Post-transcriptional Regulation of Tetraspanins CD151 and CD9 by micro-RNAs in Prostate and Breast Cancers

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BBiomed Sci (Hons)

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Declaration

The thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository**, subject to the provisions of the Copyright Act 1968. **Unless an Embargo has been approved for a determined period.

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Danielle Bond

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Table of Contents

List of figures

List of tables

Abstract

Tetraspanins CD151 and CD9 play important roles in cancer progression and metastasis. CD151, referred to as a metastasis enhancer, is typically upregulated in solid malignancies including breast and prostate cancers. In contrast, CD9 is commonly considered a metastasis suppressor, with downregulation of CD9 protein levels in advanced stage cancers. Therefore, CD151 and CD9 are potential targets for new therapeutics to combat cancer progression. However, CD151 and CD9 are not typical "druggable" targets, therefore other ways to change tetraspanin expression such as manipulation of tetraspanin regulation are required. Regulation of CD151 and CD9 expression has been minimally investigated. Therefore, the aim of this thesis was to investigate post-transcriptional regulation of CD151 and CD9 by miRNAs in non-tumourigenic and tumourigenic prostate and breast cell lines.

CD151 mRNA and protein levels were found to inversely correlate in prostate cell lines, with highly tumourigenic prostate cancer cells expressing high levels of CD151 protein. Breast cancer cell lines displayed low levels of CD151 mRNA and protein compared to non-tumuorigenic breast cells, however triple negative MDA-MB-231 breast cancer cells showed similar CD151 protein expression to that of non-tumourigenic breast cells. The degree by which the CD151 3'UTR regulates protein expression was determined with a dual luciferase assay, with greater repression of protein expression found in the tumourigenic cell lines. Bioinformatic analysis of miRNA predicted to bind CD151 together with miRNA expression profiling in prostate cells was used to identify miRNA

that had expression levels matching the luciferase output. miR-637, which was upregulated in prostate cancer cell lines, was shown to regulate expression at the CD151 3'UTR, with transfection of miR-637 mimic into RWPE1 and DU145 prostate cells resulting in a 10-20% decrease in CD151 protein expression. However, miR-637 had no effect on CD151 protein expression in non-tumourigenic 184A1 and MDA-MB-231 breast cancer cells, suggesting a role specifically in prostate cancer.

CD9 mRNA and total protein levels were similar across all prostate cell lines, with typically slightly lower levels of CD9 cell surface levels in tumourigenic cells. In addition breast cancer cell lines displayed lower levels of CD9 mRNA, total protein and cell surface protein expression compared to non-tumourigenic breast cells. A 3'UTR luciferase reporter assay showed that the CD9 3'UTR is differentially regulated in prostate and breast cell lines, with highly tumourigenic prostate cancer cells showing more repression of luciferase compared to other cells. In the panel of breast cells, CD9 3'UTR activity was similar across all lines, however 184A1 breast cells showed increased luciferase, which suggests that the CD9 3'UTR is partly responsible for high CD9 protein levels in normal breast cells. Using the same approach to identifying miRNA as for CD151, miR-518f* was found to bind to the CD9 3'UTR *in vitro*. Overexpression of miR-518f* in non-tumourigenic prostate RWPE1 and prostate cancer DU145 cells as well as non-tumourigenic breast 184A1 and MDA-MB-231 breast cancer cells led to a significant decrease in CD9 protein expression. Furthermore, transfection of miR-518f* increased migration of RWPE1, 184A1 and MDA-MB-231 cells and decreased migration of DU145 prostate cancer cells. Moreover, overexpression of miR-518f* significantly decreased RWPE1 adhesion to fibronectin

and basement membrane extract and increased 184A1 cell proliferation and adhesion to BME, but had no effect on adhesion in other cell lines or proliferation.

In conclusion, tetraspanins such as CD151 and CD9 are at least partially regulated by miRNAs in prostate and breast cell lines. miRNAs such as miR-518f* may be novel and effective biomarkers and/or therapeutic targets for inhibiting cancer progression in the future.

Publications and conference abstracts arising from this thesis

- D Bond, J Brzozowski, K Skelding, S Roselli & J Weidenhofer. **Use of tetraspanins CD151 & CD9 as biomarkers for breast cancer,** *Breast Cancer Management*, March 2014 Vol. 3 issue 2.

- Bond D, Cairns M, Ashman LK & Weidenhofer J. **Post-transcriptional regulation of tetraspanins CD151 & CD9 in breast & prostate cancers** (poster), AACR 2014 San Diego, USA.

- Bond D, Cairns M, Ashman LK & Weidenhofer J. **Post-transcriptional regulation of tetraspanins CD151 and CD9 by micro-RNAs in prostate cancers** (oral presentation), Hunter Translational Cancer Conference, Newcastle city hall 2013.

- Bond DR, Cairns MJ, Ashman LK & Weidenhofer J. **Post-transcriptional regulation of CD151 and CD9 in prostate and breast cancers** (oral presentation), ComBio 9 September – 3 October 2013, Perth, Australia.

- Danielle Bond, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Investigating miRNA Regulation of Tetraspanins CD151 & CD9 in Prostate and Breast Cancers** (poster presentation), ASMR NSW Scientific Meeting, 3rd June 2013, Australian Technology Park Redfern NSW, Australia.

- Danielle Bond, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Post-transcriptional Regulation of CD151 & CD9 in Prostate Cancers** (oral presentation), The 5th Annual Hunter Cancer Research Symposium, 5th November 2012, Newcastle NSW, Australia.

- Danielle Bond, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Post-transcriptional Regulation of CD151 and CD9 in Breast and Prostate Cancer** (oral & poster presentation), 5th European Conference on Tetraspanins, 26-28 September 2012, Nijmegen, the Netherlands.

- Danielle Bond, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Investigating micro-RNA Regulation of Tetraspanins CD151 & CD9 in**

Prostate and Breast Cancers (poster), 22nd IUBMB and 37th FEBS Congress: From Single Molecules to Systems Biology, 4th-6th September 2012, Sevilla, Spain.

- Danielle Bond, Matthew J Bowman, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Investigating Regulation of Tetraspanin Expression in Breast and Prostate Cancers** (poster), ASMR NSW Scientific Meeting 2012, Australian Technology Park, Redfern, NSW, Australia.

- Danielle Bond, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Investigating Regulation of Tetraspanin Expression in Prostate Cancer** (poster), HMRI Cancer Research Symposium, 4th November 2011, Newcastle, NSW, Australia.

- Danielle Bond, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Investigating Micro-RNA Regulation of Tetraspanins in Prostate Cancer** (poster), Australian Society for Medical Research (ASMR) XIX NSW Scientific Meeting, 6th June 2011, The University of Sydney, Camperdown, NSW, Australia.

- Danielle Bond, Matthew J Bowman, Murray Cairns, Leonie K Ashman & Judith Weidenhofer, **Investigating Regulation of Tetraspanin Expression in Breast and Prostate Cancers** (poster), 11th Hunter Cell Biology Meeting, 22-25 March 2011, Hunter Valley Vineyards, NSW, Australia.

Abbreviations

Abbreviation	Word
BC	Breast cancer
BME	Basement membrane extract
cDNA	Complimentary DNA
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
Exp.	Expression
FN	Fibronectin
miRNA	Micro ribonucleic acid
NTC	Non-targeting control
PC	Prostate cancer
RBP	RNA binding protein
Refs	References
RNA	Ribonucleic acid
TEM	Tetraspanin-enriched microdomain

Chapter 1: Literature Review

1.1 Breast and prostate cancer statistics

Breast and prostate cancer are two of the most common epithelial malignancies affecting men and women, respectively. In 2011, prostate cancer was the second highest cause of cancer-related mortality in men and accounted for the highest cancer incidence rates across both genders in Australia [1]. Similarly in women in 2007/2008, breast cancer was the second leading cause of cancer-related mortality and had the highest cancer incidence rates among Australian women [2, 3].The high incidence of breast and prostate cancer in recent years is most likely a result of improvements in early detection through the use of mammography for early detection of indolent breast tumours in women, and serum PSA level testing with digital rectal exam for early detection of indolent prostate tumours in males, which would otherwise have gone undetected. However, improvements in early detection have not resulted in the anticipated major decline in mortality rates [4-7].

Although five-year survival rates for breast cancer and prostate cancer patients have marginally improved over time (~90% in 2010/2011) [1, 3], there are still at least 10% of patients who develop aggressive cancer, which metastasizes to other organs around the body, ultimately resulting in death. It is this group of patients with metastatic disease for which there are currently no effective or curative long-term treatments. Therefore, breast and prostate cancer are both major health issues and continued research is necessary to determine the factors that are related to cancer cell behaviour and therefore could be used to predict disease course, in particular those that specifically drive the progression of localised cancer to metastatic disease.

1.2 The pathogenesis of breast and prostate cancer

The pathogenesis of breast and prostate cancer is somewhat similar due to the fact that they are both derived from epithelial cells. Whilst both breast and prostate cancers can arise in basal or ductal epithelium, the majority in both cancers are adenocarcinomas and thus show similarities in cellular architecture, such as polarised epithelial cells lining the ducts which form compact acini structures [8] (Fig 1.1A & B). They are both heterogeneous diseases that are at least initially hormonally-driven, which is a fundamental requirement of normal breast and prostate glandular development [9]. However in malignancy, hormones begin to fuel excessive growth and division of cancer cells, resulting in a primary, localised tumour mass.

Cancer, whether it originates in the breast or prostate gland, is thought to begin when a single cell acquires a number of genetic, epigenetic and/or environmental insults, causing transformation into a malignant cell that is capable of uncontrolled division. Some of the genes commonly altered in breast cancer include the oestrogen receptor (ER), HER2, c-MYC, RAS, p53 and PTEN [10], and in prostate cancer, Nkx3.1, PTEN and p53 are commonly mutated [11]. These changes ultimately result in the formation of a primary tumour mass that is initially localised within the confines of the breast or prostate duct (Fig 1.1). In the majority of cases, the cancer remains in the primary site and does not spread to other organs.

Patients with localised, primary breast or prostate cancer are usually treated with surgery in combination with radiotherapy and/or anti-hormone therapy [12-15]. Therefore, patients within this group are usually cured of disease or continue to live with a slow growing, indolent tumour that does not lead to mortality. However, 40% of

these patients will relapse and present with metastatic disease several years post-treatment [16]. Along with patients who have relapsed, ~10-15% of patients [16] are diagnosed with or quickly develop advanced metastatic cancer. This is not a result of a lack of early diagnosis as early detection methods for breast and prostate cancer such as mammography and PSA testing have been effective [17, 18]. Instead it is likely a consequence of our limited understanding of the mechanisms that drive cancer metastasis leading to inappropriate choice of treatment and/or lack of effective treatment options.

Once a diagnosis of breast or prostate cancer has been made, there are no definitive prognostic markers to determine which patients require aggressive treatment (i.e. patients at risk of developing advanced, metastatic disease) compared to those who may require little if any treatment (patients diagnosed with localised, non-life threatening disease)[19] [20, 21]. This is a major health problem as many cancer patients are either being over-treated and are therefore experiencing unnecessary pain, discomfort and possibly long term side effects leading to decreased quality of life, or are not being treated effectively and are therefore at high risk of developing life-threatening, metastatic disease. Therefore, novel prognostic markers which allow stratification of cancer patients into treatment groups, which will be effective in the long term are desperately needed. The tetraspanin family consists of a group of proteins that are suggested to have prognostic significance in cancer [22] and may therefore be suitable prognostic markers for breast and prostate cancer.

A

B

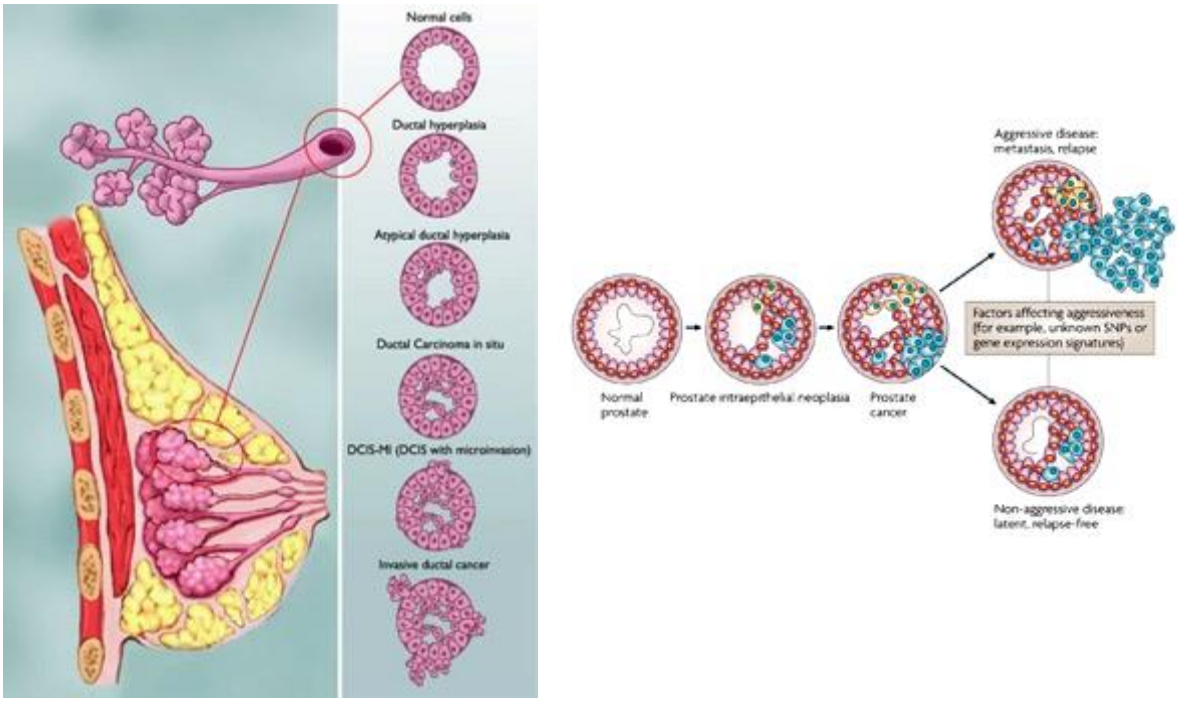

Figure 1.1 Schematics of breast and prostate adenocarcinoma progression. A) In normal mammary gland development, polarised epithelial cells of the mammary ducts form acini structures with hollow lumens. Over time, these cells can undergo hyperplastic changes, which are thought to predispose to Ductal carcinoma *in situ* or localised breast cancer. Once breast cancer is established it can then further progress and involve micro-invasion of the acini and/or invasion of the surrounding mammary gland, leading to metastasis. B). Similarly, in the prostate gland, normal prostate epithelial cells form acini with hollow lumens and these cells can undergo changes resulting in intra-epithelial neoplasia which is considered a pre-cursor to prostate cancer. Prostate cancer can then stay localised to the confines of the prostate gland or can undergo further modifications resulting in aggressive and metastatic prostate cancer. Figure A from **http://www.breastcancer.org/symptoms/types/dcis/diagnosis.jsp** and figure B adapted from [23].

1.3 Tetraspanin superfamily: structure and function

The tetraspanin superfamily consists of small transmembrane proteins that are expressed in a range of organisms including fungi, fly, mouse and humans [24-27]. The first tetraspanin was identified in 1990 [28] with 33 tetraspanins identified in humans to date [29]. As their name infers, tetraspanins contain four membrane-spanning domains, a short and large extracellular loop (SEL & LEL) as well as short intracellular amino

and carboxy terminal tails (Fig 1.2). This specific structure along with highly conserved cysteine residues within it such as the CCG motif in the LEL (Fig 1.2), distinguishes tetraspanins from other four transmembrane domain proteins. Furthermore, highly conserved cytoplasmic cysteine residues are sites for palmitoylation, which is thought to influence tetraspanin homo-oligomerisation e.g. tetraspanin CD9-CD9 homo-oligomers as well as secondary interactions between tetraspanins and other cellular proteins [30-32]. The LEL has been shown to be the main binding site for other cellular proteins such as integrins [33] and EWI proteins [34].

Unlike most transmembrane proteins, many tetraspanins do not act as traditional transmembrane receptors with known ligands. Instead, tetraspanins are considered adaptor proteins and therefore functionally link multiple cellular proteins into multimeric structures in the plasma membrane, which have been termed tetraspanin-enriched micro-domains (TEMs) [35, 36] (Fig 1.3 & 1.4). This is achieved by direct and in-direct interactions between the same tetraspanin molecule, between different tetraspanins, and various membrane and cytosolic proteins, which further aggregate to form TEMs (Fig 1.3 & 1.4). These TEMs then serve as outside in signalling platforms cytosol, thereby modulating an array of cellular functions such as cell adhesion, migration, proliferation and cell signalling [35, 37](Fig 1.4). Given the vast repertoire of tetraspanin partners (e.g. laminin-binding integrins [38], growth factors [39], growth factor receptors [40], immunoglobulin superfamily proteins [41] and signalling molecules [42]), it is not surprising that TEMs are involved in modulating many important cellular processes.

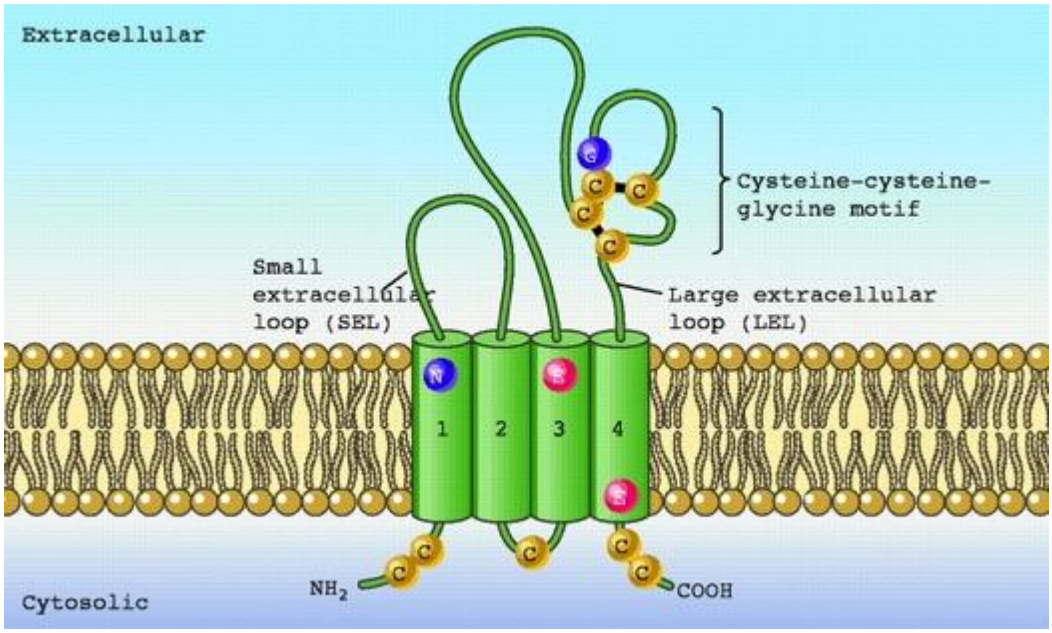

Figure 1.2. The general structure of Tetraspanins. Tetraspanin proteins are composed of four transmembrane domains (green cylinders), which contain conserved residues. Between transmembrane domains 1 and 2 is the small extracellular loop (SEL), and the large extracellular loop is flanked by transmembrane domains 3 and 4. The LEL contains highly conserved cysteine residues, which are connected by disulphide bonds forming the cysteine-cysteine-glycine motif. Furthermore, tetraspanins also contain a short cytoplasmic amino-terminus, short intracellular loop and carboxy-terminus, all of which harbour cysteine residues that are potential sites for palmitoylation. Figure adapted from [32].

