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# Transcriptional regulation of S100B and identification of novel melanoma biomarkers

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# **Transcriptional regulation of S100B and identification of novel melanoma biomarkers**

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Thesis submitted to the Royal College of Surgeons in Ireland and presented to the faculty of Medicine for the degree of Doctor of Medicine

September 2010

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I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree, Doctor of Medicine (MD), is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed *C de Blacam*

Student Number 06122264

Date *05.11.2010*

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## Abbreviations

$\alpha$ -MSH – alpha-melanocyte-stimulating-hormone

ATCC- American Type Culture Collection

AJCC – American Joint Committee on Cancer

BCA – bicinchoninic acid assay

bp – base pair

Ca<sup>2+</sup> - calcium

CDK4 – cyclin dependent kinase 4

CDKN2A – cyclin dependent kinase inhibitor 2A

ChIP – chromatin immunoprecipitation

CoA – coactivator

DAB – Diaminobenzidine

dH<sub>2</sub>O – distilled water

dNTP – deoxyribonucleotide triphosphate

DNA – deoxyribonucleic acid

DTIC - dacarbazine

EGF – epidermal growth factor

ER – estrogen receptor

FCS – fetal calf serum

H<sub>2</sub>O<sub>2</sub> – hydrogen peroxide

HOX – homeobox genes in human

Hox - homeobox genes in mouse

IFN- $\alpha$  - interferon- $\alpha$

IgG – immunoglobulin

IL-2 – interleukin-2

IMS – industrial methylated spirits

IP – immunoprecipitation

kb – kilobase

kDa – kilodalton

MA – mili amperes  
MAPK – mitogen activated protein kinase pathway  
MC1R – melanocortin receptor 1  
MM – malignant melanoma  
MMP – matrixmetaloprotinase  
mRNA – messenger ribonucleic acid  
NM – nodular melanoma  
PAGE – polyacrylamide gel electrophoresis  
PBS – phosphate buffer saline  
PCR – polymerase chain reaction  
PTEN – phosphatase and tensin homologue  
qRT PCR – quantitative real time polymerase chain reaction  
RAGE – receptor for advanced glycation endproducts  
RIPA - radioimmunoprecipitation assay (buffer)  
RT PCR – real time polymerase chain reaction  
SDS – sodium dodecyl sulphate  
SPE – S100 protein element  
SRC-1 – steroid receptor co-activator 1  
SSM – superficial spreading melanoma  
TAA – tumour associated antigen  
TRPM1 - melanocyte-specific gene melastatin  
VGP – vertical growth phase

## **Summary**

### ***Introduction***

Cutaneous melanoma is an aggressive disease, which is recognised as the most common fatal skin cancer worldwide. Approximately 450 cases are diagnosed in Ireland per year. Between 1994 and 2004, melanoma demonstrated a higher rate of increase in mortality than any other cancer in this country. Although the prognosis for early melanoma is favorable, less than 20 percent of patients with metastatic melanoma survive for five years.

S100B is a calcium sensor protein that modulates biological activity via calcium binding, which is routinely used in histological diagnosis of malignant melanoma and which is also a well-recognized serum marker of the disease. HOX proteins are members of the homeodomain family of transcription factors, which are involved in a host of cellular functions including organogenesis, cellular differentiation, cell cycle and apoptosis. As transcription factors, HOX proteins require co-activator proteins to achieve their full function. SRC-1 is one such coactivator and our group has extensively explored its function. In particular, functional interactions between SRC-1 and HOXC11 in breast cancer cell lines and tissue have been described. HOXC11 is known to enhance expression of the secreted serum marker S100B. Given the strong association between S100B and malignant melanoma, we believe that this pathway may also have a role in melanoma tumourgenesis.

S100B is a well-described biomarker in melanoma and serum levels have been shown to correlate with disease stage and response to treatment. In spite of this and the availability other prognostic indicators, many patients go on to develop an unpredictable disease course. For this reason, identification of novel biomarkers is an active area of melanoma research. In the second

part of this work, an autoantibody microarray screen was undertaken to identify differentially expressed biomarkers in sera from patients with melanoma.

### ***Hypothesis***

- Production of S100B in malignant melanoma is regulated by the transcription factor HOXC11, in cooperation with coactivator SRC-1.
- Protein microarray technology may provide a useful means of identification of autoantibody biomarkers in serum from patients with melanoma.

### ***Aims***

- To define the molecular role of HOXC11 and SRC-1 in the transcriptional control of S100B in malignant melanoma.
- To characterise the effect of manipulation of HOXC11 and SRC-1 on S100B expression.
- To identify new biomarkers in sera from patients with malignant melanoma.

### ***Results***

Expression of S100B, HOXC11 and SRC-1 protein in primary (SKMel28) and metastatic (MeWo) melanoma cell lines was confirmed by Western blotting and quantitative Real Time PCR (qRT-PCR) analysis. Colocalisation of HOXC11 and SRC-1 in melanoma cells was confirmed by immunofluorescence. Co-immunoprecipitation was carried out and demonstrated interaction of HOXC11 and SRC-1 in cell lysates. Paraffin-embedded melanoma and nevi samples were examined by immunofluorescence and a significantly higher nuclear expression of HOXC11 and SRC-1 was observed in the melanoma cohort. Colocalisation of the two proteins was also demonstrated in a series of melanoma primary culture specimens.

Chromatin-immunoprecipitation was employed to confirm recruitment of HOXC11 to the promoter region of the S100B gene. To determine the ability of HOXC11 to regulate expression of S100B, HOXC11 was transfected into the SKMel28 cell line and it was found to significantly increase the expression of the target gene S100B. When concomitant HOXC11 and SRC-1 knockdowns were performed, a significant reduction in the presence of S100B was noted. Treatment of cell lines with the phospho-Src inhibitor, dasatinib, resulted in decreased coassociation between HOXC11 and SRC-1 in both primary and metastatic cell lines as well as decreased expression of S100B in SKMel28 cells.

Protein microarray analysis of sera from patients with melanoma and control patients was carried out. A series of differentially expressed autoantibodies was identified and the non-receptor tyrosine kinase, BMX was chosen for further study. Elevated expression of anti-BMX autoantibody in sera from a larger cohort of melanoma patients was confirmed. Furthermore, expression of BMX protein in melanoma cell lines and frozen tissue samples was confirmed by Western blotting.

### ***Conclusion***

In the absence of effective treatment for advanced melanoma, elucidation of novel signalling pathways and therapeutic targets remains at the forefront of molecular research. In this work, translational techniques have provided an insight in to the transcriptional regulation of S100B in melanoma.

Furthermore, protein microarray analysis has been utilised to identify potentially useful autoantibody biomarkers.

These findings constitute a small fragment of all the potential genetic aberrations that may be implicated in melanoma tumourgenesis. Advanced melanoma is likely to present a significant therapeutic challenge to clinicians

and academics for many years to come. What is certain is that translational research methods, as have been employed here, are essential in pushing forward the boundaries of our molecular understanding of this fascinating disease.



# 1 General introduction

## ***1.1 Introduction to melanoma***

Melanoma was first described as a disease entity by the French Physician, René Laennec in 1806 (Laennec, 1806). Malignant melanoma is a solid tumour which arises from melanocytic cells and primarily involves the skin. In 1840, Samuel Cooper made the first formal acknowledgement of advanced melanoma as an untreatable condition, stating that “the only chance of benefit depends on the early removal of the disease” (Cooper, 1840). In spite of many years of intensive laboratory and clinical research, this situation remains largely unchanged.

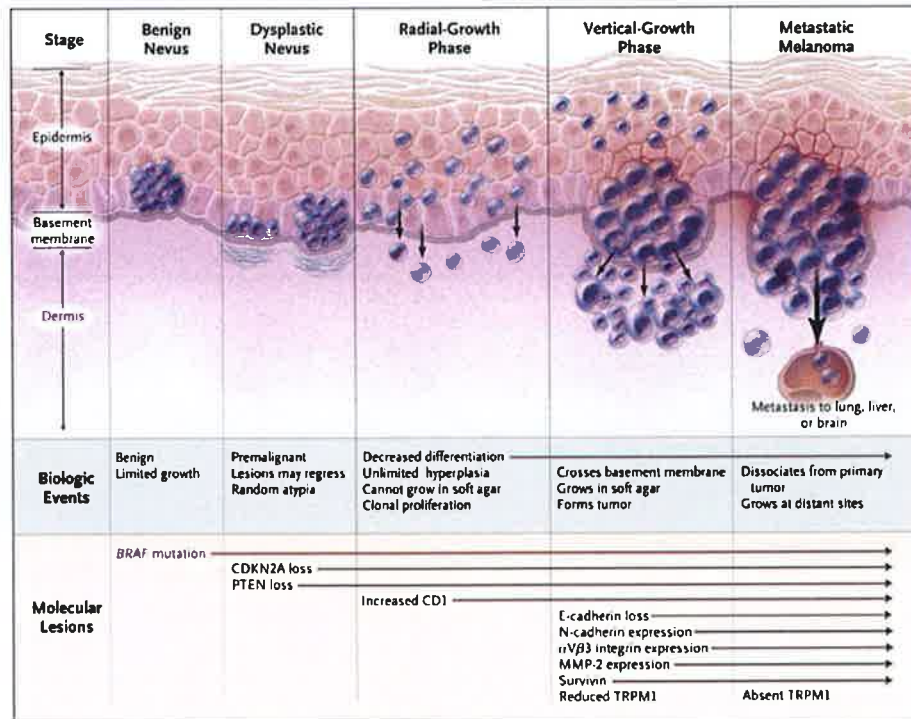
Cutaneous melanoma is an aggressive disease, which is recognised as the most common fatal skin cancer worldwide (Nestle and Carol, 2003). Approximately 450 cases are diagnosed in Ireland per year. Between 1994 and 2004, melanoma demonstrated a higher rate of increase in mortality than any other cancer in this country (Comber, 2006). Although the prognosis for early melanoma is favourable, the 5-year survival falls from over 90% to less than 20% for a deep lesion.

While much remains to be understood about the causes of melanoma, it is clear that both environmental and genetic factors play a role. The strongest risk factors for melanoma are a family history of the disease, multiple benign or atypical nevi and a previous melanoma (Bliss et al., 1995, Miller and Mihm, 2006).

## ***1.2 Molecular development of melanoma***

According to the widely accepted Clark model of tumorigenesis, development of melanoma is depicted as a progression from proliferating melanocytes that form nevi to dysplasia, hyperplasia, invasion and metastasis (Clark et al., 1989). The critical moment in melanoma development is when a normal melanocyte converts in to a vertical growth phase, enabling its progeny (malignant melanoma cells) to invade the deep dermis. With the advent of

genomic and proteomic techniques, multiple somatic genetic alterations have been related to the pathogenesis of melanoma. These are summarised in Figure 1.1.



**Figure 1.1 Biologic events and molecular changes in the progression of melanoma**

BRAF mutation and activation of the mitogen-activated protein kinase (MAPK) pathway occurs at the stage of the benign nevus. The cytologic atypia in dysplastic nevi reflect lesions within the cyclin-dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways. Progression from dysplastic nevus to melanoma is associated with decreased differentiation as well as the decreased expression of melanoma markers regulated by microphthalmia-associated transcription factor (MITF). Changes in the control of cell adhesion are characteristic of the vertical-growth phase and progression to metastatic melanoma. Changes in the expression of the melanocyte-specific gene melastatin 1 (*TRPM1*) correlate with metastatic propensity. Other changes include the loss of E-cadherin and increased expression of N-cadherin, V3 integrin, and matrix metalloproteinase 2 (MMP-2).

*From The New England Journal of Medicine (Miller and Mihm, 2006)*

Not all melanomas show the same set of genetic events, but some changes are more common than others. If we follow the Clark model, the first event is the proliferation of structurally normal melanocytes, leading to the appearance of a benign nevus. BRAF mutation and activation of the mitogen-activated protein kinase (MAPK) pathway are present at high frequency in benign nevi and persist in melanomas also (Pollock et al., 2003, Curtin et al., 2005). Up to 70% of melanomas express mutated BRAF (Davies et al., 2002). Melanomas also tend to contain activating mutations of NRAS, which again stimulates MEK-MAPK pathway. Another element of this pathway is MYC which is over-expressed in up to 40% of melanomas (Kraehn et al., 2001). Other pathways that stimulate proliferation include CDK4/cyclin D1 (CCND1) and  $\beta$ -catenin/AKT (Curtin et al., 2005, Delmas et al., 2007). Both MAPK and AKT pathways can be activated by amplification or activating mutations of receptor protein tyrosine kinases (RTKs). An example of such a RTK is KIT, which is mutated in acral and mucosal melanomas (Curtin et al., 2006).

Most nevi remain static for years and never gain invasive potential. Therefore, further alterations are required for a melanoma to develop. The next step toward melanoma is the development of cytologic atypia, which are seen as dysplastic nevi. Dysplastic nevi may arise from pre-existing benign nevi or as de novo lesions. Molecular abnormalities at this stage lead to alterations in cell growth, DNA repair and susceptibility to cell death. One such mutation is the inactivation of cyclin-dependent kinase inhibitor 2A (CDKN2A) (Kamb et al., 1994, Harland et al., 2005). Deletion of the CDKN2A locus on chromosome 9 leads to suppression of INK4A, a protein that blocks the cell cycle at the G1-S checkpoint by inhibiting cyclin dependent kinases. Phosphatase and tensin homologue (PTEN) (Guldborg et al., 1997) is located on chromosome 10 and is also frequently deleted in melanoma. PTEN encodes a phosphatase that attenuates signalling by a variety of growth factors that use phosphatidylinositol phosphate (PIP3) as an intracellular signal. One of its downstream targets is AKT, which is allowed to

increase in the absence of PTEN. This leads to prolonged cell survival and proliferation.

In normal melanocyte differentiation, cells exit the cell cycle and express genes that encode proteins necessary for pigment production. Both of these processes are dysregulated in malignant melanoma and constitute the next stage of Clark's melanoma progression. The microphthalmia-associated transcription factor (MITF) regulates the differentiation of melanocytes and appears to contribute to survival by increasing expression of Bcl-2, a key anti-apoptotic factor (Garraway et al., 2005, McGill et al., 2002).

Having converted from a normal melanocyte in to an undifferentiated melanoma cancer cell, the next stage in the Clark model is the development of invasive characteristics – the transition to vertical growth phase. Normally, cell adhesion controls cell migration and tissue organisation (Johnson, 1999); its disturbance contributes to tumour invasion, tumour-stroma interactions and tumour-cell signalling. In melanoma, changes in cell adhesion influence the vertical growth phase and metastatic phase of the disease (Haass et al., 2005). Specifically, changes in the expression of melanocyte-specific gene melastatin (TRPM1) correlate with metastatic propensity, but the function of this gene remains unknown. Other changes at this stage include the loss of E-cadherin and increased expression of N-cadherin (Danen et al., 1996, Hsu et al., 1996, Felding-Habermann et al., 2002), and matrix metalloproteinase 2 (MMP-2) (Hofmann et al., 2000).

In summary, development from normal melanocyte to malignant melanoma depends on changes to a precise set of molecular pathways. The genetic events which affect these pathways are multiple and are complex in their interactions. As well as the primary events described here, a whole host of secondary outcome events also play an important role in melanoma tumourgenesis (Bennett, 2008). Distinct genetic changes are identified in the different subtypes of melanoma and also in melanomas occurring in different

body sites (i.e. chronically versus intermittently sun-exposed or mucosal and acral skin).

### **1.3 Current treatment**

The primary treatment of melanoma is surgical excision (Garbe et al., 2009) and this provides high cure rates. Excisional biopsy is preferred and a definitive surgical excision with safety margins should be performed within 4-6 weeks of initial diagnosis. Surgical excision provides excellent cure rates for thin melanomas (<1 mm). If lymph node metastasis is identified either clinically or by ultrasound, radical lymph node dissection is indicated. In patients with a melanoma deeper than 1 mm and no evidence of lymph node metastasis on either palpation or ultrasound, a sentinel lymph node dissection is the standard of care.

Following surgical excision, adjuvant therapy is offered to patients without evidence of metastases but at high risk for further tumour spread – i.e. patients with AJCC stages II and III. European guidelines recommend that there is no indication for adjuvant systemic chemotherapy outside the context of controlled studies (Garbe et al., 2008). The cytokine, Interferon- $\alpha$  is the only agent which has been shown to confer a significant improvement in disease-free survival when used in the adjuvant setting. There is however no clear benefit in terms of overall survival and unfortunately many patients can not tolerate the treatment due to its formidable toxicity.

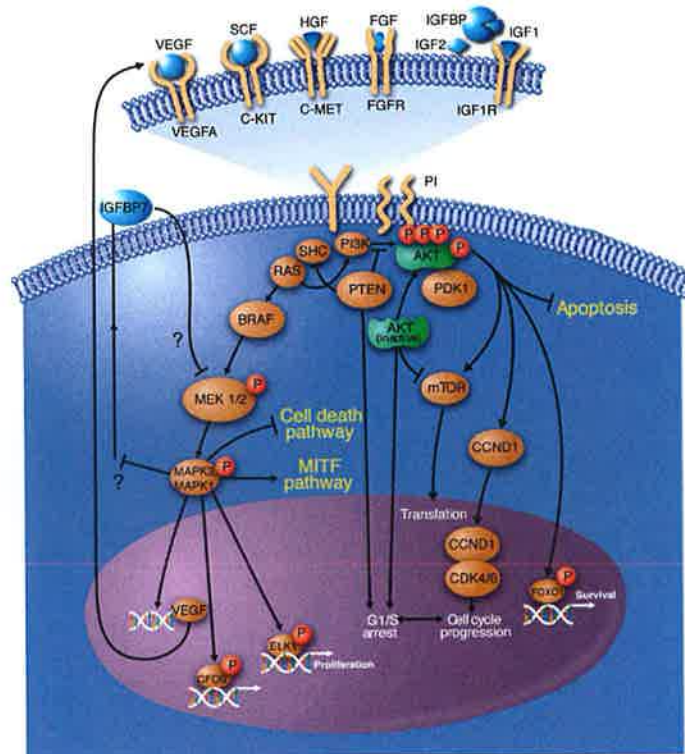
Primary systemic chemotherapy and chemoimmunotherapy are reserved for patients with inoperable primary tumours or inoperable regional or distant metastases. In Stage IV disease, complete response is extremely rare and any treatment undertaken is essentially palliative. The best established monotherapy drug is dacarbazine (DTIC) but recorded remission rates vary widely (Schadendorf, 2002). Cytokines, IFN- $\alpha$  and interleukin-2 have also been evaluated in advanced disease but are both associated with high toxicity. No randomized controlled trial has shown significant *overall* survival

advantage with the use of any specific drug or combination of drugs, including dacarbazine, interferon and interleukin-2 (Wheatley et al., 2003).

#### ***1.4 Targeted molecular therapies***

In the context of this dearth of effective therapeutic options, the current hope for melanoma is molecularly targeted therapy. Targeted therapies comprise small molecule inhibitors that block the activity of oncogenes responsible for driving melanoma growth and progression. Figure 1.2 summarises the principle oncogenes in melanoma and the pathways that lead to their activation.





**Figure 1.2 Principle molecular pathways in malignant melanoma**

Activation of various receptor tyrosine kinases (VEGFR, C-KIT, C-MET, FGFR, IGF1R, and others) by their ligands (VEGF, SCF, HGF, FGF, and IGF1/2, respectively) leads to proliferation and survival of melanoma cells through two main interacting signal transduction pathways - MAPK and PI3K/AKT. FGF = fibroblast growth factor; HGF = hepatocyte growth factor; IGF = insulin-like growth factor; P = phosphate; VEGF = vascular endothelial growth factor; ? = mechanism unknown.

*From Mayo Clinic Proceedings (Sekulic et al., 2008)*

In terms of therapy, one of the most promising targets is mutant BRAF<sup>V600E</sup>, which is found in up to 70% of all melanomas. However, initial research has shown limited therapeutic benefit in targeting BRAF alone (McDermott et al., 2008). While this may be due to the lack of sufficiently targeted therapeutics, it is more likely reflective of the fact that multiple oncogenic pathways are activated in melanoma. It is probable that combination-targeted therapy is required to achieve maximal therapeutic benefit.

Aside from BRAF, other potential therapeutic targets include c-KIT, Bcr/Abl, VEGF, EGFR and RAS (Sosman and Puzanov, 2006). There is evidence of impressive responses to the KIT inhibitor imatinib in small numbers of patients whose melanomas were identified as harbouring activating KIT mutations (Hodi et al., 2008).

### **1.5 Melanoma biomarkers**

Diagnosis in melanoma is typically based on histopathological characteristics of the tumour, with Breslow thickness, ulceration and extent of lymph node involvement being important indicators of prognosis. However difficulty can arise in distinguishing subsets of benign nevi from melanoma. Furthermore some patients who have a thin melanoma, for whom surgical excision should be curative, do progress to metastatic disease. Therefore, identification of these patients, who are apparently free of disease but at high risk of relapse, represents a key issue for treatment optimization. For this reason, the search for prognostic biomarkers is an active field in melanoma research.

The 'gold standard' routine immunohistochemical markers for the diagnosis of histologically unclear and/or amelanotic melanomas are S100B, melanocyte lineage-specific antigen gp100 (silver homolog; also known as HMB-45 and PMEL17), and melan-A protein (MLNA; also known as MART-1) (Haass and Smalley, 2009). Many of these proteins contribute to melanocyte pigmentation. MLNA, S100B and HMB-45 show high sensitivity for melanoma but these markers are also found in melanocytic nevi (Ohsie et al.,

2008). Therefore, their specificity to distinguish melanoma from nevi is low but they are widely used to distinguish melanoma from other non-melanocytic tumours.

A recent meta-analysis (Gould Rothberg et al., 2009) yielded a number of potentially useful tissue markers, most of which are linked to either growth or invasion/ microenvironmental escape. These included melanoma cell adhesion molecule (MCAM, also known as Mel-CAM, MUC18 and CD146), matrix metalloproteinase-2 (MMP2), the proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA), and cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as tumor suppressor p16INK4A).

