
of media was performed to replenish nutrients, stabilise pH and remove waste
products from the cell culture. Since adherent cells were maintained at 37°C,
fresh media was warmed to 37°C in a water bath prior to feeding to avoid
causing a temperature shock to the cells. Care was taken to add media away
from the cell monolayer to avoid flushing cells into suspension. All
manipulation of cell cultures was performed in a Class II biosafety cabinet.
Volumes of media varied according to culture vessel and cell density however
in all instances it was critical that the monolayer was completely covered in
media. Care was taken to ensure that culture vessels were incubated on a level
surface to avoid unnecessary stress on cells due to uneven distribution of media
over the cell monolayer.

2.1.7 Passage of cells

Background

Cells were subcultured into new vessels when 80-90% confluent and not later
than a maximum of 10 days in one vessel regardless of confluency. The
objective of subculturing cells is to maintain them in the logarithmic phase of
growth and to avoid senescence. Passaging is done using the enzyme Trypsin
EDTA (T/E) which cleaves peptides responsible for binding adherent cells to
the surface of the culture vessel. It should be noted that T/E, which works best
at 37°C, is inhibited by fetal bovine serum (FBS) as well as by magnesium and
calcium (Ca2+ and Mg2+) ions.
Method

To passage cells, appropriate media, Ca²⁺ and Mg²⁺ free Phosphate Buffered Solution (PBS) and 0.25% Trypsin-EDTA were warmed to 37°C. Media was decanted from cultured cells which were subsequently washed with PBS to remove traces of FBS. Warmed T/E was added to the adherent cells and incubated for 1 min at 37°C. T/E was then decanted from the flask and the cells were incubated 37°C for 2-5 mins in the remaining traces of T/E. At this point cells were examined macroscopically and microscopically to determine if they had detached completely. Clumped or adherent cells were incubated for a further 2 mins. Once the adherent cells moved freely in a single cell suspension complete media was used to wash the remaining adherent cells free and to stop the action of the T/E. Cells were then counted as described in section 2.1.8 and seeded at an appropriate density for expansion or experimental purposes.

2.1.8 Counting Cells using a Nucleocounter

Background

Cells were counted using a Chemometec Nucleocounter® NC-100™. This counter works on the basis that fluorescent Propidium iodide (PI) intercalates with genetic material. Initially immobilised in the flow channel of the nucleocounter cassette shown in Figure 2.1, PI is dissolved by uptake of the single cell suspension becoming free to intercalate with DNA of permeable cells. Green light is used to interrogate the intercalated PI-DNA in the measurement chamber. This excites the fluorescent PI-DNA molecule which emits a red fluorescent signal detected by a charged coupled device camera. The limits for accuracy are 3 x 10⁵ cells/ml – 2 x 10⁶ cells/ml.
Figure 2.1 Chemometec nucleocassette. Uptake of sample releases PI from the flow channel allowing it to enter porous membranes and bind with DNA [290]. Living cells are impermeable to PI whereas non-viable cells are not due to their porous membrane. This allows nonviable cells to be counted separately as shown in Figure 2.2a. In order to count non-viable cells, the cell suspension is directly loaded into the nucleocassette. To determine the total cell number, cells are treated with a lysis buffer to permeabilise the membrane and provide access for the PI to the nuclear DNA and subsequently treated with stabilising buffer (Figure 2.2b). The number of viable cells can then be extrapolated from these two values.
Figure 2.2 Principles of nucleocounter. 

a) The process for non-viable cell counts allows PI contact with the nuclei of cells which already possess porous membranes. 

b) The process for total cell counts artificially permeabilises the membranes of all cells present ensuring PI contact with all cells [291].
Method

To determine the total number of cells (viable and nonviable) 100 μl from the homogenous cell suspension (prepared by trypsinisation as described in section 2.1.7) was transferred to a 1.5 ml eppendorf tube. To this an equal volume of Lysis Buffer A100 was added to the cells. This was followed by 15 sec vortexing. An equal volume of Stabilising Buffer B was subsequently added followed by another 15 sec vortex. 50 μl of sample was then loaded into the Nucleocassette. The result returned represents a 1/3 dilution of the cell suspension. This value was multiplied by 3 to get the actual concentration of cell per ml.

To determine the number of nonviable cells 100 μl of sample was transferred to a round bottomed 2 ml eppendorf. 50 μl of this neat sample was loaded into the nucleocounter cassette and analysed.

The Viable Cell Count was calculated as follows according to the formula

\[ C_{VC} = (C_{TC} \times 3) - C_{NV} \]

Calculation of Percentage Viability

\[ \%V = \left( \frac{C_{VC}}{C_{TC}} \right) \times 100 \]

(C = concentration cell/ml, VC = Viable cell count, TC = Total cell count, NV = Nonviable cell count, V = Viability)

2.1.9 Cryopreservation of cells

Background

Frozen stocks of mammalian cell lines were stored in liquid nitrogen at -196°C. This extreme temperature minimises variation in the characteristics of pre- and post-storage cell populations. The formation of ice crystals is the chief culprit responsible for damaging healthy cells during cryopreservation. The effects include mechanical (crushing, puncture of membrane) damage, concentration
of solutes by exclusion from newly formed ice, and dehydration of cells by sequestration of \( \text{H}_2\text{O} \) in ice crystals. Addition of Dimethyl Sulfoxide (DMSO) reduces the formation of ice crystals and increases the recovery potential of frozen cell populations. DMSO is however toxic to mammalian cells at 37\( ^\circ \text{C} \) so rapid processing of cells is critical. Cooling at a slow constant rate (approx -1\( ^\circ \text{C}/\text{min} \)) also helps reduce formation of intracellular ice crystals by allowing \( \text{H}_2\text{O} \) to cross the cell membrane to the freezing extracellular fluid.

To prepare frozen stocks, cells were trypsinised and counted as described in section 2.1) and a cell suspension of 6.25 x 10\(^5\) cells/ml was prepared. The cell suspension was then dispensed into 2 ml Sarstedt Cryovials (1.6 ml aliquots-1 x 10\(^6\) cells/ vial). DMSO was added at a final concentration of 5 % immediately prior to placing stocks in a Nalgene Cryo 1\( ^\circ \text{C} \) Container (Mr Frosty) and incubating at -80\( ^\circ \text{C} \). Frozen stocks were transferred to liquid Nitrogen storage after 3 hrs.

### 2.2 PKH26 labelling of MSCs

**Background**

PKH26 Red consists of a yellow-orange fluorescent dye linked to long aliphatic chains. These aliphatic chains allow the molecule to stably incorporate itself into the lipid regions of the membrane. In doing so, a population of cells can be reliably distinguished from other populations under the right conditions. Because of the stability of the fluorescent dye, PKH26 Red is ideally suited to staining cells which need to be introduced to \textit{in vivo} models.

**Method**

In order to label MSCs with PKH26, a cell suspension was prepared which was centrifuged for 5 mins at 400 x g with the supernatant subsequently aspirated. The pellet was resuspended in diluent C (PKH26GL kit Sigma) at a concentration of 8 x 10\(^5\) cells per 490 1 diluent. Under darkened conditions 10 1 PKH26 label was added for every 490 1 of diluent C-cell suspension. After
mixing the suspension was incubated for 5 mins at room temperature (RT) inverting gently at minute intervals to ensure homogenous staining. The reaction was stopped by adding 10 mls of complete media contain 10 % fetal bovine serum. MSCs were centrifuged for 5 mins at 400 x g, the supernatant was removed and the pellet was washed in basal medium. The cells were resuspended in basal medium at a concentration of 2 x 10⁷ cells/ml in preparation for intravenous tail vein injection to mice (as described in section 2.10.8).

2.3 Investigation of the effect of β-estradiol, all trans Retinoic acid and l-thyroxine on NIS expression

Background

In this experiment the true role of estrogen, retinoic acid and thyroid hormone receptors in the regulation of NIS expression in breast cancer cell lines was investigated as a result of evidence from a gene expression study of human breast cancer tissue. β-estradiol (E₂), all trans Retinoic acid (ATRA) and l-thyroxine (T₄) were selected to stimulate the estrogen, retinoic acid and thyroid hormone receptors respectively.

While E₂ is easily soluble in water, ATRA required dissolution in Dimethyl Sulfoxide (DMSO), and T₄ in 4M Ammonium hydroxide solution (NH₄OH) in methanol before adjusting to appropriate concentrations. This variation in solvent also meant that appropriate solvent controls needed to be included.

Method

To investigate the effects of E₂, ATRA, and T₄ on NIS expression, T47D, Sk-Br-3 and MDA-MB-231 cells were seeded in triplicate at a density of 2.4 x 10⁴/cm² in T25 cm² flasks. 16 hrs later each cell line was treated as per Table 2.1.1 with E₂ (1 nM, 10 nM, 50 nM), ATRA, (0.1 μM, 1 μM, 5 μM) or T₄ (0.1 μM, 0.5 μM, 1 μM) for 24 or 72 hrs. Media and stimulants were replenished at
24hr intervals, and control cells were cultured in the appropriate diluent used for each stimulant e.g. 0.1 % NH₄OH for T₄ and 0.5 % DMSO for ATRA.

Cells were harvested following 24 or 72 hrs incubation, centrifuged at 1000 rpm for 4 mins and the cell pellet stored at -80°C. RNA was extracted and expression of NIS and the endogenous controls PPIA and MRPL19 was determined using RQ-PCR as described in section 2.7. The data was expressed relative to cells grown in appropriate diluent controls.

The experiment was then repeated in triplicate with T47D and Sk-Br-3 cells using 10 nM E₂, 1 µM ATRA and 0.1 µM T₄ individually and in combination over 24 hrs (Table 2.1). Combinations of diluents were also used for controls where necessary.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>E₂ (10 nM)</th>
<th>ATRA (1 µM)</th>
<th>T₄ (0.1 µM)</th>
<th>Controls: NFW, DMSO, NH₄OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual</td>
<td>*</td>
<td>*</td>
<td></td>
<td>NFW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*</td>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td>NH₄OH</td>
</tr>
<tr>
<td>Combinations</td>
<td>*</td>
<td>*</td>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
<td></td>
<td>NH₄OH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*</td>
<td></td>
<td>DMSO, NH₄OH</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
<td>*</td>
<td>DMSO, NH₄OH</td>
</tr>
</tbody>
</table>

**Table 2.1** Stimulation of T47D and Sk-Br-3 cells with E₂, ATRA and T₄ individually and in combination over 24hrs

Again the level of NIS gene expression was quantified by RQ-PCR relative to endogenous controls and normalised to appropriate diluents controls.
2.4 MSC differentiation assays

Background

An important trait of MSCs is their ability to differentiate into osteogenic, adipogenic and chondrogenic lineages [118]. Along with characterisation of surface markers this ability is often used to confirm that a population of cells are in fact MSCs [126]. If kept in standard conditions these multipotent stem cells have an excellent capacity for self renewal. However changes in environmental conditions such as growth media or mechanical stresses can initiate differentiation resulting in the formation of cells belonging to different lineages, e.g. osteoblasts [119], adipocytes [292], chondrocytes [293].

When osteoblasts are formed from stem cells the process is known as osteogenic differentiation. This osteogenic phenotype can be simulated in MSC using a combination of Dexamethasone, Ascorbic acid and β glycerophosphate which changes the gene expression profile of MSCs pushing them towards an osteoblast phenotype [119].

Newly differentiated osteoblasts produce an organic matrix called osteoid. They also control the mineralization of this osteoid with phosphates, calcium, copper, sodium zinc and other elements.

In an osteogenic differentiation assay, the proportion of cells with an osteoblast phenotype can be quantified by measuring the mineralization process they control. Both phosphates and calcium can be used for this purpose.

Von Kossa staining utilises a precipitation reaction in which silver ions react with phosphate under acidic conditions to form silver phosphate. After exposure to light this silver phosphate degrades to silver highlighting the areas of mineralization in the population of cells with brown to black staining.

The StanbioTotal Calcium LiquiColor assay targets calcium mineralization by disassociating the calcium from proteins under acidic conditions and combining it with ortho-cresolphalein complexone (OCPC). This Ca-PCPC complex turns purple in alkaline medium which can be quantified at 550 nm
using a Labsystem Multiscan RC plate reader. When compared to a calcium/colour standard curve accurate quantification of calcium content per well can be achieved.

Method

To commit MSCs specifically to an osteogenic lineage cells were seeded in a 6-well plate at a seeding density of $3 \times 10^4$ cells/well. MSCs were incubated and allowed to adhere overnight. Osteogenesis was induced in assigned wells by replacing complete growth media with 2 ml of osteogenesis media comprised of the following: DMEM (LG), Dexamethasone 100 nM, Ascorbic acid 2-P 50 $\mu$M, $\beta$ glycerophosphate 10 mM, FBS 10 %, Penicillin 100 U/ml, Streptomycin 100 U/ml. Control conditions were established by maintaining 2ml of standard growth media on the MSCs. Cells were fed twice a week for 17 days at which point they were tested for the mineralization characteristic of osteoblasts using two different methods.

2.4.1 Von Kossa calcium staining

To assess mineralization or more specifically phosphate accumulation using a Von Kossa assay adherent cells were first fixed using 10 % formalin for 10min-1 hr. This was then washed off and 1 ml 3 % silver nitrate solution was added per well (6-well format) followed by a 10 min incubation in the dark. The wells were rinsed 3 times and exposed to bright warm light for 15 mins. The wells were rinsed a further 3 times and cells were then inspected by microscopy looking for evidence of mineralisation, e.g. dark brown staining of the cell layer indicated the presence of calcium deposits.

2.4.2 StanBio Calcium assay

To perform a StanBio Total Calcium LiquiColor assay the media was aspirated from cells which were then washed twice with PBS. Cells were scraped from
each well in 2 separate 0.5 ml volumes of 0.5 M HCl. Both 0.5 ml cell-HCl suspensions were pooled into the same eppendorf which was agitated overnight at 4°C.

Equal volumes of Colour Reagent and Base Reagent (700 μl per sample) were mixed to prepare a working reagent. This solution allowed to stand for 15 mins at RT before use. A serial dilution of calcium standard (100 mg/L) was prepared (0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 μg/ml). The previously described cell-HCL suspension or standard were added to the working solution in a 1:20 ratio on a 96-well plate. Absorbance at 550 nm was detected using Labsystem Multiscan RC plate reader and calcium concentrations in test samples were calculated based on a standard curve.

2.5 Transfection of MSCs with Adenovirus

2.5.1 Background

Viral infection of mammalian cells requires that the target cell has a surface receptor which facilitates cell entry. In the case of Adenovirus the Coxsackievirus and Adenovirus receptor (CAR) is the primary receptor that facilitates attachment by the capsid bound fibre of the Adenovirus particle to mammalian cells [294]. Following attachment, internalisation of the Adenovirus occurs as a result the affinity of the arginine-glycine-aspartic acid (RGD) peptide found on the capsid of the Adenovirus for surface integrins of mammalian cells [294, 295].

MSCs however do not express a high level of this CAR receptor leading to inefficiencies of transfection with Adenovirus [296, 297]. To overcome this, a transfection protocol is used which incorporates centrifugation [92]. This offsets the issue of low CAR expression by increasing contact between cells and virus, and more importantly between surface integrins and RGD peptides, resulting in increased infectivity. Fetal bovine serum interferes with
Adenovirus binding to its cellular receptors and so for this reason adenovirus infection protocols were performed in the absence of FBS.

The viral construct, shown in Figure 2.3 is a genetically modified adenovirus type 5. It is replication deficient due to a deletion of the E1 region of its genome which encodes proteins responsible for regulating all the early functions of adenovirus infection. Inserted in this deleted E1 region is the sodium iodide symporter (NIS) transgene under the control of a Cytomegalovirus (CMV) promoter. CMV promoter control of NIS results in constitutive expression of the gene. The resultant viral construct is abbreviated as Ad5/CMV/NIS.

![Diagram of Ad5/CMV/NIS](image)

**Figure 2.3** Replication deficient Adenovirus type 5 carrying the human sodium iodide symporter under the control of CMV promoter (AD5/CMV/NIS).

This Ad5/CMV/NIS vector was provided by Professor John Morris, Mayo Clinic, Rochester, Minnesota, U.S. and was amplified in the Regenerative Medicine Institute (REMEDI), NUI Galway.
2.5.2 Ad5/CMV/NIS infection

To infect MSCs with Ad5/CMV/NIS, cells were trypsinised, and washed twice with serum free media (SFM) to remove any residual FBS, counted and placed in a 50 ml falcon. Adenovirus was added at multiplicities of infection of 0, 100 and 200, i.e. MOI 100 represents a ratio of 100 viral particles per cell. The volume of virus needed was calculated using the following formula.

\[ V_{Ad5} = \frac{(C_c \times V_c \times MOI)}{C_{Ad5}} \]

- \( V_{Ad5} \): Volume of adenovirus,
- \( C_c \): Concentration of Cells,
- \( V_c \): Volume of Cells,
- \( MOI \): Multiplicity of Infection,
- \( C_{Ad5} \): Concentration of adenovirus.

The cells were then centrifuged at 2000 x g for 90 mins in the presence of Ad5/CMV/NIS under serum free conditions. After centrifugation cells were washed twice to remove free Ad5/CMV/NIS particles from the cell suspension and subsequently cultured as needed. All Adenovirus-contaminated waste was transferred directly to a container of bleach.

For analysis of NIS gene expression cells were cultured in T75 cm\(^2\) culture flasks (4 x 10\(^5\) cells/flask) or 6-well plates (5 x 10\(^4\) cells/well), followed by RNA extraction and RQ-PCR as described in section 2.7. For analysis of NIS protein expression by immunohistochemistry MSCs were cultured in 4-well Millicell\(^\text{®}\) EZ chamber slides (3 x 10\(^4\) cells/chamber) followed by immunostaining using a Discovery Ventana and microscopic analysis as described in section 2.8.
2.5.3 Ad5/CMV/GFP infection efficiency

**Background**

To determine adenovirus transfection efficiency a construct containing the reporter gene for green fluorescent protein (Ad5/CMV/GFP) was used. Using this vector, Ad5 transfected cells could be accurately quantified using flow cytometry with high levels of green fluorescence, compared to nontransfected cells. Since both Ad5 vectors differ only in terms of the genes for NIS and GFP, the transfection efficiency of Ad5/CMV/NIS can be inferred from that of Ad5/CMV/GFP.

To determine the transfection efficiency of Ad5/CMV/GFP, MSCs were infected as previously described with the virus at a range of MOIs from 0-150. The cells were cultured for 3 days in chamber slides and T75 cm² flasks for analysis by fluorescent microscopy (as described in section 2.8) and flow cytometry (as described in section 2.9).
2.6 Iodide $^{131}$ cytotoxicity assay

**Background**

$I^{131}$ releases beta (90%) and gamma (10%) radiation which are capable of damaging cellular DNA resulting in mutation at low doses, or cell death at higher doses. Beta radiation has a shorter tissue penetration with a destructive radius of up to 2mm [298]. Gamma radiation, which is also capable of damaging DNA, travels much further and is detectable beyond the tissues in which it has been accumulated giving it value as an imaging reagent [299, 300]. Cells with the capacity to accumulate iodide act as the epicentre of the radioactive damage. Additionally a bystander effect is also observed with $I^{131}$ whereby cells not accumulating $I^{131}$ but adjacent to cells that do, display a similar genotoxic response. In the following $I^{131}$ cytotoxicity assay the purpose of the assay is to determine the survival of T47D and MSCs expressing NIS co-culture in the presence or absence of $I^{131}$.

**Method**

To perform an $I^{131}$ cytotoxicity assay, MSCs were transfected with Ad5/CMV/NIS (as described in section 2.5) at MOIs of 0 and 100. They were then seeded into T175 cm$^2$ flasks at a density of 9 x $10^5$ cells per flask and incubated for 24hrs. At Day 1 MSC (MOI 0), MSC-NIS (MOI 100) or T47D cells were trypsinised and seeded individually and in co-culture in T75 cm$^2$ flasks as described in Table 2.2. Each condition was prepared in duplicate to accommodate each $I^{131}$ treatment with a relevant treatment-free control. Cells were cultured in complete MSC media.
<table>
<thead>
<tr>
<th>Condition</th>
<th>MSC (MOI0)</th>
<th>MSC-NIS</th>
<th>T47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSC alone</td>
<td>3.5 x 10^5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>MSC-NIS alone</td>
<td>---</td>
<td>3.5 x 10^5</td>
<td>---</td>
</tr>
<tr>
<td>T47D alone</td>
<td>---</td>
<td>---</td>
<td>3.5 x 10^5</td>
</tr>
<tr>
<td>MSC-NIS T47D 1:1</td>
<td>---</td>
<td>1.75 x 10^5</td>
<td>1.75 x 10^5</td>
</tr>
<tr>
<td>MSC-NIS T47D 1:3</td>
<td>---</td>
<td>8.75 x 10^4</td>
<td>2.63 x 10^5</td>
</tr>
<tr>
<td>MSC-NIS T47D 1:10</td>
<td>---</td>
<td>3.18 x 10^6</td>
<td>3.18 x 10^7</td>
</tr>
</tbody>
</table>

Table 2.2 Individual and co-culture seeding densities for I\textsuperscript{131} cytotoxicity assay.

The cell cultures were incubated for a further 48hrs to allow NIS expression in transfected cells to reach an optimum level. At Day 3, cells designated for I\textsuperscript{131} treatment were incubated with 0.8 mCi Na\textsubscript{131}-I in HBSS supplemented with 10 M NaI and 10 mM HEPES for 7 hrs. Controls were incubated in HBSS supplemented with 10 M NaI and 10 mM HEPES for 7 hrs. All cells were then washed with HBSS and replenished with complete MSC media. Cells were maintained and observed for a further 7 days. At Day 10, cells were harvested and counted by flow cytometry as described in section 2.9, and survival rates were compared between I\textsuperscript{131} treated and untreated groups.
2.7 Analysis of gene expression

2.7.1 Background

Quantification of messenger RNA (mRNA) is currently the most popular way of quantifying expression of specific genes. This is achieved by extracting the mRNA from cells or a homogenised sample of tissue, reverse transcribing the mRNA to complimentary DNA (cDNA) and specifically amplifying the cDNA which corresponds to the gene in question. Amplification of cDNA fragments is achieved by the process known as polymerase chain reaction (PCR). When conducting real-time PCR (RT-PCR,) cDNA is amplified in repeated PCR cycles until it reaches a threshold quantity which can be determined by the level of fluorescence of the PCR product due to fluorescent labelling of the DNA components. The number of PCR cycles required to achieve the threshold quantity is indicative of the original amount of the gene of interest. This is known as a Ct value. This method of quantifying cDNA in referred to as absolute quantification. It is prone to error mostly contributed by the operator or variation in reagents. An alternative method known as relative quantification-PCR (RQ-PCR) reduces such error and is illustrated in Figure 2.4. This method expresses the Ct value as a function of that of a reference gene whose level of expression is dependably consistent in the cell. This reference gene is known as a housekeeping (endogenous) control gene. In conjunction with the use of appropriate controls this technique can be very accurate and reliable and has become the gold standard of gene expression analysis.
Figure 2.4 Overview of Gene expression analysis. RNA is extracted from lysed cells. The quantity of RNA is determined by Nanodrop as well as levels of purity. Reverse transcription is used to convert mRNA to complementary (c) DNA. cDNA is amplified by repeated cycles of PCR to a discrete number of copies. The number of PCR cycles required is related to the initial quantity of cDNA. Expressing the quantity of a target gene as a function of a reference gene improves the reliability of data. Image modified from Vierstrate 1999 [301].

2.7.2 Tissue homogenisation in preparation for RNA extraction

Background

Tissue homogenisation is critical to achieving the optimal RNA extraction levels from biopsies. In this regard homogenisation is all about breaking down the tissue into smaller, finer pieces. In doing so the total surface of the biopsy is dramatically increased. This allows for more penetration of lysis buffer into
the cells and subsequently a larger amount of RNA is released into the cell lysate which can then be isolated and purified.

Minimising the duration of homogenisation is important since this process introduces heat to the system which could speed up RNA degradation. For more robust biopsies intermittent cooling of the sample in liquid N₂ is advisable. Homogenised lysates should always be returned immediately to liquid nitrogen.

**Method**

To homogenise tissue biopsies a Polytron PE 1600 E homogeniser was set up in a clean environment with autoclaved drill bits. Frozen tissue specimens were maintained in liquid nitrogen to preserve sample integrity. Immediately prior to homogenisation samples were added to an appropriate volume of TRIzol lysis buffer (Invitrogen)(1-4 ml depending on type and size – e.g. human breast usually 1ml, mouse liver usually 3-4 ml) in an 8 ml polypropylene tube and blitzed with the Polytron at 3 x 10^5 RPM. While homogenising the tube was manoeuvred repeatedly to optimise the effectiveness of the drill bit. When the TRIzol-tissue mixture appeared to have a creamy to pasty appearance it was suitably homogenised and was returned to the liquid nitrogen immediately. Tissues which took longer to blend were dipped in liquid N₂ to keep the temperature low. Before proceeding to a subsequent sample, the drill bit was washed with ddH₂O, followed by RNase away, then EtOH and finally in ddH₂O. Thorough inspection of the bit was also performed to check for potential sources of cross contamination.

**2.7.3 Preparation of cultured cells for RNA extraction**

Cultured cells were trypsinised, counted and suspensions centrifuged using an Eppendorf Centrifuge 5810 R at 1000 x g for 4 mins as described in section 2.1. The supernatant was decanted and any remaining media was removed by
pipetting. Cell pellets were immediately transferred to a -80°C freezer for storage.

Pellets were then thawed for RNA extraction. As a result of freeze thaw action and media pooling from the inside of the falcon, a layer of media was usually apparent on top of the cell pellet. Where possible this media was removed prior to RNA extraction procedure as incomplete removal of media inhibits lysis of cells and may reduce RNA yields.

2.7.4 RNA extraction

Background

Extraction of RNA from the homogenised TRIzol-tissue (or TRIzol-cell pellet) lysate is critical for accurate quantification of gene expression. This process removes deleterious proteins and contaminating DNA from the sample which results in a more pure RNA sample with better stability, and more reproducible and reliable results.

The addition of chloroform to the lysate, followed by centrifugation, allows the formation of a biphasic mixture whereby the RNA collects in the upper aqueous phase, DNA at the interphase and protein in the lower, inorganic phase. This aqueous phase can then be applied to the RNeasy® Mini Kit (Qiagen) for RNA extraction resulting in improved purity and quality.

The principle of this RNeasy® process is that RNA treated with ethanol precipitates out of solution and is restricted by a silica based membrane in a high salt buffer system. While RNA is bound to the surface of the membrane, contaminants can be removed by repeated washes. This includes unwanted genomic DNA which can be digested and sluiced through the membrane by treatment with DNase 1 and further washes. The membrane bound RNA can then be eluted in a small volume of H₂O.
mRNA is highly sensitive to degradation by RNases. These are present within the sample as well as the environment. When extracting RNA, additional precautions should be taken to limit the introduction of these enzymes to samples or reagents. These include operating within a biosafety cabinet, use of nuclease decontaminant such as RNase Away (Molecular BioProducts), and dedicated pipettes for RNA work accompanied by sterile filter tips. A large risk of environmental RNase contamination can be eliminated by using proper personnel protective equipment such as powder free gloves and clean lab coats.