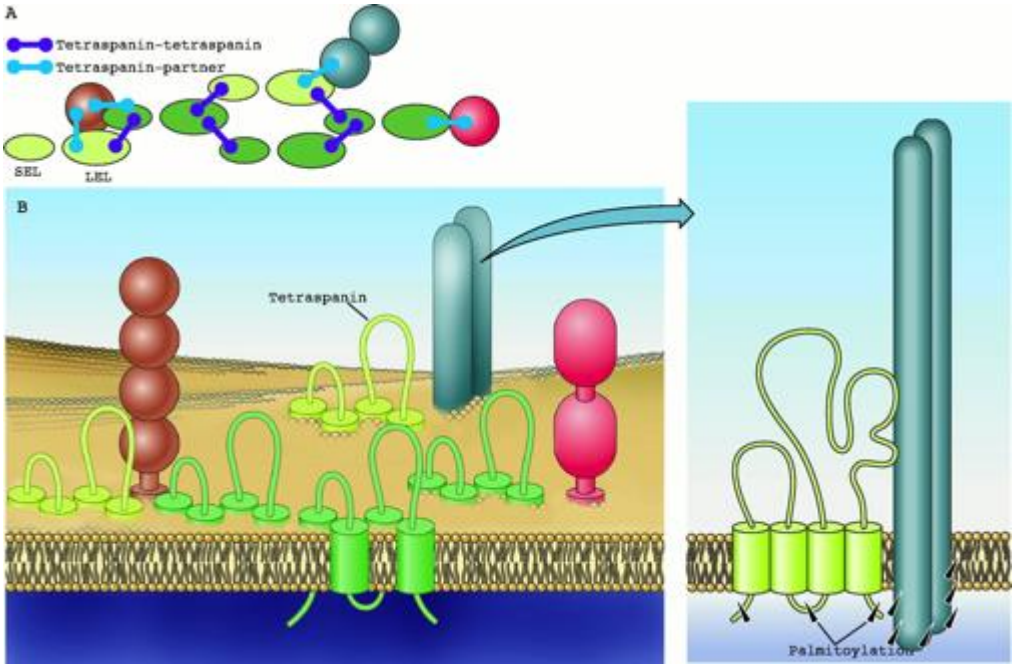

Figure 1.3. Tetraspanin Protein Interactions in the Plasma Membrane. A. Top view of tetraspanin – tetraspanin and tetraspanin – protein partner interactions in the membrane. B. Side view of these direct primary interactions which further associate to form tetraspanin-enriched microdomains that signal into the cell and modulate many cellular functions. Interactions between tetraspanins (shades of green) and other cellular proteins such as integrins (blue) and members of the immunoglobulin superfamily (red & brown) are facilitated by palmitoylation of intracellular cysteine residues of the tetraspanins and partner proteins Figure adapted from [32].

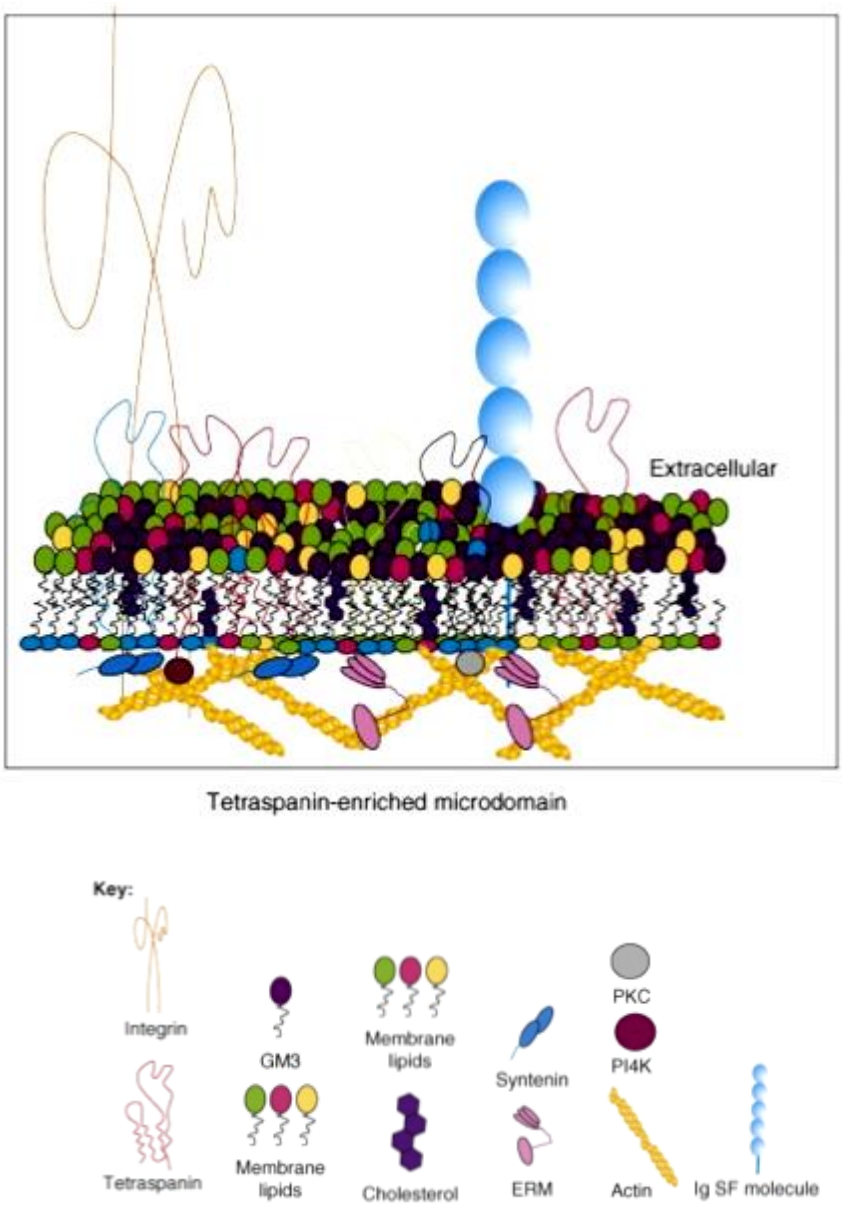

Figure 1.4. Tetraspanin-Enriched Microdomains. Tetraspanin proteins form lateral associations with other tetraspanins and many cellular proteins such as integrins and immunoglobulin superfamily proteins. These complexes then form secondary interactions with other proteins such as PKC, actin and ERM proteins as well as membrane lipids to form tetraspanin-enriched microdomains (TEMs) in the plasma membrane. TEMs are complex dynamic structures, important in regulating many cellular processes such as cell migration, adhesion and cell signalling. Figure adapted from [36].

1.4 Tetraspanins and cancer

Many tetraspanins such as CD9, CD151, CD82, CD63 and CD81 appear to be almost ubiquitously expressed, indicating that they are involved in cellular processes that are common to all cell types [43]. Many of the tetraspanins within this group are involved in cell-cell and cell-matrix adhesion, cell migration and invasion as well as cell signalling, all of which are cellular processes that are deregulated in cancer [34]. Therefore, it is no wonder that these tetraspanins have been shown to strongly influence cancer cell behaviour and progression. In particular, CD151 and CD9, which are the focus of this thesis, appear to play prominent roles in many cancer types.

1.4.1 Tetraspanin CD151

The human *CD151* gene is located on chromosome 11p15.5 and has many aliases including PETA3, SFA1 and TSPAN24 [44]. There are 4 transcript variants that encode the same CD151 protein [44]. The *CD151* gene has 9 exons, with alternate splicing resulting in transcripts that only differ in their 5'UTR [44]. Mutations in CD151 are not commonly reported for disease, with only 1 reported to date occurring in 3 individuals of the same family, resulting in a severe disease phenotype affecting multiple organs [45].

CD151 was firstly discovered in 1995 due to its role in platelet aggregation during wound healing [46, 47]. Since then, CD151 has been shown to be involved in other diseases such as kidney disease [45, 48, 49], pulmonary fibrosis [50], atherosclerosis

[51] and myocardial infarction [52-55] and many different types of solid malignancies including gastric [56-58], bladder [59], skin [60], hepatocellular [61-63], endometrial [64], renal [65] and Merkel cell cancers [66]. Over the past two decades, much attention in the tetraspanin research field has been allocated to teasing out CD151 binding partners and the role/s of CD151 in these diseases. CD151 was the first tetraspanin postulated as a metastasis enhancer, through a study showing that administration of an anti-CD151 monoclonal antibody inhibited metastasis of a cancer cell line *in vivo* by reducing migration of a human epidermoid carcinoma cell line [67].

1.4.2 CD151 and prostate cancer

The clinical significance and functional role of CD151 in prostate cancer progression has not been well defined, but recent studies have started to unravel the importance of CD151 in prostate cancer (for more detail see Ch3 introduction). CD151 is typically expressed by cells of the basal membrane of normal prostate glands and on the apical surface of prostate epithelial cells [68]. Immunohistochemical analysis of primary prostate cancers has provided evidence that CD151 is over-expressed in primary prostate cancer compared to non-tumourgenic prostate tissue [69]. Patients with tumours expressing low levels of CD151 had significant increased overall survival rates and CD151 expression was more predictive of patient outcome than Gleason scoring [69]. Therefore, CD151 protein expression is increased in prostate cancer and high expression is indicative of lower survival rates. In addition, a particular subpopulation of CD151 that is not associated with integrins (CD151free) appears to correlate with poor patient outcome in prostate cancer, with prostate cancers displaying expression of CD151free compared to no expression in normal prostate tissues [70].

Furthermore, Ang et al (2010) [71] showed that CD151 plays a pivotal role in prostate cancer cell migration and invasion *in vitro*. Overexpression of mutant CD151 lacking ECL2 decreased the migratory and invasive potential of LNCaP prostate cancer cells (lymph-node metastasis of prostate cancer), whereas this was increased on overexpression of wild-type CD151. Moreover, knockdown of CD151 in a more aggressive prostate cancer cell line, PC3 (bone metastasis of prostate cancer), which express a high level of CD151, also led to a significant reduction in cell migration and invasion [71]. CD151 has also been shown to be involved in prostate cancer invasion and lymphangiogenesis in vivo. PC3 and DU145 prostate cancer cell mouse xenografts developed lymph node metastases and CD151 expression was higher in primary tumours that went on to develop metastases [72].

More recently, our lab provided the first evidence that genetic ablation of CD151 inhibits spontaneous metastasis to the lung in a model of *de novo* prostate tumourigenesis, with no change in primary tumour growth [73]. Therefore CD151 expression is an important determinant of site-specific prostate cancer metastasis.

1.4.3 CD151 and breast cancer

There are many reports implicating CD151 in breast cancer and its progression to metastatic disease (for more detail see Ch5 introduction). CD151 protein expression is commonly upregulated in breast cancers [74-76], particularly all subtypes involving ER and PR negativity [74, 75]. Increased CD151 protein expression is associated with decreased survival rates in breast cancer patients [75, 76]. Moreover, CD151 deletion in a mouse model of breast cancer showed a trend towards delayed tumour onset and there

was heterogeneity in the numbers of lung metastases in the CD151 positive group that was not observed in the CD151 negative group [77].

CD151 has also been shown to play an important role in breast cancer cell migration, invasion and drug resistance *in vitro*. CD151 knockdown in MCF10A and MDA-MB-231 cells resulted in decreased migration and invasion [74, 76], and when injected into mice, CD151 knockdown MDA-MB-231 breast cancer cells displayed less tumourigenesis and vascularisation *in vivo* [76]. CD151 interactions with integrins have been shown to affect breast cancer cell growth and cell polarity in 3D cell culture models [78]. In addition, CD151 expression combined with integrin alpha3beta1 expression is a predictor of poor survival in patients with invasive ductal carcinoma [78]. CD151 expression along with the expression of integrins has also been shown to be involved in breast cancer drug resistance to Herceptin, where knockdown of CD151 in resistant ErbB2 positive breast cancer cells, re-sensitized them to ErbB2 inhibitors [79]. High CD151 and high ErbB2 expression also predicts a reduction in metastasis free survival in breast cancer patients, and CD151 knockout mice crossed on to an ErbB2 breast cancer mouse model displayed delayed tumour onset, impaired tumour cell survival and decreased invasiveness [80]. These results suggest that CD151 and ErbB2 are intricately involved in breast cancer progression.

1.4.4 Tetraspanin CD9

CD9 is also a member of the tetraspanin family of transmembrane proteins. CD9 is sometimes also referred to as TSPAN29, MRP-1 or p24 [81]. The human *CD9* gene is located on chromosome 12p13.3 and there is one reported CD9 transcript with 8 exons

and two predicted transcripts that have not been validated [81]. There has been one report in prostate cancer of CD9 mRNA point mutations and deletions in some prostate cancer cell lines and prostate cancer tissues, however in all samples wild type mRNA was also present [82].

CD9 was originally described as a 24kDa protein expressed on B-lineage-derived acute lymphoblastic leukeamia cells and B lymphocytes during development [83]. CD9 is also highly expressed on platelets and has been shown to be involved in platelet aggregation and activation [83]. It is also required for sperm-egg fusion (for a review see [84]) and is involved in co-stimulation of naïve T cells [85-87], activation of eosinophils [88], modulating HIV-1 infections [89], cell adhesion, cell motility, tumour metastasis (see review by Xuan *et al.* 2014 [90]) and is considered a marker of exosomes (see review by Zoller 2009 [34]). One of the first studies implicating CD9 in cancer progression, showed that an antibody recognising CD9 inhibited motility of a lung cancer cell line [91] and overexpression of CD9 in lung cancer cells suppressed cell motility and reduced metastasis in a mouse melanoma model [92]. These early studies suggested that CD9 also plays a crucial role in cancer progression and metastasis.

1.4.5 CD9 and prostate cancer

There are few studies that have investigated CD9 expression and/or functions in prostate cancer (for more details see Ch4 introduction). A reduction or loss of CD9 protein expression was seen in primary prostate adenocarcinomas and metastases, particularly in advanced stage tumours and lymph node metastases [82]. This highlights

that a loss of CD9 may be important to allow prostate cancer progression and metastasis. PC3 prostate cancer cells overexpressing CD9 underwent mitotic catastrophe with continued cell culture and this was shown to involve an interaction between CD9 and Mortalin, which is a heat shock protein. Therefore, CD9 is implicated in mitotic catastrophe of prostate cancer cells [93]. Although CD9 is usually considered a metastasis suppressor in many cancer types, there is one study in prostate cancer that opposes this notion. Overexpression of CD9 in PC-3M-LN4 metastatic prostate cancer cells led to no difference in prostate cancer development or progression following orthotopic injection in mice [94]. In fact, these prostate cancer cells displayed increased invasion *in vitro* compared to controls [94]. The lack of an *in vivo* effect may be due to the presence or absence of different tetraspanin binding partners or may involve factors from the tumour microenvironment. Moreover, increased invasion *in vitro* following overexpression of CD9 may also be a result of CD9 interactions with other proteins as the c-terminus of CD9 was tagged in this study, a region of CD9, which has been shown to be important for cell adhesion and spreading [95].

1.4.6 CD9 and breast cancer

There is a wide range of studies that have investigated the prognostic value of CD9 expression and the function of CD9 in breast cancer (see Ch5 introduction for more detail). CD9 expression significantly correlates with ER/PR and histologic grade in metastatic breast cancer [96]. CD9 gene and protein expression is typically downregulated in breast cancer compared to normal breast tissue [97-102], and breast cancer patients with tumours expressing high levels of CD9 tend to have better treatment outcomes and higher survival rates compared to patients with breast tumours

expressing low levels of CD9 [96]. There are also a couple reports of CD9 protein expression in breast tumours associating with lymph node metastasis, with low CD9 expression typically seen in lymph node metastases [99, 103]. Therefore, CD9 expression is commonly lost with breast cancer progression and is predictive of patient outcome.

The functional role of CD9 in breast cancer has been minimally investigated to date. Studies have shown conflicting effects of CD9 on breast cancer cell migration / motility. CD9 knockdown MDA-MB-231 cells showed increased motility due to impaired localisation of Talin to focal adhesions [104], whereas increased CD9 cell surface protein expression in MDA-MB-231 cells enhanced cell migration [105]. Therefore, CD9 is involved in modulating breast cancer cell migration, however the exact role of CD9 appears to be dependent on other factors such as its sub-cellular localisation, the presence of CD9 protein partners and the surrounding microenvironment.

1.5 Regulation of tetraspanin expression

Given the strong link between CD151 and CD9 protein expression and prognosis in epithelial cancers, it is important to understand how tetraspanins are normally regulated and what mechanisms lead to their deregulation in cancer. There are multiple levels of gene and protein regulation and these are usually sub-divided into epigenetic, transcriptional, post-transcriptional and translational / post-translational mechanisms.

Epigenetic changes are often heritable and include changes in chromatin structure due to histone modifications which are commonly directed by DNA methylation and histone acetylation [106]. Transcriptional regulation involves control of gene expression by transcription factors, repressors, activators and enhancers, which all influence how much DNA is transcribed to mRNA [107, 108]. Therefore epigenetic changes influence the accessibility of the DNA by transcriptional regulators, hence controlling the amount of DNA that is transcribed.

Once DNA has been transcribed to mRNA, post-transcriptional regulators will control the stability and distribution of mRNA transcripts and therefore influence translation of proteins. These include RNA-binding proteins that control various post-transcriptional events such as RNA capping, splicing, addition of a poly(A) tail, RNA editing and control by micro-RNAs (miRNA) [109-111]. Translation of mature mRNA transcripts is also highly regulated through the recruitment of ribosomes and protein synthesis initiation and elongation factors required for translation. However, once a protein has been synthesised, its expression and localisation are controlled by protein degradation pathways and post-translational modifications, respectively. As a result, gene and protein regulation is very complex and involves many facets. Therefore it is no wonder that aberrant gene and/or protein regulation plays a critical role in many disease states such as cancer [112, 113].

Given the complexity of gene and protein regulation, it is likely that all levels of control influence tetraspanin expression to different extents in normal and cancer cells. Most of what is currently known about tetraspanin regulation comes from studies of the metastasis suppressor CD82, in which transcription factors, alternative splicing and the

E3 ubiquitin ligase gp78, have been shown to regulate CD82 expression in cancer cells [114-117]. Moreover, the expression of some tetraspanins appears to be influenced by androgens and oxidative changes such as hypoxia in cancer [118-122]. However, very little is known of how other tetraspanins such as CD151 and CD9 are regulated in normal cells and thus deregulated in cancer.

1.5.1 Regulation of CD9 expression

There is evidence in multiple myeloma and non-small cell lung cancer cells that CD9 expression is downregulated as a result of promoter hypermethylation and histone deacetylation, respectively [123, 124]. However, epigenetic mechanisms of CD9 downregulation have not been explored in most cancer types and given the heterogeneity within and between different tumour types, we cannot simply assume that this is the case for other cancers. Furthermore, methylation of the CD9 gene was not observed in normal breast or breast cancer cells [100] and changes in methylation or histone modifications have not been observed between primary prostate epithelial cells and LNCaP prostate cancer cells (Susan Clarke, Garvan Institute, personal communication). This suggests that epigenetic changes are not responsible for the down-regulation of CD9 commonly observed in breast and prostate cancers.

In terms of transcriptional regulation, The RNA-binding proteins HuR [125] and CUGBP1 [126] have been shown to bind and regulate the expression of CD9 mRNA transcripts in breast cancer and cervical cancer, respectively. Moreover, in the 5'-flanking domain of the CD9 gene there are several SP1 binding sites and a consensus site for the binding of zinc-finger proteins of the Krox/EGR family [83]. Furthermore,

CD9 has also been shown to be a direct target of the transcription factor FosB [127]. Therefore, there are transcription factors that may bind and promote transcription of the CD9 gene and hence influence CD9 mRNA levels, however these have not been identified to be involved in the deregulation observed in cancer. In addition, since CD9 and CD151 mRNA are not commonly reported to be deregulated in these cancers when assessed by genome wide expression studies it suggests that the deregulation occurs either post- transcriptionally or post-translationally. In Merkel cell carcinoma, two different CD9 mRNA species were found and these differed by the 5'UTR length [66]. The long 5'UTR was particularly expressed in Merkel cell carcinoma, and the structure of this 5'UTR made it prone to inhibition of ribosome scanning and assembly, resulting in a decline in CD9 translation. Therefore, the presence of different 5'UTR structures may also influence the amount of CD9 protein in cancer cells.