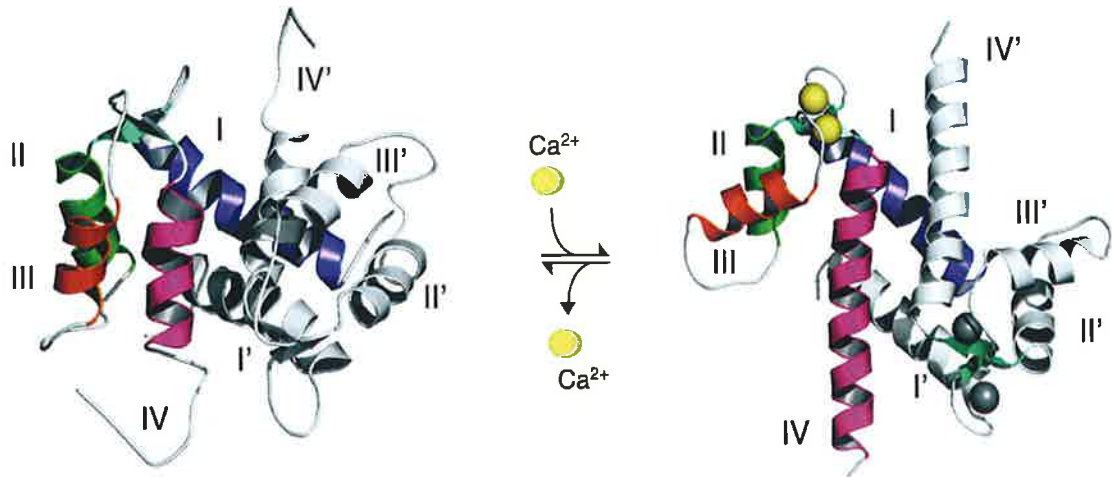
## **1.6 S100B**

### **1.6.1 S100 protein family**

S100B is a member of the S100 family of calcium binding proteins. The S100 protein family comprises 25 members, the first of which was identified in 1965 as a protein fraction in bovine brain tissue (Moore, 1965). The isolate was named S100 due to its solubility in 100% saturated ammonium sulphate solution at neutral pH. In 1978, Isobe and Okuyama demonstrated that S100 consisted of two subunits, S100 $\alpha$  and S100 $\beta$ , which could be combined in three different isomeric forms – S100 $\alpha\alpha$ , S100 $\alpha\beta$  and S100 $\beta\beta$  (Isobe et al., 1978). Subsequently, nine different gene members coding for the S100 protein family were identified on the long arm of chromosome 1 and a new system of nomenclature was introduced (Schafer et al., 1995). S100 $\beta$ , which is located on chromosome 21, became known as S100B. S100 proteins are highly conserved in their amino acid composition and are distributed in a cell-specific fashion, indicating a conserved biological function (Heizmann et al., 2002). S100 proteins have diverse roles including involvement in cell structure, cell growth, energy metabolism, contraction and intracellular signal transduction. A wide range of human diseases, including melanoma, have been attributed to dysregulation of S100 gene expression.

### **1.6.2 Structure of S100B**

S100B is a 10.5 kilodalton protein. Like other members of the S100 family, it consists of two highly conserved calcium binding sites, each made up of 12 amino acids with an alpha helix on either side, which constitutes the EF-hand. The two alpha helices are interconnected by a hinge region and the molecule also has a C-terminal extension (Donato, 2001, Heizmann et al., 2002). S100B exists as a 21 kilodalton homodimer in which two subunits are arranged in an anti-parallel fashion and are held together by non-covalent bonds. It can also exist as a tetramer, octamer or oligomer, as well as a S100B/S100A1 heterocomplex. When calcium binds, it mediates a conformational change in the protein, which results in opening up of the hinge region and exposure of the C-terminal extension (Rustandi et al., 2000) (Figure 1.3). This is thought to be the site for binding of target proteins. It is the binding of the target protein to the conformationally altered S100 protein that results in the intracellular response (Fritz et al., 2002). Other regions of the S100B dimer have also been implicated in the recognition of certain target proteins within cells and these interactions may not all be calcium-dependent (Santamaria-Kisiel et al., 2006).



**Figure 1.3 Structure of S100B; conformational change in calcium binding**

In the symmetrical dimer on the left, helices of one monomer (I–IV) are highlighted in different colours, while the other monomer (helices I'–IV') is coloured grey. As sensors, the S100 proteins experience a conformational change upon calcium binding (four atoms/dimer). The rearrangement of helix III (orange) exposes previously buried residues that are essential for target recognition (not shown) and further biological response.

*From The Biochemical Journal (Santamaria-Kisiel et al., 2006).*

### 1.6.3 Function of S100B

S100B is expressed in varying abundance in a number of different cell types. As well as melanocytes, these include astrocytes, maturing oligodendrocytes, kidney epithelial cells, neural progenitor cells, pituicytes, ependymocytes, certain neuronal populations, chondrocytes, adipocytes, Langerhans cells, dendritic cells, certain lymphocyte subpopulations, skeletal myofibers, myoblasts and muscle satellite cells (Donato et al., 2009). S100B's cytoplasmic localisation and its relatively high abundance in these cell types means that it can interact with a large number of target proteins. As a consequence, S100B exerts many intracellular and extracellular functions.

### **1.6.3.1 Intracellular functions**

S100B has been shown to take part in regulating cell proliferation (Millward et al., 1998). In particular, S100B interacts in a  $\text{Ca}^{2+}$  dependent manner with the tumour suppressor p53 inhibiting its phosphorylation by protein kinase C. This can cause downregulation of the p53 tumour suppressor mechanism, contributing to uncontrolled growth in melanoma and other cancers (Wilder et al., 1998, Lin et al., 2004).

S100B also influences cell differentiation (Marks et al., 1990) and structure (Donato, 1991). It is implicated in  $\text{Ca}^{2+}$  homeostasis (Xiong et al., 2000), protein phosphorylation and transcription, as well as the dynamics of microtubules and type III intermediate filaments (Donato, 1988, Sorci et al., 2000). S100B is also involved in regulation of cell growth, enzyme activity and metabolism (Zimmer and Van Eldik, 1986).

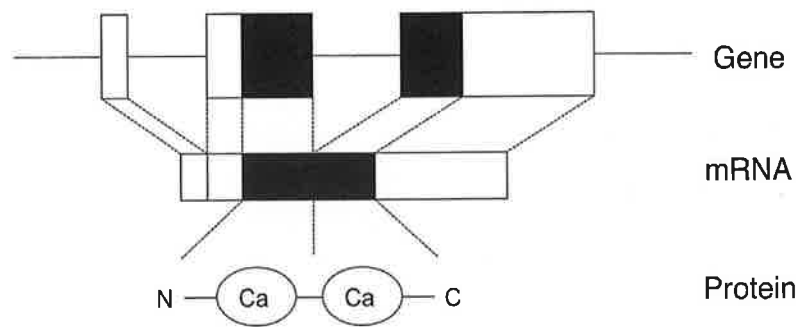
### **1.6.3.2 Extracellular functions**

As well as these intracellular regulatory activities, S100B can function as an extracellular signal. S100B is secreted by several cell types and is released by damaged cells. RAGE has been identified as an S100B receptor, transducing S100B effects on a variety of cell types with different outcomes (Huttunen et al., 2000, Hofmann et al., 1999, Schmidt et al., 2000). These effector cells include neurons, astrocytes and microglia, as well as monocytes/macrophages, T-lymphocytes and endothelial cells (Donato et al., 2009). The effect that S100B has on these varied cell types can be beneficial or detrimental and will depend on the concentration attained by the protein.

### **1.6.4 Regulation of S100 expression**

The S100 family of proteins are highly conserved among vertebrates in terms of their intron/exon organization (Figure 1.4). The first exon is not translated. The second exon encodes the amino terminal EF-hand calcium-binding domain and the third exon encodes the carboxy-terminal one. Furthermore, within the coding regions, the nucleotide sequences and their deduced amino acid sequences are highly conserved across different species. In contrast,

the sequences of the promoter regions of the genes display very little similarity (Allore et al., 1990). This heterogeneity may reflect different regulatory mechanisms for the expression of individual S100 genes.



**Figure 1.4 Schematic representation of the S100 gene**

The boxes represent exons and are separated by lines representing introns. Shaded boxes represent the coding region while open boxes represent 5'- and 3'- untranslated regions. N = amino terminal of protein; C = carboxy terminal of protein; Ca = calcium binding domain.

*Adapted from Brain Research Bulletin (Zimmer et al., 1995)*

Altered expression of S100 proteins has been implicated in many disease states including cancer. Understanding transcriptional regulation of S100 may therefore yield important information about tumourigenesis. The fact that discrepancies have been demonstrated between the mRNA and protein levels of S100 expression (Zimmer et al., 1991) suggests that gene regulation may involve translation and other posttranscriptional events as well as gene transcription.

Evidence of transcriptional regulation of S100 is also seen in the tissue-specific and cell-type specific variation in expression of different members of the S100 family (Zimmer et al., 1995). S100 genes are expressed in many

different tissues and each S100 protein is restricted to its own specific set of cell types. Individual cell types contain multiple S100 proteins that are expressed at different levels. This type of variation in expression must involve transcriptional control. Jiang et al suggest that murine S100B is under complex transcriptional control of multiple regulatory elements, including cell type specific elements (Jiang et al., 1993).

Bioinformatic analysis has assisted in gaining insight in to the regulation of S100 gene expression by identifying potential DNA regulatory elements. Jiang et al. identified one such element, known as the S100 protein element (SPE) (Jiang et al., 1993). The SPE is a 12 bp consensus DNA sequence that is located near the TATA box in human several S100 proteins, including S100B. Its role in regulating S100 gene transcription is not clear however. A cAMP regulatory element has also been identified in human S100B (Allore et al., 1990) and putative AP-1 and AP-2 elements exist in murine S100B genes (Jiang et al., 1993). Again the significance of these elements in terms of regulation of S100 expression is uncertain.

## **1.7 SRC-1**

### **1.7.1 Coregulatory proteins**

Gene regulation is influenced by ligands binding to their associated receptors. Ligand bound receptors rarely act in isolation and their function is regulated by the presence of specific coregulatory proteins. Coregulators are present at rate-limiting levels that modulate transcription (McKenna et al., 1999). Binding of these co-regulators results in activation or repression of transcriptional activity. Coregulatory proteins are thus designated as coactivators and corepressors.

Screening strategies have led to the discovery of a large number of coregulatory proteins. These proteins interact with nuclear receptors at a conserved LXXLL motif within the receptor-interacting domain of the protein to drive target gene expression (Heery et al., 1997).



### 1.7.2 Coactivator proteins

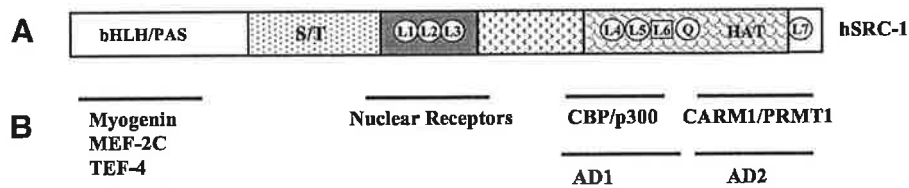
A coactivator is defined according to the biochemical analysis of its interaction with nuclear receptors (NR) and transcription factors, as well as its ability to enhance transcription in transient transfection assays. Most unbound co-activators that interact with NRs form distinct protein complexes with downstream intermediate factors. These complexes can affect chromatin remodelling or may interact with general transcription factors. Ligand-activated and DNA-bound transcription factors or NRs can efficiently recruit coactivator complexes to specific promoters. The concentration and function of each individual component in a co-activator complex can be regulated through transcriptional control and various post-translational modifications, as well as degradation by multiple signalling pathways. The usage of these co-activator complexes by transcription factors and NRs may provide platforms for sophisticated transcriptional regulation (Xu and Li, 2003).

One of the best-characterized groups of co-activators is the p160 steroid receptor coactivator (SRC) gene family, named because the constituent proteins have a molecular weight of 160 kDa. The family contains three homologous members, which serve as transcriptional coactivators for nuclear receptors and certain other transcription factors. These are **SRC-1** (Onate et al., 1995) (also known as NCoA-1 (Kamei et al., 1996, Torchia et al., 1997)), **SRC-2** (also known as TIF-2 (Voegel et al., 1996), GRIP-1 (Hong et al., 1996) or NCoA2 (Torchia et al., 1997)) and **SRC-3** (also known as ACTR (Li et al., 1997), AIB1 (Anzick et al., 1997) or TRAM (Takeshita et al., 1996)).

### 1.7.3 SRC-1

The steroid receptor coactivator 1 (SRC-1) gene is located on chromosome 2p23 (Carapeti et al., 1998), (Figure 1.5). SRC-1 was the first nuclear receptor activator to be cloned (Onate et al., 1995). It was cloned through its ability to interact with the ligand-bound progesterone receptor (PR) (Takeshita et al., 1996). SRC-1 protein has been identified in numerous tissues including the testis, brain, lung, liver, kidney, and heart (Torchia et al.,

1997).



**Figure 1.5 Structural and functional domains of the SRC-1**

**A** The letters within the structural diagram indicate structural domains: bHLH/PAS = basic helix loop helix/ Per/ARNT/Sim homologous domain; S/T = serine/threonine-rich regions; encircled L = typical LXXLL -helix motifs; boxed L = atypical LXXLL motifs; Q = glutamine-rich regions; HAT = histone acetyltransferase domains. **B** The lines under the diagram indicate domains that interact with different factors or serve as transcriptional activation domains (AD 1 and 2).

*Adapted from Molecular Endocrinology (Xu and Li, 2003)*

#### 1.7.4 SRC-1 binding partners

P160 coactivators interact with ligand-bound nuclear receptors to recruit histone acetyltransferases and methyltransferases to specific enhancer/promotor regions. This facilitates chromatin remodeling, assembly of general transcription factors, and transcription of target genes.

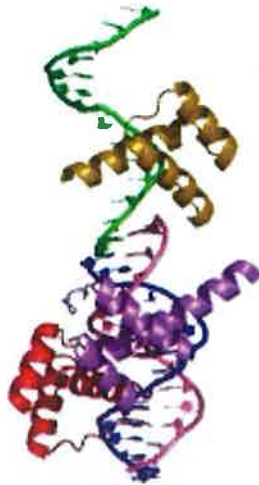
Although previously thought to exclusively bind steroid receptors, the role of p160 coactivators in regulating nonsteroidal transcription factor activity has now been established (Myers et al., 2005, Qin et al., 2008, Goel and Janknecht, 2004). In particular, our group has reported functional interactions between SRC-1 and the Ets family of transcription factors, Ets-2 and PEA3 (Al-azawi et al., 2008) as well as the homeodomain protein, HOXC11

(McIlroy et al., 2010). Interestingly, it was shown that HOXC11 and SRC-1 cooperate to regulate expression of S100B in endocrine resistant breast cancer cells.

## **1.8 HOXC11**

### **1.8.1 Homeobox genes**

Homeobox genes were discovered independently in 1983 by Walter J Gehring in Basel, Switzerland and Matthew Scott in Indiana, USA (McGinnis et al., 1984, Scott and Weiner, 1984). A homeobox is a 183 base pair stretch of DNA that is found within genes that are involved in the regulation of development of animals, fungi and plants. Homeobox genes encode proteins characterized by a highly conserved 61-amino acid motif called the homeodomain (McGinnis and Krumlauf, 1992). The homeodomain confers DNA-binding ability on the proteins and enables them to transcriptionally activate their target genes. (Levine and Hoey, 1988) The homeodomain consists of three helices that bind directly to regulatory sequences of target genes, influencing their expression (Figure 1.6). In this way, they act as transcription factors. There are approximately 200 homeobox genes that are subdivided into families based on the sequence similarity among their respective homeodomains. The most widely studied vertebrate homeobox genes in humans and mice are the HOX genes, which are homologues of the HOM-C homeotic genes first identified in *Drosophila* (Lewis, 1978).



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**Figure 1.6 Homeodomain region of protein binding to DNA**

The homeodomain has a structure of three helices that bind directly to regulatory sequences of target genes, influencing their expression. Here, the homeodomain from the drosophila paired protein is shown binding to a DNA oligonucleotide.

*From Cell (Wilson et al., 1995)*

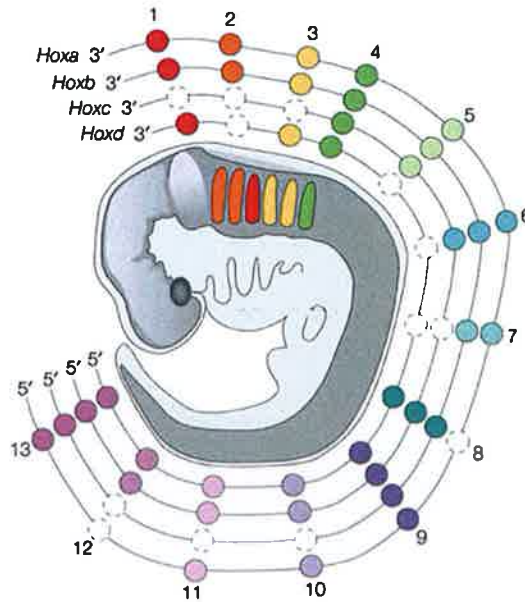
There are at least 39 HOX genes contained in human DNA. These are organized in four genomic clusters, HOX A, B, C and D, on chromosomes 7, 17, 12 and 2 respectively (Scott, 1992). HOX gene nomenclature is directly derived from their chromosomal positioning – A, B, C or D, followed by a numerical value 1-13, depending on their position in the 5' to 3' chromosomal coordinate. There is strong homology among genes in equivalent positions on different chromosomes.

### **1.8.2 HOX genes in embryogenesis**

In humans, HOX genes act as master regulators during embryogenesis and early development, when they are expressed in a spatiotemporal fashion. HOX genes are expressed as early as gastrulation (Dekker et al., 1992). HOX gene expression controls the identity of various regions along the body axis (Graham et al., 1989). HOX genes are expressed in sequence in the 3'→

5' direction along the anterior/posterior (A/P) axis of the embryo, with the lower-numbered genes, e.g. HOXA1, expressed earlier and more anteriorly than the higher-numbered genes, e.g. HOXA10. This one-dimensional representation of gene expression by a cluster of genes within the genome has been termed colinearity and is illustrated in Figure 1.7.

Loss and gain of function analyses have revealed that the vertebrate homeobox gene network regulates a broad spectrum of biological functions during embryonic development (McGinnis and Krumlauf, 1992, Mark et al., 1997). These include limb formation, axial skeleton patterning, craniofacial morphogenesis, development of the central nervous system and organogenesis including development of the gastrointestinal tract and reproductive organs. It has been shown that homozygous deletion of certain homeobox genes results in specific morphological defects in facial, neck or thoracic structures, depending on the gene mutated (Daftary and Taylor, 2000).



**Figure 1.7 Colinear cluster arrangement of homeobox genes in the human embryo**

The figure shows the four Hox clusters, with each paralogous group identified by a specific colour code. The position of each paralogous group corresponds approximately to the rostral border of its expression domain in the mammalian embryo. Detailed expression patterns are shown for Hox paralogous groups 1 – 4 in the rhombomeres [definition: transiently divided segments of the developing neural tube, within the hindbrain region, in the area that will eventually become the rhombencephalon]. The colouring in the hindbrain indicates the most rostral rhombomeres that express each gene. With the exception of the group 1 genes, the expression domains generally continue through the spinal cord to the posterior end of the neural tube.

*From Nature Reviews Neuroscience (Santagati and Rijli, 2003)*

### **1.8.3 HOX genes in adult cells**

Apart from antero-postero patterning during embryonic development, multiple cellular processes are regulated through the HOX network. These include the following: acquisition and maintenance of spatial and temporal cell allocation; establishment of cell identity, achieving specific cell phenotypes through the decoding of microenvironment signals; control of cell growth and proliferation through interaction with the cell cycle and apoptotic pathways; cell-cell communication through cross-talk with growth factors, cytokines and signal transduction pathways; maintenance of tissue specific architecture (Cillo et al., 2001).

HOX genes demonstrate tissue specific expression in normal adult human organs (Cillo, 1994, Takahashi et al., 2004). Expression of HOX genes has been described in the normal adult lung (Tiberio et al., 1994), colon (De Vita et al., 1993), kidney (Cillo et al., 1992) and breast (Cantile et al., 2003).

### **1.8.4 HOX genes and cancer**

It is widely accepted that neoplasia shares many of the same pathways as normal embryogenesis, and that tumour development is an aberrant form of organogenesis (Samuel and Naora, 2005). On this premise, a large body of literature has been produced investigating the role of the homeobox gene network in tumourigenesis.

Two trends of homeobox gene expression have emerged from the functional studies to date – namely the upregulation of certain genes and the downregulation of others. For example, HOXB5 and HOXB9 are expressed in normal kidney but not in renal cancer (Cillo et al., 1992) and overexpression of HOXC8 in prostate cancer correlates with loss of a differentiated phenotype (Waltregny et al., 2002).

Many of the homeobox genes that are downregulated or lost in tumours are normally upregulated during embryogenesis and maintained in fully

differentiated adult tissues. For example, loss of expression of HOXA5 in breast cancers correlates with loss of p53 expression, and HOXA5 has been found to activate the promoter of the p53 gene (Raman et al., 2000). Furthermore, molecular biology approaches have shown that aberrant redeployment of HOX genes converted certain cells into a malignant phenotype. Examples include forced expression of HOXA1 in breast cancer cells resulting in increased cell growth (Zhang et al., 2003b) and transfection of HOXD3 in to lung cancer cells resulting in a more metastatic and motile phenotype (Hamada et al., 2001).

### ***1.9 Interaction of HOXC11 and SRC-1; targeting S100B***

As outlined, HOX genes are expressed in embryogenesis and in adult tissues and are differentially activated in normal and malignant phenotypes. One mechanism by which HOX transcription factors might exhibit these differential phenotypic effects is through the presence or absence of different binding partner proteins.