**Method**

To extract RNA from TRIzol-tissue lysates (or cell pellets resuspended in 1 ml TRIzol), 200 µl chloroform was added to approximately 1.2 ml of homogenised mixture. This chloroform-lysate mixture was agitated by vortex and left to stand at RT for 15 mins, followed by 15mins centrifugation at 11500 rpm at RT. The upper aqueous layer of this biphasic mixture containing the majority of mRNA was removed with a pipette and added to 3.5 times its volume of pure ethanol. This aqueous RNA-EtOH solution was vortexed briefly and applied to the RNeasy Mini Spin Column (RNeasy Minikit) in 700 µl aliquots and centrifuged at 11500 rpm until the complete sample was passed through the column. The membrane bound RNA was washed with 350 µl RW1 buffer (RNeasy Minikit), then incubated with 80 µl of 12.5 % DNAse 1 in RDD buffer (Qiagen) for 15 mins at RT, followed by another RW1 wash. This was followed by two washes with 500 µl RPE buffer (RNeasy Minikit) after which the column was transferred to a 1.5 ml Eppendorf tube for collection of RNA eluted in 30 µl nuclease free ddH2O. 1.3 µl of purified RNA sample was aliquoted for analysis using the Nanodrop spectrophotometer with the remainder stored at -80°C.
2.7.5 RNA quantification

Background

The Nanodrop 1000 Spectrophotometer (Thermo Scientific) is used to accurately quantify RNA. It also provides an indication as to the quality of the RNA in the analysed sample based on absorbance ratios. The Nanodrop 1000 works by interrogating a small volume of sample drawn between 2 fibre optic ends with a pulse of light delivered by a xenon lamp. The incident light is read by a charged coupled device and converted to levels of absorbance at different wavelengths. Based on this data, RNA concentrations of up to 3000 ng/µl (3700 ng/µl DNA) can be accurately quantified. The ratio of absorbance @ 260 nm and 280 nm is indicative of RNA purity. Pure RNA has a 260/280 ratio of 2. A low 260/280 ratio could indicate the presence of contaminating factors such as proteins, phenols (from anti-RNAase reagents e.g. Qiazol) or other sources. The 260/230 ratio is also indicative of RNA purity and usually ranges in value from 1.8 to 2.2 for pure RNA samples. A low 260/230 ratio could be caused by carbohydrate, peptide, phenol, or aromatic compound contaminants.

Method

An aliquot of nuclease free water was used to blank the Nanodrop 1000 Spectrophotometer for detection of RNA. Once blanked, 1.1 µl aliquots of RNA sample were loaded onto the pedestal of the Nanodrop and the concentration was measured. In between samples the pedestal was cleaned with nuclease free water and Kimwipe tissue paper to prevent cross contamination of loaded samples. The Nanodrop was reblanked every 5 samples. When the measurement was taken for each sample a results window was displayed as shown in Figure 2.5.
Figure 2.5 Results Window from RNA-40 analysis on Nanodrop 1000. A) The absorbance versus wavelength curve of pure RNA, B) The 260/280 ratio, C) the 260/230 ratio, D) concentration of RNA in ng/µl.

This window displayed data on concentration (ng/µl), and purity (260/280 and 260/230 ratios). An absorbance versus wavelength curve for the sample was also displayed. An ideal curve representative of good quality RNA should look like that in Figure 2.5 peaking @ 260 nm and possessing an continuous even line. Deviation from this curvature such as a broken or wavy line indicates potential degradation in RNA quality.


2.7.6 RNA concentration

Where RNA samples were not of a sufficient concentration for use, a Concentrator 5301 (Eppendorf) was used. This instrument works on the basis of water evaporation using a vacuum, thereby allowing sample temperatures to remain relatively low (30°C). A general guide of 10 μl evaporation per 10 mins was used. Following concentration, RNA levels were quantified as described in section 2.3.5.

2.7.7 Reverse Transcription/ cDNA synthesis

Background

Single stranded mRNA can be used as a template for the production of corresponding double stranded DNA known as complementary DNA (cDNA). This is achieved by exploiting the natural process of reverse transcription which has been observed in retroviruses. The enzyme responsible, reverse transcriptase is commercially available as SuperScript® III Reverse Transcriptase (Invitrogen) and is sourced from the Moloney Murine Leukaemia retrovirus.

During reverse transcription an initial heating step is used to denature or remove the secondary structure from mRNA. This optimises cDNA synthesis by increasing primer and Reverse Transcriptase access to the mRNA template. The DNA polymerase binds at the site of a hybridised primer and synthesises cDNA in a 3’-5’ prime direction along the mRNA template in the presence of DNA nucleotides. This is known as first strand synthesis. A complementary strand of cDNA is then created and bound to the first strand to produce double stranded cDNA.

Different types of primers can be used such as specific, oligo-dT or random primers. Random primers were selected for use in this study because they hybridise to complementary regions at different points on the mRNA strand. They prime reverse transcription of shorter cDNA fragments from random sites.
along the mRNA strand and in doing so increase the probability of synthesising cDNA from the 5’ end. This also ensures that no one part of the mRNA is disproportionately represented by cDNA.

RNaseOUT™ (Invitrogen) is incorporated in this reaction protecting the mRNA template from degradation by ribonucleases. Dithiothreitol (DTT), supplied by Invitrogen is also used to inhibit RNase activity by reducing disulphide bonds and consequently RNase stability. Additional measures include those described in section 2.7.4.

**Method**

To reverse transcribe mRNA to cDNA, only samples with a 260/280 nm absorbance ratio of >1.8 were used. 1 μg mRNA was denatured in the presence of random primers (76 pg/μl) (Eurofins MWG Operon) and deoxyribonucleotide triphosphates (dNTPs) (770 μM) for 4mins at 55°C in a reaction volume of 13 μl. If the mRNA sample was too dilute to accommodate this volume it was concentrated as described in section 2.7.6.

A 7 μl reaction mix was then prepared consisting of First Strand Buffer (2.9 x) (Invitrogen), DTT (14 mM), RNaseOUT™ (6 U) and SuperScript® III Reverse Transcriptase (29 U) was prepared. This was added to the denatured RNA mixture making a final volume of 20 μl. This complete mixture was incubated for 5 mins at 25°C, followed by 1hr at 50°C which is the optimal temperature for SuperScript® III activity and finally 15 mins at 70°C to denature the enzyme. This cycle was performed in a Gene Amp PCR 9700 machine (Applied Biosystems). Once completed, 30 μl of nuclease free water (NFW) was added to give a final volume of 50 μl cDNA which was stored at -20°C.
2.7.8 Relative Quantitative PCR

Background

Complementary DNA (cDNA) can be amplified through the polymerase chain reaction (PCR). This involves repeated thermal cycles in the presence of DNA constituents (nucleotides and primers) and the heat resistant enzyme, DNA polymerase (Taq Polymerase) sourced from a thermophilic bacterium. Each cycle begins with a denaturing step at 95°C for 1 sec. This removes secondary structure from the DNA and splits it into single stranded DNA. The temperature is reduced to 60°C for 20 secs to allow the annealing step to take place. This involves synthesis of new cDNA strands from existing single stranded templates, in the presence of nucleotides, gene specific primers and Taq polymerase. Specific primers hybridize to sites of complementarity and direct the exonuclease activity of Taq polymerase to synthesize a new strand of DNA from that point. One denaturing and one annealing step equals one cycle (Figure 2.6). In a perfect PCR cycle the DNA is amplified by a factor of 2, although this is not always achieved.
Figure 2.6 Polymerase chain reaction. Repeat heat cycles in the presence of a DNA template, DNA polymerase and free nucleotides and gene specific primers allow amplification of target genes [302]. Permission granted to use image.

When PCR is performed in the presence of double stranded DNA binding dyes such as SYBR Green, the fluorescent signal generated by the SYBR-dsDNA complex can be quantified. This is done at the end of each cycle allowing real time quantification of the PCR product in contrast to traditional endpoint quantification. The number of PCR cycles required for a sample of DNA to produce a standard threshold of fluorescence intensity is known as the threshold cycle (Ct) value. When standard volumes of reagents and samples are used in reactions the quantity of amplified cDNA, which is dependent on the starting quantity of mRNA can be inferred using the Ct value. This is also known as absolute quantification.
However, this process is slightly flawed in that it depends largely on the efficiency of the reverse transcription reaction, and consistent measurement and aliquoting of samples and reagents. This can be largely influenced by human operators and because of this there is potential for significant error. Using the relative quantitative PCR method can reduce this error.

Relative Quantitative PCR (RQ-PCR) counters this issue by quantifying the expression of a housekeeping gene/endogenous control, which is known to have consistent levels of expression regardless of cell state. Target gene expression can be expressed relative to this gene thus alleviating the impact of human error to a large extent. Data is expressed by relative quantification by calculating Ct values for target genes as a function of endogenous controls resulting in the Ct value. The Ct value for test samples is then subtracted from that of the control sample to produce the ΔCt. Since the quantity of DNA is approximately doubled (depends on amplification efficiency) per PCR cycle, the fold difference in expression can be calculated as $2^{\Delta \Delta Ct}$ [303].

$$Ct = Ct_T - Ct_E$$

$Ct_E =$ Ct endogenous control, $Ct_T =$ Ct target gene

$$Ct = Ct_{TS} - Ct_{CS}$$

$Ct_{CS} =$ Ct control sample, $Ct_{TS} =$ Ct test sample

$$\text{Fold change} = 2^{Ct}$$

$$2^{\Delta \Delta Ct} = 2^{(Ct_E - Ct_T) - (Ct_{CS} - Ct_{TS})}$$

It is important however to ensure that target and endogenous control genes are amplified at a similar rate to each other to allow valid comparison of expression. This rate is dependent on the specific primers used for each gene amplification and can be established by performing an amplification efficiency assay as described in the next section.

An amplification efficiency assay involves the absolute quantification of a serial dilution of a DNA sample in the presence of a specific primer. When
each dilution reaches the set threshold of fluorescence the corresponding Ct value is plotted on a standard curve. The slope of this curve is used to calculate the efficiency or ability of the primer to amplify the product by a factor of 2 (increase by 100 %) in each cycle. Only primers with comparable efficiencies of approximately 2 (limits 2 ± 0.1 or 100 % ± 10 %) can be used for relative quantification.

2.7.9 Amplification Efficiency

To determine the amplification efficiency of each primer a sample known to have a relatively robust level of expression for that specific gene was used, i.e. a sample whose RT-PCR analysis would register a low Ct value (Ct15-25) for the targeted gene. A log serial dilution, from neat to 10^{-4} of this sample was prepared using NFW.

A reaction mix with a final volume of 10 µl containing 50 % v/v Mastermix, 35 % v/v H2O, and 5 % v/v PDAR was added to each well of a PCR plate followed by the final 1µl cDNA sample (10 % v/v). Triplicate wells were prepared for each dilution and target. The plate was run using the system specific software version SDS 1.2.2 on an Applied Biosystems 7900HT Fast Real-Time PCR System or an ABI Prism 7000 Sequence Detection System as an “Absolute Quantification” assay.

A maximum of 40 cycles were run per plate as follows:

7900HT Fast Real-Time PCR System: Enzyme activation: 20 sec at 95°C

40 cycles Step 1: 1 sec at 95°C (Denaturing)

Step 2: 20 sec at 60°C (Annealing)

or

Prism 7000 Sequence Detection System: Enzyme activation: 10mins at 95°C

40 cycles Step 1: 15 sec at 95°C (Denaturing)

Step 2: 1 min at 60°C (Annealing)
Ct values were generated for each reaction where the amplification curve crossed a set threshold.

Only samples with triplicate values with a standard deviation < 0.3 were used. This quality control check for PCR data increases reproducibility of experimental data.

The results were analysed and a Standard Curve was generated from the dilutions, and the PCR Cycle number at which the level of amplification crossed a set threshold (Ct value). The efficiency of the primer driving this amplification was calculated as follows.

Efficiency = \( 10^{(\frac{-1}{\text{Slope Std Curve}})} - 1 \) x 100

Primer amplification efficiencies were expressed as a percentage. Only primers with an efficiency of 100 ± 10 % were used for RQ-PCR.

All primers used are shown in Table 2.3a and 2.3b.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Full name</th>
<th>Purpose</th>
<th>AB Product code</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNIS</td>
<td>human sodium iodide symporter</td>
<td>Iodide uptake in human thyroid and lactating mammary gland</td>
<td>Hs00166567_m1</td>
<td>gcccaccaaggcagcaccctggcccccggattgttggtgggaccctgccgagagcagatcagttggc</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mNIS</td>
<td>Mouse sodium iodide symporter</td>
<td>Iodide uptake in mouse thyroid and lactating mammary gland</td>
<td>Mm00475074_m1</td>
<td>gaccggaagacatccctgtcgcaccaagaagccccctggttcagggcagaagccagcctccctatcttg</td>
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<td></td>
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</tr>
<tr>
<td>PPIA</td>
<td>Cyclophilin A</td>
<td>Accelerates protein folding. Suitable as an endogenous control</td>
<td>Hs99999904_m1</td>
<td>ccaagactgtagtgttggatgggaaggctagttgttttggccaaagatgaagaagcatatttggagc</td>
</tr>
<tr>
<td>MRPL19</td>
<td>Mitochondrial Ribosomal Protein L19</td>
<td>Mitochondrial protein synthesis. Suitable as an endogenous control</td>
<td>Hs00608519_m1</td>
<td>ttgagtcctgatcattcctggaggggaagaactatcctccaaatgaaagagataatgtgagccccag</td>
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<td></td>
</tr>
<tr>
<td>mKi67</td>
<td>Antigen Identified by mAb Ki67</td>
<td>Nuclear protein associated with proliferation.</td>
<td>Hs00606991_m1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase, catalytic, alpha polypeptide</td>
<td>Cell growth, proliferation, survival, differentiation, intracellular trafficking. Potential NIS regulator</td>
<td>Hs00907966_m1</td>
<td>acagacactatgttgtaactactccggaaatctacccaaattcctccgaagctgctgttggtaaagt</td>
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**Table 2.3a** Description of primers used
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Primer ID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ESR1</strong></td>
<td>Estrogen Receptor alpha</td>
<td>Hs00174860_m1</td>
<td>ggttgaacacagccagagagagatggg</td>
</tr>
<tr>
<td><strong>RARα</strong></td>
<td>Retinoic acid receptor alpha</td>
<td>Hs00940446_m1</td>
<td>gacgacaccagctcggagggctgaccc</td>
</tr>
<tr>
<td><strong>RARβ</strong></td>
<td>Retinoic acid receptor, beta</td>
<td>Hs00233407_m1</td>
<td>atctgtcaggagaatcaggagaacagagagacttgaagaagaagaagagacgctgactgagttgga</td>
</tr>
<tr>
<td><strong>THRα</strong></td>
<td>Thyroid Hormone Receptor A</td>
<td>Hs00268470_m1</td>
<td>gtctcttgcgcacaatccaggaacacaatcccaatcccaacctat</td>
</tr>
<tr>
<td><strong>THRβ</strong></td>
<td>Thyroid Hormone Receptor B</td>
<td>Hs00230861_m1</td>
<td>tgctgtgatgacagagaggtgctgcaagagagagctgtgtagaggaaacacgggagagacgctgc</td>
</tr>
<tr>
<td><strong>mβ actin</strong></td>
<td>Mouse beta actin</td>
<td>No longer available</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Mouse HPRT</strong></td>
<td>Mouse Hypoxanthine-guanine phosphoribosyltransferase</td>
<td>Mm00446968_m1</td>
<td>agctttgctgtgaaagacgctctcgaagtg</td>
</tr>
</tbody>
</table>

**Table 2.3b** Description of primers used
2.7.10 RQ-PCR

PCR reagent and samples were prepared as described for detection of amplification efficiency with one exception. No dilution was performed on cDNA samples. The plate was run on a 7900HT Fast Real-Time PCR System (in a 10 µl volume) or an ABI Prism 7000 Sequence Detection System (in 20 µl volume) as a “Relative Quantification” assay. Assay conditions, cycle times and quality control checks of data were the same as with absolute quantification.
2.8 Immunohistochemistry

2.8.1 Background

Immunohistochemistry (IHC) allows for the detection of specific proteins and highlights their spatial distribution within cells. It depends on the availability of antibodies specific for a protein of interest. These can be conjugated to a signalling molecule such as a fluorescent label (e.g. Allophycocyanin) or an enzyme to which a chromogenic substrate is later added resulting in a quantifiable colour change (e.g. Horseradish peroxidase and Diaminobenzidine). If the primary antibody is not conjugated to a signalling antibody it can be specifically targeted by a secondary antibody with conjugated labels.

A mouse monoclonal antibody for human NIS (hNIS) [304] was supplied by Professor John Morris (Mayo Clinic, Rochester, Minnesota, U.S.).

The process of IHC involves targeting the NIS protein in specimen tissue using the hNIS antibody. A secondary antibody conjugated to biotin is added which conjugates to the primary antibody. Enzyme-conjugated streptavidin, which has a high affinity for biotin, allows for specific enzyme activity proportionate to the amount of NIS present. For faster, automated IHC of tissue sections a Discovery Ventana is used. This machine possesses a carousel which can be loaded with 24 slides at a time. Reagents are mounted on a dispensing carousel and protocols can be programmed into the controlling software resulting in reliable and reproducible staining of tissue sections. Two enzyme-chromogenic substrate systems are used with the Discovery Ventana. They are REDMap and DABMap. In the case of the REDMap detection system, a red colour is generated by alkaline phosphatase hydrolysing Naphthol R to a phenolic compound which is coupled to chromogens Fast Red R and Fast Red 2. Using the DABMap kit, horseradish peroxidase oxidises dianinobenzidine tetrahydrachloride (DAB) in the presence of H₂O₂, the product of which is brown and insoluble in alcohol and other solvents.
2.8.2 Methanol fixation of cells cultured in chamber slides

Fixation of cells prior to histochemical analysis offers a number of advantages. Using a fixative such as methanol maintains original cell architecture by stopping biochemical processes. It also prevents specimen decay through autolysis by disabling proteolytic enzymes and provides a toxic environment to micro organisms and increasing cell adherence to slides. Contrary to the term ‘fixation’, methanol does not fasten cells to the slide. This is attributed to the adherent nature of the cell culture and the use of positively charged chamber slides.

To perform a methanol fixation, monolayer cultures of Ad5/CMV/NIS infected MSCs were chilled on ice for 15 mins and then gently washed with chilled methanol. The slides were then stored at -20°C for 15 mins in 1 ml of fresh ice-cold methanol. Methanol was removed in preparation for immediate immunohistochemistry (IHC) or alternatively slides were stored in PBS for IHC at a later date.

2.8.3 Cryosectioning of tissue

Background

Cryosectioning allows frozen tissue sections to be sliced in very thin (5-7 μm) sections suitable for histological analysis without thawing the specimen. This can be done using a cryostat which facilitates tissue sectioning between -15°C and -30°C depending on the tissue used. Tissue sections are transferred to slides coated with a positively charged polymer which binds to negatively charged sites on the tissue resulting in stable attachment of tissues. The use of frozen sections was preferred to paraffin embedded sections since the antigenic quality of snap frozen tissue is often better. A drawback of this selection is poorer tissue morphology than would be observed in paraffin embedded tissue. However since this study prioritises the presence and intracellular localisation
of NIS over tissue morphology frozen sections remained the preferred selection.

**Method**

To cryosection human breast tissue as well as mouse tissue specimens the cryostat temperature was maintained between -25°C and -30°C. Specimens were temporarily stored in liquid nitrogen when not in the cryostat. In the case of mouse tissues which may contain fluorescently labelled MSCs, the tissue and subsequent slides were protected from light as much as possible. Biopsies were mounted onto cryostat chucks using optimum cutting temperature (OCT) compound and incubated at -25°C to -30°C for 10-15 mins until the OCT hardened. Thick sections (50 μm) were first removed until an appropriate cross-section of biopsy could be achieved after which 5 μm sections were mounted onto positively charged glass slides. Where possible 5 interim sections were discarded between sections mounted on slides. This approach increased the chances of observing pockets of tissue with different characteristics to the whole tissue. Typically 3 sections were mounted on each slide.

**2.8.4 Immunohistochemistry targeting the Sodium Iodide Symporter (NIS) Protein**

Slide labels were generated for the appropriate Ventana protocol for REDMap or DABMap staining of human NIS. Labelled slides were prepared for immunohistochemistry by washing twice with PBS for 5 mins. Slides were loaded onto the Ventana Discovery carousel, flooded gently with reaction buffer and the automated protocols were initiated. Protocol summaries are as follows:

**REDMap:** Ventana Discovery proceeded with initial slide preparation and 20 min incubation. This and all subsequent incubations within the Ventana
Discovery carousel were performed at RT. 100 μl of a 1:1000 dilution of monoclonal antibody for human NIS in PBS was added manually to each test slide and incubated for 60 mins. PBS alone was added to control slides. A biotinylated universal secondary antibody capable of targeting mouse immunoglobulin G was then added with subsequent 30 min incubation. Blocker R1 was added with a 2 min incubation to prevent binding of endogenous biotin and background staining. Then Streptavidin-biotin-Alkaline Phosphatase R was added followed by a further 30 min incubation. Activator R (increases enzyme activity) and Napthol R (substrate for phosphatase) were each added with a 4 min incubation. Fast Red R (colour generator) was then added with an 8 min incubation followed by Fast Red R2 (colour generator) and another 8 min incubation. The enzymatic reaction between alkaline phosphatase and chromogen resulted in a bright red precipitant in the vicinity of the biotin linked enzyme-antibody complex. Haemotoxylin counterstain and Bluing Reagent were added sequentially with 2 min incubations to highlight the nuclei of the cell monolayer.

Dehydration of Ventana REDMap Slides was performed immediately following immunohistochemistry by first washing the slides in warm soapy water to remove oil, followed by rinsing in ddH₂O. The slides were then treated sequentially as follows; 10 dips in 75 % alcohol, 10 dips in 100 % alcohol, repeat 10 dips in 100 % alcohol, 10 dips in acetone, Xylene immersion for 3 min, repeat Xylene immersion for 3 min.

Cover slips were carefully mounted onto slides using DPX mountant and left to dry in a fume hood. Excess DPX was removed by soaking briefly in Xylene.

**DABMap:** Ventana Discovery proceeded with initial slide preparation by introducing Inhibitor D which reduces endogenous peroxidase activity, followed by a 20 min incubation. This and all subsequent incubations within the Ventana Discovery carousel were performed at RT. 100 μl of a 1:1000 dilution of Monoclonal antibody for human NIS in PBS was added manually to
each test slide and incubated for 60 mins. PBS alone was added to control slides. A Universal secondary antibody was then added with subsequent 30 min incubation. Blocker D was added with a 2 min incubation to reduce background staining. Then Streptavidin-Horseradish peroxidase conjugate (SA-HRP D) was added followed by a further 16 min incubation. DAB D and DAB H$_2$O$_2$ were added and incubated for 8 mins followed by Copper D for 4 mins. Haemotoxylin counterstain and Bluing Reagent were added sequentially with 2 min incubations to highlight the nuclei of the cell monolayer.

Dehydration of Ventana DABMap Slides was performed immediately following immunohistochemistry by first washing the slides in warm soapy water to remove oil, followed by rinsing in ddH$_2$O. The slides were then treated sequentially as follows; 3 mins in 75 % alcohol, 3 mins in 95 % alcohol, repeat 3 mins in 100 % alcohol, Xylene immersion for 3 mins, repeat Xylene immersion for 3 mins.

As described previously, cover slips were carefully mounted onto slides using DPX mountant and dried overnight in a fumehood.

2.8.5 Microscopy

Background

Tissue sections were analysed using an Olympus BX60 microscope and analySIS software. Images were captured at 40 x, 100 x and 200 x magnification. No further modifications of images were performed.

2.8.6 Fluorescent Microscopy

Where tissue sections were to be inspected for a fluorescent signal (e.g. Green fluorescent protein or PKH26) fluorescent microscopy was used instead of light microscopy. This involved a number of modifications in the treatment of slide specimens. The most important is the protection of the specimen of cells or tissue from light to prevent quenching of the fluorescent signal. This was
achieved by storage of tissue and slides in the dark, operating in darkened work spaces including biosafety cabinets and cryostat, and minimising processing time. Since Green fluorescent protein (section 2.5.3) and PKH26 (section 2.2) were either produced by the cell or integrated in the cellular structure no antibody step or chromogenic interaction (e.g. DABmap, REDmap) was required. It was however necessary to stain the nucleus of these cells to put transgene expression in context. This was done by DAPI staining (next section).

2.8.6.1 Nuclei counterstaining for fluorescent microscopy

Nuclei were counterstained using the fluorophore 4', 6-diamidino-2-phenylindole (DAPI) which emits light in the blue spectrum. DAPI binds to A-T rich sections of DNA. The fluorescent emission spectrum of DAPI (blue 455 nm) overlaps slightly with that of GFP (green 509 nm) but not to such a level that it precludes the use of both fluorophores in combination.

To counterstain nuclei with DAPI, slides containing tissue or cell culture specimens were washed with PBS under darkened conditions. They were fixed in 4 % paraformaldehyde for 20 mins at 4°C and subsequently treated with 3 x 5 min washes in PBS. In the case of cell culture specimens the chamber wall was removed at this point. Slides were immersed in 1 μl/ml DAPI for 4 mins and incubated in the dark. A further 3 x 5 min washes in PBS followed. Dry blotted slides were dehydrated as described for DABmap, mounted in DPX and dried overnight wrapped in foil to protect from light.

Slides were analysed using an Olympus BX51 microscope and analySIS software. Images were captured at 40 x, 100 x and 200 x magnification. DAPI, GFP and PKH26 signals were capture using blue, green and yellow filters respectively. Exposure was reduced with increasing magnification. No further modifications of images were performed. DAPI and PKH26 images were acquired sequentially to generate composite 2 colour images.
2.9 Flow Cytometry

2.9.1 Background

Flow cytometry allows the interrogation of individual cells in a cell suspension on the basis of size, complexity, viability, proteins markers, and physiological states. This is typically achieved by creating a hydrodynamically focussed stream of a cell suspension, allowing only one cell to occupy an interrogation space at any one time. This space is analysed by a beam of laser light. If a cell crosses this beam the incident light is deflected. Light detectors, aligned perpendicular (side scatter) and in line with laser light (forward scatter) quantify this deflection of incident light giving information on cell structural complexity and cell size respectively [305].

Additional light sources and detectors can be used to register different wavelengths of light. This allows the use of antibodies conjugated to different fluorescent markers to acquire quantitative data on surface markers proportionate to formation of antibody-antigen complexes (Figure 2.7). When monoclonal antibodies conjugated to fluorophores of complementary spectral emissions are combined, data can be obtained on several protein markers at the same time. This is particularly useful in characterising panels of cell surface markers.
Figure 2.7 Flow cytometry a) The cell suspension is hydrodynamically focussed allowing cell by cell analysis [306]. b) Fluorophore conjugated monoclonal antibodies attached to target proteins on cells emit a unique fluorescent signal when interrogated by lasers. This fluorescent signal is detected, quantified and converted to data describing expression levels of the targeted protein [307]. Permission granted to use image.

Since the light signal is quite small, each detector incorporates a photomultiplier tube (PMT) similar to that shown in Figure 2.8. Incident light travelling as photons, strike the photocathode of a detector and release photoelectrons. This is known as the photoelectric effect or primary emission. One photoelectron is produced per photon strike [308, 309]. These initial photoelectrons need to be amplified in order to register a signal which is distinct from background and instrument noise. They are guided through a sequence of dynodes with increasingly positive charges. Photoelectrons are accelerated in response to the electric field, strike the first dynode at a greater energy and in doing so produce an increased number of low energy electrons. This is known as secondary emission. These in turn are accelerated, strike the second dynode and release even more electrons. At the end of the sequence of dynodes and of the PMT the initial photoelectron has amplified exponentially.
producing a charge at the anode which is converted to a distinct current pulse [308, 309]. It is this current pulse that generates readable data from the flow cytometer. By increasing the voltage for a PMT in a flow cytometer you increase the current signal produced proportionate to the initial incident light generated in the flow cell [305]. This signal is usually portrayed as a dot plot. Adjustment of voltage allows the operator to bring signals from cell populations of varying dimensions into graphical fields of view. Signals can even be positioned within the dot plot so that they act as reference points for other cell populations.