There is even less knowledge about translational and post-translational regulation of CD9. One study has shown that the extent of post-translational modification, specifically palmitoylation will affect protein stability as treating palmitoylation-deficient HEK293 with lysosome inhibitors, rescued CD9 protein expression, whereas ubiquitin-proteasome inhibitors only had a very minimal effect [128]. This suggests that unpalmitoylated CD9 is mostly degraded by the lysosome however it is not known which form of protein degradation is responsible for the turnover of palmitoylated CD9.

1.5.2 Regulation of CD151 expression

There is a striking lack of knowledge of the mechanisms that regulate CD151 mRNA and protein expression. In liver cancer, the transcription factor SP-1 was found to bind

to the CD151 gene at three SP-1 binding sites in the 5'UTR that was identified as the core promoter [129]. SP-1 is pivotal in CD151 transcription as it confers an open chromatin configuration across the CD151 promoter, allowing promoter activity and transcription to occur. This is the only account of a transcription factor binding and regulating CD151.

Similarly, there are few studies that have investigated how CD151 protein is degraded. CD151 binds to the protease-associated (PA) domain of GRAIL (*Rnf128*) allowing the ubiquitination of lysine residues in the CD151 N-terminus [130]. This promotes proteasomal degradation of CD151 resulting in decreased cell-surface expression of CD151. Additionally, palmitoylation has been found to influence the stability of newly synthesised CD151 protein [31, 128], where it protects CD151 from lysosomal degradation.

The regulation of tetraspanins CD151 and CD9 and their deregulation in cancer is an area of research that has not been given much attention. Preliminary results in our laboratory have provided evidence to suggest that CD151 and CD9 mRNA and protein levels do not correlate in the RWPE1 prostate cell line series. This prostate cell line series consists of non-tumourigenic RWPE1 prostate cells and a number of prostate cancer cell lines which are derived from RWPE1 cells and display differing degrees of tumourigenic and invasive potential [131]. The lack of correlation between mRNA and protein levels coupled with an absence of epigenetic changes (Susan Clarke, Garvan Inst., personal communication) suggests that CD151 and CD9 may be regulated at post-transcriptional or higher levels in cancers such as prostate and breast cancer. One of the

major forms of post-transcriptional regulation, which is receiving much attention, is by small non-coding miRNAs.

1.5.3 Post-transcriptional silencing by micro-RNAs

miRNA are short, non-coding RNA molecules that are usually around 22nt in length. Much of what we used to consider as 'junk' DNA has in the last decade been found to harbour miRNA [132]. miRNA are transcribed in the nucleus from the genome via cellular machinery into primary miRNA hairpins (Pri-miRNA) which are further cleaved into Pre-miRNA (Fig 1.5). The Pre-miRNA is then exported from the nucleus into the cytoplasm where it is processed and cleaved by Dicer (Fig 1.5). The two strands that make up the Pre-miRNA are then unwound and one strand is incorporated into the RNA-induced silencing complex (RISC), where it now becomes functional [133, 134] (Fig 1.5). The miRNA can then bind to the 3'UTR of its messenger RNA target/s via partial complementarity and this can result in the inhibition of translation or mRNA cleavage, with the net effect being repression of protein translation (Fig 1.5).

The human genome encodes 1527 miRNA that we know of to date (miRBase v18), and these are thought to regulate around 60% of mammalian genes, however the exact functional implications of many of these are unknown [135, 136]. One miRNA can have multiple mRNA targets, as the region of complementarity where the 5' end of the miRNA binds to the 3'UTR of the mRNA (known as the seed sequence) is only around 6-7 nucleotides in length. Additionally, one mRNA transcript can be targeted by a number of miRNA and have multiple interaction sites for the same miRNA. Importantly, many miRNA have been shown to be important post-transcriptional

regulators of genes involved in development of different disease states such as cancer [137-140].

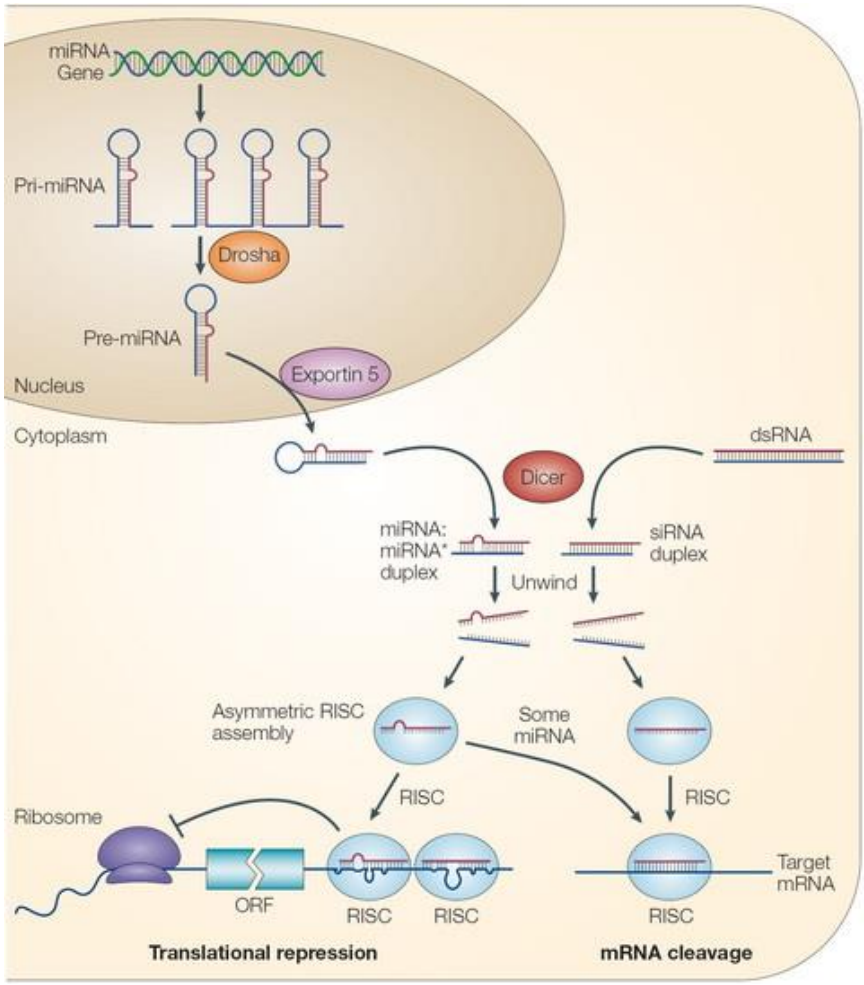

Figure 1.5. miRNA Biogenesis Pathway. miRNA are encoded in the genome within the nucleus where they are transcribed by RNA Polymerase II into large pri-miRNA hairpins. The pri-miRNA hairpins are then processed by the enzyme Drosha into shorter pre-miRNA transcripts, which are exported out of the nucleus and into the cytoplasm by Exportin 5. The enzyme Dicer then cleaves the pre-miRNA transcripts into a transient 22-nucleotide miRNA:miRNA* duplex. The two strands are separated and usually the mature strand (red) is incorporated into the RNA-silencing complex (RISC). The mature miRNA then binds to complementary sites in the target mRNA, typically in the 3'UTR which results in gene silencing through inhibition of protein translation (bottom left) or mRNA destabilisation and degradation (bottom right). Figure adapted from [134].

Since the discovery of miRNA, many have been found to play a critical role in the initiation and progression of cancer. These miRNA have been classified as tumour suppressor miRNA, onco-miRs or more recently, metastamiRs. Tumour suppressor miRNA are usually expressed in normal cells in order to repress the translation of oncogenes. Therefore in cancer, tumour suppressor miRNA are commonly not expressed or expressed at a low level, allowing the translation of oncoproteins (Fig 1.6B). Conversely, onco-miRs are typically over-expressed in cancer and these miRNA target tumour suppressor genes, resulting in the inhibition of tumour suppressor proteins (Fig 1.6C) [141, 142]. Therefore in normal cells, the lack of expression of tumour suppressor miRNA and the overexpression of onco-miRs can drive tumourigenesis and progression due to increased proliferation, migration and invasion, angiogenesis and decreased cell death (Fig 1.6). Furthermore, some miRNAs play prominent roles specifically in cancer metastasis, hence they are called metastamiRs. MetastamiRs can either promote or suppress cancer metastasis via targeting of genes that are metastasis suppressors or enhancers, respectively [143]. Therefore, metastamiRs typically regulate genes involved in cell migration and invasion, or in fact any step in the metastatic cascade.

Many onco-miRs, tumour suppressor miRNA and metastamiRs have been found to be deregulated in breast and prostate cancer progression (see table 1.1 for examples). Therefore, there has been a major emphasis on the use of miRNA as diagnostic tools and also therapeutics for cancer. Circulating miRNA have been found in the serum of healthy and cancer patients, with circulating miRNA levels closely correlating with tumour tissue miRNA levels in the latter [144, 145]. As a consequence, determination of miRNA levels in the serum of cancer patients may be an effective, non-invasive test

for early cancer diagnosis. Furthermore, given the fact that miRNA regulate a multitude of genes/proteins involved in cancer, the use of miRNA mimics and/or inhibitors may be a useful cancer therapeutic to prevent cancer progression and metastasis.

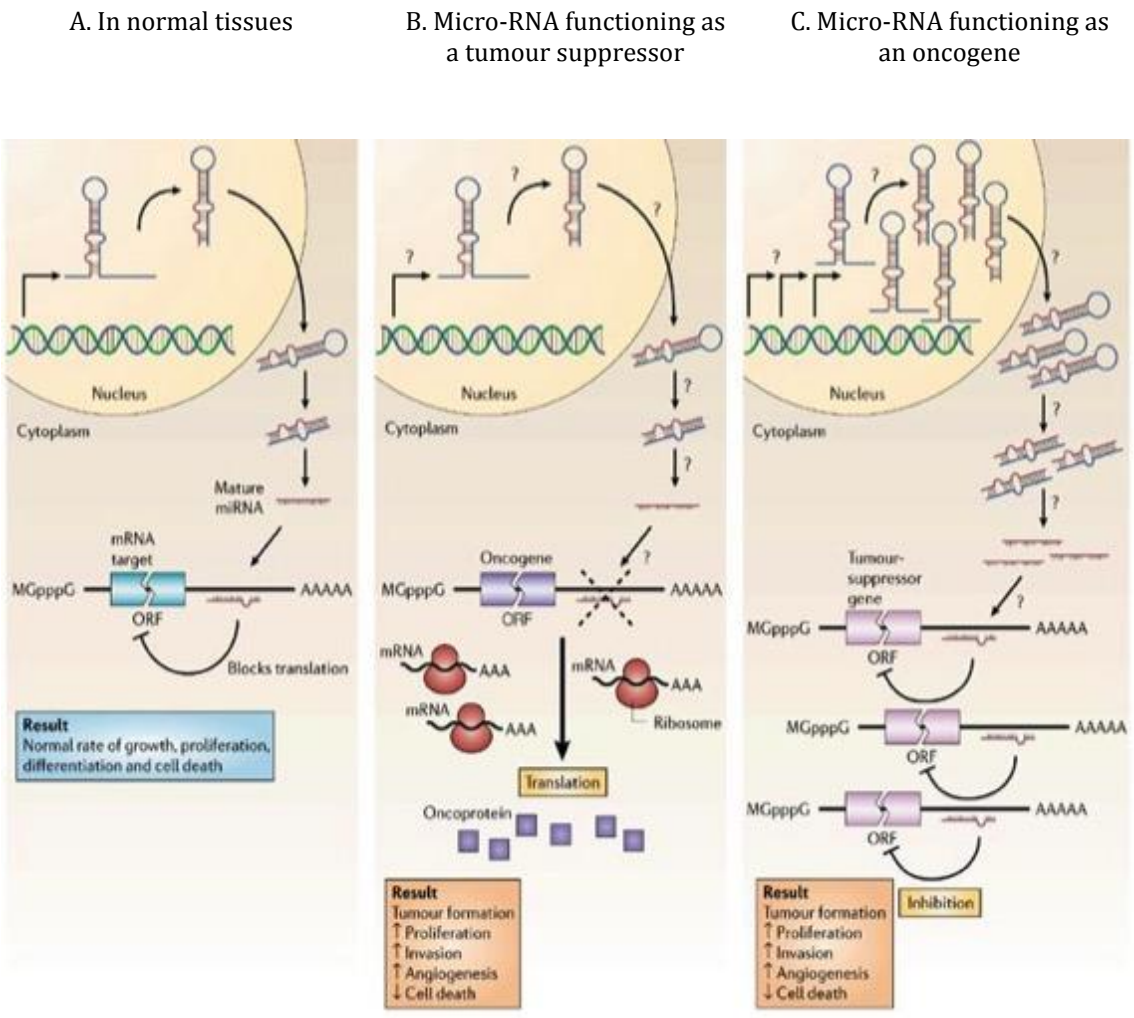

Figure 1.6. Tumour Suppressive and Oncogenic actions of miRNA A. In normal tissues miRNA are transcribed, processed and bind to complementary sequences on their target mRNA normally, resulting in gene silencing via inhibition of translation or mRNA cleavage (not shown). This allows normal cell growth, proliferation, differentiation and cell death to occur B. When the expression of a tumour suppressor miRNA is reduced or deleted due to aberrant changes at any stage of miRNA biogenesis (denoted by question marks), there is less or no miRNA - mediated repression of its oncogene target. This leads to an inappropriate increase in the expression of the oncoprotein (purple squares), resulting in tumour formation. C. The induction or overexpression of an onco-miR also leads to tumour formation. Increased expression of

an onco-miR, which targets a tumour suppressor gene (pink) leads to decreased or abolished expression of the tumour suppressor protein, which drives the progression of cancer. Figure adapted from [141].

Table 1.1. miRNA commonly altered in breast and prostate cancers.

miRNA	BC[1] &/or PC[2]	Exp.[3]	TS[4] or O[5]	Ref
miR-20a	PC	↑	O	[139, 146]
miR-21	PC & BC	↑	O	[139, 147-155]
miR-32	PC	↑	O	[139, 156]
miR-106b	PC	↑	O	[156, 157]
miR-148a	PC	↑	O	[139]
miR-34a & c	PC	↓	TS	[158, 159]
miR-15a-16 cluster	PC & BC	↓	TS	[160-162]
miR-143	PC & BC	↓	TS	[150, 151, 160]
miR-145	PC & BC	↓	TS	[138, 139, 150-152, 156, 160, 161, 163]
miR-205	PC & BC	↓	TS	[139, 152, 161, 164, 165]
miR-155	BC	↑	O	[139, 149, 150, 164]
miR-210	BC	↑	O	[139, 149-151]
miR-29b & c	BC	↑	O	[139, 151]
miR-100	BC	↓	TS	[139, 151]
miR-196a	BC	↑	O	[149-151]
miR-27b	BC	↓	TS	[166]
miR-206	BC	↓	TS	[167]

[1] Breast Cancer, [2] Prostate Cancer, [3] Expression Level, [4]Tumour Suppressor miRNA, [5] Onco-miR.

1.6 Rationale, Aims & Hypotheses

The increased incidence rates of breast and prostate cancer over the past twenty years are most likely attributable to improvements in early detection such as mammography and PSA testing, respectively. However, once a patient is diagnosed, there are currently no definitive ways to predict which patients require aggressive chemotherapy (i.e. patients who are at high risk of metastatic disease) versus patients with localised disease who only need surgery. As a consequence, some cancer patients are either not being provided with the appropriate treatment to prevent metastasis (patients at risk of metastatic disease) or are being over-treated (patients with indolent, non-life threatening disease or slow growing primary tumours).

Therefore, there is a strong need for prognostic markers to predict at diagnosis those patients who have, or are at risk of, developing metastatic disease, and also to provide critical information that can be used to stratify patients into correct treatment regimes. As a result, treatment is more likely to be effective in the long-term and improve quality of life. Moreover, for prognostic markers to be effective we also need novel therapeutic targets in order to prevent cancer metastasis, as current therapies such as hormonal therapy and chemotherapy/radiotherapy often have severe side effects and are not curative against advanced, metastatic cancer.

Previous studies have highlighted the prognostic and therapeutic potential of the tetraspanins CD151 and CD9 in a range of cancers, including prostate and breast cancers. Although the protein expression of CD151 and CD9 appears to be deregulated in cancer, the mechanisms driving this deregulation are still unknown. Yet this

knowledge will be of benefit in developing clinical tools to utilise tetraspanins as prognostic markers and potentially novel therapeutic targets. Preliminary data from our group suggested that in a human prostate cancer cell lines series, CD151 and CD9 mRNA expression levels do not correlate with their protein expression. Thus the deregulation of tetraspanins in cancer likely occurs at the post-transcriptional level or after, and hence may involve post-transcriptional regulators such as miRNA.

Therefore, ***I hypothesised that specific miRNAs targeting CD151 and CD9 are partly responsible for the aberrant expression and function of CD151 and CD9 during breast and prostate cancer progression.*** This hypothesis was investigated using the following aims:

1. *Investigate whether miRNAs are capable of regulating the expression of tetraspanins CD151 and CD9 in breast and prostate cancer cell lines*

2. *Determine which miRNAs regulate CD151 and CD9, and identify through bioinformatics whether they have a potential role in breast and/or prostate cancer independent of CD151 or CD9*

3. *Determine the expression and functional implications of miRNAs from Aim 2 in breast and prostate cancer progression*

Chapter 2: Materials & Methods

2.1 Cell culture

2.1.1 Cell lines

Primary human breast HMEC (CC-2551) and prostate epithelial PrEC (CC-2555) cells were purchased from Lonza, USA. HMEC cells were cultured in MEBM supplemented with BPE (0.4%), hEGF (0.1%), hydrocortisone (0.1%), GA-1000 (0.1%) and insulin (0.1%) (Lonza, USA). PrEC cells were cultured in PrEBM supplemented with BPE (0.4%), hydrocortisone (0.1%), hEGF (0.1%), epinephrine (0.1%), transferrin (0.1%), insulin (0.1%), retinoic acid (0.1%), triiodothyronine (0.1%) and GA-1000 (0.1%) (Lonza, USA). RWPE1 (CRL-11609) and WPE1-NB26 (CRL-2852) prostate cell lines were obtained from ATCC, and were cultured in Keratinocyte Serum-Free Media (K-SFM) supplemented with BPE (25mg) and hrEGF (2.5ug) (Gibco, AUS). PC3 and DU145 were a kind gift from Prof Rob Sutherland (Gravan Institute, NSW) and MCF7 (HTB-22), MDA-MB-231 (HTB-26) and T47D (HTB-133) cells were purchased from ATCC. All these cell lines were cultured in RPMI-1640 (Hyclone; GE Healthcare, Utah, USA) supplemented with 10% FBS (SAFC; Sigma-Aldrich AUS) and 2mM L-glutamine (Hyclone; GE Healthcare, Utah, USA). LNCaP prostate cancer cells were a kind gift from Gillian Lebrach (Garvan Institute), BPH-1 cells were a kind gift from Prof Gail Risbridger (Monash University), SKBR3 cells were a kind gift from Dr Gough Au (Newcastle University) and HEK293FT cells were a kind gift from Prof John Aitken (University of Newcastle). All these cell lines were cultured in RPM1-1640 as mentioned above.

2.1.2 Cell maintenance

All primary cells / cell lines were cultured in T75cm^2 cell culture flasks (grenier bio-one) in appropriate media at 37°C 5% CO_2. Media was replaced with fresh media every two days and cells were passaged at 80% confluence using trypsin-EDTA. Cells were counted using trypan blue and a haemocytometer to assess cell viability and either re-seeded into an appropriate sized flask or cryopreserved for later use (see 2.1.3).

2.1.3 Cryopreservation of cells

Cells were detached and counted as described above in section 2.1.2. Viable cells ($2x10^6$) were resuspended in 1mL cryovials (Nunc; Thermo Scientific) containing freezing media as recommended by the supplier (10% DMSO and 20% FBS diluted in complete media), wrapped in cotton wool and placed at -80°C for 3 days to facilitate gradual freezing. Cryovials were stored in liquid nitrogen for long-term preservation.