Understanding the interaction of HOX proteins and their target genes is also essential in elucidating their role in tumorigenesis. Over three decades since the Homeobox genes were first discovered (Lewis, 1978) the majority of direct HOX target genes remain elusive (Svingen and Tonissen, 2006). Furthermore, those target genes which have been identified have no functional similarities; they range from transcription factors (Guazzi et al., 1994) to cell adhesion molecules (Jones et al., 1992) to structural proteins (Tkatchenko et al., 2001).

Our particular HOX gene of interest is HOXC11. The differential expression of HOXC11 has been associated with several cancers including those of the colon, cervix, prostate and breast (Makiyama et al., 2005, Miller et al., 2003, Hung et al., 2003). As previously described, the coactivator protein SRC-1 has been proposed by our group as a binding partner for HOXC11 (McIlroy et al., 2010).



Several HOX responsive elements have been identified in the promoter region of the S100B gene (Zhang et al., 2007). Zhang et al. examined the effect of forced expression of HOXC11 in neuronal cells and found that it induced the expression of S100B. In transient transfection experiments, the overexpression of HOXC11 transactivated the S100B promoter-reporter construct. This information was used to perform further bioinformatic analysis. HOXC11 binding motifs (TAAT) were thereby located in the promoter region of the S100B gene.

Given the strong association between S100B and malignant melanoma, we believe that this pathway may have a role in melanoma tumourgenesis. Elucidating the pathway will provide us with potential valuable therapeutic targets. This would allow precise targeting of treatment approaches and rational treatment selection.

### **1.10 Hypothesis**

The significance of S100B in malignant melanoma is well established. Little is known about the transcriptional regulation of S100B, though it is assumed to be a complex process, given the multiple molecular pathways in which S100B is involved. Studies in cancers including those of the breast and prostate have identified the coactivator SRC-1 to be a strong indicator of disease recurrence and poor survival. While carrying out mass spectrometry discovery analysis, looking for new transcription factor hosts for the coactivator SRC-1, our group identified HOXC11. HOXC11 is known to enhance expression of S100B. To date there have been no studies investigating the role of HOXC11 in malignant melanoma. We hypothesized that production of S100B in malignant melanoma is regulated by HOXC11 in cooperation with SRC-1.

As described above, the first part of this work seeks to better refine our knowledge of transcriptional regulation of S100B. S100B is well established as a serum biomarker in malignant melanoma. As such, it has been shown to correlate with disease stage and response to treatment. In addition to S100B, a number of other serum biomarkers have been identified. In spite of our increased understanding of the significance of these biomarkers, we still fail to predict recurrence in a significant proportion of patients with malignant melanoma.

The availability of protein microarray technology presents the opportunity to screen serum samples from patients with melanoma and examine their immune response biomarker profile. Thus to follow on from our work on S100B, we sought to identify autoantibody biomarkers in a cohort of patient serum samples using a ProtoArray® protein microarray (Invitrogen, CA USA). We hypothesized that this novel technique may allow us to identify potentially useful new biomarkers in malignant melanoma.

## **1.11 Aims**

The aims and specific objectives of the study were defined as follows:

1. To define the molecular role of HOXC11 and SRC-1 in the transcriptional control of S100B in malignant melanoma.
  - a. Characterise expression of HOXC11, SRC-1 and S100B in melanoma cell lines and patient tissue samples.
  - b. Confirm interaction between HOXC11 and SRC-1 in melanoma cell lines and patient tissue samples.
2. To characterise the effect of manipulation of HOXC11 and SRC-1 on S100B expression.
  - a. Confirm recruitment of HOXC11/SRC-1 to the S100B promoter.
  - b. Investigate expression of S100B in response to suppression of HOXC11 and SRC-1.
  - c. Investigate expression of S100B in response to stimulation of HOXC11 and SRC-1.
  - d. Investigate the effect of MAPK/ Src kinase suppression on S100B expression and interaction of HOXC11 and SRC-1.
3. To identify new biomarkers in sera from patients with malignant melanoma.
  - a. Identify autoantibody biomarkers in patient sera using protein microarrays.
  - b. Validate findings from protein array by reverse ELISA on a larger cohort of patient serum samples.
  - c. Verify the protein expression of novel biomarker(s) in patient tissue samples.

## **2 Materials and methods**

## **2.1 Patient sample collection**

Local ethics committee approval was sought for the procurement and use of all patient samples. This work was carried out in close conjunction with the clinical pathology department of Beaumont Hospital Dublin.

### **2.1.1 Paraffin embedded tissue**

Archived paraffin embedded melanoma and benign nevus specimens were obtained and sectioned in full face using a microtome to 4  $\mu\text{m}$ .

### **2.1.2 Frozen tissue**

Fresh melanoma tissue samples were taken from excised specimens in the operating theatre and transported rapidly to the hospital laboratory where they were flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required. Protein was extracted from the frozen tissue by suspending the samples in a protease inhibitor/ lysis buffer solution (1:100 dilution) and sonicating on ice intermittently for 30 minutes (10 second sonication, with 5 minute break x 6).

### **2.1.3 Primary cell cultures**

Primary cell culture samples were grown from melanoma tissue specimens. Primary cultures were generated in the hospital laboratory largely according to the methods published by Stampfer et al. (Stampfer et al., 1980). Primary cell cultures were received from the hospital in 25  $\text{cm}^2$  tissue culture flasks and then plated on to coverslips in 6 well plates. Cells were treated, fixed in paraformaldehyde and stained according to standard protocols outlined below.

### **2.1.4 Serum**

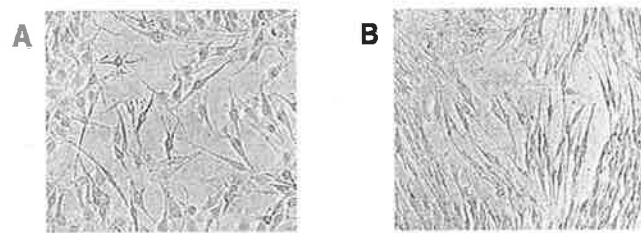
Pre-operative blood samples were obtained from patients undergoing surgery for excision of melanoma. Blood samples were also taken from age-matched patients with no previous diagnosis of melanoma to serve as controls. Blood samples were immediately spun down at 13,000 rpm and sera was extracted and stored at  $-80^{\circ}\text{C}$  until specimens were required for experiments.

## 2.2 Cell Culture

### 2.2.1 Melanoma cell lines

Two cell lines were used during the course of this study: SKMel28 cells and MeWo cells (American Type Culture Collection (ATCC), Virginia, USA) (Figure 2.1). The SKMel28 cell line is a primary melanoma cell line, initiated from the tumour of a 51 year old male patient. The cell line was initiated by T Takahashi and associates in 1979. The MeWo cell line is a malignant melanoma cell line derived from the lymph node metastasis of a white male patient aged 78 years. The cell line was initiated by Y Koderá and M Bean in 1974.

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**Figure 2.1 Cell lines used in molecular experiments**

**A** SKMel28 cells, derived from a primary cutaneous melanoma

**B** MeWo cells, derived from a melanoma lymph node metastasis

### 2.2.2 Cell culture environment

All cell culture techniques were performed in a sterile environment using a laminar airflow cabinet. All cells were maintained in a humid 5% (v/v) CO<sub>2</sub> atmosphere at 37 °C.

### 2.2.3 Culturing of cells from cryo-storage

Cryovials containing cells were removed from storage at -80°C and thawed to 4°C. The cellular contents were transferred to a sterile universal container containing 5 ml of the required culture medium. The cell suspension was

centrifuged at 1,250 rpm for 4 minutes. The supernatant was discarded and the pellet resuspended in 2 ml of fresh medium. This suspension was added to a 75 cm<sup>2</sup> tissue culture flask, to which a further 8 ml of culture medium was added. The flasks containing the cells were then incubated at 37°C.

#### **2.2.4 Routine culture**

Both cell lines were maintained in Eagle's minimal essential medium (MEM) (Invitrogen, CA USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 100 µg/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10% (v/v) fetal calf serum (Gibco® Invitrogen).

#### **2.2.5 Long-term storage of cells**

Cells were trypsinised and pelleted as described above. The cell pellets were resuspended in 2 ml of a dimethyl sulphoxide (DMSO) (Sigma-Aldrich) (10%) in FCS (90%) mix on ice. This solution was immediately aliquoted into cryovials and incubated at -80°C overnight. The vials were then placed in a cryopreserve refrigerator at -196°C.

#### **2.2.6 Cell culture treatment conditions**

Cell culture treatments were performed when the cells were 90% confluent. The culture medium was decanted from the flask and the cells were washed in sterile PBS (Sigma-Aldrich). For 24 hours prior to treatment all cells were grown in serum free MEM. The serum-free medium was decanted and the cells were then incubated under the treatment conditions outlined in Table 2.1.

**Table 2.1 Cell culture treatment conditions**

<b>Treatment</b>	<b>Final concentration</b>	<b>Treatment time</b>
Dasatinib (Sequoia Research Products, Pangbourne, UK)	100 nM in serum free MEM	2 hours (RNA) 8 hours (protein) 48 hours (migration assay)
DMSO (Sigma-Aldrich)	100 nM in serum free MEM	2 hours (RNA) 8 hours (protein) 48 hours (migration assay)
VEGF (Sigma-Aldrich)	10 ng/ml in serum free MEM	8 hours (RNA) 24 hours (protein)
dH <sub>2</sub> O - Vehicle for VEGF	10 ng/ml in serum free MEM	8 hours (RNA) 24 hours (protein)

### **2.3 Scratch wound migration assay**

Cells were seeded in 24- well plates and grown to 100% confluence. A single scratch was made in the confluent monolayer with a sterile p10 pipette tip and the wells were washed twice in PBS to eliminate detached cells. Cells were incubated for 8 hours at 37°C to allow cells to attach and then treated with dasatinib (100 nM) or DMSO (vehicle control, 100 nM) for 48 hours. The number of migrating cells at 24 and 48 hours was estimated by counting 20 fields of view of the scratch at 20x magnification.

### **2.4 Protein overexpression by transient transfection**

Introducing nucleic acids into cells facilitates the examination of genetic regulation and protein function within cells.

HOXC11 was purchased as pCMV-SPORT6.1-HOXC11 (Invitrogen, Clone ID 3462682). HOXC11 was then cloned in house in to an Invitrogen expression vector pcDNA-DEST47 (7.7 kb) using Gateway® Technology.

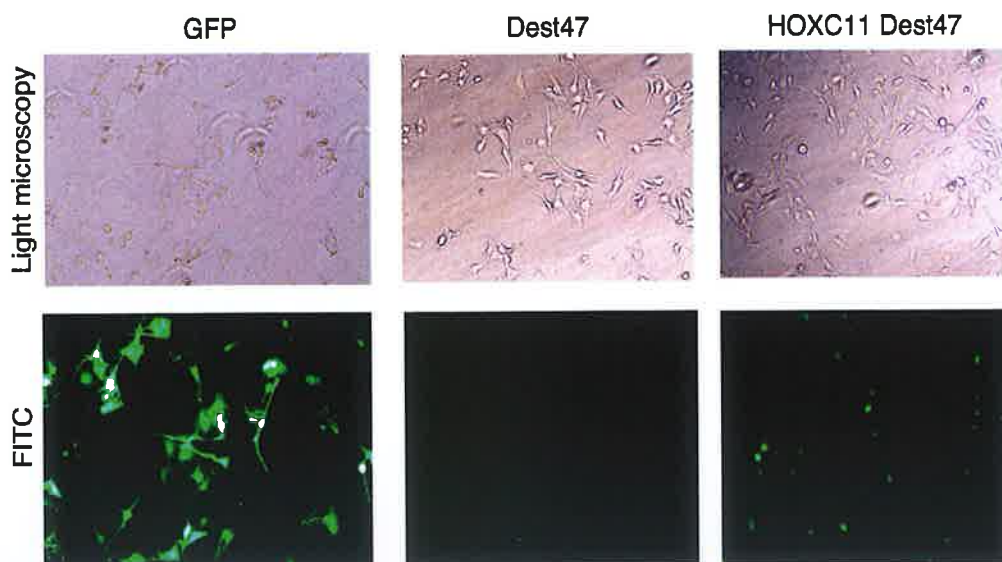
5x10<sup>6</sup> cells were grown in 6- well plates in antibiotic free medium and incubated in a CO<sub>2</sub> incubator at 37°C until 80-90% confluent. Solutions for the



transfection experiment were prepared in 1.5 ml eppendorfs as follows:

- Solution A: 400 ng of HOXC11 in pcDNA-DEST47 diluted in 375  $\mu$ l of reduced serum medium Opti-MEM (Gibco® Invitrogen).
- Solution B: 4  $\mu$ l of Lipofectamine 2000 reagent (Invitrogen) was diluted in 375  $\mu$ l of Opti-MEM. Solutions were allowed to stand for 5 minutes at room temperature.

Both solutions were mixed gently and incubated at room temperature for 20 minutes. Cells were then washed with sterile PBS. The Lipofectamine-DNA mixture was then added to the cell monolayer in a dropwise fashion and incubated at 37°C for 4 hours after which full medium was added. Protein levels and transcriptional activity were determined 24 hours post transfection. Transfection efficiency was assessed by examining cells under an inverted fluorescent microscope (Figure 2.2).



**Figure 2.2** Transient transfection of SKMel28 cells with Dest47-HOXC11

Images demonstrating SKMel28 cells transfected with GFP fluorescent tag only (*Left*), vector Dest47 (*Centre*) and vector Dest47 plus HOXC11 (*Right*). A transfection efficiency of approximately 30% was achieved.

## **2.5 Gene silencing**

Gene silencing is when a particular gene of interest is 'knocked-down' or silenced. In this study, silencing was carried out using RNA interference (RNAi) technology as described by Elbashir et al. (Elbashir et al., 2001). Predesigned and validated siRNA directed against HOXC11 (Qiagen) and SRC-1 (Ambion) were used in the knockdown studies.

$1 \times 10^5$  cells were grown in antibiotic and serum free media for 24 hours in 6-well plates. Cells were transfected when 50% confluent. Oligomer-Lipofectamine 2000 complexes were prepared as follows:

- Solution A: 60 pmol of siRNA SRC-1 was diluted in 300  $\mu$ l of Opti-MEM serum reduced media.
- Solution B: 5  $\mu$ l of Lipofectamine 2000 was diluted in 300  $\mu$ l Opti-MEM serum reduced medium. Solutions were incubated at room temperature for 5 minutes.

The diluted oligomer was then mixed with the diluted Lipofectamine 2000 solution and incubated at room temperature for 20 minutes. The oligomer-Lipofectamine complex was added to the cell monolayer and mixed gently by rocking the plate back and forth. Cells were incubated at 37°C for 6 hours after which the transfection media was replaced with standard MEM.

## **2.6 Immunofluorescence**

Immunofluorescent microscopy for detection of HOXC11 and SRC-1 was performed on all paraffin-embedded melanoma and nevus samples using a secondary fluorochrome conjugated antibody.

### **2.6.1 Antibodies**

The antibody against SRC-1 protein is a rabbit polyclonal antibody raised against amino acids 350-690 of SRC-1 of human origin (M-341 Santa Cruz, California, USA). The antibody against HOXC11 protein is raised against the promoter element of the lactase hydrolase gene. It is an affinity isolated antibody raised in chicken (GW22000F Sigma-Aldrich, St Louis, MO, USA). Negative controls were performed with omission of the primary antibody.

## **2.6.2 Staining protocol**

Four micron thick tissue sections were cut from paraffin embedded tissue blocks and mounted on Superfrost Plus slides (BDH, Poole, UK). Sections were dewaxed by passage through containers of xylene for 3 minutes each and rehydrated by immersion in industrial methylated spirits (IMS) (Lennox Chemicals, Dublin, Ireland) of decreasing concentrations (100%, 90%, 70%) for 3 minutes each. The sections were then washed twice in PBS (Sigma-Aldrich, Steinheim, Germany) for 3 minutes. Endogenous peroxidases were blocked with 3% H<sub>2</sub>O<sub>2</sub>. Antigen retrieval was carried out by heating sections in 0.1 M sodium citrate buffer (pH 6) (Sigma-Aldrich) for 7 minutes and cooling for 20 minutes. Slides were washed twice in PBS for 5 minutes and a liquid-repellant pen (Dako, Denmark) was used to delineate the tissue. Sections were incubated in 50% goat serum for 90 minutes. Chicken anti-human HOXC11 (0.4 µg/ml) and rabbit anti-human SRC-1 (0.5 µg/ml) in 10% (v/v) human serum was placed on each slide for 60 minutes. The sections were washed in PBS and incubated with goat anti-chicken and goat anti-rabbit secondary fluorochrome conjugated antibodies for HOXC11 (TRITC labeled fluorescent antibody which emits at 488 nm) and SRC-1 (FITC labeled fluorescent antibody which emits 594 nm) respectively. Both secondary antibodies were diluted to concentration 1:1000 in PBS. Specimens were covered and incubated at room temperature for 30 minutes. Following washing with PBST, slides were incubated with 4'6-diamino-2-phenylindole (DAPI) stain (1:1000 in PBST) for 3 minutes. Sections were rinsed in tap water and mounted using fluorescent mounting media (Dako).

## **2.7 Protein biochemistry**

### **2.7.1 Protein extraction**

Pefabloc protease inhibitor (10 µl) (Roche Diagnostics, Mannheim Germany) was added to lysis buffer (1 ml) (Appendix 1) to make the protein lysis solution. Lysis solution (100 µl) was added to each cell pellet. The samples were placed on ice and vortexed at 10-minute intervals for 30 minutes. The samples were then centrifuged at 13,000 rpm for 20 minutes at 4°C. The

resultant supernatant (protein lysate) was transferred into chilled eppendorf tubes and stored at -20°C.

### **2.7.2 Protein quantification**

This procedure was performed using the Pierce Bicinchoninic acid (BCA) protein assay kit (Pierce, IL USA). A standard curve was obtained by serially diluting 5 mg/ml bovine serum albumin (BSA) in dH<sub>2</sub>O. A blank solution containing only dH<sub>2</sub>O was also included. Protein lysate samples (5 µl) were diluted 1:10 in dH<sub>2</sub>O. Standards and diluted samples (25 µl) were pipetted in duplicate into a 96 well plate. The reaction mix was made up with 49 parts of Solution 1 of the Pierce BCA protein assay kit (containing Na<sub>2</sub>CO<sub>3</sub>, NaHCO<sub>3</sub>, BCA detection reagent and sodium tartrate in 0.5 M NaOH) with one part of Solution 2 (4% (w/v) CuSO<sub>4</sub>). Standards and samples were incubated with 200 µl of reaction mix at 37°C for 30 minutes. The absorbance of the samples was analysed at 450 nm using a spectrophotometer. Linear regression analysis of the BCA standard curve (y axis absorbance, x axis protein concentrations) was used to calculate the unknown protein concentrations of each sample.

### **2.7.3 Coimmunoprecipitation**

Coimmunoprecipitation is a process that allows examination of a protein-protein interaction.

Equal concentrations of total protein (1 mg) were precleared by rotation with 50 µl of Protein A/G agarose beads (Santa Cruz) (previously blocked in bovine serum albumin 3% overnight at 4°C) for 4 hours at 4°C. Protein agarose was then eliminated from the protein lysate by 3 minutes centrifugation at 1000 rpm at 4°C. Protein was immunoprecipitated with anti-SRC-1 (6 µg) or anti-HOXC11 (6 µl g) overnight at 4°C. The precipitates were collected for 60 minutes on a Protein A/G agarose complex. The samples were centrifuged at 5,000 rpm for 60 seconds and the supernatant was discarded. The remaining cell precipitates were washed 3 times in radioimmunoprecipitation (RIPA) buffer (Appendix 1) and centrifuged at

5,000 rpm for 60 seconds. The precipitates were then resuspended in 2X Laemmli SDS sample buffer (Sigma-Aldrich), boiled at 95°C for 10 minutes and analyzed by SDS-PAGE and Western blotting.

#### **2.7.4 Chromatin immunoprecipitation**

The purpose of a Chromatin immunoprecipitation (ChIP) assay is to determine whether proteins bind to a particular region on the endogenous chromatin of living cells or tissues.

Cells were cultured in 75 cm<sup>2</sup> tissue culture flasks to approximately 80-90% confluence. Cells were fixed with 1% formaldehyde in Eagle's MEM medium lacking FBS for 10 minutes before quenching with glycine (125 mM, 5 minutes at room temperature). Cells were washed in ice-cold PBS before being collected in a solution of PBS and protease inhibitors (10 µl/ml). Sonication conditions were tested to yield DNA fragments averaging 200 – 500 bp as assessed by agarose gel electrophoresis (Figure 2.3) and were as follows: 12 sonications (10 seconds), with 2 minutes between each; output control 4-5; duty cycle 60%. Lysates were clarified, diluted 1:5 in ChIP dilution buffer with protease inhibitors (10 µl/ml) and pre-cleared with salmon sperm (75 µl) for 30 minutes at 4°C. For each Immunoprecipitation, 7 µg of HOXC11 antibody was added to the lysate. After incubation with antibody overnight at 4°C, 60 µl of a goat anti-chicken IgY-Agarose was added for an additional 90 minutes of incubation. Beads were then washed consecutively for 5 minutes on a rotating platform with 1ml of each solution: (a) low salt wash buffer, (b) high salt wash buffer, (c) Lithium Chloride wash buffer and (d) 1X TE buffer twice (Appendix 1). Protein-DNA complexes were eluted and purified and subjected to PCR for amplification of the S100B promoter.



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**Figure 2.3 Shearing of CHIP DNA by sonication**

Agarose gel electrophoresis demonstrating DNA sheared in to fragments of 200 – 500 bp in size. A 1 kb DNA ladder was used, with an effective size range of 500 bp to 10 kb.

**2.7.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)**

Electrophoresis is one of the most commonly used techniques to separate proteins according to their electrophoretic mobility or molecular weight.