**Figure 2.8** Photomultiplier tubes. They work by amplifying the number of single photoelectrons created by incident light to the point where the accumulated charge of negative electrons around the anode is detectable as a current [309]. Permission granted to use image.

Intracellular proteins can also be targeted by first permeabilizing the cell membrane and staining with a fluorescently conjugated monoclonal antibody for that protein. Without permeabilizing the antibody could not attach to the internal protein so it is critical that the nature of the target protein is understood before attempting to analyse it by flow cytometry.

Fluorescence is achieved by excitation of a fluorophore as demonstrated by the Jablonski diagram in Figure 2.9b [310]. Each excited fluorophore releases light
energy at a higher wavelength compared to the excitation light. This is referred to as Stokes Shift (Figure 2.9a) and is important for two reasons: 1) the fluorescent signal can be distinguished from excitation light and 2) each individual fluorochrome has a peak excitation and emission spectrum which allows for dual or multistaining with fluorophores not just of different colours, but of different excitation wavelengths [311].

**Figure 2.9** a) Stokes Shift [311] and b) Jablonski models of fluorescence [310]. Permission granted to use images.

If the light emission spectrum of fluorophores used for dual staining overlaps slightly it is still possible to achieve accurate data by compensating for the false positive signals detected.

Selection of antibodies is a critical step in the flow cytometry process. Ideally, monoclonal antibodies validated for flow cytometry are used. If possible they should be directly conjugated to a fluorophore to avoid the complication of using secondary conjugated antibodies. Isotype controls are designed to measure the level of non-specific background signal caused by primary
antibodies. This could be caused by nonspecific binding of immunoglobulins to the Fc receptor. Incorporating an isotype control allows for correction of this nonspecific fluorescent signal revealing the quantity of true fluorescence caused by specific antibody binding. Isotype controls were selected for each antibody type and fluorochrome e.g. a mouse IgG2b antibody conjugated to Allophycocyanin (APC) requires a mouse IgG2b isotype control conjugated to APC. It should be noted that isotype controls represent the combined fluorescence of non-specific attachment of antibodies as well as the level of auto fluorescence from the analysed cells. Auto fluorescence is usually negligible, but where cell suspensions are derived from primary cell cultures, green auto fluorescence can be sufficiently high to obscure real signals. This is attributed to the presence of flavin nucleotides which, when excited between 430-500 nm, emit fluorescence at 530-550 nm. To determine the level of autofluorescence of a cell suspension, unstained samples should be included as a control. If strong green-yellow autofluorescence is observed in an unstained control, conjugated antibodies should possess a fluorochrome with emission in the red spectrum at least (e.g. Peridinin-Chlorophyll Protein-Cyanine 5.5 (PerCP-Cy™ 5.5)), and ideally higher. Figure 2.10 illustrates the peak fluorescent emission of the fluorophores used in this study, including the peak emission range of natural autofluorescence which may occur.

**Figure 2.10.** Peak emission spectrum of fluorophores and flavin nucleotides. Fluorescein isothiocyanate (FITC), R-Phycoerythrin (RPE), Phycoerythrin (PE), Allophycocyanin (APC), Phycoerythrin Cyanine 5 (PE-Cy™ 5), Peridinin-Chlorophyll Protein-Cyanine 5.5 (PerCP-Cy™ 5.5) and Allophycocyanin-Cyanine 7 (APC-Cy7)
### 2.9.2 Graphical representation of Flow Data

Data is initially presented in the form of a dot plot representing forward and side scatter as illustrated in figure 2.11. On this graph, most debris and instrument noise can be identified and removed by setting a threshold bar. Clusters of dots could be representative of individual cell populations and can be identified using square, rectangular or oval regions. New dot plots can be created which view only the cells defined within a region. This is performed by gating the new graph using that region. In doing so cell populations identified using one combination of parameters can be analysed using additional parameters in different combinations, i.e. populations initially identified using a dot plot using FSc and SSc for axes could be gated and viewed in a new plot examining any relevant combination of new parameters such as Green, Yellow, Red, NIR, FSc width, FSc area or either of the original parameters, FSc or SSc.
Figure 2.11 Graphical representation of flow cytometry data. Cell populations are selected and viewed using different parameters and graphs.
2.9.3 Methods

All analysis was performed using a Guava® EasyCyte 8HT™ Flow cytometry system. The Guava® EasyCyte 8HT™ flow cytometer is a compact bench top instrument which can be used for all traditional flow cytometry analysis of cell suspensions. The major difference between guava and traditional flow cytometry is the elimination of sheath fluid normally needed to create a hydrodynamically focussed stream of cells (Figure 2.12). The guava micro-papillary technology allows for much smaller sample sizes and much less waste.

![Diagram of Traditional Sheath Fluid System vs Guava Patented Microcapillary System](image)

**Figure 2.12** Guava microcapillary system versus traditional flow cytometry [312]. Permission granted to use image.

It has eight detection parameters which include: forward and side scatter and detection of 6 fluorescent colours resulting from excitation by red and blue
lasers. The Guava® EasyCyte 8HT™ also performs automated processing after initial setup.

### 2.9.4 Antibodies

Where possible conjugated antibodies were selected with different fluorescent markers so as to allow for dual staining of cell samples. A selection of antibodies was chosen to characterise Mesenchymal Stem Cells (Table 2.4). These were chosen based literature research and on the minimal criteria for defining mesenchymal stromal (stem) cells as determined by the International Society for Cell Therapy [126]. Antibodies targeting surface markers, CD105, CD90, CD73 and CD10, for which MSCs are typically positive, and CD31, CD34, CD45, CD227 (MUC1) and CD24 for which MSCs are typically negative, were used to characterise MSC immunophenotypes. An additional antibody targeting intracellular alpha smooth muscle actin (Alpha SMA), for which MSCs are typically negative was also used. Finally, a monoclonal antibody targeting human major histocompatibility complex 1 was also used for the purpose of discriminating between human and nonhuman tissue.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein Description</th>
<th>Ab type</th>
<th>Fluorochrome</th>
<th>Colour</th>
<th>Peak Emission</th>
</tr>
</thead>
<tbody>
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<td>CD105</td>
<td>Endoglin is 180 kDa type I membrane glycoprotein located on cell surfaces - MSC marker</td>
<td>IgG1</td>
<td>PerCP-Cy™ 5.5</td>
<td>Red1</td>
<td>695nm</td>
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<td>CD90</td>
<td>Thy-1 is a 25–37 kDa heavily N-glycosylated conserved cell surface protein - MSC marker</td>
<td>IgG1</td>
<td>PE-Cy™ 5</td>
<td>Red1</td>
<td>667nm</td>
</tr>
<tr>
<td>CD73</td>
<td>5’-nucleotidase is a 140 kDa enzyme that catalyses 5’- mononucleotides to nucleosides - MSC marker</td>
<td>IgG1</td>
<td>APC</td>
<td>Red2</td>
<td>660nm</td>
</tr>
<tr>
<td>CD10</td>
<td>Membrane metallo-endopeptidase is a zinc-dependent metalloprotease enzyme - widely distributed in haematopoietic cells</td>
<td>IgG1</td>
<td>FITC</td>
<td>Green</td>
<td>520nm</td>
</tr>
<tr>
<td>CD31</td>
<td>PECAM-1 plays a key role in removing aged neutrophils from the body-endothelial marker</td>
<td>IgG1</td>
<td>FITC</td>
<td>Green</td>
<td>520nm</td>
</tr>
<tr>
<td>CD34</td>
<td>Cell surface glycoprotein, functions as a cell-cell adhesion factor - haematopoietic stem cell marker</td>
<td>IgG1</td>
<td>PE</td>
<td>Yellow</td>
<td>578nm</td>
</tr>
</tbody>
</table>

Table 2.4a Description of antibodies used.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein Description</th>
<th>Ab type</th>
<th>Fluorochrome</th>
<th>Colour</th>
<th>Peak Emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>PTPRC is a member of the protein tyrosine phosphatase family-haematopoietic stem cell marker</td>
<td>IgG2a</td>
<td>RPE</td>
<td>Yellow</td>
<td>575nm</td>
</tr>
<tr>
<td>CD227</td>
<td>Mucin penetrates cell membranes, binds to pathogens &amp; prevents infection- epithelial cell marker (MUC1)</td>
<td>IgG1</td>
<td>FITC</td>
<td>Green</td>
<td>520nm</td>
</tr>
<tr>
<td>CD24</td>
<td>Heat stable antigen CD24 is a cell adhesion molecule- lymphocyte marker</td>
<td>IgG1</td>
<td>FITC</td>
<td>Green</td>
<td>520nm</td>
</tr>
<tr>
<td>αSMA</td>
<td>Alpha Smooth Muscle Actin in important in muscle contraction- marker of myofibroblasts</td>
<td>IgG1</td>
<td>Requires 2\textsuperscript{0} Ab</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2\textsuperscript{nd} Ab</td>
<td>Goat pAb to Ms IgG</td>
<td>pAb</td>
<td>APC</td>
<td>Red2</td>
<td>660nm</td>
</tr>
<tr>
<td>MHC1</td>
<td>Major immunohistocompatibility complex 1 functions in antigen presentation to immune cells - discriminates between mice and human</td>
<td>IgG2b</td>
<td>APC-Cy7</td>
<td>NIR2</td>
<td>785nm</td>
</tr>
</tbody>
</table>

*Table 2.4b* Description of antibodies used.
2.9.5 Concentration of antibodies and Fluorophore conjugation of monoclonal antibody

The monoclonal antibody for MHC1 was not available commercially as a fluorophore conjugated Ab. MHC1 antibody was conjugated to an APC-Cy7 fluorophore (Near infra red fluorescence) using the Lynx Rapid APC-Cy7 conjugation Kit (AbD Serotec). Antibody was concentrated to 100 μg/1 by centrifugation in a Vivaspin 500 (Sartorius Stedim) spin column. Lynx modifier reagent was added to the 80 μg antibody in a ratio of 1:10 respectively. The antibody-modifier mixture was added directly to a vial of 100 g lyophilised Lynx APC-Cy7. This mixture was incubated in the dark for 3 hours at RT after which Lynx quencher reagent was added to the mixture in a 1:10 ratio and left to stand for 30 mins before use. The newly conjugated antibody was stored at an appropriate concentration in PBS containing 0.09 % Sodium azide.

2.9.6 Preparation of Cell Suspension for Flow Cytometric Analysis

To prepare a cell suspension for Guava® EasyCyte 8HT™ analysis cells were trypsinised and suspended in media. Cells were centrifuged at 1000 rpm for 4 mins, the supernatant was decanted and the cells were resuspended in PBS. Cells were counted and the suspension was diluted to an optimum concentration of 2.5 x 10^5 cells/ml. 500 μl aliquots of suspended cells were stained with fluorochrome conjugated antibodies or a sequential combination of primary/conjugated secondary antibodies. For every antibody used, an isotype control was also applied under similar conditions to a control aliquot. Once stained, the cells were washed in PBS to remove free antibody and subsequently resuspended in 500 μl PBS. Cells were maintained on ice to slow metabolism of cells and prolong viability and also in the dark to prevent quenching of fluorochromes.
2.9.7 Flow cytometric analysis of cell suspensions by Guava® EasyCyte 8HT™

The Guava® EasyCyte 8HT™ was first calibrated before each use.

A work list was prepared using InCyte software which allowed for assignment of wells to specific samples as well as specifying number of events to be counted and max duration to achieve this. Wells filled with water were assigned between sample wells to minimise any potential cross-contamination. Stained cells were then loaded into wells of a 96-well plate in duplicate. The first replicate of each sample was used to adjust settings for optimal data acquisition. This included adjusting photomultiplier tubes voltages for amplifying forward and side scatter and fluorescent signals, setting debris threshold, identification of regions and gating. Where possible fluorescent signals generated by isotype controls were pulled into the negative region of histograms to accommodate strong positive signals. Once these parameters were set, flow data was acquired on the 2nd replicate of each sample.

2.9.8 Data analysis

Cell populations were selected on the side/forward scatter dot plot using an elliptical region. Subsequent graphs were gated on this region so data only represented events within the selected cell population. Using histograms representing population and surface marker associated fluorescence the region of the graph representing positive expression was selected.

If the isotype control overlapped with the positive region of the histogram this was also expressed in similar terms and subtracted from the percent positive cells. This metric represents the number of cells expressing the surface marker in question, corrected for auto fluorescence of the cell population and non-specific staining by the antibody.
2.10 *In Vivo* Studies

2.10.1 Background

A murine model was required to determine the potential MSC migration itinerary after administration to a host with breast cancer. In terms of MSC mediated gene therapy this step is critical in highlighting the efficiency of MSCs in migrating to cancerous tissue and also the potential adverse effects if MSCs migrate and engraft to non cancerous tissue. The use of an animal was considered only as a last resort when other approaches would have been inadequate and was subject to approval by the NUI Galway research ethics committee.

When planning for this experiment the 3Rs [313] were considered:

**Reduction** - minimizing the number of animals used to obtain valid data.

**Refinement** - minimising inhumane procedures and alleviating animal suffering where possible.

**Replacement** - potential substitution of insentient material for conscious living animals

All animal experimentation was performed in accordance with the Irish Statutory Instrument 566/2002 which is an implementation of European Union Directive 86/609/EEC. Training was performed and certification for animal handling was awarded by Laboratory Animal Safety and Training-Ireland prior to animal experimentation.

2.10.2 Athymic nude mice

Athymic nude mice were selected for use as an *in vivo* model of breast cancer metastasis for a number of reasons:
They lack a thymus and so are deficient in T-cells which facilitate their use for human tumour xenografts. They are insusceptible to graft versus host disease and do not produce cytotoxic effector cells.

They have previously been used with success for breast cancer models.

They are also phenotypically hairless due to a defect in the Foxn1\textsuperscript{nu} gene which allows easier inspection of animal health and tumour formation.

2.10.3 Background on animal handling

Housing

Provision of optimal conditions for animals limits the risk of introducing confounding factors to the experimental model.

The immunocompromised mice were housed in individual ventilated caging systems. This system provides filtered air at a rate of 12-15 changes per hour and minimises exposure to transmissible diseases.

The environmental parameters are maintained between 19-23\textdegree C, 40-70 % humidity to prevent heat stress, eye and mucociliary illness. 12 hrs daylight (350-400 lux) is provided daily and noise, particularly ultrasound was minimised.

Handling

Athymic nude mice are relatively docile and easy to handle. Caution should be taken however to limit direct handling only to trained personnel and to situations where absolutely necessary. This limits the chances of accidents resulting in injury to handlers and animals. Handling incidents can lead animals to associate handling with a negative outcomes leading to stronger resistance and increased stress for animals. Although the technique of gentling helps introduce animals to handling, thereon, less handling usually amounts to less
stress in animals. For situations where painful or complicated techniques are required animals should receive anaesthesia.

**Animal Sacrifice/Euthanasia**

Animals must be euthanized in such a way that physical and mental suffering is at a minimum. Euthanasia is performed at the end of an experiment, predetermined time points or when an animal is deemed to be experiencing pain, distress or suffering exceeding acceptable levels. CO\textsubscript{2} asphyxia is a typical method used for euthanizing mice. It involves exposure to increasing levels of CO\textsubscript{2} until anaesthesia followed by 100\% to ensure death.

**Methods**

**2.10.4 Anaesthesia**

To anaesthetise animals a ketamine (75 mg/ml)-xylazine (4.8 mg/ml) solution was prepared and loaded into a syringe. The loaded syringe was checked for bubbles which were removed if present. 20\,\mu{l} of this solution was administered intramuscularly to the left posterior thigh muscle using a 27 gauge syringe as shown in Figure 2.13. Animals were monitored for changes in consciousness, skin colour, ocular appearance, respiratory action and temperature. If the temperature of an animal dropped they were placed on a heat mat to help recover body temperature. During this time the opportunity was taken to accurately weigh the immobilised mice. Before proceeding to experimental procedures a final check on tail reflex was performed to confirm dept of anaesthesia. Animals inadequately anaesthetised at this point were given extra time or a small top up injection of aesthetic depending on whether they were going deeper or recovering from anaesthesia. Once the experimental procedure was performed animals were laid out in recovery cages separate to conscious animals. Full recovery could take up to 40 mins and animals were monitored closely during this time. The site where the skin was punctured was routinely inspected for evidence of infection.
2.10.5 Tagging

Tagging was performed under anaesthesia. A unique 3 digit identifier code etched onto a light nickel copper alloy tag was sprayed with alcohol, loaded onto the applicator and applied to the base of the ear, approximately 3 mm from the edge of the ear pinna to avoid foot entanglement, tearing and excessive inflammation.

2.10.6 Timeline of In Vivo study

Animals were inspected and monitored for 2 weeks upon delivery. They were treated with MDAMB-231 cells to establish metastatic breast cancer as described in section 2.10.7. Mice were subsequently monitored for palpable tumours, enlarged nodes and other signs of disease. Once tumours and enlarged nodes were confirmed in a number of animals, PKH26 labelled MSCs were administered intravenously to test mice (n = 35) as described in section 2.10.8.
Control mice (n=6) did not receive MSCs. This was considered T₀. Mice were assigned to one of three analysis techniques (see Table 2.5). They included:

- flow cytometry quantification of MSC and MDA-MB-231 cells in digested mouse tissue,
- immunohistochemistry and fluorescent microscopy inspection of tissue sections,
- RQ-PCR to identify human gene expression in harvested mouse tissue.

Animals were sacrificed (section 2.10.10) 1, 2, 3 or 4 weeks after MSCs administration. Prior to euthanasia blood sampling was performed through cardiac puncture (section 2.10.9). Tissues were harvested as described in section 2.10.11.

<table>
<thead>
<tr>
<th>Time since MSC injection</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Fluorescent Microscopy</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2.5** Assignment of tissues harvested from sacrificed mice to analysis by Flow cytometry, RQ-PCR or Fluorescent Microscopy. All the tissues harvested from each mouse were assigned to one technique only. *Control mice were sacrificed at various time points.*
Figure 2.1 Timeline of animal study. MDA-MB-231 cells were introduced to the 2nd thoracic MFP. Upon appearance of palpable tumours or enlarged nodes PKH26 labelled MSC cells were delivered intravenously. Animals were euthanized and tissues harvested 1, 2, 3, and 4 weeks later. Each mouse was assigned for analysis by either flow cytometry, IHC or RQ-PCR.
2.10.7 Mammary fat pad injection of MDA-MB-231 cells

A suspension of MDA-MB-231 cells was prepared at a concentration of 2 x 10^6 cells/ml as described in section 2.1. The cell suspension was loaded into a syringe with a 27 gauge needle. The loaded syringe was checked for bubbles which were removed if present. 100 μl (2 x 10^5 cells) of cell suspension was injected into the 2nd left thoracic mammary fat pad as shown in figure 2.15.

![Mammary fat pad injection](image)

**Figure 2.15** Mammary fat pad injection of MDA–MB-231 cells.

2.10.8 Tail vein injection of PKH26 stained MSCs

Tail vein injections were performed only on anaesthetised animals. MSC cells at passage 6 were labelled with PKH26 Red as described in section 2.2. They were loaded into a syringe with a 25 gauge needle at a concentration of 2 x 10^7 cells/ml. The loaded syringe was checked for bubbles which were removed if present. It was carefully aligned with the lateral tail vein 2 cm from the base of the tail as shown keeping the needle as flat as possible (Figure 2.16). 50 μl (1 x 10^6 cells) of cell suspension was injected slowly into the lateral tail vein. Correct technique was confirmed by the observation that the tail vein went pale by displacement of blood. Once complete the mice were returned to their cage and monitored until fully awake and able to access food and water.
2.10.9 Blood sampling through cardiac puncture

Animals, due for sacrifice which had reached experimental endpoints had their blood sampled by cardiac puncture under anaesthesia. This was performed using a 1 ml syringe with 21 gauge needle. The needle and syringe were pressed lightly against the abdomen of the anaesthetized animal and pushed into the thoracic cavity just below the xiphoid cartilage. It was then pushed upwards and slightly to the right of the sternum until blood entered the syringe as shown in Figure 2.17.
Blood was withdrawn slowly so as not to collapse the heart. Sample volumes ranging from 200 μl to 1000 μl were achieved. If not already dead due to blood loss, animals were euthanized by CO₂ inhalation as described in the next section.

2.10.10 Animal sacrifice

Animals were sacrificed once they reached experimental or humane endpoints. Experimental endpoints were defined as weeks 1-4 following MSC administration. Humane endpoints were defined as behavioural changes, body changes, weight loss or injury which might suggest that the animal is likely to endure undue suffering or die before the experimental endpoint is achieved. Mice were humanely killed by exposure to increasing concentrations of CO₂ in a gas chamber. Mice were monitored for any signs of life for 2 mins before proceeding to dissection.
2.10.11 Animal Necropsy, Tissue Harvesting and Processing

Animals were weighed and then sacrificed as described in the previous paragraph 2.10.10. They were dissected using a ‘Y’ incision followed by sequential removal and inspection of the kidneys, spleen, liver, heart, lungs and any tumour or enlarged node observed. Harvested organs were weighed. This and any additional observations were recorded in the necropsy form shown in Figure 2.18.

<table>
<thead>
<tr>
<th>Mouse Tag No.</th>
<th>Analysis Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight @ MSC injection</td>
<td>Date of injection</td>
</tr>
</tbody>
</table>

Estimated dose of MSCs

Description

<table>
<thead>
<tr>
<th>Weight @ Necropsy</th>
<th>Date of Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>Weight (g)</td>
</tr>
<tr>
<td>Tumour</td>
<td>Tube: Total Weight: Tissue weight:</td>
</tr>
<tr>
<td>Node</td>
<td>Tube: Total Weight: Tissue weight:</td>
</tr>
</tbody>
</table>

**Figure 2.18a** Necropsy form (see following page)
Mice were assigned to one of three modes of analysis and their organs were added to an initial storage reagent suitable for that technique (Table 2.6). Organs intended for flow cytometry were rinsed first in Hanks balance salt solution (HBSS) before addition to fresh HBSS for processing.

**Figure 2.18b** Necropsy form continued

<table>
<thead>
<tr>
<th></th>
<th>Tube:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lungs</strong></td>
<td></td>
<td>Total Weight:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue weight:</td>
<td></td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td>Total Weight:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue weight:</td>
<td></td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td>Total Weight:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue weight:</td>
<td></td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td>Total Weight:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue weight:</td>
<td></td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td>Total Weight:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tissue weight:</td>
<td></td>
</tr>
<tr>
<td><strong>Cardiac Puncture</strong></td>
<td>Volume:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Technique</td>
<td>Storage reagent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Hank’s balanced salt solution (HBSS)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>RNA-later</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescent microscopy</td>
<td>4 % Paraformaldehyde*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 Storage reagents for animal tissue. * These samples were protected from direct light.

Tissues assigned to analysis by Flow cytometry were initially transported in HBSS. Within a maximum 2hrs of harvesting, tissues were minced in petri dishes using scalpels (Figure 2.7.9). They were then treated with collagenase type 1A (Sigma) incubated for 30 min and subsequently passed repeatedly through a 21gauge needle to further break up the tissue. The 30 min incubation in collagenase was repeated and the digest was again passed through a 21gauge needle. Cells were washed twice in PBS and fixed in 1 % Paraformaldehyde (PFA) for 20 mins. After fixation cells were spun at 400 x g for 5 mins. The supernatant was aspirated and the pellet was resuspended in PBS for storage and analysis.

Tissues assigned to analysis by RQ-PCR were stored overnight in RNAlater to stabilise the mRNA (Figure 2.22). This was aspirated the following day and the tissue was frozen and stored at -80°C. Tissues were later homogenised and processed for RQ-PCR as described in section 2.7.

Tissue assigned to analysis by Fluorescent microscopy was initially stored for 24hrs at 4°C in 4 % Paraformaldehyde for fixation (Figure 2.19). They were then transferred to a 30 % sucrose solution in PBS and refrigerated at -20°C for a further 24 hrs. This solution acted as a cryoprotectant. Following that, they were snap frozen in an isopentane bath chilled with liquid N\textsubscript{2}. After snap freezing they were stored temporarily in a dewar of liquid N\textsubscript{2} and then transferred to a -80°C freezer for storage. Light exposure was always kept to a minimum.
Figure 2.19 Tissue preparation from sacrificed animal model.
2.10.12 Distribution of MSCs expressing NIS transgene after systemic administration in a mouse breast cancer model.

A non-invasive model of breast cancer was generated by injecting $2 \times 10^7$ MDA-MB-231 cells in 0.2 ml 50% Matrigel/Leibowitz-15 medium subcutaneously into right flank of Female athymic nude mice. Mice were monitored for development of disease and tumour formation. Once tumours had matured to $\approx 100$mm$^3$, mice received an intravenous injection of $1 \times 10^6$ Ad5/CMV/NIS infected MSCs suspended in 50 μl Dulbecco’s modified Eagle’s medium. A control group (n=6) received an intravenous injection of naked Ad5/CMV/NIS vector (no MSC). Animals were sacrificed and tissues were harvested 3 and 7 days after MSC administration. Tumours, heart, liver lungs, kidneys, spleen, and small and large intestines were stored in RNAlater. They were homogenized with the RNA extracted, mRNA samples were reverse transcribed and the corresponding cDNA was analyzed by Relative Quantitative PCR (as described in sections 2.7) for expression of human NIS, human MRPL19, mouse NIS and mouse beta actin. Mouse and human primers were compared for homology with target genes of the nontarget organisms by BLAST search to confirm that PDARs were organism specific. Human NIS and human MRPL19 data was expressed as undetectable (-), low (+) or more robust levels of expression (++).
Chapter 3

Results and Discussion

Expression of the Sodium Iodide Symporter (NIS) and potential regulators in Breast Tissue
3.1 Introduction

The presence, relevance and regulation of the Sodium Iodide Symporter (NIS) in breast cancer are poorly understood. Despite a number of studies having identified the presence of NIS in malignant breast tissue combined with a lower prevalence in normal tissue, neither the purpose nor relevance of NIS expression in this tissue has been clearly established [42-47]. Suggestions that NIS may be relevant as a means of distinguishing between normal and malignant breast tissue, while interesting, are subject to limitations of the methodologies employed in these studies [42, 43, 45-47][44, 45]. There have also been studies which demonstrated functional NIS expression in malignant breast tissue [45, 265, 267] suggesting potential as either an imaging or therapeutic tool [267]. However, the potential role of NIS as a biomarker, and in imaging and therapy of breast cancer was subsequently undermined by the observation of NIS expression and iodide accumulation in benign breast tissue [46, 47][269].

There is also a shortfall in relevant data relating to the regulation of NIS in malignant breast tissue. A number of in vitro studies have linked various potential regulators of mammary NIS expression such as estrogen and retinoic acid receptors (ERα, RARα, RARβ) and phosphoinositol-3-kinase (PI3K) but relationships have not been shown in actual human tissue [44, 273, 275, 276, 314, 315]. Additionally, although no direct relationship has been demonstrated in breast tissue, the importance of thyroid hormone receptors (THRα and THRβ) in thyroidal NIS expression [236], and their differential expression in breast cancer suggests that further investigation is needed to clarify their potential as mammary NIS regulators [277-279].

To shed light on the presence, relevance and regulation of the NIS in malignant breast tissue, gene expression of NIS, RARα, RARβ, ERα, PI3K, THRα and THRβ was investigated in a large cohort size which encompasses a strong representation of normal and benign mammary tissue and each of the 4 epithelial subtypes of breast cancer. This was performed using RQ-PCR, which is a very sensitive, robust and trusted method of mRNA quantification. In addition, IHC was used to confirm the presence of the NIS protein. Using snap
frozen surgically excised human tissue facilitated optimal quantification of human mRNA transcripts of NIS and putative regulators which combined with a strong and diverse cohort would generate reliable and representative data on human mammary NIS expression and regulation.