2.1.4 Reviving cryopreserved cells

After retrieval from liquid nitrogen, cryovials containing frozen cells were rapidly thawed at 37°C for 2 min. Media was added drop-wise to the cell suspension to dilute out the cryo-protectant, centrifuged at 120rcf for 5 min at 24°C and resuspended in fresh media. Cells were cultured as per section 2.1.2.

2.2 RNA/miRNA studies

2.2.1 RNA/miRNA extraction & purification

Media was aspirated from confluent T75 flask and cells were washed twice with PBS. TRIzol (1mL) was added directly to the cells on ice, cells were scraped and collected into 1.5mL microtubes, DNA was sheared by passing through a 23G needle. Lysates were left at room temperature for 5 min and centrifuged at 10,000 rcf for 10 min at 4°C. Chloroform was added to the supernatant, vortexed for 5-10 s and left for 5 min to settle. Samples were centrifuged for 15 min at 10,000 rcf at 4°C and the clear top phase containing RNA was collected. Isopropanol and glycogen (Sigma-Aldrich; aids in precipitation of small RNAs) were added to the RNA, mixed by inversion, left for 10 min at room temperature, and placed at -20°C overnight to enhance precipitation of RNA and miRNA. RNA was washed in 75% ethanol, centrifuged and the RNA pellet was dried and resuspended in RNAse-free water and stored at -80°C for later use. RNA was quantitated using the nanophotometer (Implen), according to manufacturer's recommendation and RNA integrity assessed using the Bioanalyser 2000.

2.2.2 Reverse transcription & cDNA synthesis

Total RNA was reverse transcribed into cDNA using superscript II reverse transcriptase (Invitrogen) in accordance with the manufacturer's recommendations. Briefly, random primers and 10mM dNTPs were added to 500ng of RNA, heated at 65°C for 5 min, and quickly chilled on ice. 1X first strand buffer, 0.1M DTT and RNAseOUT were added, mixed and incubated at 25°C for 2 min. SuperScipt II RT (200 U) was added and

incubated at 25°C for 10 min followed by 42°C for 50 min, after which the reaction was heat inactivated at 70°C for 15 min.

SuperScript II (Invitrogen) was also used to reverse transcribe miRNA into cDNA. RNA (500ng) was treated with DNAse mix (Invitrogen) at room temperature for 15 min and inactivated with 25mM EDTA (Invitrogen) to degrade any DNA present in the sample. Samples were incubated at 65°C for 10 min, after which RT mix A was added (1X first strand buffer (Invitrogen), 100mM DTT (Invitrogen), 10mM dNTP (Bio-line), RNAse Inhibitor (Bio-line) and 3μM reverse miRNA primer mix (contains reverse primers for three housekeeping RNA (U6, U44 & U49) and up to four miRNA reverse primers). Samples were incubated at 65°C for 5 min, then 25°C for 10 min. Superscript II was added and each sample was incubated for 10 min at 25°C, then 42°C for 50 min, followed by 70°C for 15 min. All cDNA was stored at -20°C.

2.2.3 Quantitative real-time PCR

PCR set-up was conducted in a dead air box following 30 min of UV irradiation to ensure sterility. Real time PCR master mix (1x Sybr master mix, 300nM forward & reverse primer mix and nuclease free water) (see Table 2.1 for sequences) was aliquoted into a 96 well PCR plate and 0.75ng of cDNA was added into each well. The plate was sealed, briefly centrifuged to collect contents into the bottom of the well and placed in the thermocycler (ABI 7500). Standard protocol using sybr green as the detector (stage 1: 1 cycle at 50°C for 2 min, stage 2: 1 cycle at 95°C for 10 min, stage 3: 40 cycles of 15 s at 95°C followed by 1 min at 60°C) and a dissociation curve (1 cycle of 15 s at 95°C, 1 min at 60°C, 15 s at 95°C and 15 s at 60°C) was added. For assessing miRNA

expression the cycle conditions were the same except for the second part of stage 3, which was 90 sec at 50°C.

2.2.4 Quantitative real-time PCR analysis

The average of the geomean of the Ct values of 3 housekeeping genes (*n=3*) was calculated for each cell line. To calculate the delta Ct, the geomean of the housekeepers was subtracted from the miRNA or tetraspanin Ct values (*n=3*). The delta delta Ct (delta Ct of the gene of interest (test) subtracted from the delta Ct of the control (non-tumourigenic cell line)) was used to determine the fold change using the equation $2^{\wedge(\text{delta delta Ct})}$. Results are expressed as a fold change expression relative to a control cell line or as inverse delta Ct.

2.2.5 miRNA microarrays

Following extraction of total RNA from cell lines (2.2.1), 1µg of RNA underwent FlashTag Biotin HSR labelling using the FlashTag Biotin HSR RNA labelling kit in accordance with the manufacturer's instructions (Genisphere). Biotin labelled RNA was hybridised onto Affymetrix 2.0 miRNA microarrays using the GeneChip Eukaryotic Hybridisation Control Kit and the GeneChip Hybridisation, Wash and Stain Kit (Genisphere). Affymetrix miRNA microarray chips were then washed, scanned with the Affymetrix scanner (Affymetrix USA) and results were interpreted using GeneSpring software. The results were first normalised across all microarray chips using Robust Multi-array Analysis (RMA) designed and recommended by Affymetrix. The normalised values were then filtered by probe set limited to 'has' to obtain values of

only human miRNAs. The data was grouped based on the cell line's level of tetraspanin 3'UTR targeting as determined by luciferase assay (see 2.2.6) to identify miRNA likely to target the 3'UTRs. In addition miRNA that were differentially expressed between non-tumourigenic and tumourigenic cell lines were identified using unpaired t-tests with Benjamini & Hochberg False Discovery Rate post-hoc testing, *n=1*.

Table 2.1. Forward and reverse primers for the detection of mRNA and miRNA

mRNA/miRNA	Forward primer (5'-3')	Reverse primer (5'-3')
HMBS	GAGAGTGATTCGCGTGGGTA	CAGGGTACGAGGCTTTCAAT
GusB	GCCAATGAAACCAGGTATCCC	GCTCAAGTAAACAGGCTGTTTTCC
RPS18	TAGCCTTTGCCATCACTGCC	CATGAGCATATCTTCGGCCC
CD151	AACACGGAGCTCAAGGAGAA	AGCGGATCCACTCACTGTCT
CD9	TTGGTGATATTCGCCATTGA	ACGCATAGTGGATGGCTTTC
HuR	ATGAAGACCACATGGCCGAAGACT	AGTTCACAAAGCCATAGCCCAAGC
U6	CGGCAGCACATATACTAAAATTGG	GCCATGCTAATCTTCTCTGTATC
U44	TGATAGCAAATGCTGACTGA	CAGTTAGAGCTAATTAAGACCT
U49	ATCACTAATAGGAAGTGCCGTC	ACAGGAGTAGTCTTCGTCAGT
hsa-miR-15a	T+AG+CAGCACATAA	GTAAAACGACGGCCAGTCACAAACCA
hsa-miR-27a	TT+CACAGTGGCTA	GTAAAACGACGGCCAGTGCGGAACT
hsa-miR-27b	TT+CACAGTGGCTA	GTAAAACGACGGCCAGTGCAGAACT
hsa-miR-25	CATTGCACTTGTCT	GTAAAACCACGGCCAGTTCAGACCG
hsa-miR-1226	TCACCAGCCCTGTG	GTAAAACGACGGCCAGTCTAGGGAA
hsa-miR-1293	TGGGTGGTCTGGA	GTAAAACGACGGCCAGTGCACAAATC

2.2.6 3'UTR dual luciferase reporter assay

All prostate and breast cell were seeded in 96 well plates at $1x10^4$ cells/well and allowed to adhere for 24 h. 100ng of 3'UTR renilla reporter vector was co-transfected with 10ng pmiR-Report firefly luciferase vector (transfection control) into cells using

Lipofectamine LTX. 24h post-transfection, cells were lysed in 20ul of 1x passive lysis buffer (Promega) and placed at -80°C for later analysis. CD151 3'UTR, CD9 3'UTR and empty 3'UTR Renilla luciferase vectors were purchased from SwitchGear Genomics (USA). Luciferase activity was assessed on 10ul of each lysate in a white 96 well half-size plate (Corning) with renilla and firefly luminescence measured according to Promega's dual luciferase assay kit using a Biotek Synergy 2 plate reader. Results were expressed as luminescence intensity normalised to empty 3'UTR vector and firefly vector, $n=3$.

2.3.7 Co-transfection of tetraspanin 3'UTR luciferase reporter vector and miRNA mimics

Experiments were conducted as stated in section 2.3.6 above, using only HEK293FT cells. In addition to the firefly luciferase vector (transfection control) and tetraspanin 3'UTR renilla luciferase vector, 50nM miRNA mimic (Bioneer pacific) was co-transfected using Lipofectamine 2000.

2.2.8 Transient reverse transfection of miRNA mimics

miRNA mimics were purchased from Bioneer Pacific (Korea). Briefly, RNAimax and 100nM miRNA mimic were diluted separately in OptiMEM and then mixed and incubated for 20 min at room temperature to allow complex formation. Complexes were then dispensed into 12-well plates and cells ($2.5x10^5$/well) were added and mixed to facilitate transfection. Cells were incubated at 37°C with 5% CO2 for 20 h following

which fresh growth media was added. At 48h and 72h post-transfection, cells were lysed in 1% NP-40 lysis buffer (100uL/well) and protein lysates were collected and stored at -80°C (see 2.3.1).

2.3 Analysis of Protein Expression

2.3.1 Protein extraction & quantitation

Cellular proteins were solubilised in 1% NP40 lysis buffer (50Mm Tris-HCl, 150mM NaCl, 1% NP40 pH8.0) containing 1X complete EDTA free protease inhibitor cocktail (Roche, Mannheim, Germany). Sub-confluent cell monolayers were rinsed with PBS and the lysis buffer added on ice, and cells were detached with a cell scraper (BD Biosciences) and collected into a 1.5mL tube. Complete cell lysis was ensured by incubating on ice with intermittent vortexing for 30 min. Insoluble proteins were removed by centrifugation at 15000rcf for 30 min at 4°C and the total protein concentration was determined using the microBCA kit (Pierce, Rockford, IL, USA) as per the manufacturer's instructions.

2.3.2 SDS-PAGE & western blotting

Protein Samples were diluted in non-reducing sample buffer (0.35M tris pH 6.8, 30% glycerol, 10% SDS with bromophenol blue) and heated to 70°C for 3 min prior to loading. Precision Plus Dual Colour protein standard (Bio-Rad, Hercules, California, USA) (7.5µL/lane) as well as 20µg of total protein per well were loaded onto a 4-12% Novex Tris-Glycine pre-cast gel (Life Technologies) and electrophoresed at 80V for

10min then 100V for 120min in 1x MOPS running buffer (Astral Scientific, AUS). Four lanes containing 1x non-reducing sample buffer were left between the protein standard and samples to ensure no leaching of reducing agents contained in the protein standard went into the samples as the tetraspanin antibodies used only recognise tetraspanin proteins in the non-reduced form.

Proteins were electrophoretically transferred to Hybond™-C Extra Nitrocellulose membrane (Amersham Biosciences, GE Healthcare, UK) at 4°C for 2 h at 650mA in transfer buffer (25mM Tris, 192mM glycine, and 20% v/v methanol). Membranes were incubated overnight in TTBS (Tris-buffered saline with 0.1%Tween-20) at 4°C, blocked for 30 min in 5% skim milk powder in TTBS, and incubated with primary antibody (Table 2.2) in TTBS with for 2 h at room temperature. Primary antibodies were detected with HRPD-conjugated goat anti-mouse secondary antibody (Table 2.2) diluted in TTBS for 1 h, washed with TTBS and TBS and detected with enhanced chemiluminescence (25mL TBS, 2.5mM Luminal, 0.4mM PCA and 2.6mM hydrogen peroxide). Blots were photographed using a FujiFilm LAS 4000 and images viewed with the Image Reader LAS-4000 (FujiFilm). Membranes were washed in dH_2O and probed for beta actin (Table 2.2) using the same procedure to control for protein loading and even transfer. Densitometry was analysed using Multi Gauge V3.0 software (FujiFilm).

Table 2.2 Table of Antibodies and conditions used for experiments.

Antibody (clone)	Isotype	Species	Applications & concentration	Source
CD9 (1AA2)	IgG1	Mouse	WB: 4ug/mL[1] FC: 8ug/mL[2]	Ashman Lab
CD151 (11B1.G4)	IgG2a	Mouse	WB: 2ug/mL[1] FC: 8ug/mL[2]	Ashman Lab
ID4.5	IgG2a control	Mouse	FC: 8ug/mL[2]	Ashman Lab
IB5	IgG1 control	Mouse	FC: 8ug/mL[2]	Ashman Lab
Anti-beta actin – HRP (AC-15)	IgG1	Mouse	WB: 1:20000[3]	Sigma-Aldrich (A3854)
Anti-mouse HRP	IgG	Goat, anti-mouse	WB: 1:5000[3]	Biorad (172-1011)
Anti-HuR / ELAV1 (4G8)	IgG1	Mouse	WB: 2ug/mL[1]	Abcam (ab110081)
FITC-conjugated anti-mouse secondary	IgG	Goat anti-mouse	FC: 1:50[4]	Southern Biotech (1012-02)

Incubated for 2 h at room temperature[1], incubated for 1 h on ice[2], incubated for 1 h at room temperature[3], incubated on ice for 45 min in the dark[4]

2.3.3 Flow cytometry

Cells were seeded at $3x10^5$ cells/well of a 6-well plate and allowed to adhere for 24 h. Prostate cells (90% confluence) were trypsinized as stated previously. Cells were collected into a FACS tube and placed on ice, washed with 3mL cold PBA (1% BSA, 0.1% Azide in PBS) and pelleted at 220rcf at 4°C for 5 min. Cells were incubated on ice with 10% normal rabbit serum (NRS, Invitrogen) in PBA (NRS/PBA) for 15 min to block non-specific binding then incubated in primary antibody (8µg/mL) (Table 2.2) in NRS/PBA for 1 h. Cells were washed 3 times with cold PBA and incubated with a 1:50

dilution of FITC-conjugated goat anti-mouse IgG secondary antibody for 45min in the dark (Table 2.2). Cells were washed 3 times with cold PBA, resuspended by vortexing, and fixed with 200µL FACS fixative (1% formaldehyde, 2% glycerol, 0.02% azide in PBS) whilst gently vortexing. Tubes were stored at 4°C overnight in the dark and analysed the next day using the FACScalibur (BD Biosciences).

2.4 *In vitro* functional assays

2.4.1 Cell proliferation assay

To assess cell proliferation, the reduction of resazurin to resofurin which emits light at 590nm was measured [168]. This assay measures the total viable cell number/well based on the amount of metabolism of resazurin and therefore correlates with cell number/viability [168]. On day 0, cells were trypsinized and seeded at $1x10^4$ cells/well of a 96 well plate in 200µL of media in triplicate, 200µL of media was used as a blank. Plates were incubated overnight at 37°C with 5% CO_2 to allow cells to adhere and resume normal metabolic activity. After 20 h, 20µL of resazurin was added to each well and the plate returned to the incubator for 5 h. Fluorescence at 544/590nm was measured using the FLUOstar-OPTIMA plate reader (BMG labtech, Offenberg, Germany). Rate of cell proliferation was determined using number of viable cells at 24, 48, 72 and 96h time points, after subtracting background (the average of triplicate wells containing media only) results were graphed as fluorescence intensity (arbitrary units).

2.4.2 Cell adhesion assay

Prostate cells were pre-loaded with 8µM of Calcein-AM (AnaSpec, Fremont, CA, USA) for 30min at 37°C with 5% CO_2 and then seeded at $2x10^4$ cells/well in triplicate in a black tissue culture-treated 96-well plate (Costar, Corning, USA). To assess adhesion on various substrates, wells were pre-coated with human plasma Fibronectin (Sigma) and basement membrane extract (Trevigen) at 10µg/mL and 0.5x respectively, overnight at 4°C and blocked with 1% BSA in PBS at room temperature for 1 h before cell seeding. Triplicate wells containing media only (blank) and wells with cells seeded after blocking alone, were used as negative controls. Plates were centrifuged at 200 rcf for 2 min to sediment cells and incubated for 1 h to investigate cell adhesion. After 1 h, the plate was read using the FLUOstar-OPTIMA plate reader (490/520nm) to obtain an initial intensity of total cell fluorescence. Wells were then washed three times with PBS to remove non-adherent cells, 200µL of media was added to each well and the fluorescence at 490/520nm was read using the FLUOstar-OPTIMA plate reader to obtain the fluorescence intensity of adherent cells. Results are shown as percentage cell adherence.

2.4.3 Transwell migration assay

Migration assays were performed using 6.5mm, 8µm pore size 24-well Transwells™ (Costar, Corning) to measure single cell migration. Media (600µL) was added to each receiver well and cells ($5x10^5$) loaded with Calcein-AM (8uM) were added to the top chamber in 200µL media/well following overnight serum starvation. Cells were incubated at 37°C with 5% CO_2 for various time points (6, 18, 24 & 48h) to allow migration through the pores to the outer side of the insert. Medium was carefully

removed from the insert and receiver, both were washed twice with PBS and non-migratory cells were removed from the top chamber with a cotton swab. The inserts were placed in their receiver well containing trypsin (350µL) with 8µM Calcein-AM and incubated for 1h at 37°C with 5% CO_2 to dislodge migratory cells. An aliquot (200µL) from each well was then transferred to a black low-fluorescence plate and the fluorescence at 490/520nm was measured using the FLUOstar-OPTIMA plate reader.

2.4.4 Statistical Analysis

Statistical comparisons were performed using one-way or two-way ANOVA with Bonferroni multiple comparison testing for analysis of significance between different values using GraphPad Prism 6 software (San Diego, CA). All values are expressed as mean ± SEM and differences were considered significant at a *p*- value of less than 0.05.

Chapter 3: Post-transcriptional regulation of CD151 in prostate cancer

3.1 General introduction

Prostate cancer is a very heterogeneous disease that affects 1 in 7 men worldwide [169]. Currently there are no specific therapies for advanced stage prostate cancer and hence this leads to mortality. Therefore, we need novel treatments for prostate cancer that will inhibit or slow progression to metastasis. Several members of the tetraspanin family of proteins are potential candidates as new drug targets for prostate cancer, in particular CD151. Tetraspanin CD151 is considered a metastasis enhancer, as its protein expression is commonly upregulated in many solid malignancies. Moreover, *in vitro* studies have provided evidence that CD151 plays an important role in cancer cell migration, invasion and adhesion [34]. However, it is not known how CD151 is regulated in normal prostate and prostate cancer cells. This is important to elucidate, as it may uncover new ways to target CD151 as a therapy for inhibiting prostate cancer progression without harming normal prostate cells.

There are minimal studies that have focused on the expression and/or role of CD151 in prostate cancer. CD151 is typically expressed by cells of the basal membrane of normal prostate glands and on the surface of prostate epithelial cells, where it co-localises with integrin alpha3 and/or alpha6 to form adhesion complexes [68]. However, in prostate cancer, the interaction between CD151 and alpha3 is lost and instead CD151 interacts with alpha6, which appears to play an important role in prostate cancer progression [68].

CD151 protein expression is typically increased in prostate cancer compared to benign prostatic hyperplasia, which is thought to be a precursor to prostate cancer [69]. CD151 protein expression was found to be highest in poorly differentiated tumours compared to

highly differentiated prostate tumours [69]. This is important as poorly differentiated tumours tend to be more advanced and are at a higher risk of becoming metastatic. Patients with tumours expressing low levels of CD151 had significantly increased overall survival rates compared to patients with high levels of CD151 [69]. Importantly, this finding was independent of patient age or pre-operative PSA levels, and was a better predictor of overall survival than traditional methods such as Gleason scoring [69].