Polyacrylamide gels of varying composition were cast for each protein in an ATTO gel system (Atto Corporation, Tokyo Japan) and allowed to polymerize for 20 minutes. Molecular weight markers (8  $\mu$ l) (Bio-Rad, CA USA) were loaded directly onto the gel. Protein samples (50 - 100  $\mu$ g) were made up with 2X reducing Laemmli SDS sample buffer and heated to 95°C for 2 minutes. Samples were loaded in to lanes and the gel was run in chilled running buffer (Appendix I) according to the conditions summarized in table 4.2 Gels were then transferred on to a nitrocellulose membrane using an ATTO semi-dry transfer system. Protein transfer was confirmed by rinsing the nitrocellulose membrane with Ponceau S solution (Sigma-Aldrich).

### **2.7.6 Western blotting**

Non-specific binding sites on the membrane were blocked by incubating the membrane with 5 % molecular-grade powdered milk (milk details) in 0.05% Triton X-100/Tris buffered saline (TBST) (Sigma-Aldrich) for 60 minutes with continuous rocking at room temperature. The membrane was incubated overnight with primary antibody, made up as outlined in Table 2.2. The housekeeping proteins  $\beta$ -Actin and HSP70 were used to ensure correct loading of samples. The membrane was then washed 3 times for 10 minutes each with 0.05% TBST prior to incubation with a Horseradish Peroxidase (HRP) conjugated secondary antibody at concentrations outlined in Table 2.2. The membrane was again washed for 10 minutes 3 times with 0.05% TBST. Protein bands were detected by the addition of a chemiluminescent substrate. Light emitted during an enzyme-catalysed decomposition reaction was captured by exposure to Fuji X-ray film for 30 seconds to 10 minutes. The size of the bands was determined using the molecular weight markers.

**Table 2.2 Western blotting protocols**

<b>Protein</b>	<b>% Gel</b>	<b>Transfer</b>	<b>Primary antibody</b>	<b>Secondary antibody</b>	<b>Chemiluminescent reagent</b>
HOXC11 33 KDa	10	45 minutes Semi-dry	Sigma Anti-HOXC11 (Cat No GW22000F) 1:2000 in PBS (Note: wash in PBST)	Genway GAYFC- HRP Goat Anti- Ch (Ref 25-288- 11000) 1:1000 in PBS	ECL x 1 minute
SRC-1 160 KDa	6	90 minutes Semi-dry	Santa Cruz Rb poly Anti-SRC-1 (Ref sc-8995) 1:100 in 2% milk	Anti-Rb 1:2000 in 2% milk	PICO x 1 minute
S100B 10.5 kDa	15	30 minutes Semi-dry	Abcam Ms (Ref ab8330-100) Anti-S100 1:1000 in 2% milk	Anti-Ms 1:10,000 in 2% milk	Supersignal x 1 minute
BMX 80 KDa	10	45 minutes Semi-dry	BD Biosciences Anti-BMX (Cat No 610793) 1:1000 in 2% milk	Anti-Ms 1:5000	ECL x 1 minute
$\beta$ -Actin 45 KDa	-	-	Anti B-Actin 1:7500 in 2% milk	Anti-Ms 1:7500 in 2% milk	ECL x 1 minute
HSP70 70 KDa	-	-	Anti HSP-70 1:2000 in 2% milk	Anti-Ms 1:2000 in 2% milk	ECL x 1 minute



## **2.8 Nucleic acid biochemistry**

### **2.8.1 mRNA purification**

mRNA was purified from cell lysate using a RNeasy Mini Kit (Qiagen, West Sussex UK), which included pre-mixed buffer solutions. RLT buffer and mercaptoethanol (1%) solution (350  $\mu$ l) was added to cell lysate samples and homogenised using a needle and syringe. An equal volume of 70% ethanol was added to each sample and applied to the spin column. The column was centrifuged at 10,000 rpm for 15 sec. Flow-through was discarded and the column was washed with each of the following solutions: Buffer RW1 (700  $\mu$ l), 2 X Buffer RPE (500  $\mu$ l). RNA was eluted from the column by adding 30  $\mu$ l of RNase free water directly on to the membrane. Samples were stored at  $-80^{\circ}\text{C}$ .

### **2.8.2 Nucleic acid quantification**

An NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Willmington, DE USA) was used to assess and quantify DNA and RNA. The ratio of absorbance at 260 nm and 280 nm of 2  $\mu$ l of sample was used to assess the purity of DNA and RNA. A ratio of  $\sim 1.8$  was accepted as "pure" for DNA; a ratio of  $\sim 2.0$  was accepted as "pure" for RNA.

### **2.8.3 Reverse transcription**

Reverse transcription is a process in which single stranded mRNA is reverse transcribed in to complementary DNA (cDNA). Using a Gradient cycler (Biosciences, Dublin Ireland), 1  $\mu$ g of RNA was denatured with oligo dT primers (1  $\mu$ l) at  $70^{\circ}\text{C}$  and quickly chilled on ice. RNase inhibitor (1  $\mu$ l), dNTPs (10 mM, 1  $\mu$ l), M-MLV reverse transcriptase (1  $\mu$ l), 5X First Strand (FS) Buffer (4  $\mu$ l) and 0.1 M dithiothreitol (DTT) (2  $\mu$ l) were added to the reaction mix and the RT reaction was extended at  $37^{\circ}\text{C}$  for 50 minutes. The enzyme was inactivated by heating the reaction mix to  $70^{\circ}\text{C}$  for 15 minutes. Samples were stored at  $-80^{\circ}\text{C}$ .

## 2.8.4 Polymerase chain reaction

Polymerase chain reaction (PCR) is a rapid means of producing a relatively large number of copies of DNA molecules from minute quantities of source DNA material. There are three major steps in a PCR; denaturation (94°C), annealing (60°C) and extension (72°C) which are each repeated for 30 or 40 cycles.

Levels of HOXC11, SRC-1 and S100B mRNA were assessed by quantitative real-time PCR (LightCycler, Roche Laboratories). A reaction mix of cDNA (2  $\mu$ l), Sybergreen Quantitect PCR reagent (10  $\mu$ l, Qiagen), primer mix (1  $\mu$ l) and dH<sub>2</sub>O (10  $\mu$ l) was used. Primers were designed using Oligoperfect software (Invitrogen) and were purchased from Invitrogen. Forward and reverse sequences for all primers used are summarized in Table 2.3.

**Table 2.3 Primers for PCR**

Gene	Forward primer sequence	Reverse primer sequence
S100B promoter	TGGCAGAGGAGAGAAGCTC	TTCCTGAGCGTCCTCTTGG
Non-promoter S100B	TGGTGAGGTTGTATCCACGA	CCACTCATGCAATGACCGTA
SRC-1	TTGACAGCTTGAGTGTAACCA	CATCGTCATCAGTTGTTGATTTC
HOXC11	AACACAAATCCCAGCTCGTC	AAAAACTCTCGCTCCAGTTCC
B-Actin	TCACCCACACTGTGCCATCTA	CAGCGGAACCGCTCATTGCCA

DNA products from ChIP experiments were amplified using an automated thermocycler (Biosciences, Dublin Ireland). The PCR reaction mix was made up in a final volume of 50  $\mu$ l, as shown in Table 2.4. Thermocycler settings for amplification of the S100B promoter region are outlined in Table 2.5.

**Table 2.4 ChIP PCR reaction mix**

Reagent	Volume ( $\mu$ l)
10X PCR buffer	5
MgCl	1.5
dNTP mix	1
Forward primer mix	2.5
Reverse primer mix	2.5
Taq polymerase	1
Input DNA	17
Output DNA	34
dH <sub>2</sub> O	(Up to total 50)

**Table 2.5 Thermocycler settings for amplification of S100B promoter**

Step No.	Temperature ( $^{\circ}$ C)	Time
1	95	2 minutes
2	94	45 seconds
3	62	45 seconds
4	72	1.5 minutes
5	Repeat Step 2-4 x 34 cycles	
6	72	15 minutes
7	4	Forever

### 2.8.5 Preparing and running standard agarose DNA gels

Agarose powder (Promega, Mannheim Germany) was dissolved with heating in 1X tris acetate EDTA (TAE) buffer (Appendix I) at a concentration of 1.5% (0.75 mg agarose in 50 ml TAE). After cooling, ethidium bromide was added to the gel (final concentration 0.5  $\mu$ g/ml) and the solution was allowed to polymerise at room temperature in a casting tray containing a sample comb. Samples were loaded in to the wells with loading dye. The gel was run at 100 V for 45 minutes in 1X TAE buffer. The gel was placed on an ultraviolet transilluminator and the image recorded.

## **2.9 Enzyme-Linked ImmunoSorbent Assay (ELISA)**

ELISA is an enzyme immunoassay used for the measurement of a secreted antigen – in this case, S100B in conditioned media. It is a heterogenous, solid phase assay that requires the separation of reagents. The technique used in the S100B kit is a sandwich or double antibody technique. Initially the antibody is bound to the well with the antigen to be measured. An enzyme conjugate is then added to the well with bound antigen-antibody or immune complex. Following this a substrate is added to the enzyme conjugate which is bound to the immune complex. If there are changes due to the presence of the enzyme conjugate bound to the immune complex, a positive test or colour change will occur (Monroe, 1984).

### **2.9.1 Preparation of conditioned media**

Cells were incubated in 75 cm<sup>2</sup> flask with Eagle's MEM. Following the relevant treatment / transfection, media was aspirated and centrifuged at 1250 rpm for 4 minutes. Samples were stored at -80°C.

### **2.9.2 Preparation of reagents**

The unopened reagents were allowed to reach room temperature before use. Reagents were prepared immediately prior to use. Calibrators and controls were reconstituted in 1 ml dH<sub>2</sub>O and allowed to stand for 20 minutes. Fresh 1X wash buffer was prepared in dH<sub>2</sub>O. Other reagents were ready for use from the kit.

### **2.9.3 ELISA Protocol**

A Sangtec® 100 ELISA (DiaSorin, Stillwater MN USA) was used for the quantitative measurement of S100B protein in conditioned media from the knockdown experiments and from cells treated with VEGF and Dasatinib. The Sangtec® 100 ELISA is a two-site, one-step enzyme linked immunosorbent assay. Calibrators, controls and unknown samples were pipetted in to the wells (50 µl). 150 µl of conjugate was added to the wells and the plate was covered and incubated for 2 hours at room temperature on a plate shaker (800 rpm). The wells were washed 3 times with 300 µl of 1X

wash buffer and tetramethylbenzidine substrate (100  $\mu$ l) was added to all wells. The plate was covered and incubated 15 +/- 2 minutes on the plate shaker (800 rpm) at room temperature. The reaction was stopped by adding 100  $\mu$ l of Stop Solution in the same order and speed as the TMB substrate. Optical absorbance was read immediately using a microplate reader at 450 nm. The mean absorbance values of the standard solutions were calculated and a standard curve was plotted on semi logarithmic graph paper. The average absorbance of each sample was used to determine the corresponding value by simple interpolation from the standard curve.

### **2.10 Protein microarray**

Serum from 5 patients with melanoma and 4 controls were screened using ProtoArray<sup>®</sup> human protein microarrays for immune response biomarker profiling (PAH05241020, Invitrogen). In a chilled 4-chamber incubation tray, arrays were incubated with 5 ml chilled blocking buffer (Appendix 1) on a plate shaker set at 50 rpm for 1 hour at 4°C. Arrays were washed with 5ml chilled washing buffer (Appendix 1) for 5 minutes at 4°C. Arrays were incubated with 5 ml patient serum (1:500 dilution in washing buffer) for 90 minutes at 4°C on a plate shaker set at 50 rpm. Serum was aspirated and arrays were washed 4 x 5 minutes with 5ml washing buffer. Arrays were incubated with 5 ml AlexaFluor<sup>®</sup> 647 antibody solution (1  $\mu$ g/ml) for 90 mins at 4°C on a plate shaker set at 50 rpm. Antibody solution was aspirated and arrays were washed 4 x 5 mins with 5ml washing buffer. Arrays were transferred from the incubation tray to a slide holder and rinsed 3 times in dH<sub>2</sub>O. Arrays were placed in to 50 ml conical tubes and centrifuged at 200 G for 1 minute at room temperature to dry. Images were captured using a fluorescent microarray scanner and analysed using GenePix<sup>®</sup> Pro microarray acquisition and analysis software (Invitrogen).

### **2.11 Reverse ELISA**

Detection of antibodies to purified recombinant human BMX in the serum of 14 patients with melanoma (5 from protein array and 9 new patients) and 12 control patients (4 from protein array study and 8 new patients) was

performed using a direct enzyme-linked immunoassay (ELISA). The entire assay was carried out at room temperature. The purified protein (50  $\mu$ l at 5  $\mu$ g/ml) was added to 48 wells of a 96 well plate (Thermo Scientific, Waltham, MA) and incubated for 2 hours. The plate was washed 6 times with PBS containing 0.1% Tween 20 (PBST) and blocked for 2 hours with PBST containing 3% BSA. After a further 6 washes, 50  $\mu$ l of each serum sample (1:500 dilution in PBST containing 0.1% BSA) was added to a well that was coated with antigen and a well that was not and incubated for 1 hour. The plate was washed 6 times with PBST and 50  $\mu$ l of HRP-conjugated polyclonal rabbit anti-human IgG (IS512; Dako), diluted 1:5000 in PBST, was added to each well and incubated for 1 hour. The plate was washed 6 times in PBST and 50  $\mu$ l of 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulphonic acid] (ABTS; Calbiochem, Schwalbach, Germany) was added for 30 minutes. Absorbance was read at 450 nm using a microplate reader. The results for each serum sample were calculated by subtracting the reactivities of the well containing no antigen from the well containing the antigen.

### **2.12 Statistical analysis**

Statistical analysis was carried out using SPSS version 18.0 for Mac (SPSS Inc., Chicago IL USA). Univariate analysis was performed using Fisher's exact test for categorical variables and Wilcoxon's test for continuous variables. A P value of less than 0.05 was considered significant.

### **3 Characterisation of SRC-1 and HOXC11 expression in melanoma cell lines and tumour specimens**

### **3.1 Introduction**

S100B has a well-established and clinically significant role in melanoma. It is widely used to differentiate malignant melanoma from other tumours of non-melanocytic origin (Trocha et al., 2002). S100B is a secreted protein and serum levels are used as a marker of tumour burden, response to treatment and prognosis: high serum levels of S100B at initial diagnosis predict poor outcome (Harpio and Einarsson, 2004, Hauschild et al., 1999b, Mocellin et al., 2008).

Translational studies from our group and others have provided substantial evidence for the significant role of p160 Steroid Receptor Co-activator (SRC) proteins as strong indicators of disease recurrence and poor survival in breast and prostate cancer (Myers et al., 2004, Gregory et al., 2001). As distinct from other oncogenes, a specific role for SRC-1 in the development of breast cancer metastasis has also been described (Wang et al., 2009). Coactivator proteins interact with a variety of different transcription factors. Recently our group has characterised the interaction of SRC-1 with the homeobox protein HOXC11 (McIlroy et al., 2010).

In addition to their control over axial specification during embryogenesis, Hox genes also regulate skin morphogenesis. A number of groups have demonstrated expression of several Hox genes, particularly those in the Hox-b cluster, in fetal and adult murine skin (Detmer et al., 1993, Mathews et al., 1993, Bieberich et al., 1991, Rieger et al., 1994). In 1998, Stelnicki et al demonstrated changing expression of the 39 human HOX genes during embryonic development and in adult human skin (Stelnicki et al., 1998). Furthermore, it is thought that HOX genes may regulate melanocyte topographic differentiation and positional memory (Chang et al., 2002). For example, palmar and plantar skin is hypopigmented and this can partly be accounted for by the fact that fibroblasts in these areas are heterogenous in their HOX gene expression.



The aforementioned work from our lab identified S100B as a specific target gene of the SRC-1 – HOXC11 complex (McIlroy et al., 2010). In line with this, Zhang et al. have previously reported that forced expression of HOXC11 in neuronal cells induced expression of S100B protein (Zhang et al., 2007). On this background, the expression of HOXC11 and its coactivator SRC-1 was investigated in melanoma cell lines and tumour tissue, which are known to express S100B.

### **3.2 Aims**

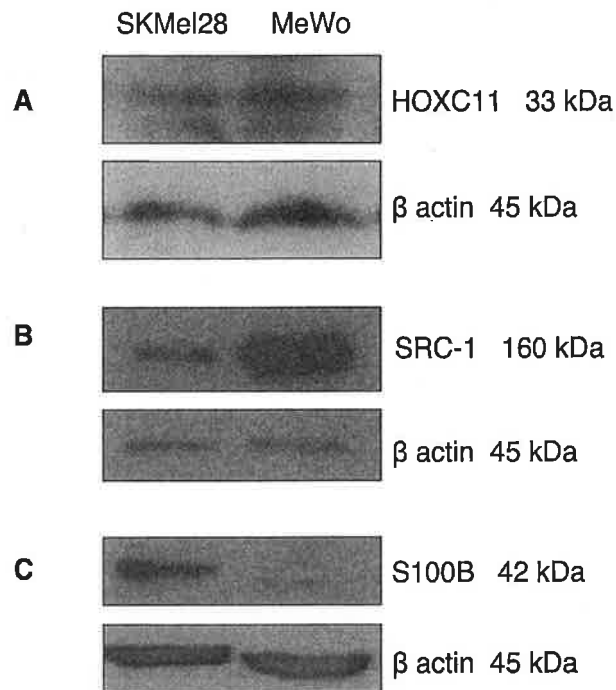
The aims of this chapter relate to the first aim of the study: to define the molecular role of HOXC11 and SRC-1 in the transcriptional control of S100B in malignant melanoma. The following specific objectives were defined:

- a. Characterise expression of HOXC11, SRC-1 and S100B in melanoma cell lines and patient tissue samples.
- b. Confirm interaction between HOXC11 and SRC-1 in melanoma cell lines and patient tissue samples.

### 3.3 Results

#### 3.3.1 Expression of HOXC11, SRC-1 and S100B in melanoma cell lines

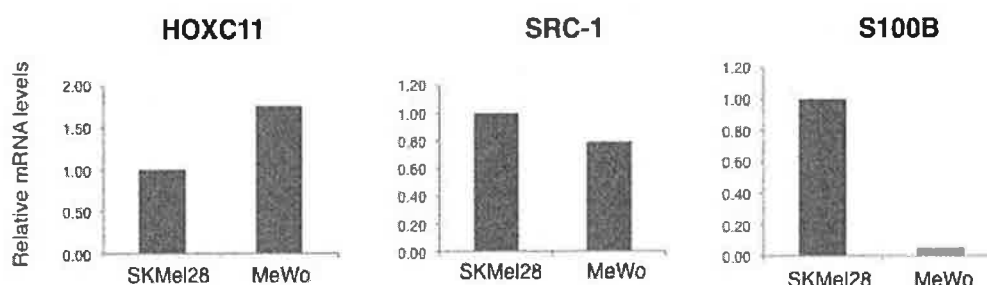
Western blotting was performed to investigate the protein expression of HOXC11, SRC-1 and S100B in melanoma cell lines. As shown in Figure 3.1, equal expression of HOXC11 was observed in both the primary SKMel28 cells and the metastatic MeWo cells. Higher expression of SRC-1 was seen in the more invasive MeWo cells. S100B was observed as a 42kDa tetramer and was more abundantly expressed in the SKMel28 cells.



**Figure 3.1 Expression of HOXC11, SRC-1 and S100B in two melanoma cell lines.**

**A** Expression level of HOXC11 was the same in both the primary (SKMel28) and metastatic (MeWo) cells. **B** Higher expression of SRC-1 was demonstrated in MeWo cells. **C** S100B was demonstrated in a higher concentration in SKMel28 cells. Results shown are representative of three separate experiments.

Relative mRNA levels were also investigated, using quantitative real time PCR (qRT-PCR). Results are shown in Figure 3.2. Higher levels of HOXC11 were observed in MeWo cells and higher levels of SRC-1 in SKMel28 cells. In keeping with the protein findings, higher S100B was seen in the SKMel28s.

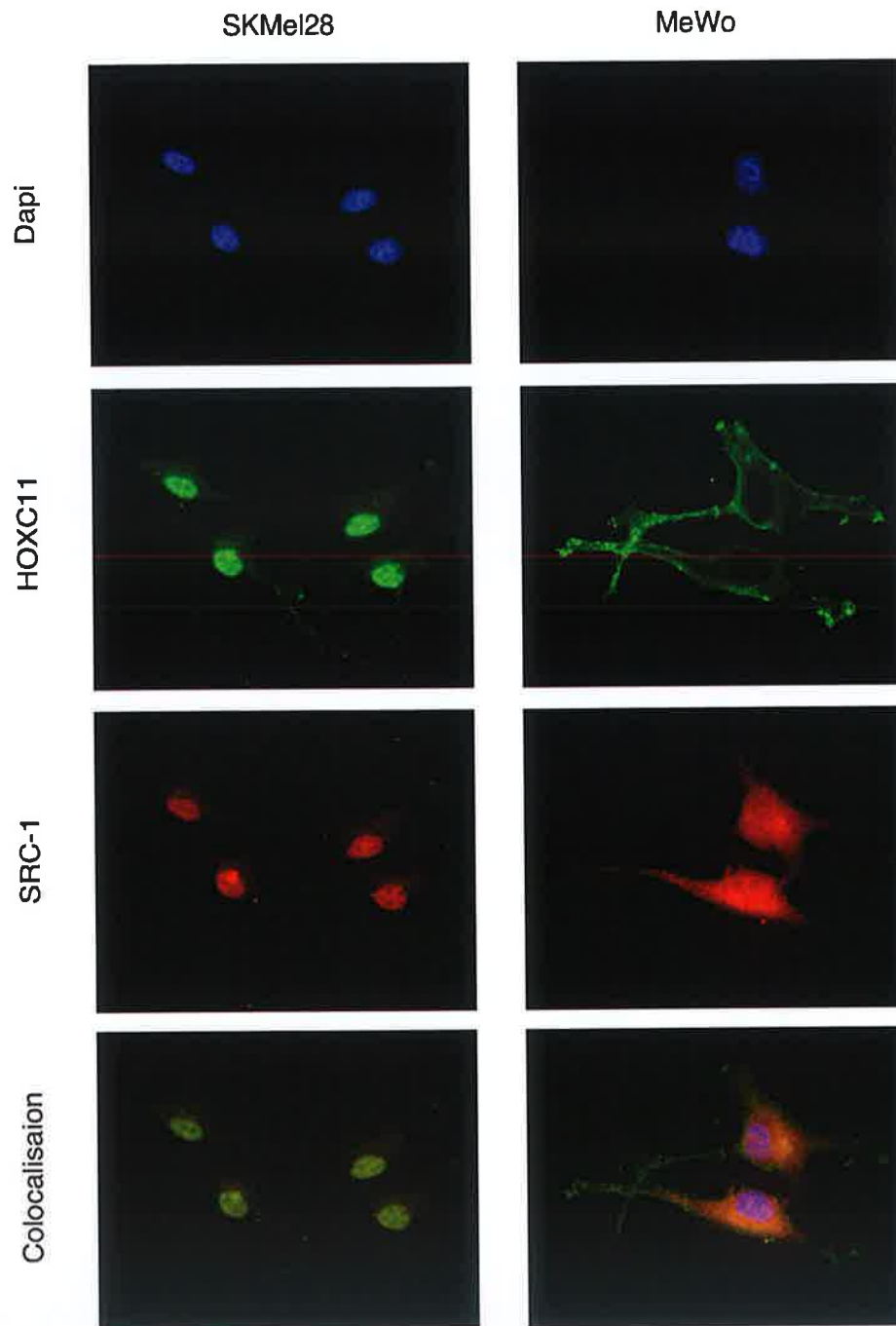


**Figure 3.2** Relative mRNA levels of HOXC11, SRC-1 and S100B in two cell lines.

Higher HOXC11 mRNA levels were seen in MeWo cells with higher S100B in SKMel28 cells and comparable levels of SRC-1 in the two cell lines.