3.2 Objectives

- To quantify the levels of native NIS gene expression in normal, benign and malignant breast tissue.
- To investigate the relationship between NIS expression levels and tumour epithelial subtypes and patient clinicopathological details.
- To confirm NIS protein expression in selected breast tissue specimens.
- To quantify and compare expression levels of putative regulators of NIS, RARα, RARβ, ERα, PI3K, THRα and THRβ, in normal, benign and malignant breast tissue.
- To investigate the relationship between correlate expression of putative NIS regulators and NIS expression.

3.3 Materials and Methods

Breast tissue specimens (n = 100) were obtained at the University College Hospital in conjunction with the principles expressed in the Declaration of Helsinki. Each specimen was snap frozen within minutes of excision, and transferred to storage at -80°C. Sections of each tissue were homogenized and RNA was subsequently extracted. cDNA was generated from this RNA template. This cDNA was then analyzed for expression of NIS, RARα, RARβ, ERα, PI3K, THRα and THRβ using RQ-PCR. Gene expression data was normalised to endogenous controls PPIA and MRPL19 and expressed relative to the lowest expresser of each target. The relative expression data was interrogated by statistical methods for significant differences in the level of NIS expression in tissues subdivided by clinicopathological criteria. Similarly
differences in RARα, RARβ, ERα, PI3K, THRα and THRβ expression were investigated in the context of clinicopathological data. Potential relationships between targets were also investigated using scatter plots and Pearson Correlation Coefficients. Finally, selected tissue samples were examined for expression of the NIS protein. This was done by cryosectioning breast specimens, mounting them on slides and probing them with a NIS specific monoclonal antibody.

3.4 Patient cohort

Of the 100 tissue specimens, 75 represented malignant mammary tissue, 15 were normal mammary tissue biopsies harvested at reduction mammoplasty and 10 were fibroadenoma biopsies. The demographics of this patient cohort are further described in Table 3.1.
<table>
<thead>
<tr>
<th>Patient cohort details</th>
<th>Breast cancer</th>
<th>Fibroadenoma</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>75</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Pre menopausal</td>
<td>24</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Post menopausal</td>
<td>51</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>57(35-90)</td>
<td>36(20-50)</td>
<td>48(33-78)</td>
</tr>
<tr>
<td>Pre menopausal</td>
<td>46(35-59)</td>
<td>34(20-49)</td>
<td>44(50-78)</td>
</tr>
<tr>
<td>Post menopausal</td>
<td>62(41-90)</td>
<td>50</td>
<td>62(33-48)</td>
</tr>
<tr>
<td>Histology</td>
<td>Ductal</td>
<td>Lobular</td>
<td>Other</td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Epithelial Subtype</td>
<td>Luminal A</td>
<td>Luminal B</td>
<td>HER2</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>Disease Stage</td>
<td>Stage 1</td>
<td>Stage 2</td>
<td>Stage 3</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>35</td>
<td>15</td>
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<tr>
<td>Disease Grade</td>
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<td>Grade 1</td>
<td>Grade 2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table 3.1** Patient demographics of breast tissue specimen cohort.
3.5 Confirmation of Normal distributions of gene expression data.

All gene expression data sets, including those of NIS, RARα, RARβ, ERα, PI3K, THRα and THRβ were subjected to normality tests to determine if the distribution of the data was normal (gaussian) or non-normal. This included the use of histograms, normal distribution and probability plots and the Arlington-Darling test for normality. Knowledge of the distribution of the data allowed for appropriate selection of parametric or non-parametric statistical tests.

Figure 3.1 shows clear evidence of normal distribution in NIS, RARα, RARβ, ERα, PI3K, THRα and THRβ gene expression data. Datasets were plotted against a theoretical normal distribution represented by a straight line in probability plots (Figure 3.1 a-1, b-1, c-1, d-1, e-1, f-1, g-1). Visually there was little deviation between real data and the theoretical normal distribution and this was confirmed by Arlington-Darling (AD) analysis which suggested than no data set was significantly non-normal. Similarly a frequency distribution histogram plot of data revealed normal patterns of distribution compared to a theoretical normal curve (Figure 3.1 a-2, b-2, c-2, d-2, e-2, f-2, g-2). As a result, the parametric Anova and Pearson correlation coefficient tests were used to analyse this data as they are best suited to analysing normally distributed data.
Figure 3.1 Probability (PP) and histogram plots (HP) displaying normality of gene expression data including Arlington Darling analysis. a-1) PP NIS, a-2) HP NIS, b-1) PP RARα, b-2) HP RARα, c-1) PP RARβ, c-2) HP RARβ, d-1) PP ERα, d-2) HP ERα, e-1) PP THRα, e-2) HP THRα, f-1) PP THRβ, f-2) HP THRβ, g-1) PP PI3K, g-2) HP PI3K.
3.6 NIS gene expression in human breast tissue.

NIS was detected in 98 of the 100 breast tissue specimens analysed. Expression levels shown in Figure 3.2 were found to be significantly higher in breast cancer (Mean (SEM) 1.18 (0.07) Log$_{10}$ RQ, p<0.05) and fibroadenoma tissue (1.69 (0.21) Log$_{10}$ RQ, p<0.005) than normal tissue (0.70 (0.12) Log$_{10}$ RQ.). The highest level of NIS expression overall was observed in fibroadenoma tissue. When divided on the basis of epithelial subtype, the highest level of NIS expression was observed in the Her2 subgroup (1.43 (0.11) Log$_{10}$ RQ, Figure 3.2). Expression of NIS in the Her2 subtype was significantly higher than that of normal tissue (p < 0.05).

NIS gene expression data was also subdivided on the basis of prognostic indicators such as disease stage, tumour grade, menopausal status, estrogen, progesterone, and Her-2/neu status, with no statistically significant relationship observed between NIS and these clinical characteristics.
Figure 3.2 NIS gene expression in normal, benign (fibroadenoma) and malignant breast tissue. Results have been normalised to endogenous controls PPIA and MRPL19 and expressed relative to the lowest detectable sample. Breast cancer data is also stratified by epithelial subtype.
3.7 NIS protein expression in human breast tissue.

Tissue sections were prepared from a selection of snap frozen biopsies from the same cohort and investigated for NIS protein expression using a mouse anti-human monoclonal NIS antibody and DABMap staining. The stained tissue was examined by microscopy and is presented in Figure 3.3 at 400x magnification.

Thyroid tissue which was used as a positive control to confirm the effectiveness of the NIS antibody was strongly immunopositive for the NIS protein (Figure 3.3a). This image also serves as a clear example of membrane bound localisation of NIS protein, a characteristic which is critical for NIS function. Antibody-free controls were included for each tissue analysed to check for nonspecific staining and these were all confirmed to be negative. An example of an antibody free control for fibroadenoma tissue is shown in Figure 3.3b using a lower magnification of 200x to facilitate a wider field of view.

Strong membrane bound staining was observed in fibroadenoma tissue (Figure 3.3c). The level of staining appeared to be comparable to that of the thyroid tissue section (Figure 3.3a). Selected tissues from all four epithelial subtypes of breast cancer were also shown to be immunopositive for NIS protein (Figure 3.3d-g). Of these tissues Luminal A appeared to stain most strongly for NIS (Figure 3.3d). Evidence of NIS membrane localisation is also apparent. Membrane bound NIS staining was also observed in Her2 tissue (Figure 3.3f). Positive staining for NIS protein was also observed in the Luminal B (Figure 3.3e) and Basal subtypes (Figure 3.3g), however, evidence of NIS membrane localisation was not observed.
Figure 3.3 Immunohistochemical detection of NIS protein expression in 5 µM sections of selected tissues from the patient cohort. a) Thyroid tissue 400x magnification (positive control). b) Fibroadenoma without NIS antibody 200x (negative control). c) Fibroadenoma with NIS antibody 400x. d) Luminal A subtype 400x. e) Luminal B subtype 400x. f) Her2 subtype 400x. g) Basal subtype 400x.
3.8 RARα and RARβ expression in human breast tissue and relationship with NIS expression.

Gene expression of RARα and RARβ was highest in fibroadenoma tissue (Figure 3.4a). In the case of RARβ, this was significantly higher than both normal and malignant tissue (p<0.001, p<0.005 respectively, Figure 3.4b), which displayed similar levels of RARβ expression. In the case of RARα, expression was also significantly higher in tumour compared to normal tissue (p<0.005, Figure 3.4a), with no significant difference between malignant and fibroadenoma tissue levels detected.

The scatter plots with linear regression and Lowess smoother lines in Figure 3.4c and Figure 3.4d demonstrate linear relationships between NIS and both RARα and RARβ expression respectively. Calculation of the Pearson correlation coefficient (r) confirmed a positive relationship between NIS and RARα (r = 0.29, p < 0.05). The relationship was even stronger between NIS and RARβ (r = 0.38, p < 0.0001).
Figure 3.4 RARα and RARβ expression in breast tissue samples and their relationship with NIS. a) RARα and b) RARβ expression in normal, benign and malignant breast tissue. Results were normalized to the endogenous controls (PPIA, MRPL19) and expressed relative to the lowest detectable sample. Correlation of NIS gene expression with RARα (c) and RARβ (d) with Pearson correlation coefficient (r) and p values also shown.
3.9 ERα and PI3K expression in human breast tissue.

ERα expression followed a similar expression profile to NIS, being higher in both fibroadenoma and malignant breast tissue compared to normal (Figure 3.5a). The levels of expression were not however significantly different. PI3K expression was significantly lower in both fibroadenoma and malignant compared to normal breast tissue (p<0.0005, p<0.0001 respectively, Figure 3.5b). This expression profile appeared to be the inverse of that of the NIS gene.

Again, using scatter plots with linear regression and Lowess smoother lines, linear relationships were observed between NIS and ERα (r = 0.22, p < 0.05 Figure 3.5c) and NIS and PI3K (r = -0.21, p < 0.05 Figure 3.5d) expression.
Figure 3.5 ERα and PI3K expression in breast tissue samples and their relationship with NIS. a) ERα and b) PI3K expression in normal, benign (fibroadenoma) and malignant breast tissue. Results have been normalised to the endogenous controls PPIA and MRPL19 and expressed relative to the lowest detectable sample. Correlation of NIS gene expression with ERα (c) and PI3K (d) with r and p values is also shown.
3.10 THRα and THRβ expression in human breast tissue.

Both THRα and THRβ were significantly lower in malignant compared to normal tissue (p<0.001, p<0.005 respectively, Figure 3.6). THRβ expression was even higher in fibroadenoma tissue compared to normal and as such was significantly higher than malignant tissue (p<0.0001).

There was no significant relationship observed between NIS and either thyroid hormone receptor α or β in the complete cohort. However a robust linear relationship can be seen in Figure 3.6c between RARβ and THRβ using scatter plots (PCC of r = 0.47, p < 0.0001).

Additionally, when stratified on the basis of ER status it was found that THRβ expression was significantly higher in ER positive breast cancer tissue compared to ER negative (Figure 3.6d, p<0.05).
Figure 3.6  a) THRα and b) THRβ expression in normal, benign (fibroadenoma) and malignant breast tissue. Results have been normalised to the endogenous controls PPIA and MRPL19 and expressed relative to the lowest detectable sample. c) Correlation of THRβ gene expression with RARβ. d) THRβ in stratified by ER status.
3.11 Additional correlations in gene expression.

A number of additional correlations in gene expression were observed in breast tissue. These are shown in table 4.1. They include relationships of varying strengths between RAR\(\alpha\), RAR\(\beta\), ER\(\alpha\), THR\(\alpha\), THR \(\beta\) and PI3K.

<table>
<thead>
<tr>
<th>Relationship</th>
<th>Pearson correlation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAR(\alpha) → ER(\alpha)</td>
<td>0.40</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>RAR(\alpha) → PI3K</td>
<td>-0.23</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>RAR(\beta) → ER(\alpha)</td>
<td>0.20</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>RAR(\beta) → THR(\alpha)</td>
<td>0.31</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>ER(\alpha) → THR(\alpha)</td>
<td>0.22</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>ER(\alpha) → THR(\beta)</td>
<td>0.29</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>THR(\alpha) → THR(\beta)</td>
<td>0.50</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>THR(\alpha) → PI3K</td>
<td>0.40</td>
<td>&lt; 0.000</td>
</tr>
<tr>
<td>THR(\beta) → PI3K</td>
<td>0.22</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Table 3.2 Additional significant Pearson correlations observed between genes expressed in breast tissue.
3.12 Discussion

Previous studies demonstrated elevated NIS expression in malignant breast tissue compared to normal tissue [42-47] and for this reason it was suggested that NIS might serve as a biomarker capable of diagnosing malignant over normal breast tissue. However, the strength of these studies may have been limited by the methodology used. Such studies include those by Wapnir [46], Tazebay [44], Renier [42], Rudnicka [43] and Upadhyay [45] which used immunohistochemistry (IHC) to determine levels of NIS protein expression. IHC analysis is vulnerable to error both quantitatively and qualitatively. In terms of quantitative analysis IHC is very subjective, and depends too much on the operator to categorize levels of positivity as is the case in a study by Wapnir et al. [46] of 202 breast tissue cores. In this instance, while the results were very interesting, suggesting increased NIS protein expression in malignant compared to normal tissues, a clear quantification of this difference in NIS protein expression could not be achieved. In a qualitative capacity, IHC may be liable to over or underestimation of target protein expression. For example, whereas RQ-PCR give a representation of gene expression for all of the tissue processed, it is only feasible to analyse a few sections of tissue from the total piece by IHC. In the event where NIS protein expression is not uniformly distributed throughout the tissue this may result in NIS protein positive tissue being assigned a false negative and vice versa. Other studies used RT-PCR and RNAse protection assays to determine levels of NIS mRNA but these techniques also lack an objective quantitative output [45, 47]. A study by Kilbane et al [47], used RT-PCR to demonstrate NIS gene expression in both benign (n = 2), and malignant (n = 7) breast tissue, however, the size of this cohort was inadequate to definitively establish a pattern of NIS gene expression. The gene expression data presented in this chapter was derived by RQ-PCR which is currently the gold standard of mRNA quantification [316, 317]. Using a large patient cohort to increase the power of the data, it was clearly shown that while the level of NIS expression in malignant breast tissue is higher than in normal tissue, it is even higher in fibroadenoma tissue. NIS was not significantly higher in fibroadenoma compared to malignant tissue. As a consequence, it is clear that NIS gene expression alone cannot be reliably
used as a marker of breast cancer. This data is supported by previous observations of this trend in NIS protein and NIS mRNA [46, 47].

One drawback to RQ-PCR is that it accounts for mRNA and not the protein product. There is also conflicting data on how well NIS gene expression correlates with the protein expression. A study using breast tumour tissue suggests that there is a positive correlation [265], while the opposite is suggested in a study of thyroid tissue [318]. To compensate for this issue, NIS immunohistochemistry was performed to confirm the presence of NIS protein.

In selected tissues of each of the 4 different breast cancer epithelial subtypes, Luminal A and B, Her2 and Basal, as well as fibroadenoma tissue, the presence of NIS protein was confirmed. A recent study examining NIS protein expression in fibroadenoma specimens demonstrated either mild (n = 2), moderate (n = 10) or intense (n = 7) positivity in 19 of the 20 specimens examined [319]. This confirms the potential that strong NIS mRNA levels detected in fibroadenoma tissue in this study may be translated into robust protein levels.

Increased NIS gene expression in fibroadenoma relative to normal and malignant tissue may also imply a tumour suppressor role for NIS as suggested by Gol Choe et al [320], who demonstrated NIS transgene inhibition of oncogenic RAS-mediated signalling. Due to comparatively low levels of breast cancer in Japanese women who consume a diet containing iodide rich seaweed it has been suggested that dietary iodide may play a role in protecting against breast cancer [321]. Japanese women who emigrate or live on a western diet have shown increased cancer rates [322, 323]. A study by Funahashi et al [324], showed that mutagenic stimulation of mammary cancer in rats was suppressed by coadministration of Lugol’s iodine or iodine-rich Wakame seaweed. Other studies have shown that iodide may produce a protective effect by reducing expression of estrogen responsive elements and increasing expression of estrogen metabolising elements in MCF-7 breast cancer cells [325]. This was also supported by the observation that iodide-deficient rats develop areas of atrophy, necrosis, dysplasia and atypia in mammary glands which are hyper sensitive to estradiol [326]. Venturi et al [327, 328], have also suggested that iodide might play an ancestral antioxidant function in all iodide-concentrating cells. In thyroid tissue higher tumour stages (stages >I versus
stage I) were associated with lower expression of NIS (P = 0.03) [329]. In contrast, an interrogation of NIS gene expression data in the context of clinicopathological characteristics such as stage, grade or epithelial subtype did not identify a similar trend within the breast cancer cohort of this study. This data would appear to contradict the association of NIS, dietary iodide and lower rates of breast cancer. It should be noted however, that all tissue specimens were obtained from Irish women and previous studies have suggested that dietary intake of iodine by Irish women is significantly less than the level recommended by the World Health Organisation [330, 331]. As such, there remains the possibility that levels of endogenous NIS expression, if even slightly functional, may provide a protective effect against breast cancer as long as iodide is available in the diet. Conversely, Lacoste et al. [332] have reported NIS enhanced cell migration and invasion of tumours by binding to a Rho guanine exchange factor that activates the small GTPase RhoA. Reduced membrane localisation and sequestration of NIS in intracellular compartments further increased this effect. However, these observations cannot be supported by the current study which found no evidence of a correlation between NIS and tumour stage, which accounts for levels of metastasis and invasiveness.

The question of the therapeutic relevance of NIS is more complicated. While iodide accumulation has already been demonstrated in breast cancer [265], and may potentially result in a level of radioablation if used in combination with radiiodide, this is not a green light for its medical application. Its important to highlight that a minimal level of functional iodide accumulation is required to safely treat a tumour using radiiodide. In the treatment of thyroid cancer it has become standard practice to use higher doses of $^{131}$I than required in order to ensure that the entire area is ablated preventing the risk of recurrence or secondary cancers through survival of $^{131}$I damaged cells [333-335]. In this context, even if it were possible, variation in NIS expression across different types of breast cancer, makes the use of native NIS in radioablation therapy an unlikely prospect. Despite this issue a number of laboratories are examining the potential of increasing endogenous NIS expression in breast tissue for the purposes of $^{131}$I tumour ablation [336]. For this to be successful it is important that the regulatory pathways of mammary NIS expression are clearly established.
In this study, analysis of potential regulators of mammary NIS lead to a number of interesting observations.

The elevated level of NIS and also RARα and RARβ in the fibroadenoma compared to the normal tissue indicates that a relationship may exist between these genes. This pattern is supported by more direct comparisons of NIS and RARα and RARβ expression. Linear relationships observed in scatter plots of gene expression and statistically significant Pearson correlations show for the first time in human breast tissue that relationships exist between NIS and both retinoic acid receptors. It confirms previous observations from studies performed in vitro and in vivo whereby NIS expression was increased in response to treatment with retinoic acid in MCF-7 cells and in an MCF-7 xenograft murine model of breast cancer [270, 271, 337]. A study by Kogai et al [270] also suggests that stimulation of the beta receptor increases NIS expression more significantly than stimulation of the alpha receptor. This is confirmed by the higher PCC observed for RARβ than that of RARα. In the context of potentially enhancing endogenous NIS expression to therapeutic levels, the confirmation of the retinoic acid receptor role in mammary NIS regulation is highly promising since retinoic acid is a metabolite of Vitamin A which can be taken without prescription. While a number of clinical trials have investigated the therapeutic potential of retinoic acid in conjunction with other therapies for breast cancer did not highlight an additive effect, the safety data from these trials did show that using retinoic acid is relatively safe [338-341]. As such retinoic acid may represent the first and safest stepping stone to enhancing endogenous NIS expression in humans. This may initially gain a medicinal foothold not in a therapeutic role but through a cancer prevention role if used in conjunction with iodide. A study of minimal risk to patient health, involving dietary supplementation of Vitamin A and iodide could potentially amplify functional NIS expression increasing delivery of iodide to breast tissue and potentially emulating Funahashi’s observations of iodide tumour suppression in rats [324]. It is also interesting that RARβ2 over expression has previously been shown to result in decreased metastatic potential of MDA-MB-435 in vivo [342]. Since RARβ expression was highest in benign tissue it may be possible that this increase in expression may be part
of a protective anti-tumour response in the benign tissue. The similar expression profile between NIS and ERα is interesting since ERα has previously been shown to activate mammary NIS transcription in estrogen receptor positive breast cancer cell lines [274]. The same may also be true in fibroadenoma tissue. The linear relationship and positive correlation between NIS and ERα confirms the potential for ERα regulation of NIS. A study which demonstrated both downregulation of NIS expression in ERα positive breast cancer cell lines in response to ERα RNA interference as well as an estrogen responsive element in the NIS gene promoter adds further support to this relationship [274]. This correlation of expression conflicts with Stoddard’s observation which suggests that iodide interferes with estrogen responsive expression [325]. Such an anti-estrogen effect would suggest that if NIS were functional in the breast tissues examined and accumulated iodide at any level, one would expect a negative relationship between the NIS and ERα. This was clearly not the case in this study. It would be interesting to establish if the cohort of patients examined may have had insufficient iodide in their diet which might explain the lack of anti-estrogen effect in tissues expressing NIS. Based on the classification of the Irish population as iodide deficient by the WHO, it is likely that the cohort used in this study is also iodide deficient [330, 331]. Either way, given the role of estrogen in breast cancer growth and the successful exploitation of ER antagonism using Tamoxifen, it is clear that in the context of increasing endogenous NIS expression for therapeutic or chemoprevention purposes, neither Estrogen nor its chemical substitute estradiol, have a positive role to play.

Since PI3K was observed to have an inverse expression profile to that of NIS, it is possible that a negative relationship may exist between the two proteins in breast tissue. The negative linear relationship seen in scatter plots and negative correlation observed between NIS and PI3K is very interesting given the conflicting previous reports suggesting both a positive [275, 343] and negative [248] effect of PI3K on NIS expression can occur. Knostman et al. suggested that PI3K stimulation results in increased NIS expression in MCF-7 breast cancer cells [275]. This was confirmed when activation of the PI3K pathway by retinoic acid was shown to increase NIS expression in MCF-7 cells [343].
However, a negative relationship has previously been observed in thyroid tissue whereby inhibition of PI3K led to increased NIS expression [248]. It is this negative relationship which is supported in this study despite that fact that the former studies are more closely related by model and tissue type. The cohort data was examined again and in more detail and when stratified on the basis of tumour epithelial subtype an anomaly was observed. While there was an overall negative correlation between NIS and PI3K which was significant, within the Luminal A subtype which MCF-7 cells represent, there was a positive relationship (albeit not significant), between the two genes. Negative relationships (not significant) were to be seen in remaining subtypes Luminal B, Her2, and Basal as well as benign and normal tissue. This may explain the conflicting data observed and highlights the importance of using multiple cell types when attempting to represent this heterogeneous disease. Larger cohort sizes would need to be used in future studies in order to resolve this issue.

A negative relationship observed between RARα and PI3K (r = -0.23 p< 0.05) strengthens the observation that PI3K is a negative regulator of NIS gene expression in human breast tissue since RARα appears to act as a positive regulator of NIS.

Despite the distinct over expression of NIS and THRβ in fibroadenoma relative to normal and malignant tissue it is interesting that no direct relationship was observed between NIS and either of the thyroid hormone receptors in human breast tissue. This highlights the huge differences in how mammary NIS and thyroid NIS are regulated. However, the absence of a direct relationship with NIS as seen here does not preclude the possibility of an indirect relationship. In fact, the observation of a robust correlation in THRβ and RARβ expression, and the fact that the strongest correlation with NIS expression in human breast tissue was with RARβ does suggest that the THRβ may yet play a role in NIS gene expression.

The downregulation of both isoforms of THR in malignant tissue suggests that they may play a role as tumour suppressors in breast cancer and this is supported by studies suggesting a similar role in thyroid tissue [344]. More specifically THRα and THRβ have previously been shown to be downregulated
in malignant breast tissue compared to normal [345]. Expression of THRβ1 in breast cancer cells has been demonstrated in murine models to inhibit tumour progression by reducing tumour growth, invasiveness, extravasation, and metastasis formation in mice [278]. In this context the elevated level of THRβ expression in fibroadenoma compared to normal and malignant tissue may be viewed as a possible protective response against development of a malignancy.

A previous study has shown that thyroid hormone T3 enhances E2 stimulation of proliferation in ER positive breast carcinoma cells and suggests that retinoid X receptor-thyroid hormone heterodimer receptor formation may facilitate this effect [346]. However, other studies suggest mechanisms of action that don’t involve thyroid receptors [346, 347]. Additionally, the silencing mediator of retinoic and thyroid hormone receptors is required for the optimum estrogen transcription activity implying that thyroid hormone receptors aren’t involved in ER-dependent proliferation of cancer cells [348]. It is interesting that THRβ expression is highest in the ER positive population representing the less aggressive epithelial subtypes of breast cancer (Luminal A and B) compared to the more aggressive ER negative subtypes (Her2 and Basal). This again points to a possible tumour suppressor role for THRβ as suggested in the previous section as well as a possible correlation in the gene expression of both receptors.
Chapter 4

Results and Discussion

_In vitro_ stimulation of NIS expression in breast cancer cell lines.
4.1 Introduction

Regulation of the NIS gene expression in thyroid tissue has been clearly established. The critical positive regulator of NIS expression is Thyroid Stimulating Hormone (TSH) and this is counterbalanced by the negative effects of thyroid hormone and iodide accumulation, thyroglobulin and cytokine inhibition [236][253][349][350,351].

In mammary tissue, there is little to no evidence to suggest that the mechanisms above bear any relevance leaving a large gap in what is understood about mammary NIS regulation. It is known that during lactation NIS expression is optimal in the presence of the hormones oestrogen, prolactin and oxytocin [44].

In the context of breast cancer, a number of putative regulators of NIS have been identified such as RARα, RARβ, ERα and PI3K [44, 273, 275, 276, 314, 315]. Retinoic acid receptor α (RARα) has been shown to increase NIS expression in MCF-7 cells in vitro [270], and in combination with dexamethasone it has been shown to induce functional NIS expression in breast cancer xenografts [271]. Retinoic acid receptor β (RARβ) promotes a greater increase in NIS expression than does RARα [270]. Alotaibi et al. [315] highlighted activation of mammary NIS transcription in MCF-7 breast cancer cells through the unliganded ERα. This data was supported by the presence of an estrogen responsive element in the NIS gene promoter [315]. Activation of phosphoinositide-3-phosphate (PI3K) has been shown to increase NIS expression in MCF-7 cells despite having the opposite effect in thyroid follicular cells [248, 275, 276].

Thyroid hormone receptors (THRα and THRβ) have been shown to be downregulated in breast cancer compared to normal tissue and may potentially have an antitumour role in breast tissue [277-279], as well as having the ability to interact with retinoic acid receptors through dimerization [352, 353]. The latter suggests a much closer relationship to mammary NIS expression than current literature would indicate since retinoic acid receptors, as previously described are strongly implicated in mammary NIS expression.
This chapter examines the effect of stimulating breast cancer cell lines with the thyroid hormone thyroxine, as well as estradiol and all trans retinoic acid.

4.2 Objectives

- To determine the true role of retinoic acid, estrogen and thyroxine in the regulation of mammary NIS expression
- To determine the effect of combining retinoic acid, estrogen and thyroxine stimulation on gene expression levels of NIS in breast cancer cells lines

4.3 Materials and Methods

The role of Retinoic acid, Estrogen and Thyroxine in mammary NIS regulation was determined in T47D and Sk-Br-3 cell lines. Cells were incubated in the presence of each ligand at selected concentrations for 24 or 72 hrs. Since each reagent required a different diluent in its preparation, appropriate diluent controls were also prepared and cells grown under the same conditions (Estradiol: Water, ATRA: DMSO, Thyroxine: NH₄OH). Cells were harvested at the end of each incubation. The RNA was extracted and cDNA was generated. NIS gene expression levels were determined by RQ-PCR, normalised to endogenous controls and expressed relative to appropriate controls.