CD151 has also been shown to modulate prostate cancer cell migration and invasion *in vitro*, and this was found to be dependent on its interactions with non-tetraspanin protein partners. LNCaP prostate cancer cells overexpressing CD151 (lack ECL2) had decreased migratory and invasive potential, however when wild type CD151 was overexpressed, migration and invasion increased [71]. Moreover, CD151 knockdown in PC3 prostate cancer cells, which express high levels of CD151, also led to a significant reduction in cell migration and invasion [71]. This study provides evidence that CD151 plays a pivotal role in prostate cancer cell migration and invasion via its interactions with partner proteins. In addition, CD151 is important for prostate cancer invasion and lymphangiogenesis *in vivo*. Highly tumourigenic and androgen insensitive PC3 and DU145 prostate cancer cell lines expressed higher levels of CD151 compared to LNCaP cells that have low tumourigenicity and are androgen sensitive [72]. The majority of *in vivo* xenografts using these cell lines, developed lymph node metastases and more importantly primary tumours that developed metastases had significantly higher CD151 protein expression [72]. Therefore, these results suggest that CD151 may be an important regulator of interactions between prostate cancer cells and the tumour microenvironment.

In recent years, our laboratory provided the first evidence that genetic ablation of CD151 inhibits spontaneous metastasis to the lung in a model of *de novo* prostate tumourigenesis [73]. CD151 knock out mice crossed onto the TRAMP model displayed no differences in prostate cancer initiation, proliferation, apoptosis or angiogenesis in primary tumours compared to wild type and heterozygous mice [73]. However, ablation of CD151 led to a significant reduction in the number of lung metastases, but had no effect on liver metastasis [73], therefore implicating CD151 expression as a determinant of site-specific prostate cancer metastasis.

It is still unclear how CD151 protein expression is deregulated in prostate cancer, which is somewhat surprising given its functional importance. In particular, there are no studies that have elucidated post-transcriptional regulation of CD151. Recent studies have shown that miRNAs such as miR-124 and miR-506, which share the same seed sequence, are capable of downregulating CD151 protein expression in breast cancer cell lines *in vitro* [170, 171]. In addition, miR-22 and miR-152 have been shown to decrease CD151 expression in gastric cancer cell lines [172, 173]. Transfection of all of these miRNAs has resulted in decreased cancer cell proliferation, migration and invasion [170-173]. These studies suggest that CD151 is regulated by miRNAs in cancer and these miRNAs play important functional roles in cancer progression. Therefore, the aim of this chapter was to investigate if miRNAs regulate CD151 in prostate cancer using a panel of non-tumourigenic prostate and prostate cancer cell lines *in vitro*.

3.2 Results

3.2.1 Characterisation of CD151 expression in prostate cells

The analysis of CD151 mRNA & protein expression was conducted using RT-PCR, western blotting and flow cytometry respectively. As can be seen in figure 3.1A, most prostate cancer cell lines expressed low and similar CD151 mRNA levels to non-tumourigenic prostate cells, however BPH-1 and LNCaP cells expressed higher levels of CD151 mRNA. In contrast, the highly tumourigenic and invasive prostate cancer cell lines DU145, PC3 and WPE1-NB26 showed a trend towards higher levels of CD151 total protein compared to non-tumourigenic prostate cells as measured by SDS-PAGE and western blotting (Fig 3.1B & C). BPH-1 and LNCaP cells, which had the highest CD151 mRNA expression, displayed the lowest CD151 total protein levels. In fact, a significant inverse correlation between CD151 mRNA and CD151 total protein expression was observed (Fig 3.2A; R^2 0.72 (linear regression); $p = 0.01$). CD151 cell surface expression was measured using flow cytometry. Similar to total CD151 protein levels, PC3 and WPE1-NB26 prostate cancer cells showed a very high expression of cell-surface CD151 compared to the other prostate cell lines (Fig 3.1). CD151 mRNA and CD151 cell surface protein levels did not correlate although there was a slight trend towards an inverse correlation (Fig 3.2B). Moreover, there was a trend towards a positive correlation between CD151 total and cell surface protein levels (Fig 3.2C).

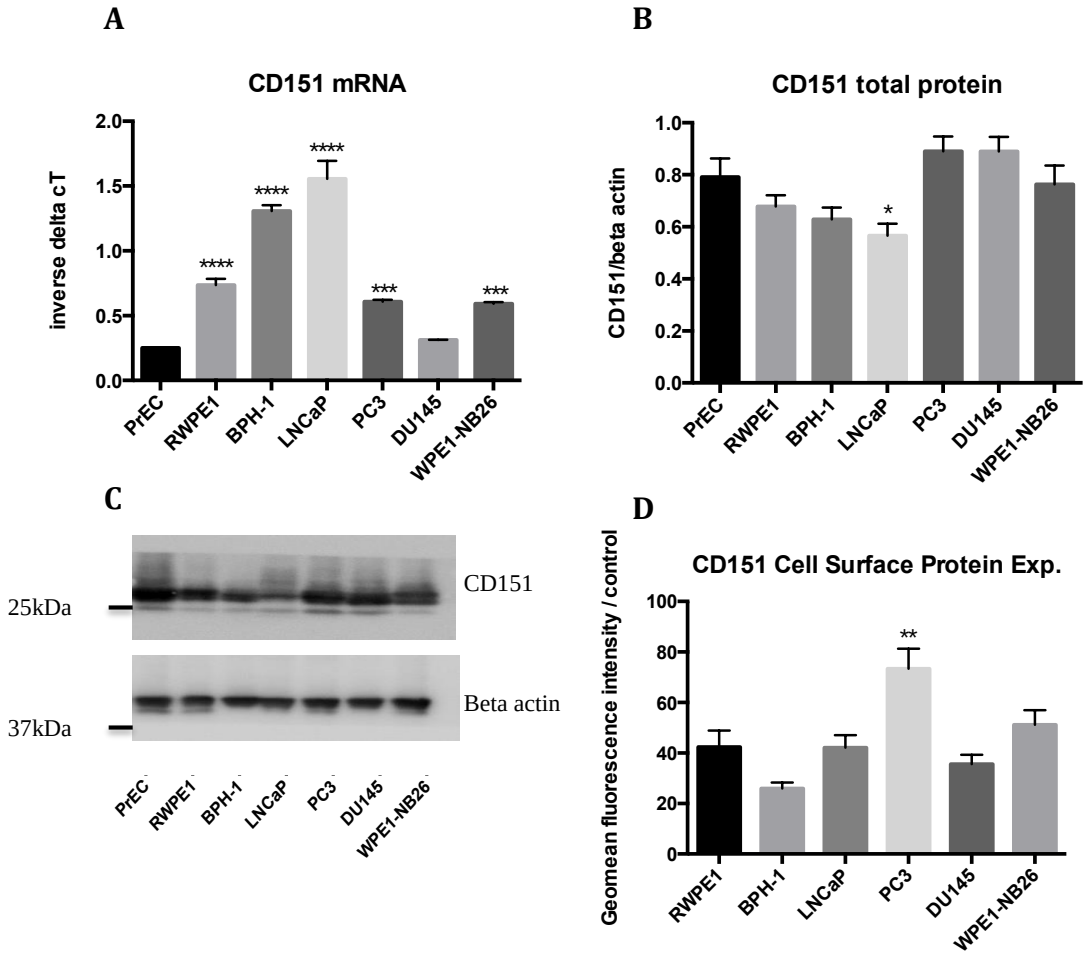

Figure 3.1. CD151 profiling of non-tumourigenic prostate and prostate cancer cell lines. A. CD151 mRNA levels in non-tumourigenic (PrEC, RWPE1, BPH-1) and prostate cancer cell lines (LNCaP, DU145, PC3 & WPE1-NB26) were quantitated using RT-PCR. RNA was extracted from low passage cells using TriZol-chloroform and converted to cDNA using superscript II. mRNA expression was normalised to the geomean of 3 housekeeping genes (HMBS, GusB and RPS18). Results are shown as inverse delta Ct. B. Low passage prostate cells were lysed in 1% NP-40 and CD151 total protein levels were measured using SDS-PAGE western blotting with 2ug/mL IIB1 antibody. Protein bands were quantitated using densitometry (Multi-gauge software) and are shown as band intensity normalised to beta actin. C. Representative western blot of CD151 total protein levels and beta actin across prostate cell lines. D. CD151 cell surface expression was measured using flow cytometry. Briefly, low passage prostate cells were incubated with 4ug/mL IIB1 antibody and 4ug/mL IgG2A control antibody (ID4.5) and cell surface expression was measured using a FACScalibur. Results are expressed as geomean fluorescence intensity normalised to control IgG2A. All graphs are *n=3* with y-axes shown as arbitrary units, *p<0.05*, p=0.001**, p=0.0001***, p<0.0001****.*

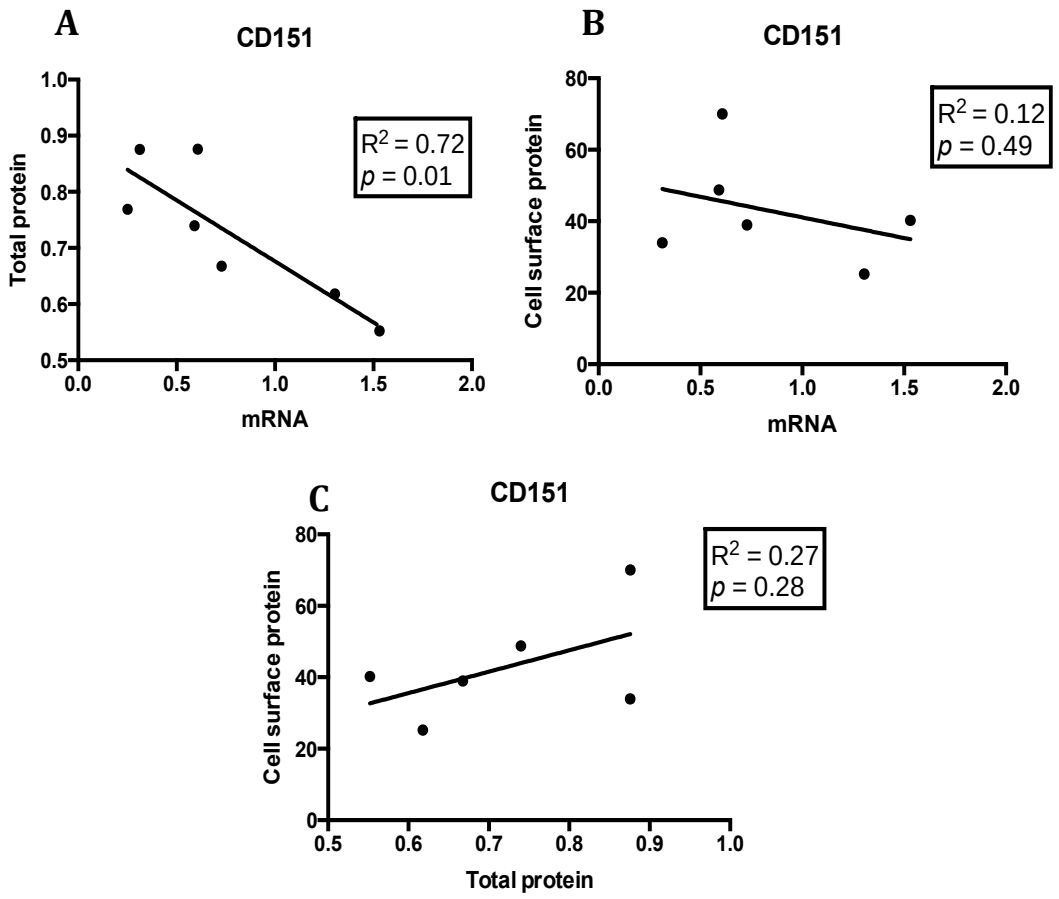

Figure 3.2. Inverse correlation between CD151 mRNA and total protein levels in a panel of prostate cell lines. A. Linear regression analysis of the geomean of CD151 mRNA levels in PrEC, RWPE1, LNCaP, PC3, DU145 and WPE1-NB26 cells (from Fig 3.1) versus the geomean of CD151 total protein expression in the prostate cells (from Fig 3.1). B. Linear regression analysis of CD151 mRNA versus CD151 cell surface expression (flow cytometry) in RWPE1, BPH-1, LNCaP, PC3, DU145 and WPE1-NB26 prostate cells (from Fig 3.1). C. CD151 total protein (western blotting) versus CD151 cell surface protein expression (flow cytometry) with linear regression analysis (values from Fig 3.1). The geomean of values from fig 3.1 were used in linear regression analyses that were conducted using GraphPad Prism 6 software. A *p-value* of less than 0.05 was considered statistically significant.

3.2.2 Many miRNAs are predicted to regulate the CD151 3'UTR

miRNA target prediction databases were used to predict which miRNA may regulate the CD151 3'UTR based on sequence homology. A number of target prediction databases including mirna.org [174], TargetScan [175], miRDB [176] and miRWalk [177] were utilised as they all involve different algorithms and are usually not consistent with each other. Just over 300 miRNA were predicted to target CD151 based on seed sequence complementarity when the results from all databases used were compiled (Table 8.1 Appendix). miRNA were selected for further study based on previous implication in prostate or other cancers, but also novel miRNA were selected to ensure novel findings to cancer were not excluded (for a summarised list please see Table 3.1).

Table 3.1. miRNA predicted to regulate CD151 and their role in prostate cancer and/or other cancer types

miRNA	Database	Expression & function in PC or other cancers	REF
miR-16	Microrna.org, miRWalk, TargetScan	Deleted in a subset of PC; inhibits metastatic PC; downregulated in PC mets; anticancer effect on PC mouse model	[160, 178-182]
miR-1226	Microrna.org	Higher exp in BC versus normal cell lines	[183]
miR-128	Microrna.org, miRWalk, TargetScan	Loss of exp in invasive PC compared to benign tissue	[184]
miR-15b	Microrna.org, miRWalk, TargetScan	Higher exp in blood of men with high risk PC (high PSA); oncomiR in BC – targets a metastasis suppressor	[185, 186]
miR-637	Microrna.org, TargetScan	Regulates HER2 signalling in BC; Tumour suppressor in hepatocellular carcinoma; decreased exp in glioma tissues	[187-189]
miR-1285	Microrna.org, miRWalk, TargetScan	Inhibits exp of p53; Tumour suppressor in renal cell carcinoma	[190, 191]
miR-124/miR-506	miRDB, miRWalk, TargetScan	Both regulate EMT in PC; miR-124 is a tumour suppressor in PC & has anti-proliferative and anti-aggressive effects on PC	[192-195]
miR-7-2*	Microrna.org, miRWalk	N/A	N/A
miR-619	Microrna.org, miRWalk	N/A	N/A
miR-548o	Microrna.org, miRWalk	N/A	N/A
miR-1909	Microrna.org, TargetScan, miRWalk	N/A	N/A
miR-1293	Microrna.org, TargetScan, miRWalk	Upregulated in gingiva buccal cancer	[196]
miR-31*	miRWalk	Changed exp in Laryngeal cancer cell line following paclitaxel treatment	[197]

Predicted miRNA were sourced from mirna.org [174], TargetScan [175], miRDB [176] and miRWalk [177]. PC = prostate cancer, BC = breast cancer and exp. = expression.

3.2.3 Regulation of the CD151 3'UTR in prostate cells

There have been minimal studies investigating post-transcriptional regulation of CD151, particularly whether miRNAs can regulate CD151. To elucidate whether miRNAs potentially regulate CD151 expression in prostate cell lines, a 3'UTR dual luciferase reporter assay was used. Prostate cells were transfected with CD151 3'UTR renilla luciferase and firefly (transfection control) luciferase vectors for 24 h to allow binding of any miRNA that are endogenously expressed and capable of binding the CD151 3'UTR. As shown in figure 3.3, the highly and moderately tumourigenic prostate cancer cell lines PC3, DU145 and WPE1-NB26 had a significantly higher level of 3'UTR targeting as measured by the decreased luminescence intensity of the renilla luciferase reporter compared to lowly tumourigenic (LNCaP) and non-tumourigenic prostate cells (RWPE1, PrEC & BPH-1).

3.2.4 miRNA expression profiling in prostate cells

In order to determine which miRNA regulate CD151, Affymetrix miRNA microarrays and bioinformatics were utilised to discern which miRNA are differentially expressed between normal and prostate cancer cell lines and which of these miRNA are predicted to target CD151. As shown in figure 3.4 very few miRNA were significantly differentially expressed between non-tumourigenic and prostate cancer cells. In fact, only miR-15b and miR-1226 were upregulated and miR-22*, miR-659, miR-1293 and miR-3177 were downregulated in prostate cancer versus non-tumourigenic prostate cells.

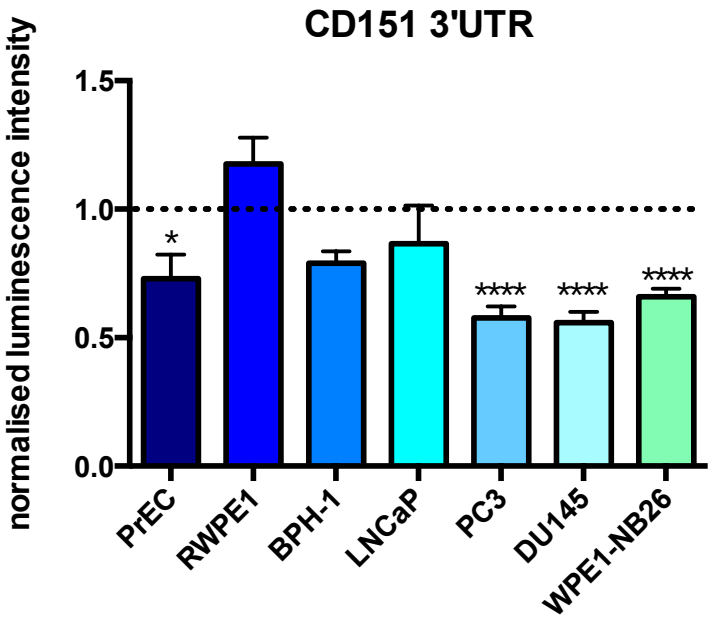

Figure 3.3. CD151 3'UTR mediated regulation in a panel of prostate cell lines.

Prostate cells were seeded in a 96 well plate at $1x10^4$ cells/well and transfected with 100ng CD151 3'UTR renilla luciferase vector and 10ng firefly vector (transfection control) for 24h to assess the effect of endogenously expressed miRNA on CD151 3'UTR activity. Luminescence was measured in a white plate using the synergy 2 luminometer. Results shown are luminescence intensity normalised to the luminescence of the transfection control and positive control (empty 3'UTR vector) *n=3; p = 0.05*, p=0.0001*****

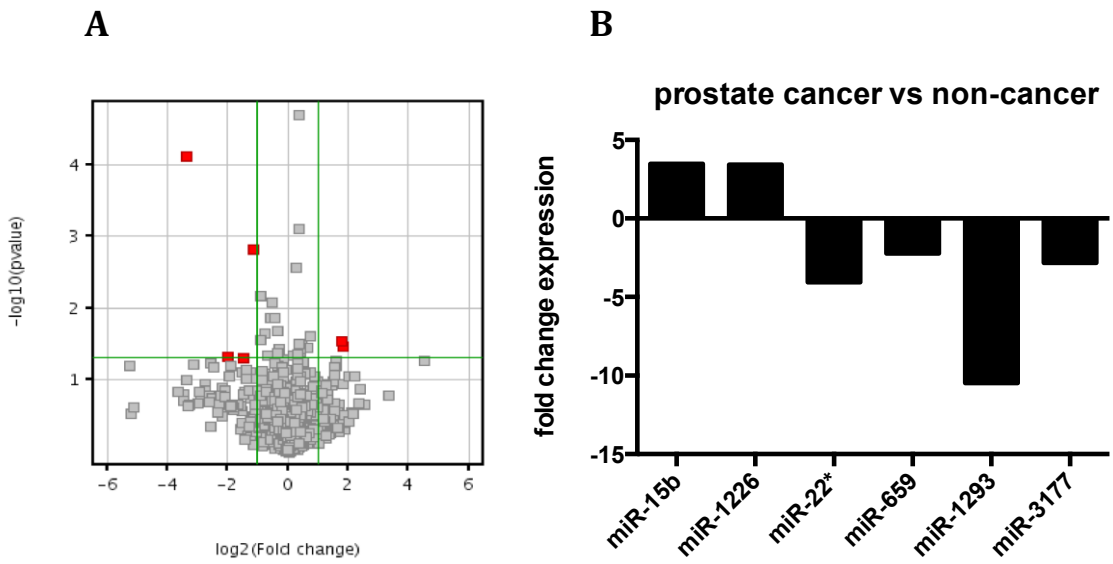

Figure 3.4 Differential expression of miRNAs in prostate cancer versus non-tumourigenic prostate cells.