### **3.3.2 Localisation of HOXC11, SRC-1 and S100B in melanoma cells**

Localisation of HOXC11 and SRC-1 in the two cell lines was investigated by immunofluorescence (Figure 3.3). In the SKMel28 cells, both HOXC11 and SRC-1 were seen predominantly in the nucleus. In the MeWo cells, HOXC11 was localised in the cytoplasm, with absent nuclear staining. SRC-1 was mainly localised to the cytoplasm.

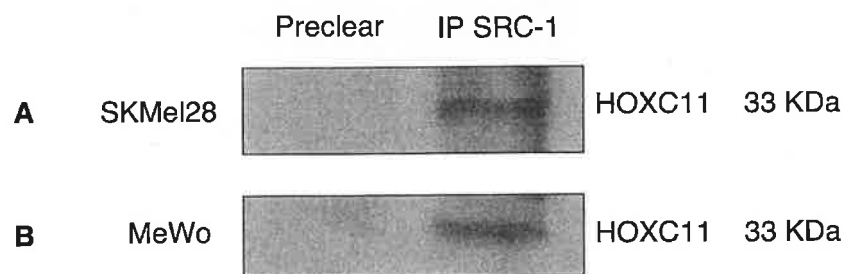


**Figure 3.3 Colocalisation of HOXC11 and SRC-1 in SKMel28 and MeWo cell lines.**

Nuclear HOXC11 and SRC-1 in SKMel28 cells; cytoplasmic HOXC11 and nuclear and cytoplasmic SRC-1 in MeWo cells.

### 3.3.3 Interaction of HOXC11 and SRC-1

To determine the level of interaction between HOXC11 and SRC-1, co-immunoprecipitation (co-IP) was performed on the two cell lines. The results showed that while there was less SRC-1 in the SKMel28 cells than in the MeWo cells, there was a similar level of interaction with HOXC11 in both cell lines (Figure 3.4).

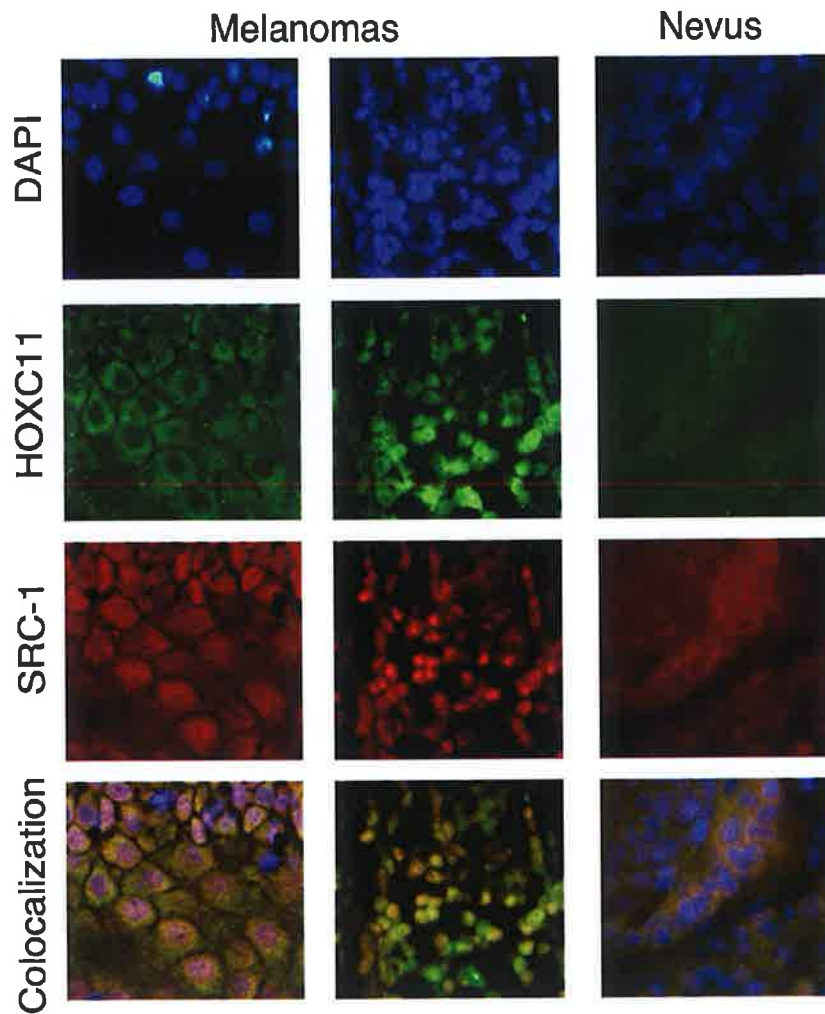


**Figure 3.4 Co-immunoprecipitation of SRC-1 to determine HOXC11 interaction.**

Immunoprecipitation was performed in two melanoma cell lines, pulling out SRC-1 using anti-SRC-1 antibody and immunoblotting for HOXC11. Results shown are representative of three separate experiments.

### 3.3.4 Co-localization of SRC-1 and HOXC11 in melanoma tissue

The immunoprecipitation experiments (n = 3) confirmed that there was an interaction between HOXC11 and SRC-1. In order to assess precisely where this interaction was occurring within the cell, co-localisation studies were performed. Immunofluorescent staining was performed in melanoma tissue samples (n = 64), as well as in a cohort of benign nevi specimens (n = 20). Variable patterns of staining were observed within the nuclei and cytoplasm of the benign and malignant tissue, representative examples of which are shown in Figure 3.5.



**Figure 3.5 Co-localization of HOXC11 and SRC-1 in melanoma and nevus tissue.**

DAPI (blue) stain depicts the nucleus; FITC (green) stain represents SRC-1 (30  $\mu\text{g/ml}$ ); TRITC (red) stain represents HOXC11 (50  $\mu\text{g/ml}$ ); Combined stain (yellow) stain represents the co-localisation of the two proteins. (Images viewed at 60x magnification).

Matched controls did not stain positive when primary antibodies were omitted. Significantly more positivity was observed in melanomas than nevi (Table 3.1).

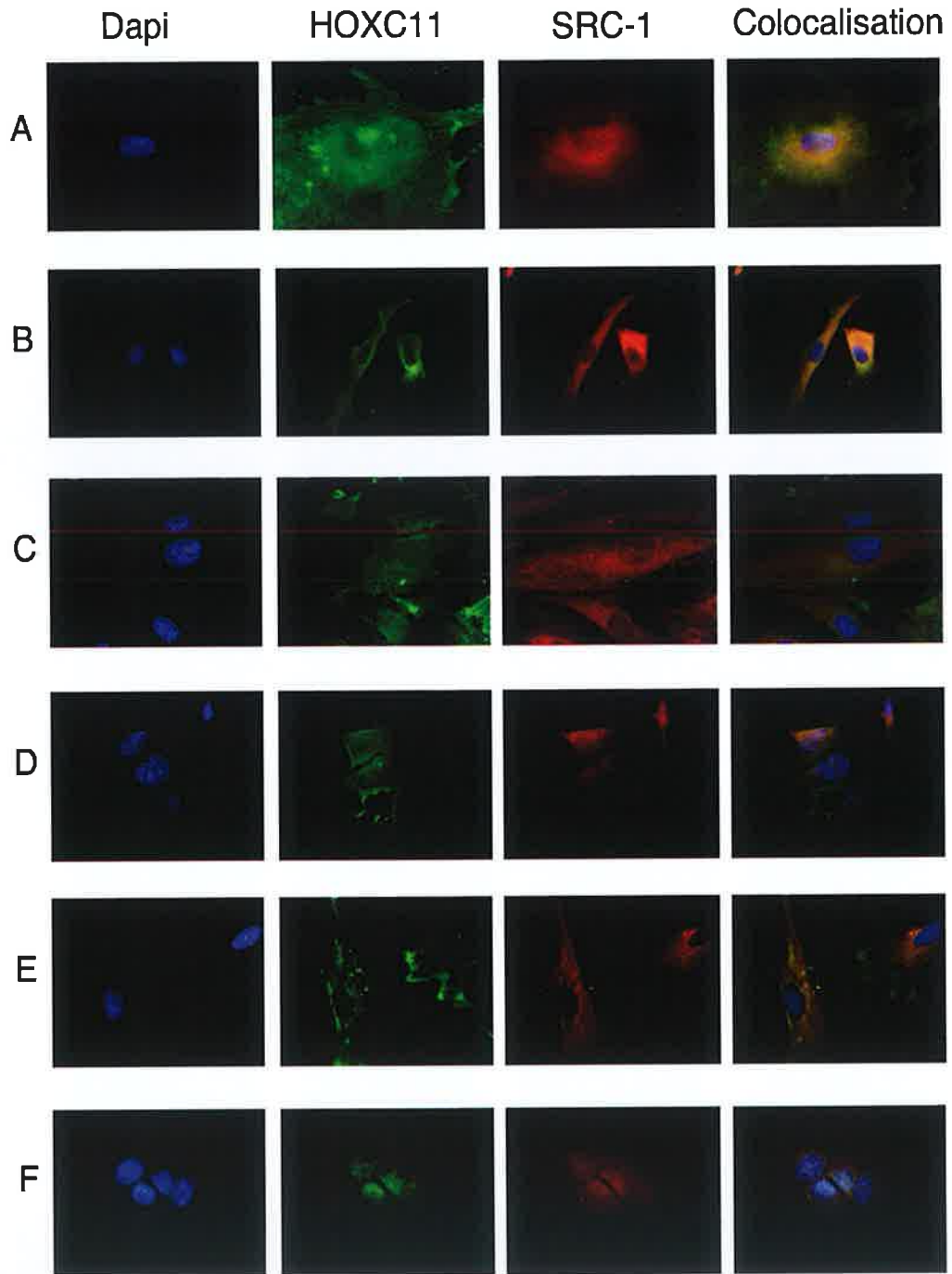
**Table 3.1 Expression of HOXC11 and SRC-1 in a melanoma and nevus tissue samples**

	HOXC11 n (%)		SRC-1 n (%)	
	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
Melanoma (n = 64)	34 (53.1)	19 (29.7)	30 (46.8)	21 (32.8)
Benign nevus (n = 20)	1 (5)	8 (40)	3 (15)	6 (30)
P value	< 0.001	NS	0.017	NS

### 3.3.5 Expression of HOXC11 and SRC-1 in primary culture samples

Immunofluorescent staining was once more employed to investigate colocalisation in a small number of melanoma primary culture samples. Both nuclear and cytoplasmic patterns of expression were observed (Figure 3.6).

In order to quantify the association of SRC-1 and HOXC11, a subset of primary culture samples (n = 4) was examined under a Zeiss LSM 510 META confocal fluorescent microscope. The mean Pearson's correlation coefficient was  $0.547 \pm 0.0042$  (mean  $\pm$  SEM).



**Figure 3.6 Localisation of HOXC11 and SRC-1 in melanoma primary cultures.**

Both nuclear (A,D,F) and cytoplasmic (B,C,E) localization was observed.



### **3.4 Discussion**

Almost 30 years ago it was documented that 100% of melanomas and benign nevi express S100B (Cochran et al., 1982). S100B is widely used as a diagnostic marker of melanocytic tumours. Rising serum levels of S100B in melanoma are associated with advancing disease stage (Mocellin et al., 2008). In this study it was postulated that the transcription factor HOXC11 in association with its coactivator SRC-1 are involved in transcriptional regulation of S100B production and may be implicated in advancing disease stage.

Differential expression of HOXC11 and SRC-1 in melanoma tissue and in nevi was investigated in order to establish variation in expression between benign and malignant lesions. Expression and colocalisation of the two proteins were also investigated in primary and metastatic melanoma cell lines, thereby focusing on potential variation in expression of HOXC11 and SRC-1 with disease progression. It is recognised that immortalised cell lines are limited in how accurately they represent in vivo disease behaviour and for this reason colocalisation of HOXC11 and SRC-1 was also examined in a small number of primary tumour specimens.

#### **3.4.1 Expression of S100B**

Throughout this work, S100B protein was consistently observed as a 42 KDa tetramer when cell lysate was analysed by SDS-PAGE. As stated in the general introduction, S100B is known to exist in oligomeric forms. This phenomenon has been examined in the context of RAGE binding by Ostendorp et al., who observed that in human brain, the most prominent form of S100B was a tetramer (Ostendorp et al., 2007). In the same paper, it was shown that SDS-PAGE analysis of different oligomeric species of S100B revealed a single 10.5 KDa band. Given this easy dissociation of the non-covalent bonds between the S100B monomers, it was concluded that the oligomeric structures were not therefore stabilised by intermolecular disulphide bonds.

In the current melanoma work, the S100B tetramer appeared to be stable to this kind of denaturation under conditions of native SDS-PAGE. Previous studies have suggested variable requirements for the disulphide linked form of S100B in carrying out its different functions in the brain (Koppal et al., 2001, Selinfreund et al., 1991). It is possible that S100B exists in a disulphide linked form in melanoma and that this accounts for the stability of the tetramer that was observed in these experiments.

### **3.4.2 Expression of SRC-1**

SRC-1 has been demonstrably associated with a reduced disease-free survival in a number of human cancers (Xu and Li, 2003). Three gene expression profiling studies have identified differential expression of SRC-1 in melanoma samples when compared to benign nevi and normal skin (Talantov et al., 2005, Critchley-Thorne et al., 2007, Riker et al., 2008). While these studies identified SRC-1 among a large panel of differentially expressed genes, a detailed investigation of SRC-1 expression in melanoma has not been carried out.

Of the current cohort of 64 melanomas, 51 (79.6%) stained positive for SRC-1, with 30 (46.8%) demonstrating nuclear localisation of the protein. Of 20 benign nevi, 9 (45%) stained positive for SRC-1, with only 3 (15%) demonstrating nuclear localisation. Only the differences in nuclear staining between melanomas and nevi were statistically significant. So, while a similar proportion of melanomas and nevi express cytoplasmic SRC-1, a significantly higher proportion of melanomas express SRC-1 in their nuclei.

In examining cell lines, both primary (SKMel28) and invasive (MeWo) melanoma cell lines were shown to express the coactivator SRC-1. When immunofluorescently stained, SRC-1 localised more to the nucleus in the SKMel28 cells and to the cytoplasm in the MeWo cells. Levels of SRC-1 mRNA were comparable between the two cell lines but the protein

expression was considerably higher in the MeWo cells, as demonstrated by Western blotting of whole cell lysate. One potential explanation of this is post-translational modification of SRC-1 in the SKMel28 cells by microRNA (miRNA), resulting in relative gene silencing. A number of publications have demonstrated the significance of miRNAs in melanoma tumourgenesis (Elson-Schwab et al., 2010, Hafliadottir et al., 2010, Sigalotti et al., 2010). Specifically, Adams et al. recently demonstrated that miR-206 coordinately targets SRC-1 mRNA in MCF-7 breast cancer cells (Adams et al., 2009).

### **3.4.3 Expression of HOXC11**

Expression of the transcription factor HOXC11 was also observed in both SKMel28 and MeWo cell lines. Equal protein expression was seen in the two cell lines, though there appeared to be higher levels of mRNA in the invasive MeWo cells. Several studies have examined expression of various HOX genes in melanoma. Caré et al looked at the expression of HOXB cluster genes in human melanoma and demonstrated that HOXB7 was constitutively expressed in 25 melanoma cell lines as well as in vivo primary and metastatic cells (Care et al., 1996).

Expression of HOXC11 was also examined in the cohort of melanoma tissue samples and compared to expression in benign nevi samples. Similar to the observations for SRC-1, only nuclear staining was significantly higher in the melanomas. Maeda et al compared HOX gene expression patterns between melanomas and benign nevi and amongst melanomas from different body sites (Maeda et al., 2005). In a small series of 15 melanomas versus 7 benign nevi, they demonstrated that the expression levels of HOXA11, A13, B9, D12 and D13 in melanoma were higher than those in nevi.

Cillo et al classified melanoma in to two major groups – those that express genes of the HOX C locus and those that do not (Cillo et al., 1996). They found these two patterns of HOX gene expression to be reflective of the intra-tumour heterogeneity of melanoma clones and to be associated with distinct

surface phenotypes. In the current study, HOXC11 was not found to associate with a particular phenotype of melanoma. The small size of the cohort is potentially the reason that no significant association could be demonstrated.

Hypothesizing that gene expression alters in melanomas of increasing depth, Riker et al employed gene microarray analysis to evaluate primary melanomas of varying Breslow thickness (Riker et al., 2008). HOX loci were amongst the putative oncogenes identified as changing expression during this so called 'transition period'. Specifically they found HOXA10, B6, B7 and B9 to be increased in metastatic relative to primary melanomas. At least two other gene-profiling studies have suggested upregulation of HOX genes in melanomas when compared to nevi or normal skin (Talantov et al., 2005, Critchley-Thorne et al., 2007) though as yet none have looked at HOXC11 in particular.

#### **3.4.4 Co-association of SRC-1 and HOXC11**

SRC-1 was initially described as a nuclear receptor coactivator protein (Heery et al., 1997). More recently SRC-1 interactions with other transcription factors have been described, in particular those running downstream of an activated mitogen-activated protein kinase pathway (Qin et al., 2009, Al-azawi et al., 2008, Fleming et al., 2004). Such associations have been shown to be important in tumour progression and metastasis in breast and prostate cancers (Qin et al., 2009, Al-azawi et al., 2008).

Co-association of SRC-1 with HOXC11 in two melanoma cell lines was confirmed via co-immunoprecipitation with SRC-1 and subsequent immunoblotting for HOXC11. Interestingly, though a higher concentration of SRC-1 was expressed in MeWo cells, equal pull down of HOXC11 was observed in the two cell lines on immunoprecipitation with SRC-1 antibody. The SRC-1 expressed in the MeWo cells may be complexed with different proteins and in a different conformation than in SKMel28 cells and this may account for the antibody only pulling down some of the available antigen. In

future work it would be useful to repeat this assay using antibodies targeted at different SRC-1 epitopes.

As well as confirming co-association of SRC-1 with HOXC11 in two melanoma cell lines, significant colocalisation was observed in a cohort of melanoma tissue and in 6 primary culture samples – as confirmed by standard and confocal immunofluorescent microscopy. The findings presented in this chapter are consistent with our group's previous investigations in breast cancer (McIlroy et al., 2010) and raise the exciting possibility that HOXC11 in conjunction with SRC-1 may be relevant to the transcriptional regulation of S100B in malignant melanoma.

## **4 Interactions of HOXC11, SRC-1 and S100B**

## **4.1 Introduction**

In the previous chapter, HOXC11 and SRC-1 were confirmed as interacting protein partners in melanoma cell lines and tumour tissue. Previous published work from this laboratory has established S100B as a target gene of the HOXC11-SRC-1 complex in breast cancer (McIlroy et al., 2010). In this chapter, a potential functional role of HOXC11 and SRC-1 in the production of target protein S100B in melanoma was elucidated. The experiments were carried out in the SKMel28 and MeWo cell lines. Recruitment of HOXC11 to the promoter region of the S100B gene was examined by chromatin Immunoprecipitation (ChIP). Gene silencing was used to characterize the functional importance of HOXC11 and SRC-1 in the production of S100B. The effect of overexpressing HOXC11 was also investigated.

In addition to this, attempts were made to place the interaction between HOXC11 and SRC-1 in to the context of what is already known about molecular signalling networks in melanoma. Src kinase regulates key pathways in metastasis including cell adhesion, invasion and motility and certain Src family kinases (SFKs) the have been implicated in melanoma progression (Homsí et al., 2007, Qi et al., 2006). Of particular interest in melanoma is the role played by SFKs in crosstalk between the MAPK and PI3K pathways (Parsons and Parsons, 2004). Under normal physiologic conditions, the MAPK pathway regulates cell growth, survival and migration through transduction of cell-surface signals to the nucleus via phosphorylation events. Constitutive activation and subsequent dysregulation of MAPK signalling is a frequent event in multiple cancers and is one of the best- described genetic mutations in melanoma (Fecher et al., 2008).