Following the establishment of optimal ligand concentrations this experiment was repeated using ligands individually and in combination for an incubation period of 24 hrs. Again relative NIS gene expression was determined.
4.4 Assessment of baseline NIS expression in selected cell lines.

In order to select suitable cell lines for studying the effect of retinoic acid, estradiol and thyroxine on NIS gene expression it was important to identify physiologically relevant models with a detectable baseline of NIS and the putative regulators RARα, RARβ, ERα, THRα, and THRβ.

NIS, being barely detectable in MDA-MB-231 cells represented the lowest detectable target across all samples and targets and so was used as the reference point for calculation of all RQ values, with all targets expressed relative to NIS in MDAMB-231 cells (Figure 4.1). T47D cells, which are defined by the American Type Culture Collection (ATCC) as ER +ve, PR +ve and Her2 –ve [354], were found to have detectable levels of NIS and all putative regulators of NIS, with ERα expression being predictably high. Sk-Br-3 cells, defined by the ATCC as ER –ve, PR –ve and Her2 +ve [355], had a similar profile with the exception of ERα expression which was predictably low. Interestingly RARβ expression was low in both T47D and Sk-Br-3 cells compared to levels of RARα, THRα and THRβ. As expected, ERα gene expression was also very low in the triple negative (ER –ve, PR –ve, Her2 –ve [356]) cell line MDA-MB-231. NIS was undetectable in MSCs (not shown).

On the basis of reasonably detectable baseline levels of NIS expression and satisfactory expression of putative regulators relative to other cells lines tested (Figure 4.1), it was deemed that T47D and Sk-Br-3 cells were suitable for stimulation experiments. Despite the low levels/absence of detectable NIS in MDA-MB-231 cells and MSCs, these cells did express detectable levels of the putative regulators of NIS and ligand stimulations of these cells were also attempted. MSCs were included to determine if stimulation of NIS expression could be achieved in cells originating from a different lineage to breast cell lines.
Figure 4.1 Baseline expression of NIS and potential regulators of NIS in T47D, Sk-Br-3, and MDA-MB-231 cells. Data was normalised to endogenous controls PPIA and MRPL19 and is expressed relative to lowest detectable target across all samples (NIS in MDA-MB-231 cells).
4.5 NIS gene expression following stimulation of T47D cells with Estradiol

T47D cells were treated with E₂ at concentrations of 1 nM, 10 nM, or 50 nM for 24 or 72 hrs after which they were harvested and interrogated for levels of NIS expression. Gene expression data was normalised to endogenous controls and expressed relative to relevant diluents controls. Each stimulation was performed in triplicate.

Increases in NIS gene expression were observed in response to treatment with all 3 concentrations of E₂ at both time points (Figure 4.2). The amplitude of these increases fell within a range of 3-6-fold relative to diluent controls. The greatest, most consistent increase in NIS expression was seen with 10 nM E₂ stimulation for 24 hrs resulting in a 6 (±2) ([Mean (SEM)]) fold change. With the exception of the 3-fold increase observed after 50 nM stimulation for 72 hrs all other conditions resulted in a 5-fold or more increase in NIS gene expression which is considered a significant change. There appeared to be consistently larger increases in NIS expression after 24 hrs compared to 72 hrs stimulation. The difference was not significant except where the highest concentration of E₂ (50 nM, p < 0.05) was used.

![Figure 4.2](image)

**Figure 4.2** Effect of various concentrations of Estradiol on NIS expression in T47D cells. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluent controls.
4.6 NIS gene expression following stimulation of T47D cells with ATRA

T47D cells were also treated with ATRA at concentrations 0.1 µM, 1 µM, and 5 µM for 24 or 72 hrs. As before changes in NIS expression were quantified relative to diluent controls.

Increases in NIS gene expression were observed in response to treatment with all 3 concentrations at both time points (range 3–10 fold increase, Figure 4.3). The largest, most consistent increase was observed with 1 µM ATRA at 24 hrs (6 (1)-fold) however, as high as a mean 10 (5)-fold increase was observed with the same concentration over a 72 hr stimulation. Again, all but one condition (5 µM for 24hrs: 3 (1)-fold) resulted in a 5-fold or more increase in NIS gene expression which is considered a significant change. Despite the very obvious difference to be seen graphically between the effect of 24 and 72 hr 5 µM ATRA stimulation on NIS expression (3 (1)-fold and 8 (3)-fold respectively), using a one tailed Student T-Test, this difference was deemed to be not significant. Where 24 hr stimulations were concerned there did appear to be a trend towards smaller increases in NIS expression as ATRA concentration was increased.

![NIS expression in ATRA stimulated T47D cells](image)

**Figure 4.3** Effect of various concentrations of all trans Retinoic acid on NIS expression in T47D cells. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluent controls.
4.7 NIS gene expression following stimulation of T47D cells with Thyroxine

In a similar fashion thyroxine (T₄) was used to stimulate T47D cells at concentrations of 0.1 μM, 0.5 μM and 1 μM for 24 or 72 hrs and its effect on NIS mRNA levels was subsequently quantified.

As with E₂ and ATRA, increases in NIS gene expression (range 4–7 fold) were observed in response to treatment with all 3 concentrations at both time points (Figure 4.4). In this case the greatest, most consistent increase was seen in T47D cells treated with 0.1 mM T₄ for 72 hrs (6 (3)-fold), although there was a lot of variation between replicates with a relatively large standard error. Both 0.1 μM and both 0.5 μM treatments resulted in at least a 5-fold increase in NIS expression which is considered a significant result. Both treatments with 1 μM T₄ resulted in a 4-fold increase. Despite the large variation between replicates, there was a very clear trend whereby increases in NIS expression declined as the concentration of T₄ was increased.

**Figure 4.4** Effect of various concentrations of Thyroxine on NIS expression in T47D cells. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluent controls.
4.8 NIS gene expression following stimulation of Sk-Br-3 cells with Estradiol

When Sk-Br-3 cells were treated with 1 nM, 10 nM, or 50 nM E₂ for 24 or 72 hrs it resulted in increases in NIS gene expression in all conditions ranging from 3–6 fold (Figure 4.5). The largest most consistent change in NIS expression was observed after treatment with 10 nM E₂ at 24 hrs (Mean (SEM) fold change 5 (1)-fold). Just the 1 nM and 10 nM treatment for 72 hrs resulted in greater than a 5-fold increase in NIS expression. NIS expression appeared to peak when treated with a concentration of 10 nM E₂.

Figure 4.5 Effect of various concentrations of Estradiol on NIS expression in Sk-Br-3 cells. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluent controls.
4.9 NIS gene expression following stimulation of Sk-Br-3 cells with ATRA

Following treatment of Sk-Br-3 cells with 0.1 μM, 1 μM, and 5 μM ATRA for 24 or 72 hrs, increases in NIS gene expression were observed ranging from 6–16-fold (Figure 4.6). The greatest most consistent changes in NIS expression was observed after 1 μM ATRA at 24 hrs (Mean (SEM) fold change 9 (2)-fold) however as high as a 16 (8)-fold increase was observed in response to 5 μM treatment for 24 hrs. This was by far the largest increase observed across all stimulations in both cells lines. All concentrations for both 24 and 72hrs of ATRA treatment resulted in a 5-fold or more increase of NIS gene expression in Sk-Br-3 cells which is considered a significant change. Although not statistically significant, a much larger increase in NIS expression was observed in Sk-Br-3 cells treated with 5 μM ATRA for 24hrs as opposed to 72 hrs. This is the opposite of what was observed in T47D cells. The benefit to the shorter incubation was not extended to the other 2 concentrations of ATRA.

Figure 4.6 Effect of various concentrations of all trans Retinoic acid on NIS expression in Sk-Br-3 cells. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluent controls.
4.10 NIS gene expression following stimulation of Sk-Br-3 cells with Thyroxine

Lastly, Sk-Br-3 cells were treated for 24 and 72 hrs with 0.1 μM, 0.5 μM and 1 μM Thyroxine resulting in increases in NIS gene expression ranging from 3–8 fold (Figure 4.7). The greatest most consistent changes in NIS expression was observed after treatment with 0.1 mM T4 at 24 hrs (Mean (SEM) fold change (7 (1)-fold). Treatment for 24 hrs with 1 μM T4 resulted in a significantly larger increase in expression compared to 72 hrs treatment (p < 0.05) although again, this divergence did not extend to the other concentrations used.

![Figure 4.7](image)

**Figure 4.7** Effect of various concentrations of Thyroxine on NIS expression in Sk-Br-3 cells. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluents controls.

The same set of ligands and incubations were used to treat MDA-MB-231 cells with subsequent analysis of NIS expression. No increases were detected.

Stimulation of NIS expression in Mesenchymal Stem Cells was also attempted to determine if the effects observed in T47D and Sk-Br-3 were limited to a breast tissue lineage. Again, no increases in NIS expression were observed.
4.11 NIS gene expression is synergistically stimulated in cells T47D using combinations of ATRA and T4

The previous experiment was repeated in triplicate using ligands individually as reference points and in combination. The objective was to determine if combined stimulation of receptors had an additive or deleterious effect. To do this only the optimal concentration for each ligand was used. These were selected on the basis of the largest most consistent increase in NIS expression observed. Based on these criteria, concentrations of 10 nM E2, 1 μM ATRA and 0.1 μM T4 were selected as optimal. As mentioned previously, there was no apparent overall benefit in exposing cells to ligands for 72 hrs so only 24 hr stimulations were performed.

Increased NIS expression was again observed in T47D cells when treated individually with E2, ATRA and T4. This further supports observations reported in sections 4.5-4.7. Interestingly, on this occasion, individual treatments with E2 and T4 both resulted in much larger increases in NIS expression (E2 13 (5)-fold, T4 14 (5)-fold) than observed in the previous set of experiments (E2 6 (2)-fold, T4 6 (3)-fold). Increases in NIS expression resulting from ATRA stimulation (T4 7 (1)-fold) were consistent with previous observations (T4 6 (1)-fold).

Combinations of ligands also increased NIS expression. The greatest increase in NIS expression resulted from stimulation of T47D (26 (2)-fold) cells with the combination of ATRA and T4. Addition of E2 to this combination did not increase the amplitude of NIS expression significantly.

While the increase in NIS expression with the combined treatment of ATRA and T4 (26 (2)-fold) was greater than the sum of the individual treatments (21 (5)-fold), the opposite was true of any combination involving E2. In fact, treatment of T47D cells with E2 and ATRA (10 (2)-fold) and with E2 and T4 (10 (1))-fold resulted in increases in NIS expression much smaller than the sum of the individual treatments (24 (5)-fold and 20 (5) respectively). The combined stimulation of T47D cells with all three ligands also resulted in a smaller increase in NIS expression (27 (3)-fold) compared to the sum of the individual treatments (34 (5)-fold).
Figure 4.8 Effect of treating T47D cells with Estradiol, all trans Retinoic acid and Thyroxine individually and in combination on NIS gene expression. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluents controls.
4.12 NIS gene expression is also synergistically stimulated in Sk-Br-3 cells using combinations of ATRA and T4

When the same experiment was conducted in Sk-Br-3 cells a similar trend was observed (Figure 4.9). Increased NIS expression was again observed following individual treatment with E2, ATRA and T4 confirming earlier observations in sections 4.8-4.10. On this occasion the increase in NIS expression in response to E2 and T4 was considerably lower in Sk-Br-3 cells (E2: 6(2)-fold, T4: 3(1)-fold) compared to T47D cells (E2: 13(5)-fold, T4: 15(5)-fold). Again the greatest increase in NIS expression resulted from stimulation of Sk-Br-3 (16(1)-fold) cell lines with the combination of ATRA and T4 and inclusion of E2 to this combination didn’t result in a significant increase in NIS expression.

Again, a synergistic effect of combined ATRA and T4 treatment on NIS expression in Sk-Br-3 cells was observed. Compared to the sum of the individual treatments (7 (1)-fold), a much larger increase in NIS was actually observed following ATRA and T4 stimulation (16 (1)-fold). As before, the combination of E2 with ATRA (6 (2)-fold) and E2 with T4 (3 (1)) resulted in increases in NIS expression which were smaller than the sum of the individual treatments (10 (2)-fold for both). Interestingly, unlike the previous observation in T47D cells, using a combination of all three ligands to treat Sk-Br-3 cell resulted in a much larger, possibly synergistic increase in NIS expression (21 (4)-fold) compared to the sum of the individual treatments (12 (2)-fold).
Figure 4.9 Effect of treating Sk-Br-3 cells with Estradiol, all trans Retinoic acid and Thyroxine individually and in combination on NIS gene expression. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluents controls.
4.13 Changes in receptor expression in response to ligand stimulation.

Changes in the level of ERα, RARα, RARβ, THRα and THRβ gene expression in response to E₂, ATRA and T₄ stimulation were also examined in T47D cells. The results of this stimulation are shown in Figure 4.10. Data was presented as Log₁₀ RQ to accommodate the large variation in gene expression. The largest increase was observed in RARβ expression in response to the presence of ATRA. ATRA alone created a 1.75 log₁₀ RQ (65-fold) increase in RARβ while the E₂:ATRA (1.64 log₁₀ RQ (47-fold)) and ATRA:T₄ (1.65 log₁₀ RQ (47-fold)) resulted in slightly smaller increases. The trio of E₂:ATRA:T₄ (1.29 log₁₀ RQ (20-fold)) stimulated a smaller increase again. RARα in comparison was only very slightly increased (0.58 log₁₀ RQ (4-fold)) in response to ATRA. No increase in RARβ was observed in response to ATRA when a similar experiment was performed in Sk-BR-3 cells.

ERα appeared to be slightly increased in expression in response to E₂ (0.58 log₁₀ RQ (4-fold)) and T₄ (0.64 log₁₀ RQ (4-fold)) with a similar effect observed with the combination of the two ligands (0.57 log₁₀ RQ (4-fold)). THRα appeared to be slightly increased in the presence of E₂ (0.49 log₁₀ RQ (3-fold)).

THRα and THRβ were downregulated by the presence of ATRA and T₄ and the combination of same. Addition of E₂ to these ligands also contributed to a reduction in RARα expression.
Figure 4.10 Expression of RARα, RARβ, ERα, THRα and THRβ in T47D cells following treatment with Estrogen, all trans Retinoic acid and Thyroxine individually and in combination. Data is normalised to endogenous controls PPIA and MRPL19 and expressed relative to appropriate diluents controls.
4.14 Discussion

Upon investigation of the role of retinoic acid, estrogen and thyroxine on NIS expression in breast cancer cell lines it was apparent that incubation with each of the 3 ligands had a consistently positive influence on NIS expression relative to appropriate diluent controls.

In the case of estrogen and retinoic acid these findings support previous investigations which showed ATRA and E$_2$ stimulation of MCF7 epithelial cells which, like T47D cells, represent the luminal A subtype, resulted in increased NIS gene expression [270, 315, 337, 343, 357, 358]. However, the observation that thyroxine increases NIS expression in T47D cells has not been reported to date. This effect is unusual in that thyroid NIS gene expression is decreased by increasing the concentration of T$_4$ [236]. This is an example of how the typical regulatory processes involved in thyroid NIS expression may not apply to breast tissue. It suggests that gaps in knowledge about the regulation of NIS in breast tissue should only be tentatively instructed by the well established processes in the thyroid until resolved by appropriate experimentation.

Alotaibi et al. demonstrated that unliganded ER$\alpha$ can interact with an estrogen responsive element (ERE) closely positioned to the TATA box of the NIS gene promoter and that this is associated with increased NIS expression [315]. It has also been demonstrated that mutations in the ERE of the NIS gene promoter actually lead to increased NIS expression (unpublished work). They suggested that this increase in NIS expression may be attributed to a “relatively complex and dynamic interplay between ER and other transcription factors operating on the NIS gene promoter” and pointed to the close proximity of the ERE to the TATA box elements which may be the reason for this behaviour. They also speculated that concentrations of E$_2$ higher than that of normal physiological levels could lead to over activation of ER$\alpha$ which might disrupt interactions of general transcription factors with the TATA element of the NIS gene resulting in downregulation of expression [264]. This concept may provide an explanation for the drop in NIS gene expression observed when treating T47D cells with the highest concentration of E$_2$ (Figure 4.2).
Less is known about the potential for ligand-receptor stimulation of NIS expression in Sk-Br-3 cells. This is in fact the first time stimulation of NIS expression has been demonstrated in the ER negative, Her2 positive cell line using E2, ATRA or T4. The 3-7 fold increase of NIS expression in response to E2 is particularly interesting since ER is deemed to be the means by which E2 effects NIS expression. As can be seen from Figure 4.4, and from what it understood to be a one of their defining characteristics, Sk-Br-3 cells, have little or no expression of ERα. Despite this, all E2 treatments increased NIS expression, with a 10 nM incubation resulting in an impressive 6(2)-fold increase after 72 hrs. This effect is comparable to the increases in NIS expression achieved in T47D cells. It suggests that E2 stimulation of NIS expression may occur independently of the ER receptor and possibly to a similar amplitude. This may be achieved through E2 stimulation of G protein couples receptor (GPR30) which is expressed in Sk-Br-3 cells [359]. Upon stimulation GPR30 has been shown to activate the PI3K and CAMP/PKA pathway both of which are involved in mammary NIS expression [359]. Interestingly MDA-MB-231 which are both ER α and GRP30 negative displayed no increase in NIS expression in response to E2 stimulation.

Overall, there appeared to be no consistent advantage in treating either T47D or Sk-Br-3 cells with ligands for 72 hrs. As a result, subsequent experimentation involving ligand stimulation was performed for 24 hrs only.

MDA-MB-231 cells showed no increase in NIS expression in response to stimulation with ligands. This may be attributable to the relatively low level of basal NIS expression compared to the Sk-Br-3 and T47D cell line. With the exception of ERα all other putative regulators of NIS were expressed at comparable levels to Sk-Br-3 and T47D cells. This suggests that there may be potential for stimulating NIS expression in MDA-MB-231 cells. However, in this case, if changes did occur they were still below a detectable level for RQ-PCR. It also introduces the possibility that another, as yet unidentified player in mammary NIS regulation is absent in MDA-MB-231 cells but not in T47D and Sk-Br-3 cells.
It is interesting that when individual treatments of T47D cells and Sk-Br-3 cells were repeated as reference points for combined ligand stimulations a much larger increase in NIS expression in response to E₂ and T₄ stimulation was observed in T47D cells compared to Sk-Br-3 cells. This conflicts somewhat with earlier observations suggesting comparable levels of NIS expression could be achieved in both cell lines despite presence (T47D) or absence (Sk-Br-3) of ER. In the later experiment the obvious explanation for a larger increase in NIS expression in T47D compared to Sk-Br-3 cells in response to E₂ stimulation is the presence of much higher levels of ER. A similar pattern observed in response to T₄ stimulation suggests that ER may be somehow involved in T₄ stimulation of NIS expression. The observation that THRβ and ERα appeared to increase in expression in response to stimulation with E₂ and T₄ respectively in T47D cells supports this observation.

It is interesting that in vitro studies revealed significant stimulation of NIS expression in cell lines exposed to thyroid hormone and that the largest and possibly synergistic increase was observed in response to stimulation with an ATRA:T₄ combination. A possible means of explaining this effect may lie with the fact that dimerization between retinoic acid receptors and thyroid hormone receptors is known to occur [352, 353]. Heterodimerization between retinoid-X-receptor and pregnane-X-receptor has been identified as the basis for carbamazepine stimulation of NIS expression in MCF-7 cells [272]. It may be possible that in the absence of the ATRA stimulation heterodimerization might still occur in response to T₄ stimulation leading to increased NIS. This is supported by Lee et al.’s observation that where heterodimers of RAR and THR were concerned, the presence of just the T₃ (Tri-iodothyronine) or ATRA ligand facilitated only a partial release of the corepressor SMRT whereas costimulation induced a complete release [353].

Since ATRA has been shown in this study to be effective in stimulating NIS, it is interesting to note that its receptor, RARβ, which is also implicated in breast cancer NIS expression [273], is strongly increased in the presence of the ligand. This suggests ATRA may be capable not only of stimulating NIS expression, but amplifying the pathway through which it acts. This may be particularly relevant in the context of T47D cells which have a relatively low
baseline expression of RARβ. While a large increase in RARβ expression was observed in the presence of ATRA, introducing E₂ or T₄ appeared to reduce its impact and the combination of all 3 ligands seem to reduce the impact of ATRA even further. In fact, ATRA alone increased RARβ as much as 3 times more than the combination of E₂, ATRA and T₄ (65-fold increase compared 20-fold). Considering that it was the combinations of ATRA:T₄ and E₂:ATRA:T₄ that induced the largest increase in NIS expression (much higher that ATRA alone) it seems unusual then that these combinations would result in a much lower increase in RARβ than with ATRA alone. It is possible that the relatively reduced level of RARβ is countered by stronger signals arising from the combination of ligands. The potential role of RARβ:THRβ heterodimerization may provide the answer to this conflicting data. It is also interesting to note that while the increase in NIS expression arising from E₂:ATRA:T₄ treatment in T47D cells was very large it was not bigger than the sum of the individual treatments as was the case with ATRA:T₄. Essentially the addition of E₂ appeared to stop the synergistic effect of ATRA:T₄ on NIS expression despite its positive role as an individual treatment. This unusual observation is supported by a recent study by Cheong et al. who observed that ATRA stimulated iodide uptake in MCF-7 cells was reduced by co-treatment with E₂ [360]. In the context of RARβ:THRβ heterodimerization theory of NIS regulation its very interesting that E₂:ATRA:T₄ treatment in T47D cells results in less than half the RARβ expression observed in ATRA:T₄ stimulated cells.

Also interesting is the possible down regulation of THRβ in the presence of ATRA or T₄. Combination of the two ligands caused a decrease in RAR α as well both thyroid hormone receptors. Addition of E₂ to this combination resulted in a decrease in both Thyroid hormone receptors as well as RARα and ERα. Knowing that the highest levels of NIS expression were seen after stimulation with ATRA:T₄ and E₂:ATRA:T₄ it is curious that the 3 putative regulators of NIS; RARα, and THRα and THRβ were downregulated in each of these stimulations. It suggests that mechanisms by which NIS is stimulated by individual ligands may be dominated or rendered ineffective by the mechanism involving RARβ which is at play in response to combined ATRA:T₄ treatment. However, the additional downregulation of ERα expression in response to
E₂:ATRA:T₄ may point to a role for ERα within this mechanism and might further explain the inability to increase NIS expression to levels as might be expected by the sum of the individual treatments.

A similar study of Sk-Br-3 cells didn’t significantly increase RARβ expression in response to ATRA. It seems that the absence of ERα plays an important role in ATRA stimulated increases in RARβ expression. Five-fold increases in ERα were observed in response to individual treatments with E₂ and ATRA but not with the combination of the two indicating that the presence of both ligands limited their individual abilities to stimulate RARβ expression. This observation adds further support to the potential for competitive stimulation of receptors and offers a clear reason why E₂:ATRA stimulation resulted in a much smaller increase in NIS expression than the sum of the individual treatments in Sk-Br-3 cells.
Chapter 5

Results and Discussion

Adenovirus-mediated expression and function of the Sodium Iodide Symporter (NIS) in Mesenchymal Stem Cells
5.1 Introduction

The partnership of native NIS and radioiodide has been used successfully both in terms of efficacy and safety for over 60 years in the treatment of thyroid cancer [240]. The jury is still out as to the therapeutic value of native NIS expression in breast cancer. Efforts to augment native NIS expression resulting in increased iodide accumulation have had a certain amount of success in cell lines and animal models [272][271]. However, questions arise as to the safety of treating patients with such combinations of drugs to induce NIS expression, what other tissues may overexpress NIS as a result, and what damage may be caused in these noncancerous tissues.

A more promising alternative is the possibility of NIS gene therapy. This involves the delivery of a NIS transgene specifically to tumour tissue. In theory this confers to malignant tissue the ability to accumulate cytotoxic iodide facilitating imaging and treatment using.

Implementation of this approach is a difficult prospect. Previous clinical trials using a variety of therapeutic genes in the therapy of breast cancer have proved disappointing [361-364]. In many cases adenovirus has been used to deliver genes. However, the inability of adenovirus to deliver therapeutic genes to tumour tissue and its highly immunogenic nature are major disadvantages [100, 102, 365][103][105, 106]. The potential to migrate to tumour sites [115, 138, 156, 234], ability to suppress the immune system [366], and suitability to adenoviral transfection make MSCs ideal candidates to overcome the deficiencies of adenovirus as a gene vector. MSCs have been used safely for decades as a constituent of bone marrow transplants. Additionally evidence is stacking up that transplanting culture expanded MSCs is safe with minimal toxicity [367-371]. Since the objective is to destroy cancerous tissue, therapeutic gene expression is only required until this has been achieved.

Adenovirus does not integrate into the host genome potentially disrupting gene expression, particularly oncogenes [372-374]. As such, it is an ideal partner to provide therapeutic short-term (days-weeks) gene expression to MSC vehicles. It remains to be seen if, other than the predictable effects of the therapeutic gene, Adenovirus causes any changes to the phenotype of MSCs. Variation in
expression of cell surface markers used to define MSCs represent a useful barometer of any phenotypic aberrations which may result from adenoviral infection, as would any changes in their characteristic osteogenic potential [126]. It is important that the level of therapeutic gene expression is sustained until therapy can be implemented. Previous studies have shown that while adenovirus vectors are efficient in delivery of a therapeutic gene to cells or tissue, expression peaks within less than a week and rapidly declines sharply thereafter [100, 101, 282, 375, 376]. Understanding the expression profile of a transiently delivered therapeutic gene is a critical step in assessing the potential of a candidate gene therapy.

A number of studies show very clearly that expression of NIS in conjunction with $^{131}I$ treatment can have a strong deleterious effect on cancer cells and if delivered directly to tumours, supports imaging and treatment of cancerous tissue.[284]. Previous descriptions of a bystander effect in which cells in proximity to cells accumulating cytotoxic radiolabelled iodide can also be ablated may help explain how this approach could be so effective [242, 298]. Further studies confirmed the therapeutic and radioimaging potential of the NIS gene therapy strategy and highlighted the potential benefit of pre-treatment imaging afforded by the NIS gene [100, 101, 283].

The aim of this chapter was to further investigate the potential of NIS-mediated imaging and treatment of breast cancer in the context of a potential tumour targeting MSC vehicle.

### 5.2 Objectives

- Establish an expression profile over time of the NIS transgene in MSCs infected with the vector Ad5/CMV/NIS
- Determine the effect of cell proliferation on transient NIS expression
- Determine the efficiency of infection of Ad5/CMV/NIS.
- Determine if introduction of transgene NIS expression effects
a) osteogenic potential

b) expression of characteristic surface markers.

- Determine the cytotoxic effects of $^{131}$I on breast cancer epithelial cell lines co-cultured with MSC-NIS

5.3 Materials and Methods

A NIS transgene expression profile was established through Ad5/CMV/NIS infection at multiplicities of infection of 0, 100 and 200 using a centrifugation method. Infected and control populations were seeded in culture vessels (including chamber slides) and harvested at various time points (3, 7, 10, 14, 21, 28 days). RNA was extracted from the harvested cells and interrogated for expression of NIS mRNA.

The effect of proliferation on NIS expression was also investigated in a similar fashion as above. However, at Day 10 the experiment was subdivided into 1) freely proliferating cells and 2) cells that remained confluent for the remainder of the experiment. As before, cells were harvested at 3, 7, 10, 14, 21 and 28 days after infection with day 14 representing the first time point at which the proliferating and nonproliferating cultures had significantly diverged. Chamber slides cultures grown in parallel with this study were used for analysing the effect of proliferation on NIS protein expression.