Total RNA from all prostate cell lines was subjected to a miRNA microarray and the results were analysed using Genespring. Cell lines were grouped on the basis of being non-turmourigenic vs tumourigenic and an unpaired t test with Benjamini-Hochsberg post-hoc was performed to determine which miRNA were significantly up and downregulated in prostate cancer cells versus non-cancerous prostate cells. A. Volcano plot generated from the GeneSpring analysis showing the fold change in expression (log scale; x axis) of miRNA expression in prostate cancer with the significance on the y axis ($-\log^{10}$ (p value)). Each square represents a specific miRNA, with miRNA displaying statistically significant changes in expression shown as red squares B. Graph showing the miRNA from the above analysis that were upregulated and downregulated in prostate cancer cells with a fold-change greater than 2 and significance level displayed as p-value less than 0.05. miR-15b (3.47 fold; $p = 0.03$), miR-1226 (3.43 fold; $p = 0.02$), miR-22* (4.06 fold; $p = 0.04$), miR-659 (2.23 fold; $p = 0.001$), miR-1293 (10.48 fold; $p = 0.0000745$) and miR-3177 (2.83 fold; $p = 0.04$).

Validation of miRNA microarray values was conducted from the analysis of miRNA expression differences between tumourigenic and non-tumourigenic groups for both prostate and breast cell lines. To identify if changes were common to both cancers qPCR was performed for miRNA regardless of which cancer the difference was originally identified in. This was of particular interest as a limitation of the study was the low number of replicates able to be conducted by microarray leading to a higher chance of false negative and positive values. In all instance the same direction of fold change was observed and the increased power in the replicates allowed statistical

differences in expression of miRNA to be identified which did not reach significance in the analysis of the microarray data. In line with results from the miRNA microarrays miR-1226, miR-15a, miR-27a and miR-27b were shown to be highly expressed in the aggressive prostate cancer cell lines (PC3, DU145 and WPE1-NB26) compared to lowly tumourigenic and non-tumourigenic prostate cells (Fig 3.5). Moreover, miR-1293 expression was downregulated in most prostate cancer cell lines compared to non-tumourigenic prostate cells as seen with the miRNA microarray analysis (Fig 3.4 & 3.5).

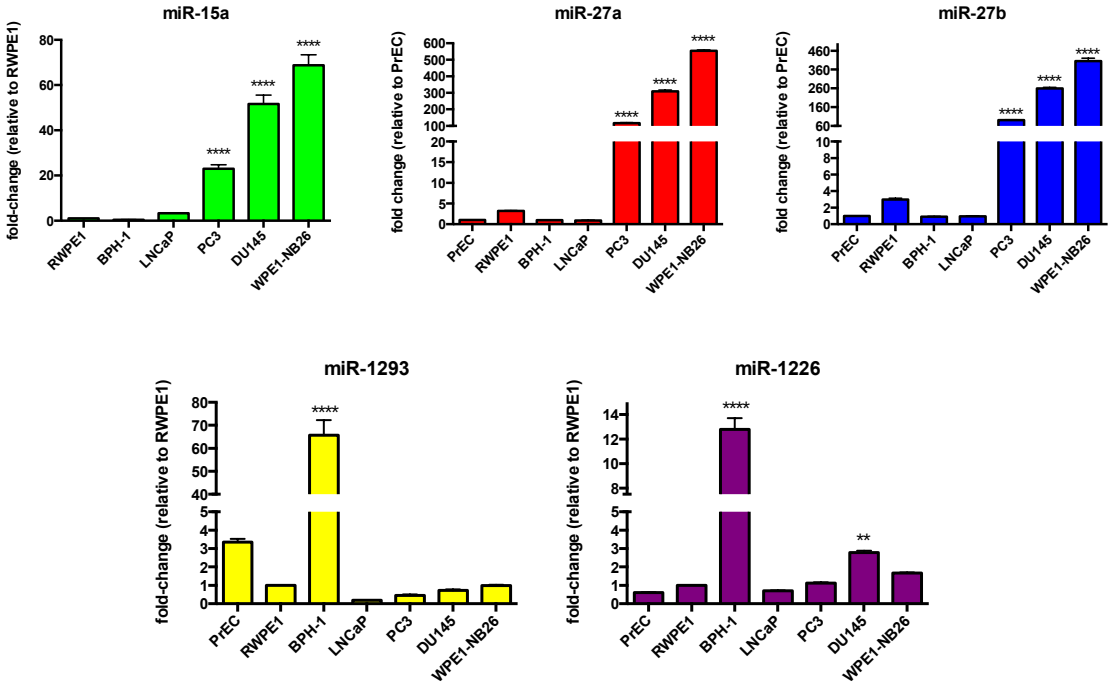

Figure 3.5. qPCR validation of miRNAs differentially expressed in prostate cancer. miRNA expression levels were quantitated using RT-PCR. RNA was extracted using TRIzol chloroform method and cDNA was synthesised using superscript II and miRNA specific reverse primers. miR-15a (green), miR-27a (red) and miR-27b (blue) were shown to be highly expressed in PC3, DU145 and WPE1-NB26 cells compared to LNCaP and non-tumourigenic lines BPH-1, RWPE1 & PrEC. Similarly, miR-1226 was highly expressed in DU145 prostate cancer cells and BPH-1 cells compared to other prostate cell lines (purple). In contrast, miR-1293 was expressed at low levels in prostate cancer cell lines however BPH-1 cells expressed significantly high levels of miR-1293 (yellow). All results were normalised to the geomean of three housekeeping

genes (U6, U44 & U49) and represented as fold change relative to RWPE1 or PrEC cells (*n=3*, SD<0.5), *p=0.004***, *p<0.0001*****).

3.2.5 miRNAs that are predicted to regulate CD151 display expression profiles which mimic effects of CD151 3'UTR regulation

To determine which miRNAs most likely regulate CD151, miRNA expression profiles of prostate cell lines from 3.2.4 were grouped based on their level of CD151 3'UTR activity (see Fig 3.3). Analysing the Affymetrix miRNA microarray data generated in section 3.2.4, with these groupings using GeneSpring software allowed identification of the miRNA that followed the profile of increased miRNA expression leading to more 3'UTR targeting/repression. Prostate cell lines were firstly grouped by whether they displayed CD151 3'UTR targeting versus no CD151 3'UTR targeting (same level of luciferase as positive control) / or stabilisation (i.e. more luciferase compared to positive control) (Table 3.2). miR-7-2*, miR-637, miR-1265 and miR-619 were upregulated in prostate cell lines (Table 3.2; Figure 3.6) with CD151 3'UTR targeting, with miR-7-2*, miR-637 and miR-619 predicted to bind to the CD151 3'UTR (see Table 3.2 for sequence alignment).

Prostate cell lines were also grouped based on the extent of CD151 3'UTR targeting i.e. high levels of 3'UTR targeting versus lower levels of 3'UTR targeting (Table 3.2). From this analysis, increased expression of miR-128, miR-1285, miR-124*, miR-548o, miR-593* and miR-148b* in prostate cell lines was found to correlate with a higher level of CD151 3'UTR repression (Table 3.2; Fig 3.7). However, only miR-128, miR-1285 and miR-548o are predicted to bind to the CD151 3'UTR (for sequence alignment see Table 3.2).

3.2.6 miRNAs bind to the CD151 3'UTR and modulate protein expression

The dual luciferase CD151 3'UTR reporter assay was used in HEK293FT cells with co-transfection of miRNA mimics in order to investigate whether specific miRNAs could bind to the CD151 3'UTR. miRNA mimics (30 including non-targeting controls) for miRNA that were predicted to regulate CD151 (3.2.2) and were differentially expressed in prostate cell lines (3.2.4) and/or breast cell lines (5.2.4), or followed CD151 3'UTR targeting profiles (3.2.5), were tested for their ability to modulate protein expression via the CD151 3'UTR. Surprisingly, miR-1226 and miR-128 increased luciferase activity regulated by the CD151 3'UTR (Fig 3.8A). In comparison, miR-1909, miR-1293, miR-619, miR-31*, miR-637, miR-649 and miR-518f* significantly repressed luciferase activity via the CD151 3'UTR to varying extents (Fig 3.8B), whereas miR-548o and miR-7-2* had no significant effect on CD151 3'UTR regulation (Fig 3.8B).

Table 3.2. Analyses of CD151 3'UTR profiles with miRNA microarray data from prostate cell lines

miRNA microarray & luciferase assay analysis	**miRNA upregulated**	**CD151 3'UTR / miRNA alignment[1]**
PrEC, BPH-1, DU145, PC3 & WPE1-NB26 (targeting) Vs. RWPE1 & LNCAP (no change or stabilisation)	miR-7-2* miR-637 miR-1265 miR-619	3' aauccaucugacccu**AAACAA**c 5' hsa-miR-7-2* |||||| 548:5' cagggccuuccuggu**UUUGUU**c 3' CD151 3' ugcgucucggGCUUUCG**GGGGUC**a 5' hsa-miR-637 :|::: :|||||| 206:5' ggguguuuugUGGGGCU**CCCCAG**a 3' CD151 3' ugacccguguuuguaca**GGUCCA**g 5' hsa-miR-619 |||||| 373:5' caucccaguuggggaag**CCAGGU**g 3' CD151
DU145, PC3 & WPE1-NB26 (more targeting) Vs. PrEC & BPH-1 (some targeting)	miR-128 miR-1285 miR-124* miR-548o miR-593* miR-148b*	3' uuucucUGGCCA**AGUGACAC**u 5' hsa-miR-128 || | |||||||| 499:5' aaugccACGUGG**UCACUGUG**c 3' CD151 3' uuucUCUGGC**CAAGUGAC-AC**u 5' hsa-miR-128 :| :: |||||||| || 556:5' uccuGGUUUU**GUUCACUG**C**UG**u 3' CD151 3' uccagagugaaaca**ACGGGUC**u 5' hsa-miR-1285 ||||||| 147:5' uugcugcgcaccaa**UGCCCAG**c 3' CD151 3' cguuuucauugacgu**CAAAAC**c 5' hsa-miR-548o |||||| 195:5' cgaaguuuggggggu**GUUUUG**u 3' CD151 3' cguuuUCAUUGACG--U**CAAAAC**c 5' hsa-miR-548o || : || | :|||||| 544:5' ggggcAGGGCCUUCCUG**GUUUUG**u 3' CD151

[1] Seed sequence alignments were obtained from www.microrna.org/ [174] and miRNAs highlighted in green are predicted to regulate CD151 (www.microrna.org/) [174]

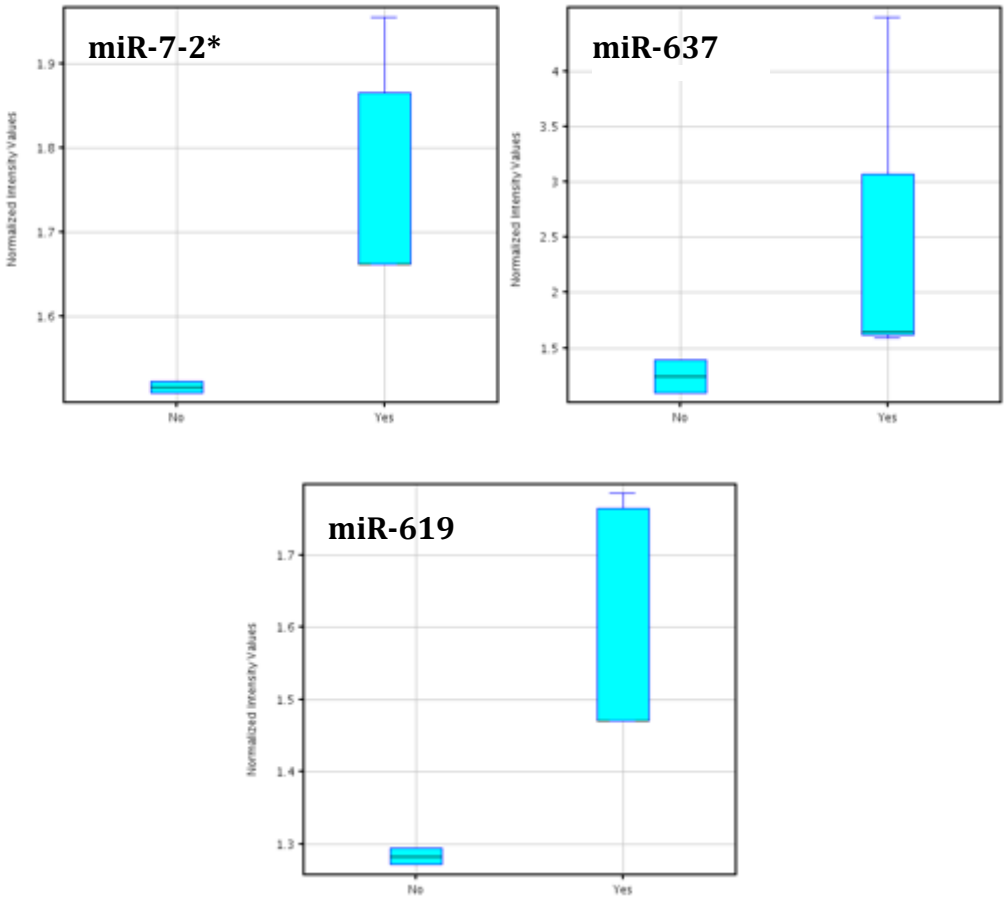

Figure 3.6. miR-7-2*, miR-637 and miR-619 have increased expression in prostate cells showing CD151 3'UTR repression. Analysis of expression data from miRNA microarrays with miRNAs that had higher expression in prostate cell lines showing CD151 3'UTR repression (Yes grouping) versus cell lines that displayed no endogenous CD151 3'UTR activity or enhanced CD151 3'UTR activity (No grouping) (see Fig 3.3 for CD151 3'UTR activity). GeneSpring software analysis revealed a small number of miRNA, miR-7-2*, miR-637 and miR-619 which follow the CD151 3'UTR activity profile and are predicted to bind to the CD151 3'UTR and therefore may regulate CD151 expression (see Table 3.2 for alignment of miRNA sequences with the CD151 3'UTR).

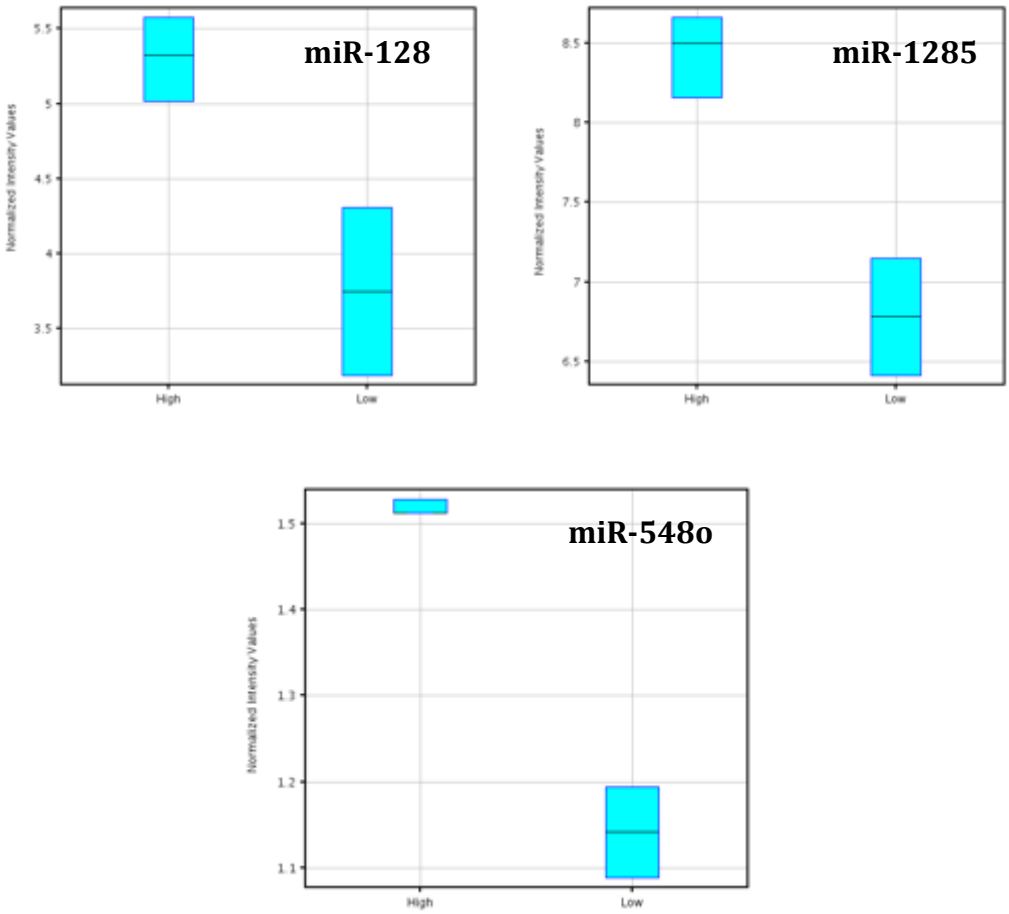

Figure 3.7. miR-128, miR-1285 and miR-548o are highly expressed in prostate cells displaying a high level of CD151 3'UTR repression. miRNA microarray expression data from prostate cell lines was grouped using GeneSpring based on their level of CD151 3'UTR activity (High levels of CD151 3'UTR repression versus lower levels of CD151 3'UTR repression). miRNAs that were differentially expressed based on this grouping were miR-128, miR-1285 and miR-548o which were overexpressed in prostate cell lines with a greater extent of CD151 3'UTR repression and are miRNAs that are predicted to bind to and regulate CD151 3'UTR activity based on bioinformatics target prediction.

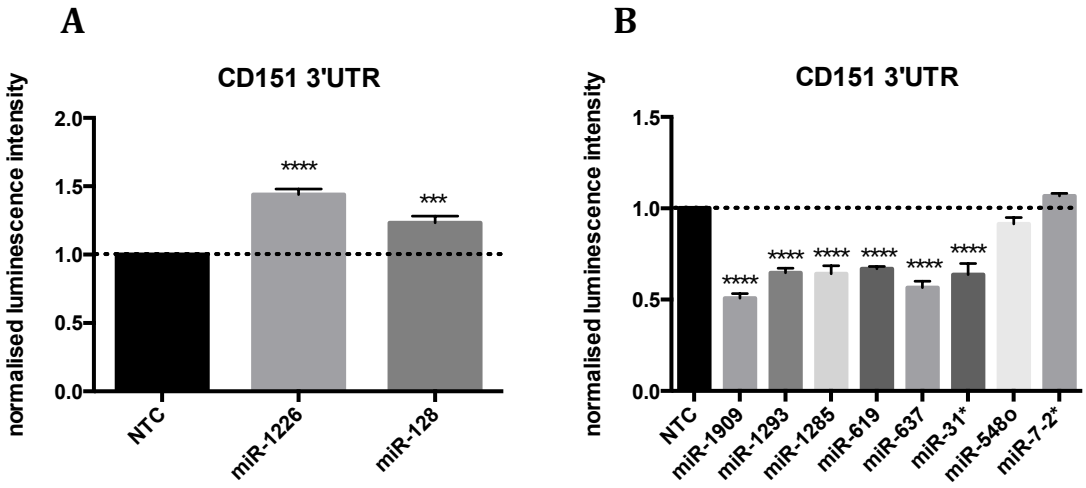

Fig 3.8. Many miRNAs bind to the CD151 3'UTR and modulate CD151 3'UTR activity A. CD151 3'UTR dual luciferase assays were conducted in HEK293FT cells with co-transfection of miRNA mimics to assess which miRNAs could alter protein expression via the CD151 3'UTR. All results are expressed as fold change of luminescence intensity relative to the non-targeting control (NTC; miRNA negative control). miR-1226 and miR-128 increased CD151 3'UTR activity by ~40% and ~20% respectively. B. Most miRNA appeared to repress protein expression via the CD151 3'UTR as seen by a decrease in luminescence to different extents depending on the miRNA. All results are shown as *n=3* with arbitrary units, *p=0.0004****, *p<0.0001*****.