The interaction of SRC-1 with its transcription factor binding partners is known to be phosphorylation dependent (Glass et al., 1997). It was therefore hypothesized that the phosphorylation cascade induced by MAPK/ Src kinase activation is important in the interaction of SRC-1 with HOXC11 and

their regulation of S100B expression in malignant melanoma. In an attempt to downregulate MAPK signalling and phosphorylation of SRC-1/HOXC11, SKMel28 and MeWo cells were treated with dasatinib (BMS-354825), a multitargeted kinase inhibitor of BCR-ABL, c-KIT, PDGFR and Src family kinases (Lombardo et al., 2004, Shah et al., 2004, Travis, 2004). Dasatinib is the most potent Src kinase inhibitor currently in clinical development and has proven efficacy in the treatment of chronic myelogenous leukaemia (Talpaç et al., 2006). Its potential in solid tumours such as melanoma remains to be fully determined (National Cancer Institute and Eastern Cooperative Oncology Group, 2009).

## **4.2 Aims**

The specific aims of the experiments in this chapter relate to the second aim of the study - to characterise the effect of manipulation of HOXC11 and SRC-1 on S100B expression. The following objectives were defined:

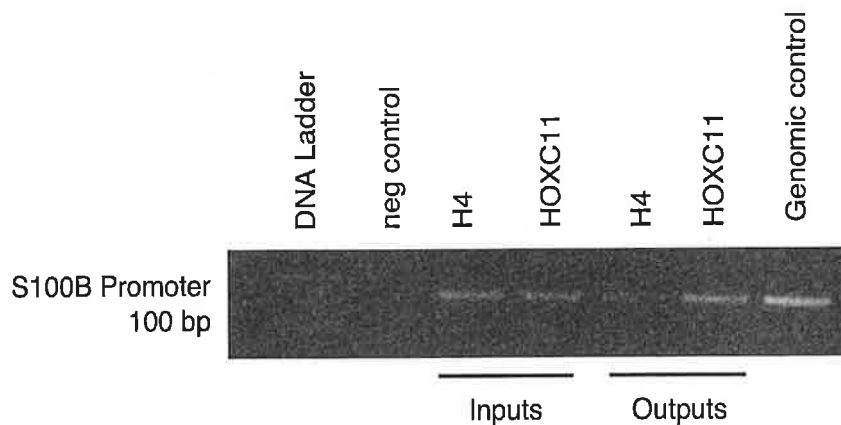
- a. Confirm recruitment of HOXC11/SRC-1 to the S100B promoter.
- b. Investigate expression of S100B in response to suppression of HOXC11 and SRC-1.
- c. Investigate expression of S100B in response to stimulation of HOXC11 and SRC-1.
- d. Investigate the effect of MAPK/ Src kinase suppression on S100B expression and interaction of HOXC11 and SRC-1.



## 4.3 Results

### 4.3.1 Recruitment of HOXC11 to the S100B promoter

To determine whether the S100B promoter is bound by HOXC11, SKMel28 cells were analyzed by a chromatin immunoprecipitation assay with anti-HOXC11 antibody. The purpose of a ChIP assay is to determine whether proteins, such as transcription factors like HOXC11, bind to a particular region on the chromatin of living cells or tissues. The HOXC11 binding site-containing region of the S100B promoter was amplified from cross-linked chromatin from SKMel28 cells that was immunoprecipitated with anti-HOXC11 antibody or H4 antibody positive control (Figure 4.1).

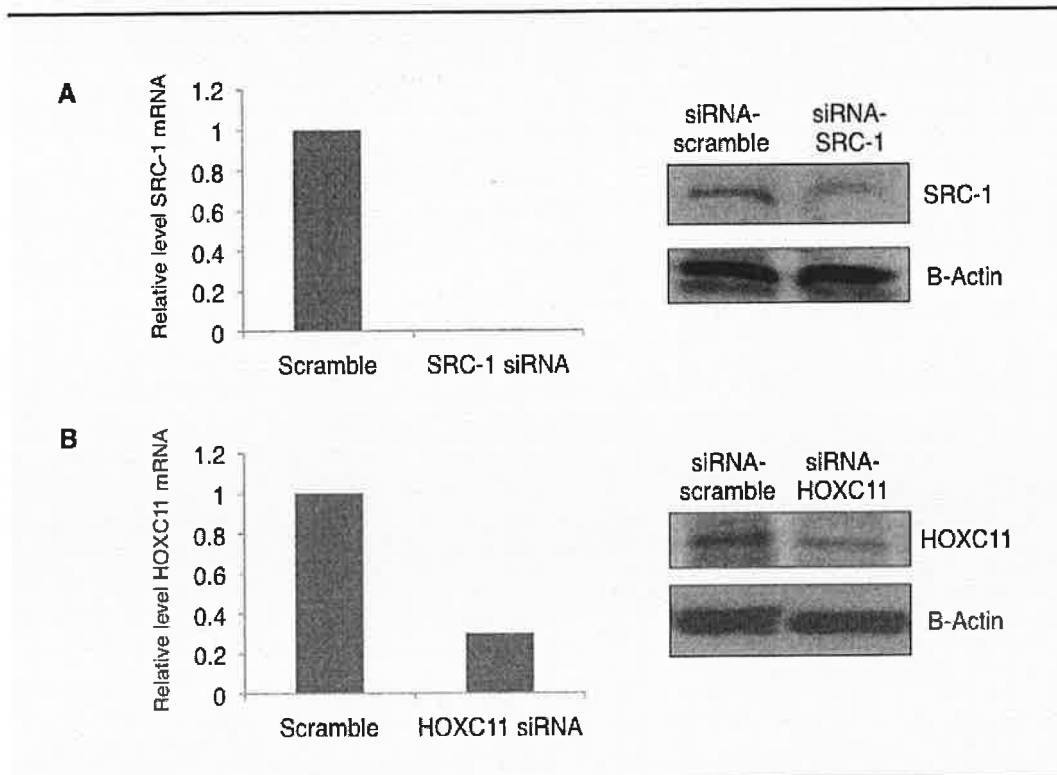


**Figure 4.1 Recruitment of HOXC11 to the S100B promoter.**

DNA gel showing amplification of the promoter region of the S100B gene. Following crosslinking and sonication, chromatin immunoprecipitation of HOXC11 was carried out. The DNA was purified and subjected to PCR amplification of the promoter region of the S100B gene. Recruitment of HOXC11 to this region was confirmed, demonstrated here in comparison to H4 control, as well as negative (no DNA) and genomic DNA control samples. Results shown are representative of three separate experiments.

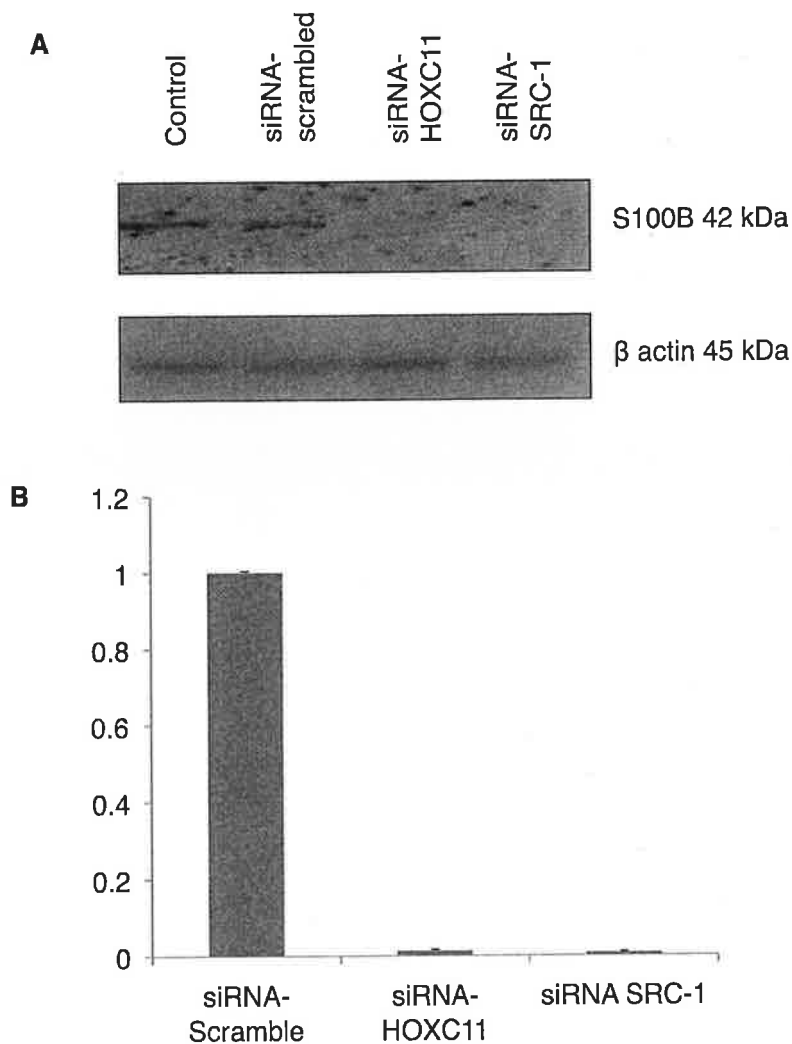
### **4.3.2 Gene silencing experiments**

If S100B is a true transcriptional target of HOXC11 and SRC-1 in melanoma cells, decreasing their expression should lead to concordant lower levels of S100B protein. To test this hypothesis SKMel28 cells were transiently transfected with siRNAs targeting SRC-1 and HOXC11. The siRNA knockdown of SRC-1 and HOXC11 was confirmed by quantitative real time PCR and by Western blot to measure SRC-1 and HOXC11 mRNA and protein levels respectively (Figure 4.2 A and B). At 24 hrs post transfection cells were harvested and lysates were subject to Western blot analysis with anti-S100B and anti-  $\beta$ -actin antibodies. Significantly lower protein levels of S100B were observed (Figure 4.3 A). At 24 hrs post transfection total mRNA was extracted and S100B transcript levels were measured by quantitative real-time PCR analysis (Figure 4.3 B).



**Figure 4.2 Down-regulation of SRC-1 and HOXC11 mRNA and protein levels.**

SKMel28 melanoma cells were transfected with siRNA targeting HOXC11 and SRC-1 or control siRNA. **A** *Left*, 24 hrs post transfection, total RNA was extracted and SRC-1 transcript levels were measured by quantitative real-time PCR analysis. *Right*, 24 hrs post transfection cells were harvested and lysates were subject to Western blot analysis with anti-SRC-1 and anti-  $\beta$ -actin antibodies. **B** *Left*, 24 hrs post transfection, total RNA was extracted and HOXC11 transcript levels were measured by quantitative real-time PCR analysis. *Right*, 24 hrs post transfection cells were harvested and lysates were subject to Western blot analysis with anti-HOXC11 and anti-  $\beta$ -actin antibodies. Results shown are representative of three separate experiments.

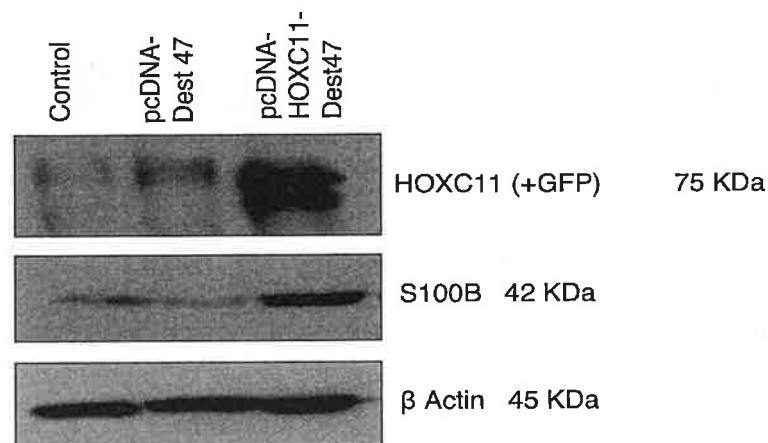


**Figure 4.3 HOXC11 and SRC-1 transcriptionally regulate S100B.**

In order to demonstrate the functional role of HOXC11 and SRC-1 in targeting S100B, the two proteins were silenced by transient transfection with siRNAs and Lipofectamine transfection reagent. **A** Western blot demonstrating decreased expression of S100B in lysate from SKMel28 cells, in which HOXC11 and SRC-1 had been silenced ( $n = 3$ ). **B** Quantitative real time PCR histogram demonstrating the effect of silencing HOXC11 and SRC-1 on production of S100B mRNA; results are mean  $\pm$  SEM ( $n = 2$ ).

### 4.3.3 Upregulation experiments

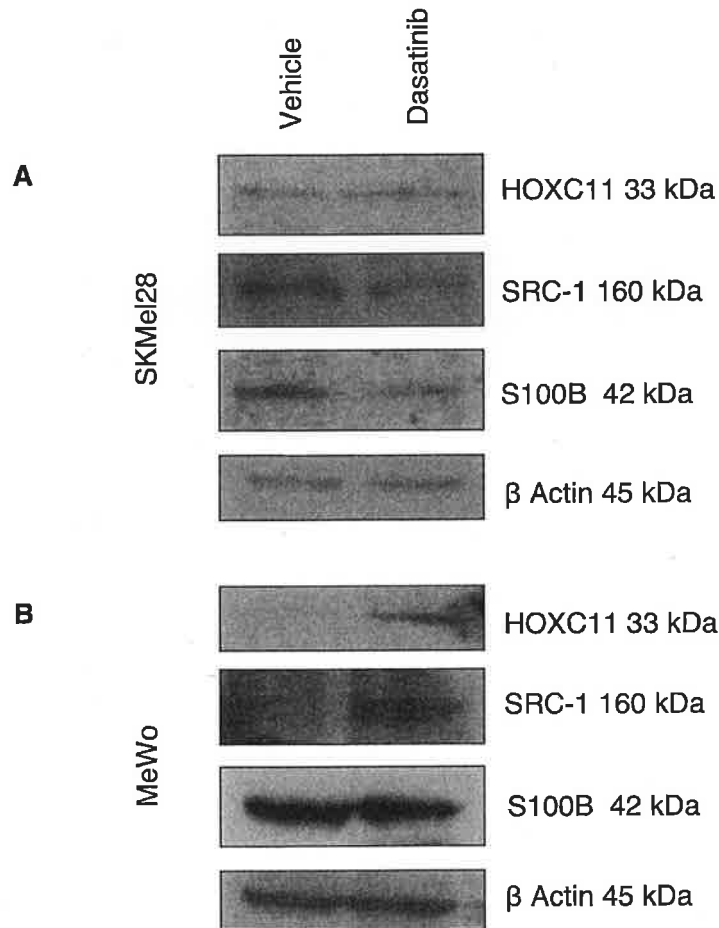
Loss of HOXC11 or SRC-1 function by siRNA knockdown lead to decreased expression of S100B. It was hypothesized that upregulation of HOXC11 would have the opposite effect. HOXC11 overexpression was achieved by transfecting pcDNA -DEST47-HOXC11 in to SKMel28 cells. At 24 hrs, cells were harvested and whole cell lysate was subjected to Western blot analysis with anti-HOXC11, anti-S100B and anti-  $\beta$ -actin antibodies (Figure 4.4). Significant increase of HOXC11 protein expression was seen in the transfected sample as compared to the empty vector. Concomitant increase in S100B protein expression was also observed, further confirming HOXC11's role in it transcriptional regulation.



**Figure 4.4 HOXC11 transcriptionally regulates S100B.**

Cells were transiently transfected with pcDNA -DEST47-HOXC11 or the empty vector pcDNA-DEST47. At 24 hrs, cells were harvested and cell lysate was subjected to Western blot with anti-HOXC11, anti-S100B and anti-  $\beta$ -actin antibodies. Significant increase of HOXC11 protein expression was seen in the transfected sample as compared to the empty vector. S100B was upregulated in a similar pattern. Results shown are representative of three separate experiments.

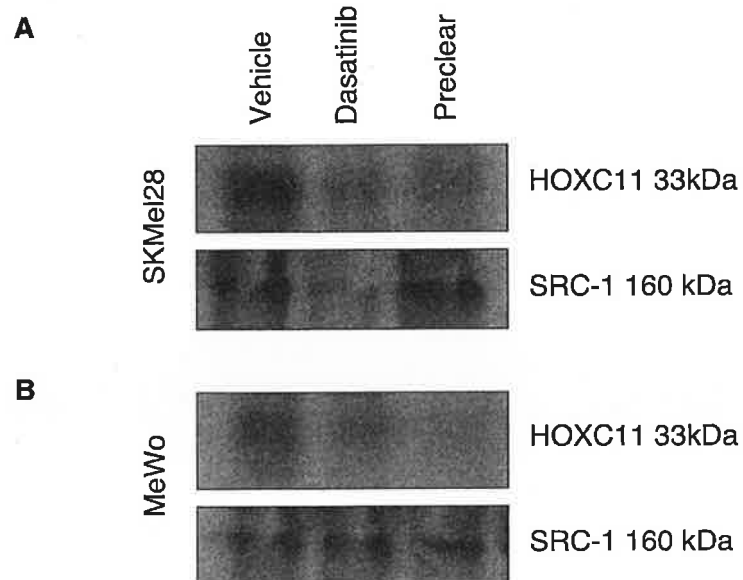
was observed in the two cell lines, as demonstrated by coimmunoprecipitation (Figure 4.7).



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**Figure 4.6 Protein expression of HOXC11, SRC-1 and S100B in response to dasatinib.**

Two cell lines were treated with dasatinib (100 nM). At 8 hrs cells were harvested and whole cell lysates were subjected to Western blot analysis with anti-HOXC11, anti-SRC-1, anti-S100B and anti-β-actin antibodies (n = 3). Varying levels of the three proteins of interest were observed in the two cell lines. **A** SKMeI28 cells showed no difference in HOXC11 protein levels and a decrease in levels of SRC-1 and S100B. **B** MeWo cells demonstrated an increase in HOXC11 and SRC-1 in response to dasatinib treatment and no difference in S100B production.

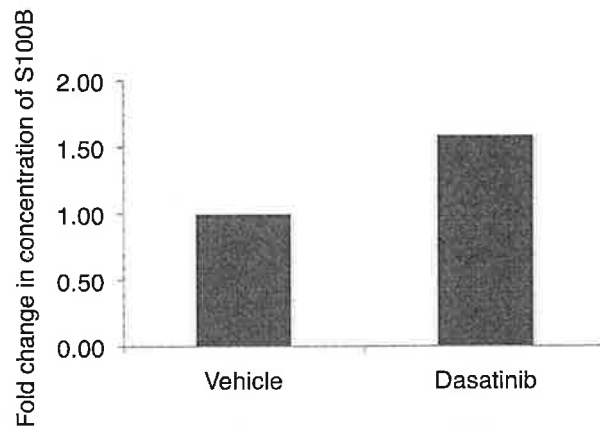


**Figure 4.7 Treatment with dasatinib and coassociation between HOXC11 and SRC-1.**

SKMel28 cells (**A**) and MeWo cells (**B**) were treated with dasatinib (100 nM) or DMSO vehicle control. At 24 hrs, cells were harvested and coimmunoprecipitation with SRC-1 and subsequent immunoblotting for HOXC11 and SRC-1 was performed. The association between HOXC11 and SRC-1 was reduced in the cells treated with dasatinib. Results shown are representative of three separate experiments.

#### **4.3.4.3 Dasatinib treatment and extracellular S100B expression**

S100B is a secreted protein and is known to exhibit different functions intra- and extracellularly. For this reason, conditioned media from treated and untreated SKMel28 cells was analysed for levels of S100B by ELISA. In contrast to intracellular S100B expression, levels of the secreted protein were observed to be higher with dasatinib treatment than under control conditions (Figure 4.8).



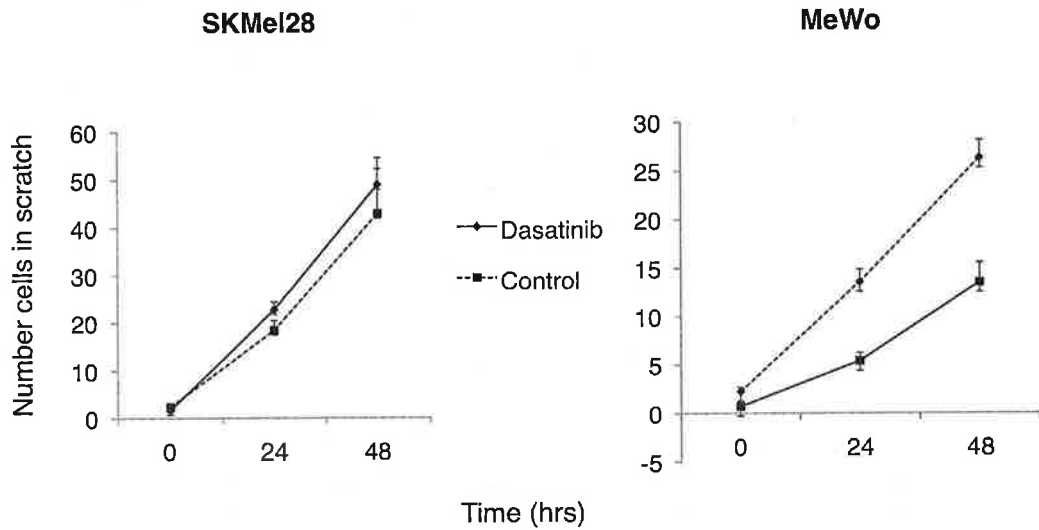
**Figure 4.8 Dasatinib increases production of secreted S100B in treated SKMel28 cells.**

Conditioned media was tested for levels of S100B by ELISA. A higher level of secreted S100B was observed in dasatinib treatment than in control conditions; results are fold change (n = 1).

#### ***4.3.4.4 Functional effect of dasatinib treatment***

The functional effect of dasatinib treatment on the two cell lines was investigated by carrying out a scratch wound migration assay. Relative numbers of cells migrating in to the scratch at 0, 24 and 48 hours were recorded and plotted (Figure 4.9).