The efficiency of Ad5/CMV/NIS infection of MSCs using centrifugation was determined by substituting an Ad5 virus similar in aspects to Ad5/CMV/NIS except that the transgene was Green Fluorescent Protein (Ad5/CMV/GFP). Infection with this virus was then accurately quantified by detecting green fluorescent cells by flow cytometry.

The effect of Ad5/CMV/NIS infection of MSCs on osteogenic potential was quantified by growing infected and uninfected cells in osteogenic media for 17 days and comparing the extent of mineralisation to non-transduced cells using StanBio and Von Kossa assays.
The cytotoxic effects of $^{131}$I on co-cultures of Ad5/CMV/NIS infected MSCs with T47D were also determined. MSCs, MSCs infected with Ad5/CMV/NIS, and T47D cells were cultured individually. In addition, co-cultures of MSCs infected with Ad5/CMV/NIS and T47D cells were performed at ratios of 1:1, 1:3 and 1:10. Three days after the infection of MSCs with Ad5/CMV/NIS all populations were treated with or without $^{131}$I for 7 hrs and subsequently grown for a further 7 days. At this point the cells were harvested by trypsinisation and quantified using flow cytometry. MSCs and T47D cells were distinguished from each other by targeting cell specific surface markers.

5.4 Basal expression in MSCs

It was important to establish baseline levels of MSC NIS gene expression before proceeding with NIS transgene expression studies. Following an initial assessment, it was found that MSCs had undetectable levels of NIS gene expression (results not shown). This was ideal for assessing NIS transgene expression as any NIS expression detected in subsequent studies could for the most part be attributed to the transgene and not native expression of NIS. However, to be thorough all transgene expression analysis was compared to relevant negative controls.
5.5 Robust NIS transgene expression in Ad5/CMV/NIS infected MSCs

MSCs were infected with Ad5/CMV/NIS. Three days later cells were harvested and analysed by RQ-PCR to establish the presence of NIS transgene expression. Further time points (7, 10, 14 and 21 days) were included up to 28 days after infection to investigate the persistence of NIS transgene expression.

NIS transgene expression was clearly present in MSCs 3 days after infection with Ad5/CMV/NIS (Figure 5.1). This represented at least a 5 Log_{10} Relative Quantity (RQ) increase on native levels of expression in MSCs. Robust NIS expression was observed up to 28 days after infection of the MSCs. At 7 days a 4.5(0.4) (Log_{10} RQ (Standard error)) increase in NIS expression was observed in infected cells (MOI 100) relative to the uninfected control (MOI 0) and to a large extent this strong relative expression was maintained right up to 28 days after infection with Ad5/CMV/NIS (3.7 Log_{10} RQ). There was no significant benefit in increasing the MOI to 200 as a very similar pattern of expression was observed compared to that of MOI 100 infected cells. Error bars are not displayed at the later time points of 21 and 28 days because these were not performed in triplicate like the others due to successive extensions of the experiment.
Figure 5.1 NIS transgene expression in MSCs infected with Ad5/CMV/NIS. NIS expression was examined over 28 days. Expression data was normalised relative to endogenous control PPIA and expressed relative to uninfected control.
5.6 NIS transgene expression in proliferating and nonproliferating Ad5/CMV/NIS infected MSCs.

To determine the effect of MSC cell proliferation on NIS transgene expression cells were infected with Ad5/CMV/NIS. Proliferating cells were cultured by repeated passaging every 7 days preventing cells from achieving confluence. Nonproliferating cells were maintained in the same culture vessel for the duration of the experiment. As with the previous experiment cells were harvested 3, 7, 10, 14, 21 and 28 days after infection with Ad5/CMV/NIS and the samples were analysed by RQ-PCR for NIS expression.

In MSCs infected with MOI 100 Ad5/CMV/NIS, robust NIS expression was observed up to 7 days after infection. After 7 days of infection the experiment was divided into proliferating and nonproliferating cells.

Confluent cells maintained a persistent level of NIS expression at day 14 (4.69 Log_{10}RQ) (Figure 5.2) compared to the robust levels expressed at day 3 (5.52 Log_{10}RQ). This continued right up to 28 days after infection (3.73 Log_{10}RQ).

NIS expression persisted at significantly lower levels in passaged cells compared to confluent cells. At day 14, the first time point at which a distinction in proliferation was confirmed between the 2 populations, NIS transgene expression had dropped to 3.55 Log_{10}RQ from peak day 3 levels (5.52 Log_{10}RQ). Expression of NIS continued to decline more rapidly in proliferating cells at later time points. However NIS was still detectable 28 days after infection (2.52 Log_{10}RQ).

Again, as with the previous experiment, infection of MSCs with MOI 200 Ad5/CMV/NIS resulted in a very similar set of data as that seen with MOI 100.
Figure 5.2 NIS transgene expression in proliferating and nonproliferating Ad5/CMV/NIS infected cells. Expression data was normalised relative to endogenous control PPIA and expressed relative to uninfected control.
5.7 NIS protein expression in proliferating and nonproliferating Ad5/CMV/NIS infected MSCs

In conjunction with the previous experiment a study of NIS protein expression was performed by culturing Ad5/CMV/NIS infected MSCs in chamber slides. MSCs were fixed 10, 14, 21 and 28 days after infection with Ad5/CMV/NIS and subsequently DABmap stained for NIS expression using a human NIS monoclonal antibody [304] and counterstained with haematoxylin.

Immunohistochemical analysis of Ad5/CMV/NIS infected MSCs shown in Figure 5.3 demonstrated robust expression of the NIS protein in cells up to 28 days after infection. The difference in protein expression between proliferating and nonproliferating appeared more pronounced than seen in the gene expression data. As early as 10 days after infection the percentage of proliferating cells expressing NIS protein compares very poorly to the pervasive NIS immunopositivity seen in confluent MSCs (Figure 5.4a and e). This theme is also seen consistently in later time points. At day 28, while the confluent MSC population maintains a reasonable frequency of cells immunopositive for the NIS protein, the corresponding proliferating population offers only a rare occurrence of cells expressing NIS protein. Figure 5.4 shows clear evidence of diffuse cytoplasmic and membrane bound localisation of NIS in proliferating and nonproliferating cells which express NIS protein. Membrane bound localisation can be seen as the darker stained membrane highlighted by the yellow arrows and the very fine cellular filaments highlighted by the red arrows (Figure 5.4).
Figure 5.3 DABmap NIS staining in Ad5/CMV/NIS infected nonproliferating (left column) and proliferating (right column). Nonproliferating MSCs, 40X (MOI 100) at (a) Day 10, (b) Day 14, (c) Day 21 and (d) Day 28. Proliferating MSCs 40x (MOI 100) at (e) Day 10, (f) Day 14, (g) Day 21 and (h) Day 28.
Figure 5.4 DABmap NIS staining in Ad5/CMV/NIS infected nonproliferating (left) and proliferating (right column) 28 days after infection. Darker stained membranes are highlighted by the yellow arrows and the very fine cellular filaments highlighted by the red arrows. a) Nonpassaged MOI 100 200x, b) Passaged MOI 100 200X.
5.8 Ad5/CMV/NIS infection efficiency in MSCs.

To quantify the ability of Ad5/CMV/NIS to infect MSCs resulting in expression of the transgene, an adenovirus vector was used similar in all aspects to Ad5/CMV/NIS except for the transgene. Ad5/CMV/GFP carries the gene for green fluorescent protein. Its infection efficiency in a population of cells is easily quantified by detecting green fluorescent cells using a flow cytometer. MSCs were infected with Ad5/CMV/GFP at MOIs of 0, 50, 100 and 150. They were cultured for 3 days and subsequently analysed for the presence of green fluorescent protein using a Guava Easycyte 8HT flow cytometer. To support this method infected populations were also cultured in chamber slides, fixed 3 days later, cell nuclei stained with DAPI and examined for green fluorescence using a fluorescence microscope.

Using flow cytometry, the uninfected control was almost completely negative for green fluorescent protein (Figure 5.5a). With an MOI of 50, 56 % of MSCs were positive for GFP (Figure 5.5b). At MOI 100, 77 % express GFP (Figure 5.5c). The highest MOI of 150 resulted in 95 % of MSCs expressing GFP (Figure 5.5d).

**Figure 5.5** Transduction efficiency of Ad5/CMV/GFP in MSCs determined by flow cytometry. a) MOI 0, b) MOI 50, c) MOI 100, d) MOI 150, Clear peaks in b), c) and d) represent MOI 0 fluorescent profile. This data was confirmed by fluorescent microscopy.

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Figure 5.6a shows no GFP expression in wild-type MSCs. DAPI staining of cell nuclei confirmed the presence of cells (Figure 5.6b). Figure 5.6c-e highlights GFP expression in MOI 50 cells. Higher magnifications shown to the right display strong GFP expression throughout infected cells. The same is true of cells infected at an MOI of 100 (Figure 5.6f-h). MSCs infected with the highest MOI of 150 appear to have the highest proportion of cells expressing GFP and again higher magnifications confirm strong levels of expression within infected cells (Figure 5.6i-k).

**Figure 5.6** Transduction efficiency of Ad5/CMV/GFP in MSCs determined by Fluorescence microscopy, a) MOI 0 GFP 40X, b) MOI 0 DAPI 40x, c) MOI 50 GFP 40X, d) MOI 50 GFP 100X, e) MOI 50 GFP 200X, f) MOI 100 GFP 40X, g) MOI 100 GFP 100X, h) MOI 100 GFP 200X, i) MOI 150 GFP 40X, j) MOI 150 GFP 100X, k) MOI 150 GFP 200X
5.9 Analysis of osteogenic potential and surface marker expression in Ad5/CMV/NIS infected MSCs

It is important to ensure that the phenotype of the host MSCs is not compromised by the introduction of Ad5/CMV/NIS. The characteristic traits of MSCs include the ability to differentiate into an osteogenic lineage and the presence or absence of MSC defining surface markers [126]. This experiment investigates these characteristics in the context of Ad5/CMV/NIS infected versus uninfected MSCs.

To determine osteogenic potential in Ad5/CMV/NIS infected MSCs, infected and uninfected cells were cultured in osteogenic conditions and after 17 days were assessed for the presence of calcium and phosphate deposits indicative of osteoblasts using StanBio calcium and Von Kossa assays. To determine the effect of Ad5/CMV/NIS infection on surface marker expression, infected and uninfected MSCs were cultured as normal for 3 days and analysed by flow cytometry for expression of CD105, CD90, CD73, CD45, CD34, CD31, CD24 and CD227.
The calcium content of cell lysates as assessed by the StanBio calcium assay is shown in Figure 5.7. It is clear that a large difference in calcium content was recorded in MSCs cultured in osteogenic compared to normal media. Levels of calcium deposition in MSCs alone were not significantly higher than that of Ad5/CMV/NIS infected MSCS.

**Figure 5.7** Quantification of calcium deposition by MSCs and Ad5/CMV/NIS infected MSCs induced to differentiate to an osteogenic lineage. Calcium was quantified using a StanBio assay.
Von Kossa staining, which highlights phosphate accumulation and is indicative of osteogenic differentiation, demonstrated that no mineralisation occurred in both MSCs and MSC-Ad5/CMV/NIS cells grown in normal media (Figure 5.8). In osteogenically induced populations, both infected (Figure 5.8d) and uninfected (Figure 5.8c) MSCs displayed similarly strong levels of dark brown staining which is indicative of phosphate accumulation and the presence of osteoblasts.

Figure 5.8 Von Kossa staining in MSCs and MSC-Ad5/CMV/NIS induced to osteogenic lineage. a) Uninfected MSC control (MOI 0) grown in normal media (40x). b) Ad5/CMV/NIS infected MSCs (MOI 100) grown in normal media (40x). c) Uninfected MSC control grown in osteogenic media (40x). d) Ad5/CMV/NIS infected MSCs grown in osteogenic media (40x)
Three days after infection with Ad5/CMV/NIS, MSCs (MOI 0) and MSC-NIS (MOI 100) were interrogated using flow cytometry for expression of specific surface markers. Both cell populations were > 98% positive for CD105, CD90 and CD73 (Figure 5.9). Additionally, both populations were < 2% positive for CD45 and CD34. Comparable results between both populations were also observed for CD10, CD31, CD227 and CD24 which were all below 2% positive.
Figure 5. CD105, CD90, and CD73 (Left), CD34, CD45, CD10 (Middle), and CD31, CD227 and CD24 (Right) expression profiles of MSCs (MOI 0) alone and MSCs infected with Ad5/CMV/NIS (MOI 100). Isotype controls and antibody treated cells are represented by clear and red peaks respectively.
5.10 Iodide$^{131}$ cytotoxicity assay in co-cultured MSC-NIS and T47D cells

To investigate the cytotoxic effects of I$^{131}$, MSCs were infected with Ad5/CMV/NIS (MOIs 100 and 0) and cultured overnight. They were then passaged and grown alone or in co-culture with T47D cells at varying ratios for 2 days. Following establishment of the co-cultures, cells were then treated in the presence or absence of I$^{131}$ for 7 hrs and subsequently allowed to proliferate for 7 days. Cells were then trypsinised and labelled with fluorescent CD105 (MSC) and MUC1 (T47D) antibodies and analysed by flow cytometry.

There was no significant difference in the number of viable cells retrieved following treatment of uninfected MSCs with or without I$^{131}$ (Figure 5.10). Conversely, there was a 29% drop in viability of T47D cells which were exposed to I$^{131}$ compared to the I$^{131}$ negative control. A clear difference in survival rates was apparent in I$^{131}$-treated MSC-NIS cells. In fact, there was almost 3 times more viable MSC-NIS cells retrieved in the control ($6.6 \times 10^5$ cells) versus I$^{131}$-treated population ($2.4 \times 10^5$ cells) of cells. Co-cultures of MSC-NIS and T47D cells seeded at a ratio of 1:1, showed a large decrease in total cell viability in I$^{131}$-treated compared to untreated populations (60% drop in viability). For populations seeded with fewer MSCs per T47D cell the difference in total cell viability was considerably smaller with a 32% drop observed with a 1:3 T47D:MSC-NIS ratio and a 24% drop with the 1:10 ratio. The proportion of MSCs and T47D cells was also quantified by flow cytometry techniques. However, an unexpected MUC1-positive population of cells was observed in pure MSC cultures (8-10%). Likewise the proportion of MSCs that were CD105 positive (range 90-92% positive) was lower than expected. The normal values for MSCs should be > 95% positive for CD105 and < 2% positive for MUC1. Since the reported values were not grossly aberrant, they were informative as to the proportion of MSC and T47D cells remaining within treated co-culture populations. With respect to the 1:1 T47D:MSC-NIS population, the MSC (CD105 positive) subpopulation decreased by 66% whereas the T47D (MUC1 positive) subpopulation decreased by 58% in response to I$^{131}$ treatment. This significant decrease in T47D viability demonstrates the potential for a bystander effect since such a viability decrease was not observed in T47D cells cultured alone. A similar decrease in MSC
subpopulation viability was seen in both the 1:3 (67 % decrease) and 1:10 (55 % decrease) co-cultures after $^{131}$I treatment while the viability of the T47D subpopulation decreased to a lesser extent in 1:3 (28 % decrease) and 1:10 (22 % decrease) in treated versus untreated cells. There appeared to be a dose dependent response in T47D $^{131}$I cytotoxicity whereby the smaller the proportion of MSC-NIS cells seeded in the co-culture, the better the viability of T47D cells after $^{131}$I treatment.
Figure 5.10 $^{131}$I cytotoxicity assay in MSC-NIS (Ad5/CMV/NIS MOI 100)-T47D co-cultures. CD105 (yellow) and MUC1 (green) were used as markers for MSCs and T47D cells respectively. $^{131}$I treated cells are highlighted with striped bars. Cell counts and changes in viability in total and individual cell populations are shown below the barchart. Total Cell Population (TCP), Difference between treated and untreated (Δ)

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Treatment</th>
<th>CD105</th>
<th>MUC1</th>
<th>TCP</th>
<th>Δ TCP</th>
<th>Δ CD105+ve</th>
<th>Δ MUC1+ve</th>
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<tr>
<td>MSC</td>
<td>Control</td>
<td>388,890</td>
<td>41,298</td>
<td>430,188</td>
<td>55,141</td>
<td>4,149</td>
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<tr>
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<td>Iodide 131</td>
<td>437,039</td>
<td>48,290</td>
<td>485,330</td>
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<td>(+12%)</td>
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<tr>
<td>T47D</td>
<td>Control</td>
<td>10,851</td>
<td>1,345,530</td>
<td>1,356,381</td>
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<td>T47D</td>
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<td>7,897</td>
<td>954,445</td>
<td>962,142</td>
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<td>243,524</td>
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<td>1,365,893</td>
<td>1,706,300</td>
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<td>832,567</td>
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<td>(-55%)</td>
<td>(-22%)</td>
</tr>
</tbody>
</table>
5.11 Discussion

The robust and persistent NIS gene expression observed in Ad5/CMV/NIS infected MSCs is unusual since most cells tend to lose expression of genes delivered by adenovirus vector much earlier, typically peaking 3-5 days after infection and rapidly declining thereafter [100, 101, 284]. The transient expression is attributed to the fact that Adenovirus doesn’t integrate into the genome and is replication incompetent due to an E1 region deletion. As a result, the transgene is not guaranteed to pass into each daughter cell with the end result being a dilution of Ad5/CMV/NIS infected cells. The robust expression of NIS at later time points in this study may be explained by the fact that MSCs undergo cell contact mediated inhibition of growth which benefited persistent gene expression. In the subsequent section this issue was examined by comparing NIS transgene expression in confluent and passaged MSCs after infection with Ad5/CMV/NIS. It was found that NIS gene and protein expression declined more rapidly in proliferating than nonproliferating cells but was still present 28 days after infection. In this context it should be noted that Sasportas et al. [184] reported that MSCs do not proliferate upon engraftment at tumour sites. The strong levels of NIS expression and particularly its persistence in non-proliferating (confluent) cells are very promising data in an overall context where this approach may be used as an imaging or therapeutic strategy in breast cancer. With NIS transgene expression so high even up to 28 days after infection of the MSCs, the timeframe in which a patient administered with MSC-NIS may be treated with radioiodide might be increased significantly. This has obvious benefits when considering the logistics and feasibility of the treatment in a medical setting.

Infection of MSCs with Ad5/CMV/NIS at MOIs of 100 and 200 consistently resulted in NIS expression levels which were very similar. This data suggests that for all subsequent experiments using the lower MOI 100 might be more appropriate. Considering that this approach may have a therapeutic potential the reduced MOI could prove beneficial in reducing any potential Ad5/CMV/NIS related toxicity which may occur in the MSCs or indeed in the recipient of the MSCs-radioiodide treatment.
Proliferation of Ad5/CMV/NIS infected MSCs appeared to impact persistence of NIS protein expression to a much greater level than seen earlier with NIS gene expression. This discrepancy between NIS gene and protein expression is not unknown, having already been reported by Neumann et al. (2004) in thyroid tissue [318]. It does however highlight the importance of substantiating NIS gene expression data with protein expression studies. This transience of NIS protein expression highlights the importance of timing in maximising the potential therapeutic effect of a MSC-NIS:radioiodide strategy in the treatment of breast cancer. Allowing infected populations to proliferate in vitro after infection would diminish NIS transgene expression within the cell population resulting in a higher MSC:MSC-NIS ratio being administered necessitating larger numbers of cells per dose. Alternatively, maintaining cells in a confluent state runs the risk of altering the MSC phenotype. MSC infection with Ad5/CMV/NIS should ideally be performed within days of administering the cells to a patient. The titration assay performed highlights how efficiently a population of MSCs can be infected with the Adenovirus vector and made to express the transgene. Where a large percentage of cells do not express NIS after Ad5/CMV/NIS infection it may be possible to enrich the NIS positive subpopulation using a NIS monoclonal antibody and fluorescence activated cell sorting or cell affinity chromatography [377].

While the frequency of cells expressing NIS protein does decrease over time, it is interesting that those cells which do maintain NIS protein expression at later time points appear to do so at a relatively similar intensity to the earlier time points. More specifically, a closer look at MSCs 28 days (Figure 5.3d and h) after infection shows that while there is a smaller percentage of cells expressing the NIS protein the intensity of staining is still quite similar to staining intensities observed at day 14 (Figure 5.3 a and e). The identification of NIS membrane localisation at day 28 in both proliferating and nonproliferating cells also strongly supports the potential for NIS functionality up to 28 days post Ad5/CMV/NIS infection.

It is clear from this data that from the time of Ad5/CMV/NIS infection to the point where the MSCs have engrafted and positioned themselves on tumour sites ready to accumulate radioiodide, that the exact proportion of cells
functionally expressing NIS will be uncertain. This makes assessment of the migratory and subsequent therapeutic or imaging effects harder to quantify. Likewise, in in vitro studies the transient nature of NIS transgene expression adds uncertainty to analysis of important parameters such as iodide accumulation per cell, iodide toxicity and ablative effect per cell or per dose. Future studies could involve the development of an MSC cell line stably expressing the NIS transgene. This could be established through plasmid transfection or through lentiviral vector infection with subsequent clonal selection. A population of MSCs, in which all cells express NIS would be extremely valuable in benchmarking the potential of MSCs expressing NIS and will prove very informative in instructing the pharmacological direction of Ad5/CMV/NIS infected MSCs.

Using flow cytometry to determine Ad5/CMV/GFP transfection efficiency revealed a dose dependent response to titrations of MOI 0, 50, 100 and 150. Since peak GFP expression was observed using MOI 150 infection (95 % positive), the use of MOIs higher than 100 (77 % positive) may be justified. Early observations of similar levels of NIS gene expression in MSCs transfected with Ad5/CMV/NIS at MOI of 100 and 200 may appear at odds with this observation however, it should be noted that different markers were investigated in these experiments. Additionally RQ-PCR quantifies mRNA and may or may not be proportional to the end protein product [318].

Comparable levels of osteoblast indicators (calcium and phosphate accumulation) in both MSC and MSC-Ad5/CMV/NIS cultured under osteogenic conditions suggested that both populations possess a similar capacity for osteogenic potential. The presence of Ad5/CMV/NIS doesn’t seem to impact MSCs ability to differentiate into osteoblasts significantly. Similar experiments showed that adenoviral infection of rat and porcine MSCs does not impact their osteogenic, chondrogenic or adipogenic potential [378, 379].

Minimal criteria for defining Mesenchymal Stromal/Stem cells [126] dictates that MSCs should be at least > 95 % positive for surface markers CD105, 90 and 73. The criteria were met by both infected and uninfected MSC populations. As dictated by the minimal criteria for defining MSCs, both cell
populations were also < 2 % positive for the pan-leukocyte marker CD45 and primitive hematopoietic progenitors and endothelial cells marker CD34 as well additional surface markers which include CD10 (tumour stromal and fibroblast marker), CD31 (endothelial cell marker), CD227 (epithelial cell marker) and CD24 (cancer stem cell marker). There was no difference in expression of the cell surface markers analysed between MSCs and MSCs infected with Ad5/CMV/NIS. This indicates that Ad5/CMV/NIS is not likely to affect the phenotype of MSCs other than that intended with actual expression of the NIS transgene.

Both results are hugely important in the context of systemically administering MSCs with the intention of tracking and treating breast cancer in human subjects. The observation that MSCs are unaffected in terms of their defining criteria [126] reduces the risk that their therapeutic value might be compromised by the adenoviral vector or transgene.

MSC-NIS cells appear to be highly susceptible to the 7 hrs I$^{131}$ treatment. Considering that the MSCs alone were unaffected whereas MSC-NIS cells were depleted by 63 % in response to I$^{131}$, it is very clear that manipulating MSCs to express NIS protein is very effective in creating a cytotoxic effect in the newly iodide accumulating cells. It is interesting that T47D cells alone appeared to suffer reduced viability in response to I$^{131}$. This may be possible because of very low levels of endogenous NIS gene expression in the T47D cell line. Future cytotoxic assays should include the use of the MDA-MB231 cell line which doesn’t have any detectable levels of NIS gene expression and are less likely to create this confounding issue.

The potential of MSC-NIS cells to impact the viability of co-cultured T47D cancer cells was very clear from the observation of a 58 % drop in T47D viability in the I$^{131}$ treated cells (T47D/MSC-NIS 1:1) relative to that of treated T47D cells grown alone (29 %). Reducing the MSC-NIS component of these co-cultures reduced the cytotoxicity observed in I$^{131}$ treated co-culture. It is important to illustrate that in a 2d cell culture, only a fraction of the I$^{131}$ beta and gamma energy harnessed by the MSC-NIS cells is directed at adjacent T47D cells. In this context, the cytotoxicity of I$^{131}$ is highly inefficient and the
58 % reduction in the viability of T47D cells cultured with an equal ratio of 
MSC-NIS cells appears to be very robust. Translated to a 3d environment this 
approach could see significant improvements in target cell cytotoxicity since 
radiating beta waves could affect adjacent cells in all directions from the source 
of iodide accumulation as opposed to just those in a single planar monolayer. 
This is supported by Carlin et al. [281] who demonstrated a 21 % clonogenic 
survival in I\textsuperscript{131} treated monolayer cultures, compared to 2.5 % in spheroid 
cultures of glioma cells expressing NIS. Dwyer et al. [115] reported that 
systemically administered MSCs migrated and engrafted with an even dispersal 
within breast tumours. Given the 2 mm range of beta wave penetration and the 
potential for a bystander effect it is possible that this distribution might be 
adequate to effectively ablate an entire tumour [241, 242]. Dwyer et al. [115] 
also indicated that MSCs may line blood vessels of the tumour. Radioiodide 
ablation of these blood vessels may also be effective in shutting down the 
blood supply to tumours.

Overall these results are very promising and suggest that the MSC-NIS: I\textsuperscript{131} 
combination may have huge potential in the breast cancer setting.
Chapter 6

Results and Discussion

In Vivo Investigation of tumour-targeted MSC migration and engraftment

A. Tracking MSC mediated transgene delivery in vivo

B. Tracking MSC migration, engraftment and persistence in vivo
6.1 Introduction

Upon systemic administration in healthy animals, MSCs have a natural tendency to migrate towards the lungs, liver and bone marrow in favour of other tissues [133]. However, with introduction of a wound to the animal model, the MSC migratory itinerary is redirected in favour of the sites of injury in response to inflammatory signals [133]. Dvorak drew comparisons between the microenvironments of wounds and the tumour stroma, labelling tumours as “wounds that do not heal” [135]. Many of the inflammatory signals implicated in cellular migration to sites of injury can also be released from tumours and it is not surprising that MSCs display the same preferential migration to tumour sites [136, 138].

Using an MDA-MB-231 pulmonary xenograft model of breast carcinoma metastases, Stoff-Khalili et al. [139] demonstrated that MSCs could in fact migrate and efficiently deliver an oncolytic virus to macroscopic nodules of MDA-MB-231 cells in the lungs. The metastases tropism highlighted here is an important result, and along with the observation that the proportion of MSCs engrafted in tumours was not dependent on tumour size [140], suggests that there is strong potential for MSC delivery of therapeutic genes to sites of metastasis irrespective of size. Further studies demonstrated extensive MSC engraftment throughout primary breast tumours and metastases [115][162][163, 164]. However, there has also been some speculation that pulmonary migration of systemically administered MSCs may be caused by trapping of MSCs rather than tumour specific migration [380][142] [381].

Given this potential ability to engraft to cancer metastases, the future of MSC-mediated cancer gene therapy might not be limited only to treating inoperable tumours. It could include the possibility of detection and treatment of tumours or sites of metastases unknown to the surgeon. Unfortunately, while studies have indicated huge potential in using MSCs to deliver therapeutic genes to in situ tumours [81, 141, 142, 215, 222-224, 227-234], there is not a lot of data available on how well the MSC tumour tropism might perform in models of tumour metastasis. A lot of questions remain unanswered. Can MSCs effectively deliver adenoviral transgene expression to target tumours? Are
MSCs capable of migrating comprehensively to all sites of metastasis? Can they migrate and engraft to sites of micrometastasis? Can MSCs deliver therapeutic genes to tumours before transient adenovirus-driven expression diminishes to levels incapable of supporting therapy? The answers to these questions will be critical in the success or failure of MSCs as vehicles for cancer gene therapy, both in terms of safety and efficacy.