3.2.7 miR-637 decreases CD151 protein expression in prostate cell lines

The dual luciferase assay with co-transfection of selected miRNA mimics showed, a number of miRNA were shown to modulate the activity of the CD151 3'UTR (fig 3.8). Of these, 3 miRNA (miR-637, miR-1285, miR-619) were identified through the analysis approach targeted to CD151 in that they were predicted to both regulate CD151 expression and had expression profiles that followed the pattern of CD151 3'UTR regulation in prostate cells. miR-637 was then chosen for further analysis from these

miRNA, as it showed the greatest numerical level of repression of CD151 3'UTR. To assess the effect of miR-637 on CD151 protein expression, miR-637 mimics were transiently transfected into RWPE1 and DU145 prostate cancer cells and CD151 protein levels assessed at 48 and 72 h post transfection. Transfection of miR-637 in RWPE1 prostate cells resulted in no change to CD151 total protein levels compared to the non-targeting control and mock transfected cells (Fig 3.9A). However, at 72h post transfection of miR-637, CD151 protein levels were reduced by 10% (Fig 3.9B). Similarly, in DU145 prostate cancer cells, transfection of miR-637 led to no change in CD151 protein expression compared to controls at 48 h, however a 20% reduction in CD151 protein expression was observed at 72 h post transfection compared to controls (Fig 3.10). miR-1285 had no effect on CD151 protein levels in DU145 prostate cancer cells following transfection with miR-1285 mimic (Fig 3.10).

3.2.8 miR-637 is predicted to regulate genes involved in cancer associated pathways

The databases miRPath [198] and PANTHER [199] were used to determine whether miR-637 targets other genes involved in cancer. miRPath takes predicted gene targets of a miRNA from DIANA and completes a pathways analysis on the list of genes. Using miRPath, miR-637 was predicted to regulate a range of genes involved in the following KEGG pathways: cell adhesion molecules (CAMs), ECM-receptor interaction and drug metabolism among others (see Table 3.3). Moreover, lists of genes that miR-637 is predicted to regulate from the database miRWalk [177] were put into PANTHER to sort genes into common functional pathways. This analysis generated a more extensive list of pathways that miR-637 may regulate. miR-637 predicted gene targets were shown to

be involved in many cancer associated pathways such as Wnt signalling, integrin signalling, p53 signalling and angiogenesis.

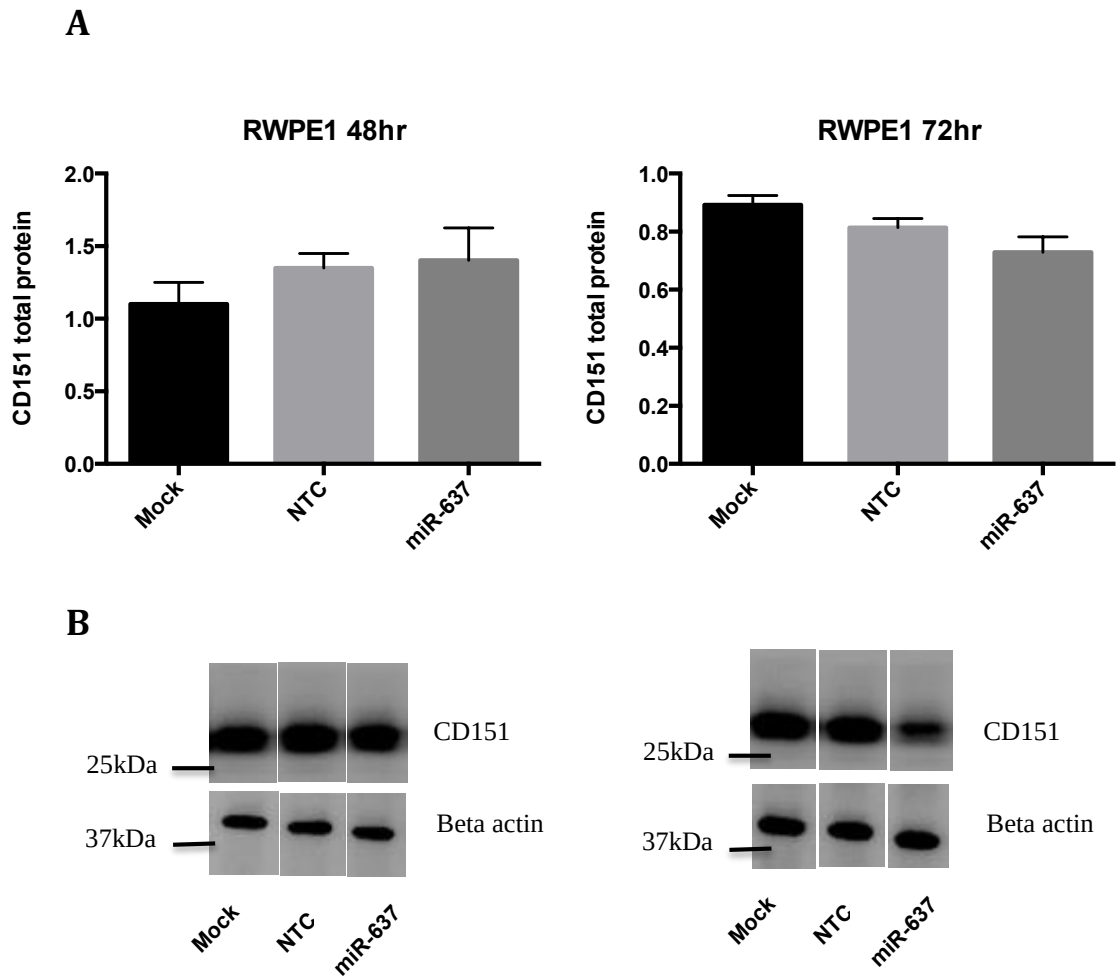

Figure 3.9. miR-637 slightly decreases CD151 protein expression in RWPE1 cells. A. RWPE1 cells were transfected with a miR-637 mimic and protein lysed in 1% NP-40 lysis buffer 48h and 72h post transfection. 10ug of total protein was loaded onto SDS-PAGE, transferred and probed for CD151 using the 11B1 antibody and beta actin. Protein bands were quantitated using densitometry, *n=3*. Data was normalised to beta actin expression (arbitrary units) in cells transfected with transfection reagent alone (Mock), cells transfected with miRNA mimic negative control (NTC) and cells transfected with miR-637 mimic, y-axes are in arbitrary units B. A representative western blot is shown below each graph depicting quantitated data. Bands were cropped to display in this order however lysates were run on the same gel.

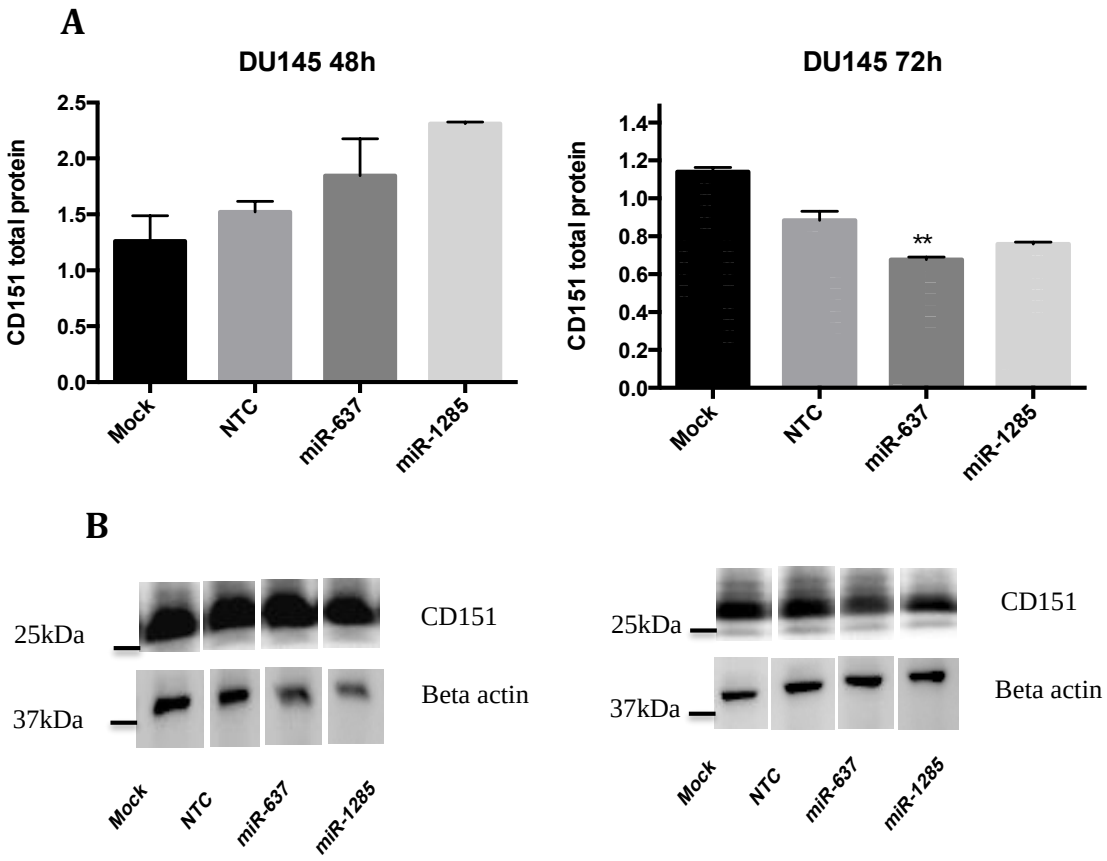

Figure 3.10. miR-637 modulates CD151 protein expression in DU145 prostate cancer cells. A. DU145 prostate cancer cells were transfected with miR-637 and miR-1285 mimics to assess their effect on CD151 protein levels. Following transfection at 48h and 72h, protein was lysed in 1% NP-40 lysis buffer and loaded onto SDS-PAGE, transferred and incubated with CD151 antibody (11B1) and beta actin. Western blots were quantitated using densitometry, *n=3*. Data was normalised to beta actin expression is account for any changes in loading. Results in graphs are shown as CD151 total protein normalised to beta actin (arbitrary units) in cells transfected with transfection reagent alone (Mock), cells transfected with miRNA mimic negative control (NTC) and cells transfected with either miR-637 or miR-1285 mimic. B. A representative western blot is shown below each graph showing quantitated results. Bands were cropped to display in this order however lysates were run on the same gel; *p=0.004***.

Table 3.3. miR-637 is predicted to regulate genes involved in cancer-associated pathways using miRPath.

KEGG pathway	***p-value***	***#genes***	***Genes***
Drug metabolism - cytochrome P450	1.56E-14	1	CYP3A7
Fatty acid elongation in mitochondria	5.26E-05	1	MECR
Circadian rhythm – mammal	5.26E-05	2	PER1, PER2
Glycosaminoglycan biosynthesis - keratan sulphate	0.000722161	1	B4GALT3
Lysine degradation	0.005903122	2	MLL, MLL2
Cell adhesion molecules (CAMs)	0.03510117	4	MPZ, NRXN2, PVRL1, NLGN3
ECM-receptor interaction	0.03738979	2	ITGA5, COL4A1

3.3 Discussion

This thesis chapter aimed to firstly characterise CD151 mRNA and protein expression in a panel of prostate cell lines and secondly investigate whether miRNAs that are deregulated in prostate cancer can regulate CD151 expression. Initially, CD151 mRNA and total protein levels were examined and found to have a significant inverse correlation. Surprisingly, aggressive prostate cancer cell lines with low levels of CD151 mRNA displayed high levels of CD151 protein, suggesting that CD151 is most likely post-transcriptionally regulated in prostate cancer. Higher CD151 protein expression in more aggressive prostate cancer cell lines compared to normal cells was expected, however the increase was only marginal. This was the first study investigating both CD151 mRNA and protein levels in a range of non-tumourigenic prostate and prostate cancer cell lines. One other study by Detchokul *et al* 2014 [72] measured CD151 mRNA and protein levels in LNCaP, DU145 and PC3 cells. They found DU145 and PC3 cells expressed high levels of CD151 mRNA and protein compared to LNCaP cells, which displayed low levels of CD151 mRNA and protein. Whilst no direct correlation analysis was performed, CD151 mRNA and protein expression did appear to correlate in these tumourigenic lines [71]. This conflicting result may be due to a number of factors such as method of RNA quantitation (real time PCR versus taqman probes) as well as cell passage and culturing conditions which may affect CD151 mRNA and/or protein expression. Importantly, in this chapter CD151 mRNA and protein expression was analysed in more prostate cell lines, including non-tumourigenic cell lines and therefore has more power to assess statistical correlations.

Previous literature has shown a role for CD151 functionally in prostate cancer progression and metastasis, with knockdown of CD151 leading to decreased migration

of prostate cancer cells *in vitro* [71]. In addition, CD151 overexpression in lowly tumourigenic prostate cancer cells has been shown to increase migration and invasion [71]. Other studies have shown a role for CD151 in prostate cancer metastasis. CD151 protein expression has been associated with lymphangiogenesis [72] and a reduction in lung metastasis has been seen in a prostate cancer *CD151*$^{-/-}$ mouse model [73]. Moreover, CD151 protein expression has been shown to be a significant prognostic indicator for prostate cancer progression [69]. However, there are no studies that have focussed on how CD151 expression is regulated in prostate cancer, with emphasis on post-transcriptional regulation.

Therefore, post-transcriptional regulation of CD151 by miRNAs was assessed in a panel of prostate cell lines. At commencement of this project, there was a lack of information with regard to tetraspanin regulation. An early paper had shown that CD151 is transcriptionally regulated by SP1 in liver cancer cells as it allows CD151 promoter activation and chromatin accessibility [129]. In addition, the ubiquitin E3 ligase, GRAIL affects CD151 protein stability [130]. However, in the last year, four papers were published implicating miRNAs in CD151 regulation. miR-124 [170] and miR-506 [171] which share the same seed region (part of the miRNA that binds mRNA 3'UTR), were shown to bind to the CD151 3'UTR and downregulate CD151 mRNA and protein expression in breast cancer cells, with observed effects on breast cancer cell proliferation, decreased migration, invasion and adhesion to various ECM substrates attributed to this decrease in CD151. Similarly, miR-152 [173] and miR-22 [172] decreased CD151 mRNA and protein expression in gastric cancer cell lines, leading to decreased cell proliferation, decreased colony formation and a reduction in gastric cancer cell migration/invasion. miR-152 and miR-22 were shown to bind to the CD151

3’UTR, and overexpression of CD151 and miR-22 or miR-152 suppressed the anti-cancer effects of transfecting miR-152 or miR-22 alone [172, 173]. Therefore, when CD151 levels were increased, miR-22 and miR-152 could no longer effectively modulate cancer processes for unknown reasons. Interestingly, gastric cancer tissues and cell lines display decreased expression of miR-152 and miR-22 but express high levels of CD151 protein [172, 173], suggesting that miR-152 and miR-22 are important for regulating CD151 expression in gastric cancers.

In line with these recent publications, many forms of evidence from this chapter suggested that CD151 is a least partially regulated by miRNAs in prostate cancer. Firstly, there was differential CD151 mRNA, protein expression and CD151 3’UTR activity between non-tumourigenic and prostate cancer cells. A significant inverse correlation between CD151 mRNA and total protein levels was found across the prostate cell lines. Moreover, more aggressive prostate cancer cell lines had repressed luciferase expression, which is a measure of CD151 3’UTR activity, suggesting that these cancer cells endogenously express miRNAs or other factors that are capable of binding to the CD151 3’UTR activity, highlighting that miRNAs may indeed regulate CD151 expression. Interestingly, the more aggressive prostate cancer cell lines displayed low levels of CD151 mRNA, more repression of the CD151 3’UTR, but high levels of CD151 total protein. This suggests that miRNAs or other factors that can bind to the 3’UTR such as RNA binding proteins may keep CD151 mRNA levels low in prostate cancer cells. However, another mechanism at the translational or post-translational level may be responsible for allowing CD151 protein levels to remain high in prostate cancer, potentially through enhancing CD151 protein stability. For instance,

altered activity of E3 Ub ligases may lead to enhanced protein half-life and work is currently underway in the laboratory to investigate this.

Secondly, bioinformatics analysis revealed that around 400 miRNAs are predicted to bind to the CD151 3'UTR. Thirdly, miRNAs that were predicted to bind CD151 and displayed expression profiles following CD151 3'UTR activity were shown to bind to the CD151 3'UTR *in vitro*. Transfection of miR-1226 and miR-128, which were upregulated in cell lines showing CD151 3'UTR repression, actually resulted in enhanced luciferase reporter expression. This was a unique finding and may be attributable to the intricate balance between all expressed miRNAs and RBPs, some of which can increase or decrease 3'UTR activity, that are capable of interacting with and regulating the CD151 3'UTR. Transfection of other miRNAs, which were upregulated in cell lines showing CD151 3'UTR repression, such as miR-637, resulted in a decrease in luciferase reporter expression. However, more experiments are needed which involve mutating miR-637 binding sites in the CD151 3'UTR to test whether miR-637 directly binds to the CD151 3'UTR. If this were true, transfection of miR-637 mimic with a mutated CD151 3'UTR luciferase construct would abolish the downregulation of luciferase expression that was observed. Most importantly, transfection of a miRNA mimic of miR-637 led to a decrease in endogenous CD151 protein expression. miR-637 is predicted to regulate a number of genes/proteins involved in various cancer associated pathways and therefore may play an important role in prostate cancer and potentially other cancer types.

miR-637 has been reported to be involved in cancers, particularly follicular thyroid carcinoma [200], malignant oral leukoplakia [201], hepatocellular carcinoma [188] and

HER2+ breast cancers [187]. These studies show downregulation of miR-637 in cancer tissues and/or cell lines and transfection of miR-637 into hepatocellular carcinoma cell lines and HER2+ breast cancer cell lines has been linked to inhibition of cell growth / induction of apoptosis [187, 188]. This has been shown to be a result of suppression of STAT3 signalling in hepatocellular carcinoma [188] and suppression of HER2 signalling in HER2+ breast cancer [187]. miR-637 has also been shown to downregulate COL4A1 mRNA and hence protein levels in primary human osteoblasts [202] and is involved in hMSC differentiation via targeting of osterix and induction of S phase arrest and suppression of growth [203]. Therefore, miR-637 may be important for communication between prostate cancer cells and the tumour microenvironment, which could impact prostate cancer progression and metastasis. Moreover, interrogation of results from a publically available dataset on high-risk ER+ breast cancer patients receiving adjuvant tamoxifen monotherapy, showed low expression of miR-637 is significantly associated with lower survival rates (see Fig 8.3 Appendix;[204]). All of these studies suggest that miR-637 acts a tumour suppressor in cancer, and induction of miR-637 leads to decreased proliferation of cancer cells due to downregulation of its cancer-associated targets such as STAT3, HER2 and now potentially CD151. This is very similar to the effects of miR-22 and miR-152 (decrease CD151) on gastric cancer cell proliferation, where an anti-cancer effect was seen with transfection of the miRNAs [172, 173]. Therefore, miR-637 may play a role in prostate cancer progression through its effects on CD151 and other mRNA targets that are important in prostate cancer.

Many miRNAs were shown to bind to the CD151 3'UTR using a luciferase 3'UTR reporter assay however there was not sufficient time to follow up these findings. The expression of these miRNAs needs to be further validated as the miRNA microarrays

were only conducted once. Moreover, the effect of these miRNAs on CD151 mRNA and protein levels and their significance to prostate cancer cell function needs to be ascertained in the future. In particular, the functional role of miR-637 in prostate cancer progression needs to be elucidated *in vitro* and *in vivo*. In addition, the effect of miR-637 transfection on CD151 protein expression needs to be investigated in other prostate cell lines such as PC3 and BPH-1 cells. Moreover, miR-637 may affect CD151 protein expression at an earlier time-point (e.g. 24h post-transfection) or may also have an effect on CD151 cell surface expression. Therefore, repeated transfection of miR-637 may lead to significant reductions in CD151 expression, which may be relevant both *in vitro* and *in vivo*. CD151 and miRNA are also shuttled out of cancer cells within exosomes [205, 206]. Therefore, overexpression of miR-637 may modulate the levels of CD151 and potentially miR-637 (if shuttled into exosomes) in prostate cancer exosomes, which has the potential to influence prostate cancer progression and metastasis to other organs.