**Figure 4.9 Dasatinib inhibits migration of certain human melanoma cells.**

*Left*, SKMel28 cells and *Right*, MeWo cells were plated in a 24 well plate and grown to 100% confluence. A single scratch was made in the confluent monolayer, floating cells were washed off, and attached cells were treated with 100 nM dasatinib or DMSO vehicle control. Each scratch was photographed at  $t = 0, 24$  and 48 hours. Cells that migrated in to the scratch were estimated by counting 20 fields of view of the scratch at 20x magnification. A significant decrease in migration with dasatinib treatment was observed in the MeWo cells only ( $P < 0.001$ ).

## **4.4 Discussion**

In the previous chapter, HOXC11 and SRC-1 were confirmed as interacting protein partners in melanoma cell lines and tumour tissue. In order to confirm that this complex actually targets S100B, chromatin immunoprecipitation of HOXC11 to the S100B promoter was carried out. To establish a functional relationship between the HOXC11:SRC-1 complex and the target gene S100B, gene silencing and upregulation experiments were conducted. Both of these approaches showed that manipulation of HOXC11 levels resulted in concomitant alteration of S100B production. The effect of treatment with the multi-target kinase inhibitor dasatinib was then explored.

### **4.4.1 Transcriptional regulation of S100B**

Altered expression of S100 genes in disease states, including cancer, has prompted interest in determining the mechanisms of regulation of S100 gene expression. Nucleotide sequences in the coding region of S100 genes are preserved across different species but sequences of the promoter region display little similarity (Allore et al., 1990). It is thought that this may account for different regulatory mechanisms for the expression of individual S100 genes (Zimmer et al., 1995). Bioinformatic analysis has been used to identify potential DNA regulatory elements of individual S100 genes and various elements have been described.

The S100 protein element (SPE) is located near the TATA box in several human S100 genes, including S100B (Allore et al., 1990). It is not clear what role this element may play in regulating S100 expression however. Other putative elements identified in the S100B gene include activator protein 1 and 2 elements (AP-1 and AP-2), which are involved in induction of specific genes by cAMP (Jiang et al., 1993). In our group, the presence of putative homeodomain binding sites in the promoter of S100B was identified (McIlroy et al., 2010). In LY2 breast cancer cells, recruitment of HOXC11 to this region was demonstrated by chromatin immunoprecipitation. Zhang et al. demonstrated that forced expression of HOXC11 alone or both HOXC6

isoform 1 and HOXC11 induced the expression of S100B in GOTO neuroblastoma cells (Zhang et al., 2007). In transient transfection experiments, overexpression of HOXC6 and HOXC11 was shown to transactivate the S100B promoter-reporter construct.

Given what is known about HOX genes and their role in embryogenesis, it is interesting to note that there is evidence to say that S100 gene expression is spatially regulated during development. De Leon et al. reported that S100B, S100A4 and S100A10 mRNAs increase 11-, 4- and 14-fold respectively during the first 3 weeks of postnatal nerve development, with each exhibiting a different tissue distribution profile (De Leon et al., 1991). During skeletal muscle development, the spatial pattern of S100A1 also changes (Zimmer, 1991).

In the current study, binding of HOXC11 to the promoter region of the S100B gene was demonstrated in melanoma cells. The experiment was carried out in SKMel28 cells, as it was found in the previous chapter that they contain a higher abundance of the target gene S100B than MeWo cells. As a coactivator, SRC-1 does not bind directly to the DNA. SRC-1 was established as a binding partner for HOXC11 in melanoma cells and it is therefore predicted that the two proteins bind as a transcription factor complex on the S100B promoter.

Through gene silencing and overexpression, we demonstrated a potential functional role for HOXC11 in production of S100B. Knocking out either HOXC11 or its coactivator SRC-1 had a dramatic effect on production of S100B mRNA and protein, as demonstrated by quantitative real-time PCR and Western blot analysis. The opposite effect was observed when HOXC11 was upregulated - high levels of HOXC11 were found in cells transfected with the expression vector when compared to those transfected with the vector backbone alone. This increase in HOXC11 expression corresponded to an increase in production of S100B. Taken together, the results of the gene

silencing and upregulation experiments implicate HOXC11 and SRC-1 in the transcriptional regulation of S100B.

While several studies have looked at expression of HOX genes in melanoma, very few have examined in detail their role in disease progression. In one paper by Caré et al, a functional role for HOXB7 was established (Care et al., 1996). It was observed that treatment of melanoma cell lines with antisense oligomers targeting HOXB7 mRNA markedly inhibited cell proliferation and specifically abolished expression of basic fibroblast growth factor (bFGF) mRNA. Direct transactivation of bFGF was demonstrated by band shift and cotransfection experiments targeting putative homeodomain binding sites on the bFGF promoter.

More recently, Morgan et al. examined the interaction between HOX genes and a second homeodomain-containing transcription factor, PBX in melanoma (Morgan et al., 2007). It was explained that HOX/PBX dimers have a greater binding affinity and specificity for target DNA sequences than HOX monomers alone. The use of a novel, cell permeable antagonist of this interaction was found to inhibit melanoma cell growth and to trigger apoptosis when administered to mice with flank tumours.

#### **4.4.2 Src kinase inhibition and S100B expression**

Src family kinases are involved in regulating a multitude of biological processes, including cell adhesion, migration, proliferation and survival. Although SFKs are currently being investigated as potential therapeutic targets in a number of cancers, the biological responses to inhibition of SFK signalling in any given tumour type are not predictable. In the experiments presented here, cells were treated with dasatinib, a multi-target Src kinase inhibitor.

Dasatinib has been previously shown to block migration and invasion of human melanoma cells without affecting proliferation and survival (Buettner

et al., 2008). Specifically, the study demonstrated that dasatinib treatment completely abolished SFK autophosphorylation activity at low nanomolar concentrations in 8 different human cell lines (including SKMel28 and MeWo cells). Dasatinib was also shown to block phosphorylation of SFK downstream substrates – focal adhesion kinase (FAK) and Crk-associated substrate (p130<sup>CAS</sup>) - in the same panel of cell lines.

While no functional effect of dasatinib was observed in the SKMel28 cells, treatment did result in altered expression of the proteins of interest. While HOXC11 levels remained unchanged, there was a significant fall in expression of SRC-1. Intracellular protein expression of S100B also dropped off, perhaps indicating that SRC-1 binding is necessary for HOXC11 to exert its full transcription regulatory effect. Secreted levels of S100B were examined and a higher concentration of S100B was observed in media from SKMel28 cells treated with dasatinib. While this result contradicts the findings in mRNA and protein, it is consistent with the complexity of S100B's intra- and extracellular functions and the multiplicity of its potential responses to drug treatment.

A different pattern was noted in the more invasive MeWo cells. mRNA levels of S100B were depressed in response to dasatinib but no change in protein expression was seen. This lack of correlation between mRNA and protein expression under various treatment conditions has been previously described and provides evidence for S100B's complex transcriptional regulation (Zimmer et al., 1995).

One finding that was common to both cell lines was the observation that when treated with dasatinib, coassociation between HOXC11 and SRC-1 was reduced, as demonstrated by coimmunoprecipitation. Binding of SRC-1 to its transcription factors is phosphorylation dependent. In the context of the results of Buettner et al., it is conceivable that dasatinib has blocked this

phosphorylation and thus caused a decreased interaction between the two proteins.

In the current study, significantly decreased migration was demonstrated in MeWo cells but not in SKMel28 cells. In fact, migration in these cells seemed to increase slightly, though this was not statistically significant. In a recent study by Eustace et al., SKMel28 cells showed an increase in proliferation when treated with dasatinib and were therefore labelled as 'dasatinib resistant' (Eustace et al., 2008).

Migration in the MeWo cells was significantly reduced but protein expression of HOXC11 and SRC-1 was actually increased. This is likely explained by the direct targeting of FAK by dasatinib. Eustace et al. reported a reduced level of FAK phosphorylation at Tyr861 in a panel of melanoma cells, thereby inhibiting migration and invasion by focal adhesion regulation but exerting no effect on cell proliferation (Eustace et al., 2008). This finding is consistent with the observations made in a previous study in colon cancer cells, in which reduced phosphorylation of FAK was implicated in dasatinib-mediated inhibition of migration and invasion (Serrels et al., 2006).

In the series of experiments presented in this chapter, an unpredictable alteration in expression of HOXC11 and SRC-1 was observed in response to SFK inhibition with dasatinib. SFKs play multiple critical roles in basic biological processes and consequently, their inhibition can induce numerous complex responses. Other groups have established dasatinib blockade of specific SFKs in melanoma (e.g. FAK, EphA2, p130<sup>CAS</sup>) (Eustace et al., 2008, Buettner et al., 2008). Investigating potential interactions between these SFKs and the HOXC11:SRC-1 complex may help to elucidate the exact mechanism of action of dasatinib in our pathway of interest. Also, primary culture samples could be treated with dasatinib in order to explore the cell line findings in an ex-vivo model.

## **5 Novel autoantibody biomarkers in melanoma**

## **5.1 Introduction**

### **5.1.1 Serum biomarkers in melanoma**

The most extensively investigated melanoma serum biomarker is S100B. Elevated serum levels of S100B have been detected in patients with malignant melanoma and the strength of expression has been shown to directly correlate with degree of malignancy (Fagnart et al., 1988, Martenson et al., 2001, Guo et al., 1995). S100B protein levels in serum have furthermore been correlated with progression and regression of disease in patients receiving anti-tumour treatment (Hauschild et al., 1999a). A recent meta-analysis suggested that serum S100B detection has a clinically valuable independent prognostic value in patients with stage I to III melanoma (Mocellin et al., 2008).

Other serum biomarkers include melanoma inhibitory activity (MIA), circulating tumour cells and lactate dehydrogenase (LDH). Of these biomarkers, only LDH has a proven prognostic value at multivariate analysis and is thereby included in the AJCC staging system (Balch et al., 2004). The value of the LDH level is however restricted to patients with metastatic disease (Neuman et al., 2008).

### **5.1.2 Serum autoantibodies as cancer biomarkers**

The presence of an immune response to solid tumours was first described by Robert Baldwin in 1955 (Baldwin, 1955). The autologous proteins of tumour cells or tumour-associated antigens (TAAs), are thought to be altered in a way that renders them immunogenic and the induced immune response functions to remove precancerous lesions (Finn, 2005). Hence the production of autoantibodies as a result of cancer immunosurveillance has been found to precede manifestations or clinical signs of tumourgenesis by several months to years (Disis et al., 1997). Thus the production of tumour autoantibodies can be detected prior to the observation of other biomarkers or phenotypic aberrations, making them indispensable markers of early



cancer. Furthermore, autoantibodies are inherently stable and resistant to proteolysis, meaning that they are detectable in serum for relatively long periods of time (Tan et al., 2009). Their long half-life (7 days) means that serum sample collection is simplified also (Tan et al., 2009).

The first tumour associated autoantibodies were in fact identified in sera from patients with melanoma (Shiku et al., 1977). Since then, autoantibodies and TAAs have been found in many cancers such as lung, colorectal, breast, prostate, pancreatic and hepatocellular carcinoma (Diesinger et al., 2002, Scanlan et al., 1998, Fernandez Madrid, 2005, Wang et al., 2005, Hong et al., 2004, Covini et al., 1997). The growing list of TAAs identified in cancers includes oncoproteins (e.g. HER-2, ras and c-MYC, (Disis and Cheever, 1996)), tumour suppressor proteins (e.g. p53, (Soussi, 2000)), survival proteins (e.g. survivin, (Rohayem et al., 2000)), cell cycle regulatory proteins (e.g. cyclin B1, (Covini et al., 1997)) and differentiation and cancer testis antigens (CTAs) (e.g. tyrosinase and NY-ESO-1, (Chen et al., 1997, Tureci et al., 2006)).

### **5.1.3 Detection of serum autoantibodies**

Initial studies of TAAs focused on a small number of antigens at a time, using techniques such as SDS/PAGE or ELISA. The development of proteomic platforms have enabled the generation of panels of TAAs, which exhibit better diagnostic value than a single TAA marker (Zhang et al., 2003a). Several high-throughput methods of autoantibody identification have been developed, which enable the concomitant discovery of many TAAs. These include serological screening of cDNA expression libraries, phage-display libraries, protein microarrays, 2D Western blots and 2D immunoaffinity chromatography.

The first of these techniques, serological analysis of tumour antigens by recombinant cDNA expression cloning (SEREX), involves the identification of TAAs by screening patient sera against a cDNA expression library obtained

from the autologous tumour tissues (Baldwin, 1966).

In the phage display method, a cDNA phage display library is constructed using a tumour tissue sample or cell line. Peptides from the tumour or cell line are expressed as fusions with phage proteins and screened with sera of cancer patients (Mintz et al., 2003).

Protein microarrays comprise purified or recombinant proteins, synthetic peptides or fractionated proteins from tumour or cancer cell lysates spotted systematically onto microarrays, which are incubated with patient sera (Anderson and LaBaer, 2005).

Another commonly used technique of serological proteome analysis is known as SERPA and uses a combination of 2D electrophoresis, Western blotting and mass spectrometry (Klade et al., 2001). Proteins from tumour tissues or cell lines are separated by 2D electrophoresis, transferred on to membranes by electroblotting and subsequently probed with sera from healthy individuals or patients with cancer. The respective immunoreactive profiles are compared and cancer-associated antigenic spots are identified by mass spectrometry.

Multiple affinity protein profiling (MAPPING) involves 2D immunoaffinity chromatography followed by the identification of TAAs by tandem mass spectrometry (Hardouin et al., 2007).

#### **5.1.4 Proteomic screening in melanoma**

Several studies have employed proteomic techniques to examine the serum of patients with malignant melanoma. Wilson et al. performed surface enhanced laser desorption/ionization (SELDI) mass spectrometric analysis of sera from patients with AJCC stage I and II melanoma and identified three proteins that accurately identified patients who developed recurrence after curative resection (Wilson et al., 2004). Caputo et al. examined the plasma

peptide component from melanoma, breast cancer and healthy individuals by a combination of reverse phase chromatography, surface enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF MS) and tandem mass spectrometry (Caputo et al., 2005). A three peak pattern was primarily observed in the melanoma samples. Takikawa et al. identified 9 proteins that were specifically expressed in melanoma plasma compared with healthy plasma, most of which had not previously been identified as plasma markers of melanoma (Takikawa et al., 2009).

In a study to examine whether molecular profiling of the serum proteome could discriminate between early- and late-stage melanoma and predict disease progression, Mian et al. analyzed over 200 serum samples from patients with melanoma of varying stages by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry utilizing protein chip technology and artificial neural networks (Mian et al., 2005). Accurate stage assignment was recorded in over 80% of the samples. It was concluded that proteomic profiling may become a valuable tool for identifying high-risk melanoma patients eligible for adjuvant therapeutic intervention.

#### **5.1.5 Autoimmune antibodies in melanoma**

Recently, a small number of studies have looked specifically at autoimmune antibody profiles in melanoma. Forgber et al. screened sera of 94 melanoma patients for 'anti melanoma activity' by 2D Western blot and mass spectrometry (SERPA) (Forgber et al., 2009). They observed seropositivity in two-thirds of the patients and 17 novel melanoma antigens were identified. Two of these proteins (enolase and galectin-3) have previously been implicated in carcinogenesis and the group concluded that proteome-based antigen discovery has the potential to identify pathologically relevant proteins.

Also using a SERPA approach, Suzuki et al. identified 5 proteins (eukaryotic elongation factor2 (EEF2), enolase1 (ENO1), aldolase A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heterogeneous

nuclear ribonucleoproteins (HNRNP)) which were differentially expressed in melanoma serum (Suzuki et al., 2010). mRNA expression of these proteins was confirmed in melanoma cell lines.

Pardo et al employed a combination of proteomic approaches, including autoantibody analysis, to look at potential biomarkers in uveal melanoma and identified cathepsin D, syntenin, and gp100 (Pardo et al., 2007).

## **5.2 Aims**

The aim of the work presented in this chapter was to identify new autoantibody biomarkers in sera from patients with malignant melanoma. The following specific objectives were defined:

- a. Identify autoantibody biomarkers in patient sera using protein microarrays.
- b. Validate findings from protein array by reverse ELISA on a larger cohort of patient serum samples.
- c. Verify the protein expression of novel biomarker(s) in patient tissue samples.

## **5.3 Results**

### **5.3.1 Differential autoantibody expression between control and melanoma patients**

Serum from 6 patients with melanoma and 5 age- and gender- matched controls were screened using ProtoArray<sup>®</sup> human protein microarrays for immune response biomarker profiling. All patients included had no known history of autoimmune disease, diabetes mellitus or other skin cancer. The control group comprised 3 females and 2 males, with a mean age of  $70.2 \pm 4.3$  years and the melanoma group comprised 3 females and 3 males with a mean age of  $70.7 \pm 7.5$  years ( $P = 0.91$ ).

Comparison of the autoantibody profiles between the melanoma and control group identified several clones that were more prevalent in the melanoma group. For each of these proteins, Table 5.1 shows the gene and cellular location, as well as the function of the protein and the number of positive melanoma and control patients.

**Table 5.1 Summary of genes identified by protein microarray screening of patient serum**

Protein name	Function	mRNA expression	Protein expression	Melanoma (n = 6)	Control (n = 5)
T-complex 10A	Unknown	Ubiquitous	Ubiquitous	4	0
BMX non-receptor tyrosine kinase	Non-receptor tyrosine kinase belonging to the Tec kinase family; implicated in several signal transduction pathways including Stat pathway; regulates differentiation and tumourgenicity of several types of cancer cells.	Highly expressed in haematopoietic cells	Highly expressed in haematopoietic cells	3	0
Cardiotropin 1	Secreted cytokine; induces cardiac myocyte hypertrophy <i>in vitro</i> ; has been shown to bind and activate ILST/gp130 receptor.	Ubiquitous	Highly expressed in heart, skeletal muscle, prostate and ovary.	3	0
Hypothetical protein MGC11082	Unknown	n/a	n/a	3	0
Myeloid/lymphoid or mixed-lineage leukaemia translocated to 6	Putative transcription factor	Ubiquitous	Ubiquitous	3	0
Oxidative-stress responsive 1	Member of Ser/Thr protein kinase family of proteins; regulates downstream kinases in response to environmental stress; play a role in regulating actin cytoskeleton.	Haematopoeitic cells	Ubiquitous	3	0
Phosphatidylinositol-5-phosphate 4-kinase, type 11, gamma	Critical second messenger regulating a number of cellular activities such as modulation of the actin cytoskeleton, vesicle trafficking, focal adhesion formation and nuclear events.	Specifically expressed in brain	Specifically expressed in brain	3	0

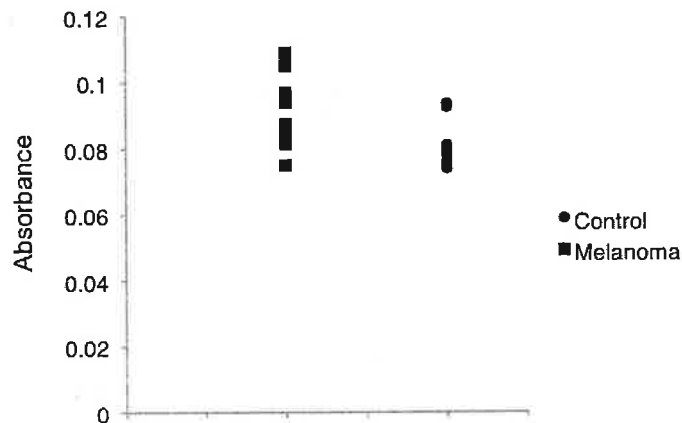
Replication factor C (activator 1) 4	Subunit of replication factor C (RFC). Both RFC proteins and proliferating cell nuclear antigen (PCNA) are required for the elongation of primed DNA templates by DNA polymerase delta and DNA polymerase epsilon.	Rapidly proliferating cells	Ubiquitous	3	0
TTK protein kinase	Essential for chromosome alignment at the centromere during mitosis; tumourgenesis may occur when this protein fails to degrade and produces excess centrosomes resulting in aberrant mitotic spindles.	Rapidly proliferating cells	Rapidly proliferating cells	3	0
Ubiquitin associated protein 1	Member of the UBA domain family, whose members include proteins having connections to ubiquitin and the ubiquitination pathway. The UBA domain family is being studied as a putative target for mutation in nasopharyngeal carcinomas.	Ubiquitous	Ubiquitous	3	0
V-erb-b2 erythroblastic leukaemia viral oncogene homolog 2 (HER2)	Amplification and/ or overexpression of this protein has been reported in numerous cancers, including breast and ovarian tumours.	Ubiquitous	Ubiquitous	3	0

### **5.3.2 Autoantibodies to BMX are elevated in sera from patients with melanoma**

A literature review was undertaken to investigate the identified proteins and to select those that were of potential interest in melanoma. It was felt that the non-receptor tyrosine kinase BMX was an attractive choice as it has been previously implicated in carcinogenesis and shown to be involved in multiple signalling pathways.

In the first instance BMX was selected for validation of the array results using reverse ELISA. Sera from 14 patients with melanoma and sera from 12 controls were screened by reverse ELISA for reactivity with recombinant BMX. Evaluation of the demographic data of the patients showed that the control and melanoma groups were age- and gender- matched. The control group consisted of 5 males and 7 females whereas the melanoma group comprised 7 males and 7 females. The mean age of the control group was  $56.2 \pm 19.6$  and the melanoma group was  $59.9 \pm 15.1$  ( $P = 0.586$ ). Figure 5.1 shows the reverse ELISA data from the serum screened for autoantibodies to recombinant BMX protein. The difference in the mean absorbance between the BMX group ( $0.0888 \text{ nm} \pm 0.0097$ ) and the control group ( $0.08025 \text{ nm} \pm 0.0064$ ) was statistically significant ( $P = 0.02$ ).





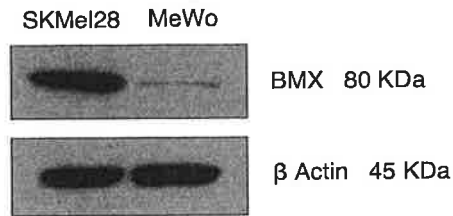
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**Figure 5.1 Reverse ELISA BMX in patient serum samples.**

Graph demonstrating relative absorbance of control and melanoma serum samples when subjected to reverse ELISA for BMX.