To address some of these issues, the migratory itinerary of MSCs engineered to express NIS following systemic administration to animals bearing breast cancer xenografts was investigated. In a second animal study, the migration of systemically administered MSCs was tracked in a metastatic model of breast cancer to determine the engraftment and persistence of MSCs in malignant tissues and to identify the pattern of MSC localisation in non-target tissues.

6.2 Objectives

- **A.** To determine the distribution over time of MSCs infected with Ad5/CMV/NIS after administration in a murine model of subcutaneous breast cancer
  - MSC distribution determined following tissue harvest by RQ-PCR

- **B.** To determine the migratory itinerary of human MSCs in a murine model of metastatic breast cancer established in the mammary fat pad
  - MSC distribution determined by IHC, Flow cytometry and RQ-PCR
Section A

6.3 A. Materials and methods

To determine the distribution over time of systemically injected MSCs infected with Ad5/CMV/NIS compared to naked Ad5/CMV/NIS in response to non-invasive/subcutaneous breast cancer, a murine model was established.

This murine model of breast cancer was established by injecting MDA-MB231 cells in 50% Matrigel/Leibowitz-15 medium subcutaneously into the right flank of female athymic nude mice (n = 24). Having given the murine models adequate time for tumours to develop, mice with tumour volumes > 100 mm³ received either an intravenous injection of Ad5/CMV/NIS or MSC-Ad5/CMV/NIS. Upon injection, MSCs had been infected with Ad5/CMV/NIS for 3 days. Mice were sacrificed 3 and 7 days later (n = 6 per time point) and a selection of tissues were harvested and stored first in RNA-later® and subsequently at -80°C for RQ-PCR analysis. This model was established primarily by Dr Roisin Dwyer with assistance given by the PhD candidate. Subsequent molecular work was performed by the PhD candidate.

These tissues were thawed, homogenised, the RNA was extracted and cDNA was generated. RQ-PCR was then used to determine the levels of human NIS, mouse NIS, and human MPRL19 expression. Human NIS expression represents the presence of Ad5/CMV/NIS infected cells. Mouse NIS acts as a control ensuring that human NIS expression data is not contaminated by mouse NIS expression. Data was expressed relative to mouse beta actin which acted as an endogenous control.

A Basic Local Alignment Search Tool (BLAST) was also used to confirm that the primers used to amplify Human NIS and human MRPL19 don’t overlap with mouse DNA sequences.
6.4 Human MRPL19 expression in tissues following systemic Ad5/CMV/NIS administration

Human MRPL19 was detected in tumours at 3 and 7 days after administration of both Ad5/CMV/NIS and MSC-Ad5/CMV/NIS (Table 6.1). This highlights the presence of human-derived cells in the tumour tissue and was expected as the tumours are derived from the human cell line MDA-MB231.

No human MRPL19 was detected in non tumour tissue harvested from mice treated with Ad5/CMV/NIS. This indicates that in the tissues tested there are no metastases confirming that the primary tumour remained in situ.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Intravenous Ad5/CMV/NIS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>Tumour</td>
<td>+</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
</tr>
<tr>
<td>Lungs</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
</tr>
<tr>
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<td>Stomach</td>
<td>-</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>-</td>
</tr>
<tr>
<td>Large Intestine</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.1 Human MRPL19 gene expression in tissues harvested from murine breast cancer models 3 and 7 days after systemic administration of Ad5/CMV/NIS. (-) Not detectable, (+) detectable.
6.5 Human NIS expression following systemic Ad5/CMV/NIS administration

A BLAST search was used to search similar sequences in the human NIS primer which were highly similar to sequences in the mouse genome. This resulted in no significant similarity so this primer was ideal for amplifying specifically human NIS cDNA. No human NIS (hNIS) expression was detected in tumour tissue 3 or 7 days after systemic administration of Ad5/CMV/NIS (Table 6.2). This indicated that the virus alone, in the absence of hMSCs, did not reach the tumour site following systemic delivery. hNIS was also absent from the spleen, kidneys, stomach and intestines. hNIS was however detected at day 3 in the lungs, heart and liver but not at day 7.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Intravenous Ad5/CMV/NIS</th>
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<tbody>
<tr>
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<td>Tumour</td>
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<td>+</td>
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<td>Small Intestine</td>
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</table>

Table 6.2 Human NIS expression in tissues harvested from murine breast cancer models 3 and 7 days after systemic administration of Ad5/CMV/NIS. (-) Not detectable, (+) detectable.
6.6 Human MRPL19 expression following systemic administration of Ad5/CMV/NIS infected MSCs

Three days following administration of MSCs infected with Ad5/CMV/NIS instead of naked Ad5/CMV/NIS, robust expression of human MRPL19 gene was detected in tumours (Table 6.3). This was expected due to the human origin of the tumours. However, robust expression was also observed in the heart and lungs of these animals, suggesting the presence of human cells. Weak MRPL19 expression was also detected in the large intestine. Seven days following injection, hMRPL19 expression had fallen to low levels in the heart and lungs but remained the same in tumour tissue. The low level of hMRPL19 expression in the large intestine was consistent over both time points. No hMRPL19 expression was observed in the liver, spleen, kidneys, stomach or small intestine.

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<th>Tissue</th>
<th>Intravenous MSC-Ad5/CMV/NIS</th>
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<td>Day 3</td>
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<tr>
<td>Tumour</td>
<td>Ct &lt; 30</td>
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<tr>
<td>Heart</td>
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<td>Large Intestine</td>
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Table 6.3 Human MRPL19 gene expression in tissues harvested from murine breast cancer models 3 and 7 days after systemic administration of MSC-Ad5/CMV/NIS. (-) Not detectable, (Ct > 30) low levels of gene expression detected, (Ct < 30) more robust gene expression detected.
6.7 Human NIS expression following systemic administration of Ad5/CMV/NIS infected MSCs

Strong levels of human NIS expression were detected in the heart and lungs of mice 3 days after systemic administration of MSC-NIS (Table 6.4). The persistence of this expression was not very robust, declining to low levels at day 7. Similarly, a low level of NIS expression was observed in the liver at day 3 and had declined to undetectable levels at day 7 following injection of NIS-expressing MSCs. However, robust human NIS expression was observed in tumour tissue both at day 3 and 7. A persistent low level of hNIS expression was also observed in the small intestine. hNIS was undetectable in the spleen, kidneys, small intestines and stomach of these animals at both time points.

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<td>Large Intestine</td>
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Table 6.4 Human NIS gene expression in tissues harvested from murine breast cancer models 3 and 7 days after systemic administration of MSC-Ad5/CMV/NIS. (-) Not detectable, (Ct > 30) low levels of gene expression detected, (Ct < 30) more robust gene expression detected.
Section B

6.8 Materials and Methods

The previous murine model investigated the distribution over time of systemically administered MSCs infected with Ad5/CMV/NIS compared to naked Ad5/CMV/NIS after administration in a murine model of non-invasive breast cancer. It is also critical to determine the migratory itinerary of human MSCs in response to metastasised breast cancer. Do MSCs localise at critical organs? Do MSCs engraft at all discernible sites of metastasis? Do MSCs become trapped in the lungs as previously described [142, 381]? This will have significant implications both in terms of the efficacy and safety of any MSC mediated gene therapy of metastasised cancer.

To investigate this, a murine model of metastatic breast cancer was established by injecting MDA-MB-231 cells into the mammary fat pad of athymic nude mice. Animals were monitored and maintained until the presence of primary tumours was established and metastasis was confirmed. Anaesthetised animals were then administered PKH26 labelled MSCs through tail vein injection. Animals were assigned to groups designated for sacrifice at 1, 2, 3 and 4 weeks after MSC-PKH26 injection. Animals from each week were also subdivided into candidates designated for analysis by flow cytometry, fluorescent microscopy or gene expression analysis. Upon sacrifice, tumours and nodes (where available) were harvested as well as heart, lungs, liver, spleen and kidney tissue. Depending on the designated analysis method, tissue from each mouse was stored in media, paraformaldehyde or RNAlater® as described in section 2.10.11.

Tissues analysed by flow cytometry were digested using collagenase and physical manipulations. Cells were then fixed using paraformaldehyde and stored in PBS. Cells were analysed for evidence of a PKH26 signal as well as the surface marker CD105 (present on MSCs) and MHC1 (present on all human cells).

Tissues designated for fluorescent microscopy were fixed in paraformaldehyde, frozen in a liquid nitrogen isopentane bath and cryosectioned @ -25°C under
low light conditions to a thickness of 5µm. Slides were DAPI stained to highlight cellular nuclei and subsequently observed using blue (DAPI) and yellow (PKH26) filters.

Gene expression analysis was performed on RNAlater® stored tissues. The tissues were homogenised, RNA extracted, cDNA was generated and RQ-PCR performed targeting mouse HPRT and human PPIA.

6.9 Fluorescent microscopy to investigate engraftment of PKH26 labelled MSCs in tissues harvested from murine models of breast cancer.

A control animal which received 50 µl α-MEM instead of PKH-26 labelled MSCs was sacrificed 2 weeks after injection. As can be seen in Figure 6.1, DAPI staining clearly highlights the presence of nuclei within the tissue section. There was no evidence of PKH26 fluorescence observed in enlarged node, lung, heart liver, kidney or spleen tissue harvested from this animal. This confirmed that no autofluorescence occurred in the tissue sections which could be confused with PKH26 fluorescence. Tumour tissue was unavailable for this animal.

The pattern of DAPI staining in kidney tissue is noticeably different to that of other tissues. This is due to the natural arrangement of glomeruli, Bowmans capsules and convoluted tubules and is not an unusual appearance for DAPI stained nuclear material in Kidney sections (Figure 6.1c).

Lung and heart tissue from a second control animal was also examined at week 3 resulting in a similar absence of PKH26 fluorescence.
Figure 6.1 Fluorescent imaging of tissue harvested from control mouse, sectioned and counterstained with DAPI (blue) to highlight nuclei. Control mice did not receive PKH26 labelled MSCs. Images taken at 100x magnification. a) node, b) lungs, c) heart, d) liver, e) kidney, f) spleen.
Kidney and spleen tissues were analysed from mice sacrificed 1 and 2 weeks after administration of PKH-26 labelled MSCs (Figure 6.2). While nuclei are clearly highlighted through DAPI staining, no MSC-specific PKH26 fluorescence was observed at either time point. Since it was most likely that human MSCs would appear in tissues at these earlier time points following systemic injection the possibility of engrafted human MSCs in kidney and spleen tissue at later time points was also discounted.

![Figure 6.2](image)

**Figure 6.2** Fluorescent imaging of kidney and spleen tissue harvested from mice up to 2 weeks after systemic administration of PKH26 labelled MSCs. Tissues were sectioned and counterstained with DAPI (blue) to highlight nuclei and images were taken at 100x magnification. **a)** Kidney, Week 1, **b)** Kidney Week 2, **c)** Spleen Week 1, **d)** Spleen, Week2.
Liver tissues harvested 1 and 2 weeks after systemic administration of PKH26 labelled MSCs were also DAPI stained and inspected for PKH26 fluorescence. However, as with kidney and spleen tissues, despite observation of very clear images of DAPI stained nuclei there was no evidence of MSC-specific PKH26 fluorescence at either time point in liver tissue (Figure 6.3).

![Week 1 and Week 2](image)

**Figure 6.3** Fluorescent imaging of liver tissue harvested from mice up to 2 weeks after systemic administration of PKH26 labelled MSCs. Sectioned tissues were counterstained with DAPI to highlight nuclei. Images were taken at 100x magnification.

There was strong evidence of MSC-specific PKH26 fluorescence in tumour tissue from animals harvested 1 week after systemic injection of MSCs. This appears as a pink/red fluorescence with an even distribution in the tumour represented by Figure 6.4a.

Enlarged nodes were also harvested from mice sacrificed at this time point. Again MSC-specific PKH26 fluorescence was observed. In this instance the fluorescence appeared to be less evenly distributed and localised around a small area within the node section shown in Figure 6.4b.

Although very faint and sparse, PKH26 signals were also detected in lung tissue analysed at this time point (Figure 6.4c).

Heart specimens from mice displayed evidence of MSC engraftment with a low frequency of PKH26 fluorescence to be seen amongst their striated morphology (Figure 6.4d).
Figure 6.4 Fluorescent imaging of tissue harvested from mice one week after systemic administration of PKH26 labelled MSCs. Tissue sections were counter stained with DAPI to highlight nuclei. Images were taken at 100x magnification. a) Tumour, b) Node, c) Lungs, d) Heart.
DAPI stained sections of tumour, node, heart and lung tissue harvested from mice sacrificed 2 weeks after systemic administration of PKH26 labelled MSCs are represented in Figure 6.5. There is very strong evidence that PKH26 labelled MSCs are present in this tissue. Upon close inspection the fluorescent image of tumour is dappled with a high frequency of pink/red fluorescence in an even distribution. No enlarged nodes were harvested from either animal.

PKH-26 signals were again observed in the lungs of mice analysed. Heart specimens also displayed evidence of MSC-specific PKH26 fluorescence. However, as with the earlier time point, PKH26 fluorescence was faint and was very sparsely distributed.

Where tumour and lung tissue were concerned, the frequency of PKH26 fluorescence observed in tissues 2 weeks after administration of PKH26 labelled MSCs, appeared to be greater than at week 1. This is particularly true of tumour tissues.
Figure 6.5 Fluorescent imaging of tumour, lung, and heart tissue harvested from mice 2 weeks after systemic administration of PKH26 labelled MSCs. All sections were counter stained with DAPI to highlight nuclei. Images were taken at 100x magnification.
Palpable tumours were not observed in mice assigned for the 3 week time point although disease was present as evidenced by enlarged lymph nodes. Enlarged lymph nodes, which are very likely cancerous were recovered from mice. Along with these specimens, heart and lung tissue sections were DAPI stained and observed under fluorescent microscopy.

Nodes from both mice showed very clear evidence of MSC-specific PKH26 fluorescence indicating the presence or engraftment of PKH26 labelled MSCs 3 weeks after systemic administration (Figure 6.6). The distribution of this fluorescence appeared to be concentrated in small clusters rather than having an even distribution through the tissue. This may represent engraftment of MSCs around vasculature.

Unlike at earlier time points there was no evidence of MSC-specific PKH26 fluorescence in either lung or heart tissue (Figure 6.6).
**Figure 6.6** Fluorescent imaging of enlarged node, lung, and heart tissue harvested from mice 3 weeks after systemic administration of PKH26 labelled MSCs. All sections were counter stained with DAPI to highlight nuclei. Images were taken at 100x magnification.
As with week 3, palpable tumours were not observed in the mice assigned for sacrifice 4 weeks after administration PKH26 labelled MSCs. Again however, enlarged nodes were available and together with heart and lung were interrogated for PKH26 fluorescence amidst a DAPI counterstain.

While there was little in the way of PKH26 fluorescence to be seen in either heart or lung tissue (Figure 6.7b and c), nodal tissue presented with MSC-specific fluorescence indicative of the presence of human MSCs (Figure 6.7a). The distribution of PKH26 fluorescence was similar to that in previous nodes, localised around one small area as opposed to a homogenous distribution.
Figure 6.7 Fluorescent imaging of enlarged node, lung, and heart tissue harvested from mice 4 weeks after systemic administration of PKH26 labelled MSCs. All sections were counter stained with DAPI to highlight nuclei. Images were taken at 100x magnification.
6.10 Detection of human and mouse specific transcripts by RQ-PCR

Murine models of metastatic breast cancer were treated systemically with human MSCs and sacrificed 7, 14, 21 and 28 days post injection. Tumours and enlarged nodes (where available), as well as lung, heart, liver, spleen and kidney tissues were harvested from at least 2 sacrificed animals per time point. Also included were control animals not in receipt of human MSCs. cDNA was generated from the mRNA template extracted from each tissue and was interrogated using probes for murine HPRT, an endogenous control present in mouse cells and human PPIA, an endogenous control present in human cells. Expression of human PPIA could indicate the presence of either MDA-MB231 cells or human MSCs.

One week following MSC injection into this metastatic breast cancer model, robust levels of PPIA were detected in tumour tissue indicating the presence of human cells (Table 6.6). Human cells were also observed in other tissues through medium levels of PPIA expression in the enlarged nodes and lungs, with lower levels observed in the heart and liver. No PPIA expression was detected in the kidney or spleen indicating the absence of human cells. PPIA expression in the tumour was observed against a backdrop of lower HPRT expression, highlighting that the tumour was predominantly composed of human cells. Robust mouse-specific HPRT expression was observed in the remaining tissues.
Following analysis of tissues from the remaining animals assigned for RQ-PCR, further human PPIA transcripts were not detected, which was unexpected. Consistent levels of HPRT were detected in the analysed tissues. Increasing the mRNA template from 1µg to 5 µg did not result in detectable levels of PPIA. Since HPRT was reliably detected it appears that cDNA quality was not an issue. Further to this, the quality of the mRNA template was also confirmed with good concentrations of RNA recovered, and absorbance ratios of 260/280 $\geq$ 1.8 and 260/230 between 2.0-2.2. The functionality of the human PPIA probe was confirmed by applying it to a sample with known levels of PPIA expression. Further controls included the use of a no template control, to eliminate doubts about false positives arising from contamination as well as the stringent inclusion of triplicate data which had to be within a standard deviation of $\leq$0.3 of each other.
6.11 Flow-cytometry based analysis of cellular components of ex vivo murine tissue samples

Tissues from murine models of metastatic breast cancer were harvested 1, 2, 3 and 4 weeks after systemic injection of MSCs. The tissues were digested to form a single cell suspension, incubated with human-specific MHC1 and CD105 antibodies, and analysed by flow cytometry. Human MHC1 indicates the presence of human cells while human CD105 indicates the presence of MSCs. Isotype controls were also run in conjunction with each antibody to account for nonspecific fluorescence. The raw data retrieved was analysed using Flowjo analytical software. Events were observed in a scatter plot on the basis of Red1-log fluorescence (CD105) and NIR2-Log (MHC1). Using the relevant isotype control for each sample, a quadstat was established delineating positive and negative zones for Red1 and NIR2 fluorescence. Figure 6.8 highlights an example of such for tissue harvested from a mouse sacrificed 2 weeks after injection of MSCs. It includes Red1-Log and NIR2-log fluorescence scatter plots for tumour, lung and heart tissue cell suspensions incubated with antibodies or isotype controls. In each chart double positive fluorescence was represented by the top right section of each quadstat. As shown in the calculations table in Figure 6.8, the true percentage of double positive fluorescent cells in each tissue was established by subtracting the double positive value in the control sample from the test sample. A similar process was undertaken in order to establish the percentage of NIR2-Log (MHC1) positive, Red1 (CD105) negative cells. MHC1 negative cells were then calculated by adding the true percentages of both MHC1 positive populations and subtracting from 100. For example, within the tumour harvested at week 2 (Figure 6.8), the CD105: MHC1 positive and MHC1 positive populations account for 3.7 % and 75.8 % of the total population. However, when corrected for nonspecific staining observed with isotype controls these values become 3.1 % and 73.8 %. The MHC1 negative population is then calculated by subtracting 3.1 % and 73.8 % from 100 % to give 23.1 %.
Figure 6.8 Format for calculating the percentage MHC1 positive, MHC1 and CD105 positive, and MHC1 negative cells in digested tissue specimens from MSC treated mice bearing metastatic breast cancer. All data was corrected for isotype control positivity. Tumour, lung and heart tissue harvested from a mouse 2 weeks after systemic injection of MSCs is used as an example.
When this format was applied to all relevant specimens, the percentages of CD105:MHC1 double positive (indicating human MSCs), MHC1 positive (indicating MDA-MB-231 cells) and MHC1 negative cells (indicating non-human cells) were plotted in a stacked bar chart shown in Figure 6.9. It is important to highlight first that lung and heart tissue from the control animal had a CD105:MHC1 double positive population which would be indicative of the presence of human MSCs. However, since no human MSCs were administered in this animal these double positive subpopulations (0.4 % in lungs, 0.5 % in heart) are clearly an indication of the potential error built into this analytical process rather than the presence of engrafted human MSCs. As such, only populations greater than 0.5 % positive were considered significant.

The tissue type with the highest percentage of human MSCs as indicated by CD105:MHC1 double positivity was tumour. A hMSC population of 3.6 % was observed in a tumour analysed one week after systemic injection of MSCs. Not all cells were analysed in each tissue digest due to time and cost restraints. However, by applying this percentage to the total number of cells present in the tumour digest it is estimated that over $9 \times 10^5$ PKH26 labelled hMSCs were present in the tumour. However, a second tumour processed from an alternate mouse at this time point had a much lower hMSC population, accounting for 1.4 % of the total tumour (Estimated $1.7 \times 10^4$ hMSCs). Tumours harvested at week 2 had hMSC subpopulations accounting for 3.1 % (Estimated $3.8 \times 10^5$ PKH26 labelled MSCs) and 3.0 % (Estimated $2.1 \times 10^5$ hMSCs) of the total tumour, while at week 3, a tumour presented with a 2.0 % hMSC population (Estimated $1.5 \times 10^4$ hMSCs).

The proportion of hMSCs found in lung tissue relative to total cells analysed was consistent over the 3 weeks (1.0-1.4 %) with the exception of lungs harvested at week 2 which was relatively high in comparison (2.3 %) (Estimated $2.7 \times 10^5$ hMSCs). Lungs from a second animal had approximately $2.8 \times 10^4$ hMSCs. Two pairs of lungs harvested at week 1 had an estimated $6.1 \times 10^4$ and $2.2 \times 10^4$ PKH26 labelled MSCs. Lungs from week 3 had $2.4 \times 10^4$ hMSCs.
Where heart tissue was analysed the hMSC population was relatively low (1.1 %) (Estimated $2.6 \times 10^3$ PKH26 labelled MSCs) at week 3 and comparable to the control levels at week 2 (0.7 %) (Estimated $1.1 \times 10^3$ PKH26 labelled MSCs).

The MHC1 positive, CD105 negative population of cells which indicates the presence of MDA-MB-231 cells confirmed that each tissue analysed was malignant. This confirms the effectiveness of establishing a metastatic breast cancer model by injecting the mammary fat pad with MDA-MB-231 cells.

It is interesting that the tumour with the smallest subpopulation of MDA-MB231 cells also had the smallest population of hMSCs. In fact these 2 values appear to correlate in each of the tumour tissues analysed. However, since the sample size is quite small a statistical tool cannot be used to confirm this potential relationship.
Figure 6.9 Percentage of MHC1 positive (MDA-MB231), MHC1 and CD105 positive (hMSC), and MHC1 negative (Murine) cells in tumour, lung and heart specimens from hMSC treated mice bearing metastatic breast cancer. Control mouse not in receipt of hMSCs was included at week 3.
6.12 Discussion

The current chapter describes analysis of MSC distribution in breast tumour bearing animals using a variety of approaches. Engraftment in tumour and non-target tissue was determined over time, along with a comparison of efficiency of gene delivery between administration of adenovirus alone, compared with adenovirus-infected MSCs. In the first in vivo study, in situ models of breast cancer were treated with Ad5/CMV/NIS. Here, it can be assumed that all of the MRPL19 expression is accounted for by the human MDA-MB 231 cells which initiated the tumour since Ad5/CMV/NIS doesn’t carry this gene and animals did not receive MSCs. The absence of human MRPL19 in tissues other than the primary tumour is evidence that metastasis of the human component of the tumour did not occur in these mice. This is also supported by the absence of enlarged nodes in all animals. Where MSC-Ad5/CMV/NIS cells were administered, it can be assumed that the larger proportion of human MRPL19 expression originates from MDA-MB-231 cells but a smaller amount could also stem from engrafted MSCs. This strong MRPL19 signal emphasises the successful generation of a tumour model derived from human cells.

The lack of hNIS expression in the tumour tissue of these animals treated with Ad5/CMV/NIS is important. It definitively highlights the inability of naked Ad5/CMV/NIS in targeting NIS expression to tumour tissue for the imaging and therapy of breast cancer. This lack of tumour-specific tropism is a major obstacle to systemic administration of adenoviral-mediated cancer gene therapy. Additionally, where hNIS expression did occur, it was found in tissues such as heart, liver, and lungs. If patients were exposed to I\(^{131}\), following this profile of expression there is a strong probability of long term adverse effects for the recipient. The transient expression of hNIS resulting from naked Ad5/CMV/NIS administration is also very clear, since the low level expression observed at day 3 was gone by day 7. This is not a surprise since Ad5/CMV/NIS is a replication-deficient, non-integrating virus. The highly immunogenic nature of adenovirus is unlikely to impact this study since the murine models lack a thymus and so are deficient in T-cells and do not produce cytotoxic effector cells. However, when applied to a human model strong immune responses can be expected which would significantly impact
persistence of therapeutic gene expression [104, 382, 383]. Based on this data, three clear issues are confirmed with the use of naked Ad5/CMV/NIS; lack of tumour targeting, infiltration of unintended targets, and an apparent lack of persistent transgene expression where it occurs. To resolve these issues Ad5/CMV/NIS was introduced into MSCs. It was hoped that the ability of MSCs to migrate to tumour sites [136], their immune privilege status [117] as well as the capacity for extending the persistence of transient Ad5/CMV/NIS transgene expression [384] may remedy these issues.

Robust expression of hMRPL19 was expected in the tumour since this tissue is derived from MDA-MB-231 cells. As a result, the presence of hMSCs can neither be confirmed nor discounted in tumour tissue based on hMRPL19 expression alone. However, since the murine model of breast cancer employed was non-invasive, hMRPL19 expression detected in heart and lung tissue was more likely to have been produced by systemically administered MSCs. Additionally, hMRPL19 expression was not observed in the heart and lungs of animals that did not receive MSCs. hMRPL19 expression decreased by day 7 in the heart and lungs. Data showing the distribution of human NIS expression following systemic administration of Ad5/CMV/NIS infected MSCs indicated that for the most part, hMRPL19 expression was collocated with hNIS. This strongly supports the presence of hNIS expressing MSCs in such tissues. The observation of hMSCs residing in lungs shortly following systemic administration is supported by a previous report by Kidd et al. which tracked MSCs expressing luciferase after I.V. injection in mice [116]. It was also suggested that MSCs egress from the lungs over time resulting in a decreasing signal. In this study, levels of NIS at day 7 confirm an egress of hMSCs from initial residence in both the lungs and heart and confirm the persistence of MSCs at the tumour site implying engraftment of hMSCs. The prolonged residence of MSCs at tumour sites is also strongly supported by observations with gliomas [142]. Nakamizo et al. [142] demonstrated that injection of hMSCs in the carotid artery of mice bearing glioma xenografts lead to tumour engraftment up to 7 days after injection. Some hMSC filtering was observed in the lungs which is not an unusual observation [234, 381]. This pulmonary trapping of hMSCs was much higher following a tail vein injection of hMSCs
into the same model and lead to significantly reduced tumour localisation
highlighting the value of choosing the right vasculature for hMSC delivery. It
is possible that further time points, if performed, might demonstrate hMSC
egression from the lungs and show a continued presence of MSCs in the
tumour tissue. In fact, additional work performed in this laboratory by Dwyer
et al. [384] highlighted weak $^{99m}$Tc (a surrogate of iodide) accumulation at the
tumour site 3 days after administration of MSC-NIS using a SPECT $\gamma$-camera.
This tumour $^{99m}$Tc accumulation, attributable to engrafted MSC-NIS cells
strengthened over time and was still evident at day 14, at which point there was
no apparent uptake in non-target tissues including lungs.