### **5.3.3 BMX is expressed in melanoma cell lines and frozen tissue samples**

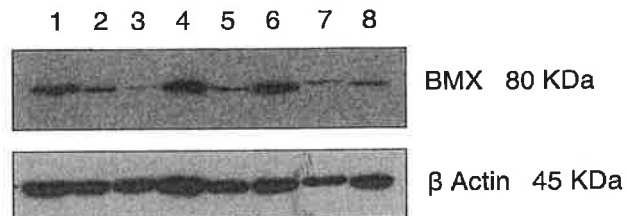
To investigate the protein expression of BMX in melanoma cell lines, lysate was extracted, resolved by SDS PAGE on a 2D gel and probed with anti-BMX antibody. Higher expression of BMX was observed in the primary cell line, SKMel28 (Figure 5.2). Snap frozen tissue from melanoma tumour samples was also tested for expression of BMX protein and variable levels of expression were observed (Figure 5.3).



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**Figure 5.2 Protein expression of BMX in melanoma cell lines**

Western blot demonstrating protein expression of BMX in SKMel28 and MeWo cell lines. Results shown are representative of three separate experiments.



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**Figure 5.3 Protein expression of BMX in snap frozen tissue samples**

Western blot demonstrating protein expression of BMX in a panel of melanoma frozen tissue specimens. Results shown are representative of three separate experiments.

## **5.4 Discussion**

In this chapter, protein microarray technology was employed to identify potential melanoma autoantibody markers in patient serum. One particular marker - the non-receptor tyrosine kinase, BMX - was selected for further investigation. The differential expression of this autoantibody in a larger cohort of patient serum was demonstrated. Expression of BMX protein in melanoma cell lines and patient tissue was also confirmed.

A relatively small number of papers have reported the presence of autoantibodies in patients with melanoma (Forgber et al., 2009, Suzuki et al., 2010, Pardo et al., 2007). In these studies, sera from patients with melanoma and normal controls were tested against western blots of antigens and the IgG patterns analysed by multivariate statistical techniques. Subsequent statistically significant antigens were identified by mass spectrometry. However, this method is biased towards autoantibodies against antigens that have a high level of expression. Protein arrays represent an alternative approach for identifying potential markers in a high throughput manner, as more than 10,000 different proteins can be simultaneously analysed. This method enables the detection of autoantigens regardless of the level of expression in their native host tissue.

Complex autoantibody repertoires exist in seemingly healthy people and are enormously diversified (Li et al., 2006). Due to the diversity, large numbers of patients need to be screened to find disease specific proteins. Specifically in melanoma, this diversity is also manifest in the range of histological subtypes of the disease, as well as the varying outcomes associated with melanomas of increasing depth. In this study, serum screened on the microarrays came from patients who had either superficial spreading or nodular type melanomas. The depth of lesions was variable however (mean 4.9 mm  $\pm$  5.3) and this is reflective of patient throughput and sample availability in the hospital. The need for gene signature studies to be specific to particular subtypes and stages of melanoma was highlighted in a recent review by

Timar et al. (Timar et al., 2010). In spite of these limitations, a panel of uniquely expressed autoantibodies was generated from the microarray analysis.

#### **5.4.1 Autoantibodies identified on protein microarray**

A panel of differentially-expressed autoantibodies was identified by screening melanoma and control patient serum on protein microarrays. A literature review was carried out in order to identify potentially useful markers of melanoma tumourgenesis.

The list included genes coding for protein kinases (BMX, ERBB2, OXSR1, TTK), transcription factors (MLLT6) and cytokines (CTF1). Of these, the non-receptor tyrosine kinase, BMX was chosen for further investigation and is discussed in greater detail below. The ERBB2 gene codes for HER2/neu, a member of the epidermal growth factor receptor family, which has been extensively researched in breast cancer and which confers a more aggressive phenotype in approximately 15-20% of patients (Coussens et al., 1985, Olayioye, 2001). HER2/neu has been implicated in the progression of melanoma and recent studies have investigated the potential use of ErbB receptor kinase inhibitors as a novel treatment strategy (Djerf et al., 2009).

OXSR1 codes for the enzyme serine/threonine– protein kinase OSR1 and belongs to the Ser/Thr protein kinase family of proteins. It regulates downstream kinases in response to environmental stress and may play a role in regulating the actin cytoskeleton (Tamari et al., 1999). TTK codes for a protein kinase that is closely related to the SPK1 serine, threonine and tyrosine kinases and has been shown to be associated with cell proliferation (Mills et al., 1992). MLLT6 is a putative transcription factor which has been associated with poor prognosis in infantile leukaemias of either lymphoid or myeloid lineage derivation (Taki et al., 1996, Mitterbauer-Hohendanner and Mannhalter, 2004). CTF1 codes for cytokine Cardiotrophin-1 (CT-1). It is a

member of the IL-6 cytokine family and has been associated with the pathophysiology of cardiac diseases (Pennica et al., 1996).

The potential role of these antigens in the molecular pathways responsible for melanoma tumourgenesis is not clear. Although the antibodies may not be directly responsible for the disease, they could be useful as biomarkers, either individually or in a panel, to aid in the diagnosis and monitoring of melanoma. With further research, they may also provide therapeutic targets for the specific prevention of tumour progression in patients with melanoma.

#### **5.4.2 BMX**

Bone Marrow tyrosine kinase in chromosome X (BMX) was first identified by Tamagnone in a bone marrow library in 1994 (Tamagnone et al., 1994) and belongs to the Btk family of non-receptor tyrosine kinases. Non-receptor tyrosine kinases serve multiple roles in diversifying and amplifying signals emanating from receptors located on the cell surface. Bruton's tyrosine kinase (Btk) is the most investigated of the Btk family, as its mutation is associated with x-linked agammaglobulinaemia in humans (Satterthwaite and Witte, 2000).

BMX Polypeptide is 80 kDa in size and is cytoplasmic in location. BMX cDNA contains a long open reading frame of 675 amino acids and comprises four structural modules; Src homology 3 (SH3), Src homology 2 (SH2), tyrosine kinase (SH1) and pleckstrin homology (PH) (Tamagnone et al., 1994). Each of these modules interacts with a number of signaling molecules to form signal complexes. Therefore it is to be expected that these kinases impact multiple signal pathways and generate pleiotropic effects. Potential transcription factors that can be activated include the following: AP-1 (Activator Protein 1, which regulates gene expression in response to various stimuli), NFkB (transcription factor involved in cellular response to stress, cytokines, free radicals, UV radiation and implicated in cancer and inflammation), Forkhead (Fox or Forkhead Box proteins are proteins which

regulate expression of genes involved in cell differentiation, growth, longevity and embryonic development), SRF (Serum Response Factor, binds to serum response element in promoter of target genes and is involved in cell cycle regulation, apoptosis, cell growth and differentiation) and STAT3 (phosphorylated by tyrosine kinases and then translocates in to nucleus to act as transcription activator of genes involved in cell growth and apoptosis) (Qiu and Kung, 2000).

BMX is implicated in various biological processes including proliferation, differentiation, cell migration and apoptosis (can be either pro- (p38MAPK) or anti-apoptotic (Akt, NFkB)) (Chen et al., 2004). BMX has been found to be highly expressed in prostate (Qiu et al., 1998) and breast tumour cell lines (Chen et al., 2004). Interestingly BMX is strongly upregulated in keratinocytes during wound healing and has been shown to induce chronic angiogenesis and inflammation in the skin (Paavonen et al., 2004). Expression of BMX in melanoma cell lines or tissue samples has not been previously described.

In the current study, a significantly higher level of BMX was observed in the serum of patients with melanoma than in matched controls. There was however some overlap seen with the non-melanoma group at the lower end of the scale. This highlights the limitation of autoantibodies as tumour markers in terms of their potential activation by other signalling processes apart from malignancy. Though every effort was made to ensure a 'clean' control group, it is possible that certain patients may have had undetected an autoimmune condition. Furthermore, patients with varying depths of melanoma were included in the cohort and their levels of BMX may have been only marginally higher than baseline. Future studies using this type of technology need to be based on rigorous sample collection and divergent histological types of melanoma should not be analysed universally.

As with the other markers identified in this chapter, the role that BMX potentially plays in melanoma is yet to be elucidated. With further

investigation in a larger cohort, of carefully selected patients, BMX may emerge as an important component in melanoma signalling, and may even have potential as a therapeutic target. At minimum, BMX may contribute to a panel of biomarkers useful in discerning patients with melanoma from healthy individuals.

## 6 General discussion



Melanoma is a highly aggressive and unique cancer. Even a minute increment in the Breslow thickness of a primary melanoma can result in rapid transformation from a relatively curable disease to a highly aggressive and often fatal one (Hearing and Leong, 2006). This sets melanoma aside from other malignancies. In breast cancer for example, a T2 lesion is 2 cm in size and more aggressive clinical behaviour only develops with significant tumour burden (Verschraegen et al., 2005). Melanoma thus creates a particular challenge for researchers and clinicians alike. In terms of molecular biology, melanoma both intriguing and humbling in its complexity.

Although a definitive treatment strategy for advanced melanoma is still sought, significant advances have been made in recent years in elucidating the molecular mechanisms underlying the disease. Given the phenotypic diversity seen in melanoma, it is not surprising that all melanomas do not follow the same set of genetic events. Certain molecular disruptions have been well described however. Up to 70% of melanomas express mutated BRAF for example and it is known that MAPK kinase signaling is upregulated as a result of this (Fecher et al., 2008). Activating mutations of NRAS and CDK4 can also stimulate proliferation via MAPK signalling (Miller and Mihm, 2006). The receptor tyrosine kinase, c-KIT has shown significant promise as a novel target in acral and mucosal melanomas and GNAQ has recently been implicated in uveal melanoma (Curtin et al., 2006, Van Raamsdonk et al., 2009). As a result of these advancements in molecular understanding, targeted therapies have become the new hope for definitive management of melanoma. Due to the multiplicity of disrupted pathways, it is likely that combination targeted therapy will be required to achieve maximal therapeutic benefit however.

## **6.1 Transcriptional regulation of S100B**

Melanoma is characterised by an unpredictable disease course. At diagnosis, prognosis is estimated on the basis of lesion depth and lymph node status. However, a significant number of patients with thin melanomas will go on to develop disease recurrence and distal metastases (Balch et al., 2009). For this reason, there is ongoing research interest in melanoma biomarkers. The calcium-binding protein S100B is one of the most widely researched biomarkers in melanoma and its serum expression has been shown to correlate with both disease stage and response to treatment (Martenson et al., 2001). While there is substantial evidence to support transcriptional regulation of S100B, little is definitively known about its regulators. In this research, the role of the coactivator protein SRC-1 and its transcription factor binding partner, HOXC11 in targeting S100B was explored.

SRC-1 is a well-described coactivator protein and its differential expression has been associated a number of different cancers (Xu and Li, 2003). Recently, the transcription factor HOXC11 was identified as a novel binding partner for SRC-1 and the two proteins in complex were shown to regulate expression of S100B in breast cancer cells (McIlroy et al., 2010). In this work, it was hypothesized that production of S100B in melanoma is regulated by HOXC11 in cooperation with SRC-1.

The first aim of the study was to define the molecular role of HOXC11 and SRC-1 in the transcriptional control of S100B in malignant melanoma. Specifically, expression of HOXC11, SRC-1 and S100B was confirmed in two melanoma cell lines, SKMel28 and MeWo, both at mRNA and protein levels. Interaction of HOXC11 and SRC-1 was confirmed by co-immunoprecipitation and protein colocalisation was established by immunofluorescent staining. Nuclear localisation of HOXC11 and SRC-1 was observed in the SKMel28 cells, whereas a more diffuse distribution was seen in the MeWo cells.

Significantly more nuclear staining was observed in tissue samples of melanoma rather than nevi, indicating that expression of the transcription factor and its coactivator may be associated with a more aggressive phenotype. In order to confirm targeting of S100B by this transcription factor complex, recruitment of HOXC11 to the S100B promoter was demonstrated by chromatin immunoprecipitation.

The functional relationship between the HOXC11-SRC-1 complex and target gene S100B was explored by gene silencing and overexpression experiments. Both approaches showed that manipulation of HOXC11 levels resulted in alteration of S100B production, thereby implicating the transcription factor and its coactivator in the transcriptional regulation of S100B.

The next step in understanding this novel molecular interaction was to investigate its potential as a therapeutic target. To this end, cells were treated with dasatinib, a multi-target Src kinase inhibitor. Dasatinib has previously been demonstrated as exerting a functional effect on melanoma cell lines. Here we sought to investigate its effect on targeting of S100B by HOXC11-SRC-1. It was found that the coassociation between HOXC11 and SRC-1 was reduced when cells were treated with dasatinib, potentially as a result of disruption of the Src kinase phosphorylation cascade.

Taken together these results suggest that HOXC11 and SRC-1 may have a role in the production of S100B in malignant melanoma. As such, this transcription factor complex may be implicated in a more aggressive disease phenotype. Further work is required to verify these findings however. In particular, demonstration of expression in a large cohort of patients with clinical follow-up is essential. Not only would this data provide crucial insight in to the expression of HOXC11 and SRC-1 in varying stages of melanoma, but it would also verify any potential association with clinical outcome.

## **6.2 Identification of novel melanoma biomarkers**

The second part of this research sought to identify new biomarkers in sera from patients with malignant melanoma. To date, only LDH has been included in the AJCC staging system and its value is restricted to patients with metastatic disease. Recent consensus appears to be that a panel of biomarkers - as opposed to any one marker alone - are likely to be the optimum tool in predicting patients' prognosis and response to treatment (Haass and Smalley, 2009). A number of biomarker-screening techniques have been employed as a result. In this work, an autoantibody expression screen, using protein microarray technology, was undertaken.

A panel of differentially expressed autoantibodies was identified in a small cohort of melanoma and control patients. Of these, the non-receptor tyrosine kinase, BMX was selected for further investigation. BMX is implicated in a number of biological processes and has previously been found to be highly expressed in prostate and breast cancer cell lines (Chen et al., 2004, Robinson et al., 1996). It was felt that of the proteins identified, BMX offered the most potential as a novel tumour marker in melanoma. Varying expression of BMX was identified in two melanoma cell lines and across a panel of frozen tissue samples. Further investigation is required to establish the exact role of BMX in melanoma. Verification in a larger patient cohort is also necessary to establish its utility in prediction of disease stage and clinical outcome.

## **6.3 Conclusion**

In this research, an insight in to the transcriptional regulation of S100B in malignant melanoma has been identified and a novel autoantibody biomarker of the disease has been proposed. Melanoma is hugely complex in its molecular biology and while these results are potentially important, it is important to view them in the context of what is already known about the disease, as well as the course that molecular research in melanoma has taken to date.

Melanomas carry hundreds of mutations. Those that occur at high frequency and that have been shown to be important in tumour development are BRAF (expressed in 50 - 60% of melanomas), NRAS (15%) and c-KIT (2 – 4%) (Miller and Mihm, 2006, Curtin et al., 2006). It is also widely understood that not all melanomas are the same and that only the minority relies on a single molecular driver. For this reason, elucidation of novel signaling pathways and therapeutic targets remains at the forefront of molecular research in melanoma.

For the last thirty years, melanoma has managed to elude all attempts at chemotherapeutic intervention. While some treatments have managed to slow disease progression, none has succeeded in significantly prolonging overall survival. Even more so than other cancers, melanoma therefore requires individualised therapy. In the past year, the first complete melanoma sequence was published (Plesance et al., 2009). It is estimated that sequencing of a further 500 specimens is required to create a comprehensive catalogue of all somatic mutations occurring in melanoma (Herlyn, 2009). While this may seem a monumental task, it is likely that the key to effective therapy in melanoma lies in the elucidation of an individual's somatic profile. It can be anticipated that in the future this information will be used to guide therapeutic selection for individual patients.

The findings presented in this work constitute a small fragment of a very large puzzle. Advanced melanoma is likely to present a significant therapeutic challenge to clinicians and academics for many years to come. What is certain is that translational research methods, as have been employed here, are essential in pushing forward the boundaries of our molecular understanding of this fascinating disease.

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## **8 Appendix**

### **8.1 Immunohistochemistry**

#### **Phosphate buffered saline**

Dissolve one PBS tablet per 200 ml dH<sub>2</sub>O

Each PBS tablet contains:

0.1 M phosphate buffer

0.0027 M potassium chloride

0.137 M sodium chloride

Autoclave and filter prior to use.

#### **Hydrogen peroxide solution**

10 ml dH<sub>2</sub>O

1ml 30% H<sub>2</sub>O<sub>2</sub>

3,3'-Diaminobenzidine

Dissolve set of tablet in 1 ml of dH<sub>2</sub>O

Each set of tablets contains

Di-amino-benzidine 0.7 mg/ml

Urea hydrogen peroxide 1.6mg/ml

Tris buffer 0.06M

#### **Sodium citrate buffer**

Dissolve 1.4mg of sodium citrate in 500 ml of dH<sub>2</sub>O

Final concentration of 0.01 M sodium citrate, pH 6.

### **8.2 Cell Culture**

#### **Fetal calf Serum (FCS) (GiBcoBRL®):**

Minimal essential medium MEM Medium 500 ml volume (GiBcoBRL®)

5 ml of Pen/Strep solution (50 U/ml Penicillin and 50 U/ml Streptomycin)

5 ml L-glutamine (200mM ; 2mM final concentration)- Renewed every two weeks.

50ml of FCS

**Trypsin-EDTA**

Trypsin 10 X liquid (25g/l GiBcoBRL®)

HBSS (GiBcoBRL®)

0.02% EDTA

20MM HEPES

1ml of trypsin and 1 ml of 0.02% EDTA was made up to 10ml HBSS filter prior to use

through Acrodisc® 32 filters (0.2µm pore size)

**RIPA buffer (Pierce)**

Used to lyse cultured mammalian cells.

1 ml of cold RIPA Buffer for every ~20 µl of packed cells, which is equivalent to ~40 mg of cells.

**8.3 Western Blotting****Tris Buffered saline (TBS) (20X):**

121.1 g Tris

175.5 g NaCl

Made up to 1 litre with dH<sub>2</sub>O

Use at 1X final concentration, pH 8.3

**Wash buffer:**

1X TBS

0.05% Triton® X-100

**Blocking buffer:**

1X TBS

0.05% Triton® X-100

5% Molecular grade skimmed milk

**1 M Tris.HCl, pH 6.8**

157.6g Tris- HCl

Made up to 1 litre with dH<sub>2</sub>O, pH 6.8

**1.5 M Tris.HCl, pH 8.8:**

236.4g Tris- HCl

Made up to 1 litre with dH<sub>2</sub>O, pH 8.8

**Transfer buffer:**

2.93 Glycine

5.8 g Tris Base

0.375 g SDS

200 mL Methanol

Made up to 1 litre with dH<sub>2</sub>O

**Sample buffer (5X)**

2 g SDS

5 ml 1M Tris.HCl (6.8)

3.0 ml dH<sub>2</sub>O

8 ML Glycerol

2 ml 0.1% Bromophenol Blue

Make up to 20ml with dH<sub>2</sub>O

5% β-mercaptomethanol

**Running buffer (10X)**

288 gm Glycine

60.6 g Tris Base

20 g SDS

Made up to 2 litres with dH<sub>2</sub>O

**Acrylamide/Bis-acrylamide 30%**

Liquid easigel 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide was used directly.

### **20% SDS**

20 g SDS was dissolved in 100 ml of dH<sub>2</sub>O

### **10% Ammonium Persulphate**

100 mg/ml was dissolved in dH<sub>2</sub>O.

## **8.4 Chromatin Immunoprecipitation**

### **ChIP Dilution Buffer**

50 µl 10% SDS

0.55 ml Triton X 100

22.334 mg EDTA (= 1.2 mM EDTA in 50 mls)

131.97 mg NaCl (= 167 mM in 50 mls)

49.4 mls dH<sub>2</sub>O

pH 8.1

### **LiCl Immune Complex Wash Buffer**

529.88 mg LiCl (= 0.25 M in 50 mls)

0.5 ml IPEGAL NP40

0.5 g deoxycholic acid

18.61 mg EDTA (= 1 M EDTA)

78.8 mg Tris HCl (= 10 mM in 50 mls)

49.5 ml dH<sub>2</sub>O

pH 8.1

### **SDS Lysis Buffer**

5 ml 10% SDS

186.12 mg EDTA (=10 mM EDTA)

394 mg Tris HCl (= 50 mM in 50 mls)

45 ml dH<sub>2</sub>O

pH 8.1

### **Low Salt Immune Complex Wash Buffer**

500  $\mu$ l 10% SDS  
0.5 ml Triton-X 100  
37.22 mg EDTA (= 2 mM in 50 mls)  
157.6 mg Tris HCl (= 20 mM in 50 mls)  
438.3 mg NaCl (= 150 mM in 50 mls)  
49 ml dH<sub>2</sub>O  
pH 8.1

#### **High Salt Immune Complex Wash Buffer**

37.22 mg EDTA (= 2 mM in 50 mls)  
500  $\mu$ l 10% SDS  
0.5 ml Triton-X 100  
157.6 mg Tris HCl (= 20 mM in 50 mls)  
1461 mg NaCl (= 500 mM in 50 mls)  
49 ml dH<sub>2</sub>O  
pH 8.1

#### **TE Buffer**

18.6 mg EDTA(= 1mM in 50 mls)  
78.8 mg Tris HCl (= 10 mM in 50 mls)  
50 mls dH<sub>2</sub>O  
pH 8.0

### **8.5 Protein microarray**

#### **Blocking buffer**

5 ml HEPES, pH 7.5  
4 ml NaCl  
0.8 ml Triton X-100  
50 ml glycerol  
610 mg reduced glutathion  
10 ml synthetic block

0.1 ml DTT  
to 100ml dH<sub>2</sub>O

**Washing buffer**

100 ml 10X PBS  
10 ml Tween 20  
100 ml synthetic block  
to 1000 ml dH<sub>2</sub>O