The ability to track in real time, the distribution of systemically administered
MSC-NIS cells using $^{99m}$Tc highlights the benefits of this approach in
predetermining the tissues potentially targeted by subsequent $^{131}$I treatment.
This has obvious benefits in terms of patient safety but may also provide a
means of tracking cancer progression in a minimally invasive fashion.

Concerning the initial distribution of MSCs to the heart, lungs and sites of
inflammation (e.g. Tumours, wounds) it is interesting to note that either
ketamine-xylazine or gaseous isoflurane anaesthesia was used in this and the
referenced studies [116, 142]. Both ketamine and isoflurane are known to have
anti-inflammatory effects [385-389] and concurrent anaesthesia at the time of
MSC administration may temporarily mute inflammatory signals from wounds
or tumours. This may result in delayed mobilisation of MSCs to inflammatory
sites. An exaggerated version of this effect has been shown previously where
MSC suppression of pancreatic tumours was reversed by introduction of anti-
inflammatory treatment potentially through downregulation of CCL3 and
CCL25 [141]. Both chemokines are known to be involved in MSC migration
[390, 391]. This represents a potential confounding issue with MSC migration
studies in murine models which would not be encountered in human patients
since intravenous administration of MSCs wouldn’t require anaesthesia. It
should however be carefully considered in the design of further studies with the
introduction of anaesthetics with less potential for suppression of inflammatory
signals.
It is interesting that hNIS was detected in the liver at day 3 but without a corresponding hMRPL19 signature after I.V. injection of MSCs infected with Ad5/CMV/NIS. This suggests the presence of naked Ad5/CMV/NIS in liver tissue possibly due to leakage from or death of MSCs. The low level hNIS expression at day 3 and absence of NIS expression 7 days after administration of MSC-NIS mirrors the hNIS/hMRPL19 expression profile of naked Ad5/CMV/NIS shown earlier. Evidence of free virus in the liver highlights the importance of delivering viable healthy Ad5/CMV/NIS transfected MSCs to the patient in order to avoid potential toxicity in untargeted tissues.

hMRPL19 expression was detected at a low levels in the large intestine 3 and 7 days after systemic MSC injection indicating a persistent low level MSC presence. Persistent low level hNIS expression over 7 days further supports evidence of MSC migration to the large intestine. This may be due to hMSC taking up residence in the natural MSC niche in the lamina propria of the large intestine [392, 393]. Dwyer et al. [384] highlighted $^{99m}$Tc in the intestines 3 days after administration of MSC-NIS cells but this had dissipated by day 14 suggesting that low level of MSC –NIS occupation of the large intestine does not persist beyond 7 days after intravenous injection.

In the second in vivo study, wherein MSCs were administered systemically in a metastatic model of breast cancer, it was observed using fluorescent imaging that PKH26 labelled hMSCs were present in the lungs, heart, enlarged node and tumour tissue of mice one week after they were administered. As mentioned previously, the observation of hMSCs in lung tissue after systemic injection in mice is not unusual [116]. Nakamizo et al. [142] highlighted pulmonary trapping of hMSCs following intravenous injection in a murine glioma model. The study by Kidd et al. [116] suggested that hMSCs egress to the liver after 3 days but there was no evidence of hMSC liver engraftment in this metastatic model. Kidd et al. [116] also suggested this is most likely because hMSCs mobilised from the lungs move preferentially to sites of injury, in this case, malignant tissue in mice. There was very clear evidence of hMSCs localised to tumour and enlarged nodal tissue. The frequency of PKH26 fluorescent hMSCs was highest in tumour tissue, followed by enlarged nodal tissue compared to a much lower frequency in the lungs and heart. Inspection
of tissue sections harvested from animals 2 weeks after systemic injection of hMSCs showed that the distribution frequency of MSCs increased in both tumour and lungs while staying the same in the heart tissue. However, at further time points (Week 3 and 4) lung tissue appeared to be completely clear of hMSCs. The delay in egress from the lungs at week 2 may be indicative of engraftment in lung metastases in the mice analysed. This is not an unreasonable assumption since MSCs have been previously been shown to migrate and engraft onto pulmonary metastases of an MDA-MB231 SCID mouse model of breast cancer resulting in suppression of metastases through virotherapy [139].

The appearance, at a low frequency, of hMSCs in the hearts of mice 1 and 2 weeks after systemic administration is interesting. This is also supported by the observation of hMRPL19 and hNIS expression in heart tissue 3 and 7 days after systemic administration of MSCs expressing human NIS in the non-invasive model of breast cancer described earlier in the chapter. A decline in expression of both these genes from day 3 to 7 indicated the mobilisation of hMSCs from heart tissue in this model. It is interesting that this trend is also reflected in the metastatic model whereby hearts harvested from animals 3 and 4 weeks after administration of hMSC showed no evidence of hMSC engraftment. It is possible that the small presence of hMSCs in the heart may be attributable to the intravenous method of administration leading to most hMSCs passing through the heart. This high level of MSC traffic through the heart might present the opportunity for a small number of cells to transmigrate across the vasculature of the heart. Evidence of hMSC migration to normal heart has been reported by Jasmin et al. [394] in a mouse model of Chagas disease which causes cardiomyopathy. There, it was clearly demonstrated that 2 days after tail vein injection of fluorescently labelled hMSCs, significantly increased fluorescence could be observed in the hearts of diseased animals. More specifically they also showed that there was a slight increase of fluorescence in the healthy hearts of control animals indicating that some hMSC migration does also occur to normal healthy hearts. While tumours were not available for week 3 and 4, enlarged nodal tissue was. Considering that this was a metastatic model of breast cancer, there is a strong probability that
the nodes were enlarged due to malignancy although this was not confirmed. The presence of hMSCs in these nodes is a strong indication of the potential for MSCs to collocate with sites of metastases up to four weeks after administration. A similar model of pancreatic cancer supports this potential by demonstrating co-localisation of hMSCs with a primary tumour and sites of metastasis in mice through overlapping fluorescent signals from rLuc expressing PANC-1 cells and red fluorescent protein labelled hMSCs [141]. This observation is further supported by Dwyer et al. [115] who also described engraftment of fluorescent PKH-26 labelled MSCs throughout multiple solid tumours that had formed in the nodes of murine models as well as in localized primary tumours.

Based on flow cytometry data which indicated the proportion of mouse, MDA-MB231 and hMSC in analysed tissue, metastasis of MDA-MB231 cells was present in each tissue analysed confirming the model of metastasis. Given that control tissues not in receipt of hMSCs showed a baseline level of 0.5 % positivity for hMSCs only levels higher than this were considered significant. Interestingly hMSC levels in lung tissue over 3 weeks were consistently higher than the baseline level observed in control tissue. A slight peak in hMSCs levels of 2.4 % was observed in one mouse at week 2 may support the higher week 2 hMSC distribution observed earlier through fluorescent microscopy. While the presence of hMSCs does appear to persist in lungs up to 3 weeks after administration of hMSCs this appears to coincide with the presence of MDA-MB231 cells. If this were the case it would certainly represent a positive attribute in terms of MSC-NIS treatment of cancer metastasis.

The largest hMSC subpopulation of 3.6 % was observed in a tumour analysed one week after systemic injection of MSCs. Estimates of hMSCs numbers indicate that over 9 x 10^5 MSCS were present in this tumour representing the majority of the MSCs introduced through tail vein injection. The fact that over 9/10 hMSCs reached this primary tumour is very promising. However, a much smaller subpopulation of 1.4 % (Estimated 1.7 x 10^4 PKH26 labelled MSCs) was seen in a tumour of second mouse analysed at this time point indicating the variability of this model. It will be important for future studies to identify the cause of this variation and the fate of hMSC which are unaccounted for. The
variation between these two models may be accounted for by the size of the MDA-MB231 population whereby the tumours with the smaller subpopulation of MDA-MB231 cells also had the smaller population of hMSCs and vice versa. Much larger numbers of tumours would be needed to confirm this relationship. The substantial hMSC population observed in tumours appeared robust with tumours harvested at week 2. In fact, hMSCs accounted for 3.1 % and 3.0 % of the total cell population in these digested tumours. At week 3, hMSCs accounted for 2 % of the total cells found in a tumour.

While the evidence from RQ-PCR, flow cytometry and fluorescent microscopy data described in this chapter delivered useful information which supports the potential for MSC-NIS treatment of breast cancer, a number of limitations and issues were encountered in the methodology.

Where fluorescent microscopy was used to identify PKH26 labelled MSCs there was no data provided on co-localisation of MDA-MB231 cells which might have shown stronger evidence of a tropism for metastases. Another issue was the use of snap frozen OCT embedded rather than paraffin embedded tissue sections. Paraffin embedded sections maintain tissue architecture more clearly than snap frozen sections embedded in OCT and as such it may have been easier to associate MSC engraftment with specific tissue features where clustering of MSCs was observed. However, with improved tissue architecture may come the potential negative effect of masking binding sites associated with paraffin embedding.

Where flow cytometry was used a number of problems were encountered. The biggest issue was caused by autofluorescence of single cell suspensions of digested tissue. This autofluorescence attributed to the presence of flavenoids produced a very strong green fluorescent signal. This not only limited the use of antibodies labelled with green fluorescent probes, but may also have obscured the PKH26 red fluorescence which despite the name has peak emission in the yellow spectrum, adjacent to green. The lack of a PKH26 signals necessitated the use of fluorescent antibodies to detect the presence of hMSCs. No commercially available anti-human CD105 (marker MSCs) had been confirmed not to bind to mouse CD105 so an anti human MHC1 antibody
was used to identify the human population in tissue digests. This approach helped to highlight MSC and MDA-MB231 populations within digested tissue sections but not with the clarity and certainty that had been hoped for.

To address the issues highlighted with both fluorescent microscopy and flow cytometry in this study a relatively easy modification could be introduced which may deliver data which is better defined in terms of cell specificity, tissue architecture and co-localisation of human cells in the mouse model. Firstly, instead of MDA-MB231 cells, a modified MDA-MB231 cell line stably expressing a Red florescent protein (RFP) such as tdTomato (peak emission 581nm) could be used to establish the metastatic cancer model [395, 396]. Following that, MSCs could be labelled using lipophilic tracers which fluoresce at a higher peak emission than the RFP used such as Invitrogen’s DiD (peak emission 665 nm) or DiR (peak emission 780 nm) [397, 398]. Since the use of antibodies may not be needed to identify MSC and MDA-MB231 cells in this instance, there would be no requirement to use snap frozen tissues for better antigen exposure. As such, where fluorescent microscopy is concerned paraffin embedded tissues could be used leading to better tissue section architecture, and fluorescent MDA-MB231 and hMSCs could be detected and their spacial relationship elucidated over time. Tissue analysed by flow cytometry could provide absolute quantification of MDA-MB231 and MSCs. If needed, known MSCs expressing RFP could be characterised on the basis of antibody targeting of MSC markers either concurrently or following isolation of this subpopulation using a cell sorter.

Fluorescent microscopy has shown in this study that hMSCs migrate to tumour sites and sites of metastasis. When further studies were performed in this lab Dwyer et al. [384], showed that systemic administration of the same dose of hMSCs expressing NIS in combination with radioiodine could be used to visualise and treat tumours in an in situ model of breast cancer. This indicates that the efficiency of the natural MSC tumour tropism may be adequate to facilitate an anticancer strategy in an in situ model. However, in a metastatic model, the same dose of hMSCs may be stretched over a wider number of malignant sites potentially reducing the therapeutic effect. This makes it hugely important to optimise the tumour targeting capacity of hMSC, ensuring that as
many of them as possible deliver their therapeutic payload to a malignant site. Approaches have already been described which may facilitate this optimisation. MSCs are known to migrate to sites of inflammation and it has been shown that tumour irradiation results in inflammatory signals CCL2, CCR8, TNFα, and platelet derived growth factor, which in turn enhance hMSC migration to tumour sites [156]. This was further supported by observations of increased hMSC tumour-specific migration following irradiation of breast and colon xenograft models [140]. In this context, delivery of an initial round of hMSC-NIS mediated radiation to malignant tissue could potentially provide the inflammatory signals necessary for increased and more efficient hMSC-tumour targeting in subsequent rounds of therapy. In an alternative approach manipulation of the hMSC cell surface has been considered in order to improve upon the natural tumour tropism [158, 159, 399]. Introduction of epidermal growth factor receptor (which binds to transforming growth factor alpha and epidermal growth factor) into hMSC led to increased migration to glioma and melanoma sites in vivo [159]. By immobilizing sialyl Lewis X onto MSCs, Sarkar et al. [158, 399] showed it was possible to increase cell rolling on a P-selectin surface which may facilitate better mobilisation of MSCs across vasculature on their migratory path to sites of inflammation. Another possible approach has been described whereby epigenetic upregulation of urokinase plasminogen activator enhanced the tumour tropism of umbilical cord blood and bone marrow derived MSCs in an in vitro model [160]. Lastly it is important to highlight how subpopulations of MSCs can have different migratory itineraries. Bensidhoum et al. [161], highlighted how a Stro-1 positive population of MSCs preferentially migrated to spleen, muscles, bone marrow and kidneys while Stro-1 negative cells were found in the lungs in a diabetic mouse model. Further investigation of MSC subpopulations on the basis of clearly defined markers may help identify a set of hMSCs with a higher tropism for cancer tissues than standard heterogeneous MSC populations.

The persistence of hMSCs in enlarged nodes and the absence in other tissues indicate the potential for selective targeting of malignant tissues harbouring MSCs after 2 weeks while other tissues remained relatively unharmed. This
approach requires the use of a gene therapy whereby the deleterious effects of the therapy can be delayed until the MSCs are appropriately engrafted. Such an approach might suggest the use of enzyme-prodrug strategies but the optimal candidate for this role is NIS. Potentially, administration of radioactive iodide ($I^{131}$) for uptake by hMSCs expressing NIS may be performed at an appropriate time in the migratory itinerary of MSCs for specific ablation of malignant tissue [384]. Additionally, unlike all other strategies, this approach may also facilitate a pretreatment safety assessment (using $I^{125}$ or technetium-99m) of the location of therapeutic hMSCs providing practitioners with vital information which could limit toxicity in non-malignant tissue. This potential was confirmed by Dwyer et al. [384] with detection of technetium-99m pertechnetate uptake in tumours of a non-invasive model of breast cancer 14 days after administration of hMSCs. Tumour bearing mice were subsequently treated with cytotoxic $I^{131}$, and showed a significant reduction in tumour growth in test animals compared to controls, with no adverse reactions reported [384]. Considering that this strategy is only in its infancy, the potential for improved efficacy is immense. Further understanding of the migratory itinerary can only serve to increase the efficiency of this MSC-mediated imaging and therapeutic strategy and optimise its safety profile.
Chapter 7

Discussion and Conclusion
7.1 Discussion

Breast cancer kills over 600 Irish women and 465,000 women worldwide each year [2, 400]. While early stage breast cancer is treatable, treatment is largely palliative for metastatic cancer. Identification of effective biomarkers for breast cancer which deliver faster diagnosis are paramount to more successful treatment. Even if successful, treatments regimes take a huge physical toll in terms of a plethora of side effects and represent a substantial financial burden to patients. It is critical that current research strategies deliver better biomarkers of breast cancer, and more effective and targeted therapies which eliminate tumours and metastases with minimal side effects.

The Sodium Iodide Symporter (NIS) has facilitated the imaging and successful radioiodide treatment of NIS positive thyroid cancers with a strong safety profile [240]. As a result, the prognosis of patients with thyroid cancer is significantly superior to breast cancer. Reports within the last decade have discovered elevated levels of NIS expression in malignant compared to normal breast tissue [42, 43, 45-47]. It has been suggested that this may be the key to identifying malignant breast tissue and could facilitate radioiodide imaging or treatment of breast cancer.

This study undertook to explore the presence, relevance and regulation of native NIS expression in breast cancer. In tandem, a mechanism of delivering NIS transgene expression to breast tumours and metastases was also investigated using MSCs as delivery vehicles.

While informative, previous studies which demonstrated elevated NIS expression in malignant compared to normal breast tissue were subject to experimental limitations, chiefly, the use of immunohistochemistry which is a subjective methodology [42, 43, 45-47] and small cohort sizes particularly where NIS gene expression was determined [44, 45]. For the first time in a large human cohort RQ-PCR was used to confirm the presence of NIS gene expression in 98 of 100 breast tissue samples. A significant increase in NIS gene expression was observed in malignant compared to normal breast tissue. This study also identified an even higher level of expression in benign
fibroadenoma tissue compared to malignant tissue. This observation was supported by previous reports of NIS expression and iodide accumulation in benign breast tissue [46, 47]. Furthermore when subdivided on the basis of epithelial subtypes, only the HER2 epithelial subtype had significantly higher NIS gene expression that normal tissue. Given this profile of gene expression in normal benign and malignant breast tissue, NIS cannot serve as a useful biomarker of breast cancer.

The imaging or therapeutic potential of mammary NIS expression was similarly in question. Assuming that functional NIS expression correlates with levels of NIS mRNA (and this is not always the case [265, 318]), at least 3 out of 4 epithelial subtypes of breast cancer could not be selectively imaged or treated using radioiodide. The HER2 epithelial subtype however did possess a significantly higher level of NIS gene expression compared to normal breast tissue. Although statistically significant this novel difference in NIS expression was observed using a relatively small HER2 cohort (n = 13). A further study focussing on normal and HER2 breast biopsies would allow for larger cohorts and more definitive data. Additionally it would be very interesting to see the outcome of a gamma camera scan of radioiodide accumulation in a patient with a confirmed HER2 positive tumour. However, in order to prevent the risk of recurrence or secondary cancers through survival of I\(^{131}\) damaged cells [333-335], it has become standard practice to use higher doses of I\(^{131}\) than required to ensure that the entire area is ablated. This raises the demands on mammary NIS expression and functionality in breast tissue in order to facilitate effective radioiodide treatment and casts doubt on the potential for HER2 mammary NIS expression to do so.

Gene expression of putative regulators of mammary NIS, previously implicated through \textit{in vitro} and \textit{in vivo} studies, was also examined in the tissue samples. Similar gene expression profiles across normal, malignant, and benign breast tissue were observed between NIS and RAR\(\alpha\) and RAR\(\beta\), and ER\(\alpha\). All four genes were higher in fibroadenoma compared to normal tissue, with NIS, RAR\(\alpha\) and RAR\(\beta\) significantly elevated. Significant positive correlations were also observed between NIS gene expression and that of RAR\(\alpha\) RAR\(\beta\), and
ERα. A stronger correlation between NIS and RARβ was observed than with RARα. This was the first time these relationships were demonstrated in human breast tissue but they are supported by previous in vitro observations. The RARα and RARβ relationship with NIS is supported by Kogai et al. [273] who used MCF-7 cells to show that RAR stimulation with receptor specific ligands could increase NIS expression. In addition they showed that RARβ specific ligands induced a larger increase in NIS expression than RARα. The significant relationship between ERα and NIS is supported by Alotaibi’s [274] in vitro observation that the unliganded ERα activates transcription of the mammary NIS expression. An inverse relationship was also identified between NIS and PI3K which is contrary to previous in vitro reports of PI3K activation leading to increased NIS expression in MCF-7 cells [275, 276, 343]. It does however appear to reflect the observations of the inhibitory role PI3K plays on NIS expression in the thyroid [249, 401]. Comparison of NIS gene expression with THRα and THRβ indicated that no relationships existed. However, further investigation of data revealed similar increases in expression of NIS, RARβ and THRβ in fibroadenoma compared to normal tissue and the largest correlation of gene expression was observed between THRβ and RARβ.

Although significant correlations in gene expression were observed they did not prove a causal effect between NIS and the putative regulators. To do this, in vitro experiments were performed using cancer cells lines and ligands targeting putative receptors.

More specifically, the effects of ATRA, T4 and E2 on NIS expression were investigated in T47D and Sk-Br-3 cell lines. Using a T47D cell line representing the Luminal A epithelial subtype it was confirmed that the previously reported effect of E2 and ATRA on NIS expression is not specific to the MCF-7 cell line [271, 315, 358, 402]. In addition, it was demonstrated for the first time that both E2 and ATRA significantly increased NIS expression in Sk-Br-3 cells which represent the estrogen receptor negative HER2 epithelial subtype. This data conflicts with earlier observations by Alotaibi who suggested that ATRA-responsive NIS expression is confined to ERα positive cell lines [274]. This study using Sk-Br-3 cells clearly indicates that both E2
and ATRA can stimulate NIS expression either in the presence of very low levels, or completely independently of the estrogen receptor.

A link was initially proposed between THRβ and NIS expression on the basis of similar expression profiles in normal, benign, and malignant breast tissue. This was supported by a significant correlation between THRβ and RARβ which in linked to mammary NIS expression. This hypothesised link was strongly boosted when T4 stimulated increased NIS expression in both T47D and Sk-BR-3 cell lines. Interestingly, when ATRA and T4 were combined a synergistic effect was observed whereby levels of NIS expression were higher than the sum of that observed with individual treatments. This may be explained by heterodimerization which is known to occur between retinoic acid receptors and thyroid hormone receptors [352, 353]. In fact, heterodimerization involving retinoic acid receptors has been previously implicated in carbamazepine stimulation of NIS expression in MCF-7 cells [272]. The fact that T4 does stimulate NIS expression in mammary cell lines shows how distinct mammary and thyroid NIS expression are from each other since T4 is known inhibitor of thyroid NIS expression. Some reports have considered hyperthyroidism to be protective against breast cancer [403]. Other reports have linked the presence of anti-TPO and anti-Tg in cases of autoimmune thyroiditis to increased risk of breast cancer [404]. Both TPO and Tg are involved in thyroid hormone synthesis. Although speculative, this might be explained by the increased levels of T4 in patients with hyperthyroidism stimulating increased iodide uptake in the breast, amplifying the potential anticarcinogenic role of iodine in the breast [321-324, 405].

A number of research groups have speculated that mammary NIS expression could potentially be stimulated to levels which facilitate imaging or treatment using radioiodide [271, 336]. Wapnir et al. [267] have suggested that the prevalence of NIS expression in numerous carcinomas might be used for radioiodide treatment and that tumour ablative effects could be minimised in the thyroid by administering thyroxine and methimizole. Considering this, thyroxine which could potentially play a dual role in thyroid NIS suppression and mammary NIS stimulation, and ATRA, which stimulates expression of its
own receptor and mammary NIS, may represent ideal candidates for stimulating mammary NIS expression \textit{in vivo}. Since NIS gene expression is already significantly higher in the HER2 breast tissue compared to normal, this epithelial subtype may represent the ideal breast cancer for initial experimentation on stimulating NIS expression to therapeutic levels \cite{271, 336}. For this reason it is important that future \textit{in vitro} studies include Sk-Br-3 or other HER2 derived cell lines and not just the MCF-7 cell line which is so common in such studies. However systemic administration of drugs such as ATRA and thyroxine present a significant risk of adding to the plethora of side effects potentially experienced by cancer patients \cite{406, 407}. Further studies will be needed to determine if any potential benefit might outweigh the associated risks.

The most likely method of acquiring therapeutic levels of functional NIS expression in tumours involves gene therapy approaches. Delivery of NIS expression using adenoviral vectors, the most commonly used vehicle of cancer gene therapy, has majors drawbacks \cite{105-107, 382, 383}. These include an inability to specifically target tumours and strong host immune responses. It is speculated that the use of MSCs as vehicles for adenoviral gene therapy may overcome these issues, through their immune privilege and tumour tropism \cite{114, 136, 408}. This project shows that MSCs infected with adenovirus carrying the NIS gene under control of the CMV promoter show no difference in characteristic surface membrane expression nor in osteogenic potential compared to controls. MSCs were demonstrated to express the NIS gene and protein in a robust and persistent fashion for up to 28 days. This outcome was very different to previous observations in other cells lines whereby expression peaks at day 3 with rapid decline thereafter \cite{100, 101, 409}. Proliferation of cells does serve to dilute the frequency at which NIS positive cells occur however it is has also been reported that tumour engrafted MSCs do not proliferate, so \textit{in vivo} expression up to 28 days may be possible \cite{184}. Given that membrane localisation of the NIS protein was also observed at day 28, this suggests that if MSC tumour engraftment could be achieved, there may be a window of at least 28 days in which to perform MSC tracking, tumour imaging and therapy. In terms of therapeutic potential, it was demonstrated for the first
time using NIS expressing MSCs, that a radioiodide bystander effect can induce cell death in a co-cultured cancer cell line. This supports the potential that tumour engrafted MSCs may ablate surrounding malignant tissue particularly given reports that beta radiation can be strong enough to penetrate cells up to 2 mm away from the source of accumulation [241]. This study has also investigated the potential for MSC-mediated delivery of the NIS gene in a non-invasive murine model of breast cancer. It was confirmed that systemically administered MSCs could deliver transgene expression to tumours whereas naked adenoviral vectors failed to do so. As observed with some other studies, localisation of therapeutic MSCs to the lungs and heart was detected using a NIS signature 3 days after systemic administration [116, 134, 136]. However this declined over time, while a similar signature detected in malignant tissue remained robust. Expansion of this study by Dwyer et al. showed that by 14 days after administration of MSCs expressing NIS, Technecium-99 accumulation was detected in the tumours and not in any other untargeted tissue [384]. Low level expression of NIS in the small intestine also did not result in iodide accumulation at this time point. This is a critical characteristic of MSC mediated delivery of NIS gene expression and emphasises how important it is that MSCs maintain robust, persistent NIS expression in order to facilitate clearance from critical organs prior to potential radioiodide ablation of MSC engrafted tumour tissue. Where additional animals from the same study were treated with MSCs expressing NIS followed by radioiodide, tumour growth rates were significantly slowed resulting in final tumour volumes 75% smaller than that of control animals [384].

However, when an in situ carcinoma in detected in the breast, the vast majority can be removed surgically with excellent success rates. This is particularly relevant with early stage cancers. The value of a MSC mediated NIS gene therapy will potentially lie in more advanced cancer involving metastasis, where there is extensive disease spread, or the tumour location is unknown, or surgery involves too much risk. To examine this potential, PKH-26 labelled MSCs were systemically administered in a murine model of metastatic breast cancer. The results were very promising. Fluorescent microscopy revealed MSC localisation in the lungs, heart, tumour and enlarged nodal tissues within
up to 2 weeks following systemic administration of PKH26 labelled MSCs. Beyond this time point, MSCs were detected only in cancerous nodes and not in any normal tissues. This suggested a therapeutic window in which $^{131}$I treatment could be administered without damaging noncancerous tissue. In some instances MSCs appeared to engraft in clusters possibly indicating vascular engraftment. Radioablation of these engraftment sites might explain how MSC-NIS-$^{131}$I treated mice presented with final tumour volumes 75 % smaller than observed in untreated control groups despite the small proportion of therapeutic MSCs to tumour cells [384]. The application of flow cytometry to this metastasis model was technically challenging. However the data returned, specifically that which indicated increased MSCs engraftment in tumours, does highlight potential for this technique in further studies in this area.

### 7.2 Conclusion

This thesis provides new insight into the presence and relevance of mammary NIS expression in human tissues. It also supports a regulatory role for estradiol and retinoic acid, and introduces the potential for thyroid hormones to stimulate mammary NIS expression. However, while it may be possible to stimulate increased expression by exploiting the regulatory controls of mammary NIS, gene therapy alternatives may provide much stronger options. The huge potential of MSC mediated NIS imaging and therapy of breast cancer demonstrated here and in related studies represents a substantial step forward from standard vector-based strategies. The potential for MSCs to target multiple tumour types, the extensive safety record of radioiodide therapy in the treatment of thyroid disease, the ability to selectively suppress thyroid NIS, and the ability to noninvasively track MSC migration prior to therapy make this strategy an extremely promising prospective. With further understanding of MSC migratory itinerary and the extent of their involvement in tumour progression, this approach may be poised to deliver an effective solution to the current inadequacies of treatment in numerous cancer types.


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Chapter 9

Appendices