The expression of all miRNAs was also assessed in the prostate cell panel, in order to determine which miRNAs are differentially expressed in non-tumourigenic versus prostate cancer cell lines. miR-15b and miR-1226 were significantly upregulated in prostate cancer cell lines compared to non-tumourigenic prostate cells. Circulating miR-15b has been found to be part of a miRNA signature that acts as a biomarker to predict risk groups in prostate cancer based upon PSA testing, with higher levels of miR-15b seen in the high risk group compared to low or intermediate risk groups [185]. Moreover, miR-15b is typically overexpressed in some cancer tissues and in the circulation of patients with different types of cancers such as lung, endometrial, melanoma, ovarian and bladder cancer [207-211]. miR-1226 has been previously shown

to be expressed in human breast cancer cell lines and non-malignant breast cells and is known to regulate the oncoprotein MUC1 [183]. In addition, miR-22*, miR-659, miR-1293 and miR-3177 had significantly decreased expression in prostate cancer cell lines versus non-tumourigenic prostate cells. There is no published literature on miR-22*, and miR-659 has not been implicated in cancer before but does appear to be involved in obesity in pregnant women [212] and diabetes mellitus [213]. Moreover, miR-1293 is implicated in buccal cancer [196] and in regulating viral IL-6 from Kaposi's sarcoma herpes virus [214, 215] and one study showed miR-3177 to be upregulated in colorectal cancer [216]. Therefore, the novel changes identified here in miRNA expression in prostate cancer versus non-tumourigenic prostate cells may show promise as new biomarkers for prostate cancer. However, further validation by real time PCR is required using more prostate cell lines as well as prostate cancer and normal prostate tissues to ascertain the effectiveness of using these miRNAs as prostate cancer biomarkers in the future.

In summary, CD151 mRNA and protein levels were found to significantly inversely correlate and differential activity of the CD151 3'UTR was observed across the panel of prostate cell lines. These results together with bioinformatics analysis and miRNA profiling suggested that miRNAs regulate CD151 expression. A number of miRNAs were found to alter protein expression through the CD151 3'UTR, however transfection of miR-637 mimic significantly decreased CD151 total protein levels only in DU145 prostate cancer cells with only a slight decreased in CD151 expression in RWPE1 prostate cells. miR-637 is predicted to regulate a myriad of genes involved in cancer pathways and therefore it may play a crucial role in prostate cancer progression.

Chapter 4: Post-transcriptional regulation of CD9 in prostate cancer

4.1 General introduction

Like CD151, CD9 expression is deregulated in many types of cancers. CD9 is often referred to as a metastasis suppressor, however it has been found to enhance progression in some tumour types such as melanoma [217], fibrosarcoma [218] and some types of breast cancer [219]. In prostate cancer, the expression and function of CD9 is relatively unknown. An early study by Zvieriev *et al* 2005 [94], found that overexpression of wildtype CD9 tagged with His/Myc increased invasiveness of a prostate cancer cell line that spontaneously metastasises to lymph nodes *in vitro*. However, when orthotopically injected into nude mice, overexpression of CD9 had no statistical effect on tumourigenicity or metastasis *in vivo*. This suggested that CD9 may not play an important role in prostate cancer progression and metastasis. In contrast, a recent publication from our laboratory showed for the first time that CD9 knockout mice crossed onto the TRAMP model of prostate cancer led to no changes in primary tumour growth but significantly diminished metastasis to the liver but not the lung [220]. Therefore, endogenous expression of CD9 either in the tumour or the stroma influences prostate cancer progression and metastasis, however the mechanism needs to be clarified. The discrepancy between results from Zvierev *et al* 2005 and Copeland *et al* 2013 could be attributed to the fact that the C-terminus of CD9 was tagged, which is likely to influence binding of CD9 to partner proteins and hence affect its functions such as cell adhesion and cell spreading [95]. Moreover, loss of CD9 expression in other organs is likely to have contributed to the drastic decrease in metastasis to the liver in the Copeland study, suggesting that CD9 may play a role in communication between tumour cells and the microenvironment.

CD9 protein expression is typically significantly decreased or lost in advanced prostate cancer and its associated bone and lymph node metastases compared to normal prostate tissues [82]. CD9 protein expression was inversely correlated with a range of clinicopathologic variables such as PSA level at diagnosis, Gleason score and metastasis. However, the mechanism/s responsible for the reduction of CD9 protein expression in prostate cancer are not clear. CD9 mRNA modifications involving deletions or missense mutations have been found in a small percentage of prostate cancer patients and some prostate cancer cell lines, with CD9 protein expression not detected in the majority of these cases. Moreover, in Merkel cell carcinoma cell lines CD9 mRNA species with different 5'UTR lengths were found, with the longer 5'UTR inhibiting ribosome scanning and translation which may explain the decreased levels of CD9 in metastases from Merkel cell carcinomas [66]. However, deregulation of CD9 in prostate cells remains to be fully elucidated.

The previous chapter showed many forms of evidence that CD151 expression is at least partially regulated by miRNAs. This chapter aimed to elucidate whether CD9 is also regulated by miRNAs in prostate cancer.

4.2 Results

4.2.1 Characterisation of CD9 expression in non-tumourigenic and prostate cancer cells

Tetraspanin CD9 is commonly referred to as a metastasis suppressor as its protein expression is usually decreased in advanced stage cancers. However, until now there has been no study that has measured CD9 mRNA and protein levels in a range of non-tumourigenic and tumourigenic prostate cells. In this study, CD9 mRNA levels as assessed by real time PCR, were found to be quite similar across a panel of non-tumourigenic (PrEC, RWPE1 & BPH-1) and tumourigenic prostate cell lines (LNCaP, PC3, DU145 & WPE1-NB26) (Fig 4.1A). Interestingly, the more aggressive prostate cancer cell lines DU145 & PC3 displayed the highest levels of CD9 mRNA (Fig 4.1A). In stark contrast, these aggressive prostate cancer cells had the lowest levels of CD9 cell surface protein (Fig 4.1C) and total CD9 protein expression (Fig 4.1B & C) compared to non-tumourigenic RWPE1 cells. Moreover, LNCaP cells (derived from a lymph node metastasis) that have low tumourogenicity expressed the highest levels of CD9 cell surface and total protein expression (Fig 4.1B-D). CD9 mRNA and CD9 total protein/cell surface protein levels showed an inverse trend that did not reach significance (Fig 4.2A & B). Likewise CD9 total protein and cell surface protein expression showed a trend towards a positive correlation (Fig 4.2C).

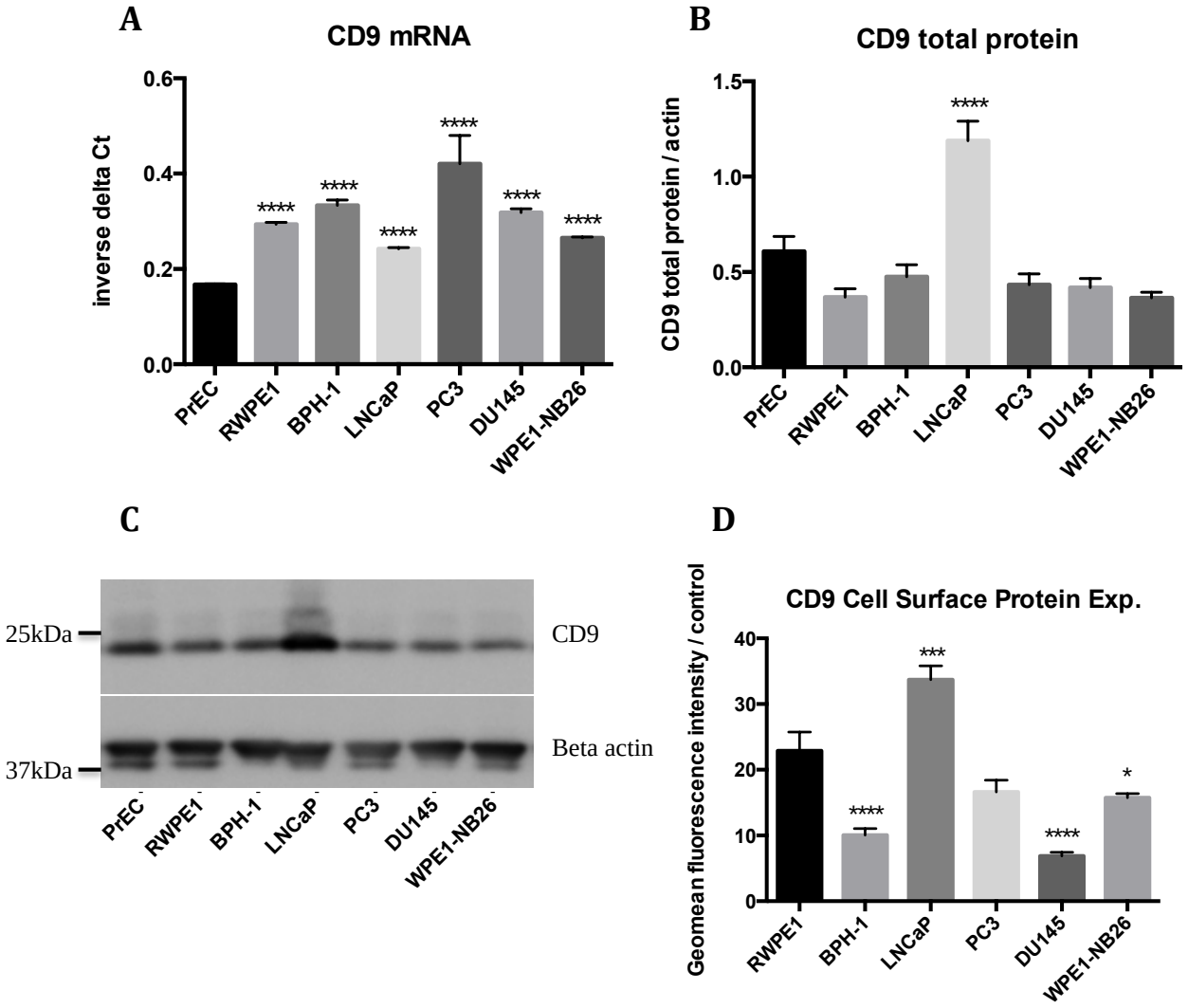

Figure 4.1. Characterisation of CD9 mRNA and protein expression in prostate cell lines. A. RNA was extracted from prostate cell lines using TriZol-chloroform, reverse transcribed and quantitated using real time PCR. Results are shown as inverse delta Ct normalised to the geomean of 3 housekeeping genes. B. Protein was lysed in 1% NP-40 lysis buffer and loaded onto 12% SDS-PAGE, transferred to nitrocellulose and probed with 1AA2 (CD9) antibody. Protein bands were quantitated using densitometry and are shown as pixels minus background/mm^2 and are normalised to beta actin to account for differences in loading. C. A representative western blot showing CD9 total protein expression across prostate cell lines. D. Flow cytometry was used to measure CD9 cell surface protein expression. Prostate cells were incubated with 8ug/mL 1AA2 (CD9) primary antibody followed by incubation with a FITC conjugated secondary antibody. All samples were analysed using a FACScalibur with cell quest software. Results are shown as geomean of FITC fluorescence minus geomean of isotype matched control antibody. All experiments were conducted with *n=3*; y-axes of graphs are shown as arbitrary units, *p = 0.05*, p = 0.01**, p = 0.001***, p< 0.0001*****.

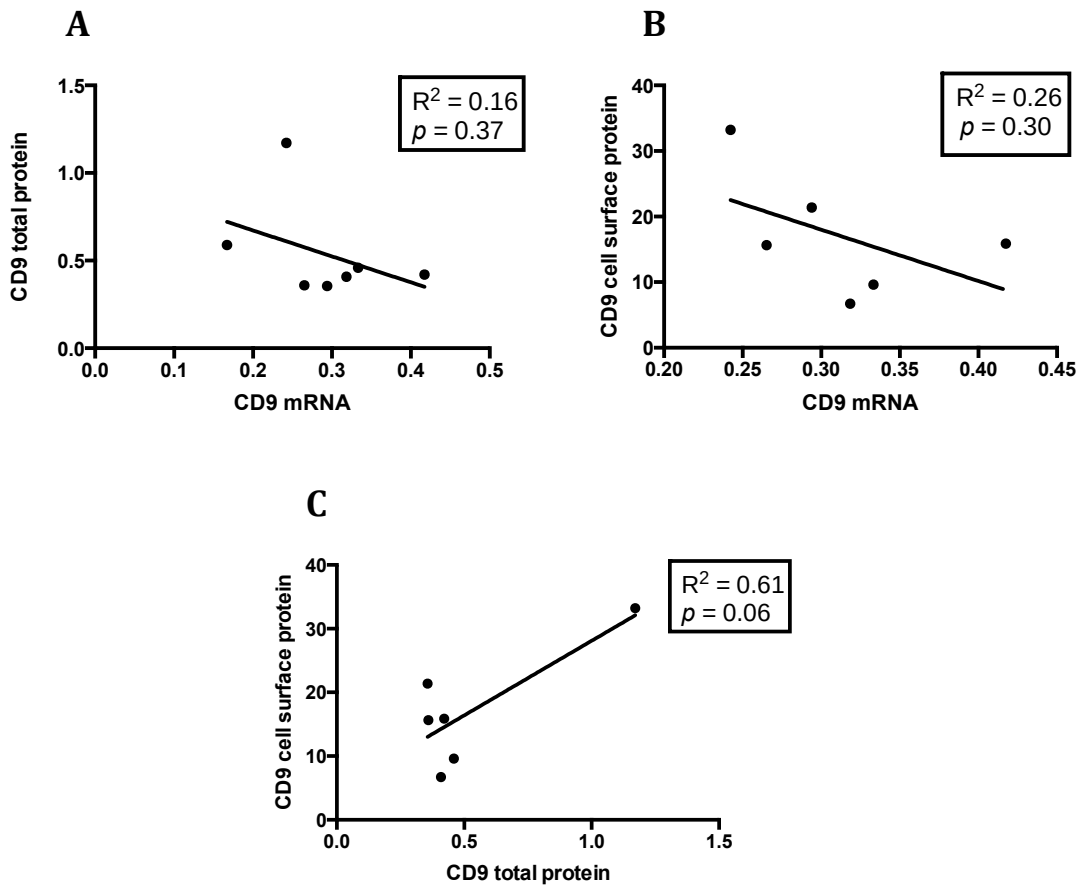

Figure 4.2. Linear regression analysis of CD9 expression in prostate cell lines.
A. Geomean of CD9 mRNA levels versus geomean of CD9 total protein levels (from Fig 4.1). B. Geomean of CD9 mRNA expression compared to geomean of CD9 cell surface expression. C. Geomean of CD9 total protein versus geomean of CD9 cell surface levels. For all combinations (A, B & C), a linear regression analysis was performed with 95% CI and a *p-value <0.05* was considered statistically significant. All results are shown as arbitrary units.

4.2.2 Regulation of the CD9 3'UTR varies across a panel of prostate/prostate cancer cells

To determine the likelihood that miRNAs regulate the CD9 3'UTR, a dual luciferase reporter assay consisting of transfection of the CD9 3'UTR into the panel of prostate cell lines was used. The aggressive DU145 and PC3 prostate cancer cells displayed repressed activity of the CD9 3'UTR which is indicative of miRNA regulation, compared to the other prostate cell lines which showed no repression (BPH-1 & LNCaP) or enhanced activity of the CD9 3'UTR (PrEC, RWPE1 & WPE1-NB26) (Fig 4.3).

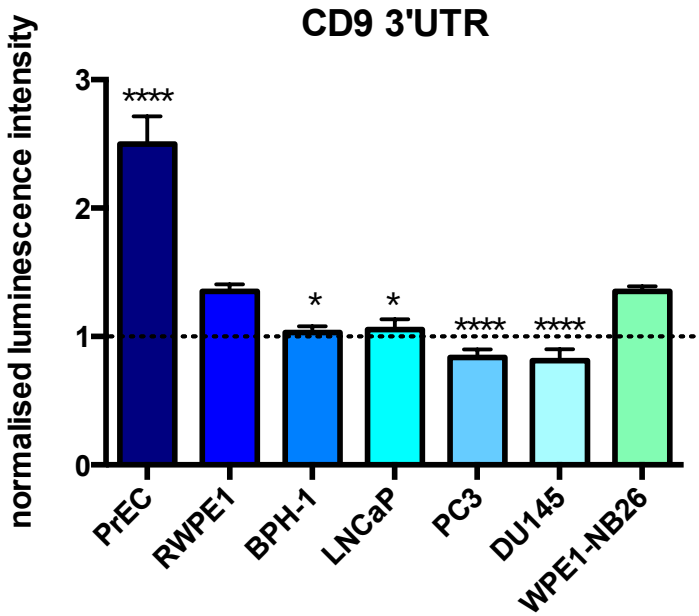

Figure 4.3. Prostate cancer cell lines naturally display repressed CD9 3'UTR activity compared to non-tumourigenic prostate cells. Prostate cell lines were transfected with a CD9 3'UTR renilla luciferase reporter construct for 24 h to assess CD9 3'UTR activity. Cells were lysed and luminescence intensity was measured using the Promega dual luciferase kit and the Synergy 2 luminometer with automatic injection of reagents. Renilla luminescence intensity was normalised to a firefly luciferase vector (transfection control) and the 3'UTR empty vector (positive control);*n=3*, y-axis shown as arbitrary units; *p=0.01*, p<0.0001*****.

4.2.3 Many miRNAs that are deregulated in cancer are predicted to regulate the CD9 3'UTR

miRNA target prediction databases were used to determine which miRNAs may regulate CD9 as there are currently no studies implicating miRNA in CD9 regulation. Similar to CD151, around 400 miRNAs were predicted to bind to the CD9 3'UTR in at least one site (see Table 8.4 Appendix for a full list). Of these, many have already been implicated in cancer progression and some are novel miRNA with no known function (see table 4.1). To shorten this list, prostate cell lines were grouped using GeneSpring firstly based on whether they displayed a decrease in CD9 3'UTR activity in the luciferase assay compared to no change or enhanced 3'UTR activity (analysis 1). The results from analysis 1 are shown in table 4.2 and fig 4.4. From this analysis, only miR-106a*, miR-548c-5p and miR-4289 were upregulated in prostate cancer cells showing CD9 3'UTR repression (PC3 & DU145) and predicted to bind to the CD9 3'UTR using bioinformatics (see table 4.2 for CD151 – miRNA predicted sequence complementarity).

The prostate cell lines were then grouped based on whether they showed an increase or decrease in CD9 3'UTR activity versus no change in CD9 3'UTR activity (analysis 2). From this analysis, only miR-518f* was found to be upregulated in prostate cell lines displaying altered CD9 3'UTR activity and predicted to bind to the CD9 3'UTR (Fig 4.4; table 4.3).

Table 4.1 miRNAs predicted to bind to and regulate the CD9 3'UTR

miRNA	Database	Role in PC or other cancers	Refs
miR-25	Microrna.org, miRWalk	OncomiR/overexpressed in PC, differential exp through PC progression	[180, 221]
miR-345	Microrna.org, miRWalk	Part of a serum miRNA signature that predicts adverse pathology in PC patients eligible for active surveillance	[222]
miR-518f*	Microrna.org, miRDB, miRWalk	No known role in cancer	N/A
miR-4289	Microrna.org	No known role in cancer	N/A
miR-106a*	Microrna.org	No known role in cancer	N/A
miR-548c-5p	Microrna.org, miRWalk, miRDB	Anti-cancer effect on liver cancer stem cells	[223]

microrna.org/ [174], miRDB (mirdb.org/) [176] and miRWalk [177]; PC = prostate cancer, Refs = references, exp = expression.

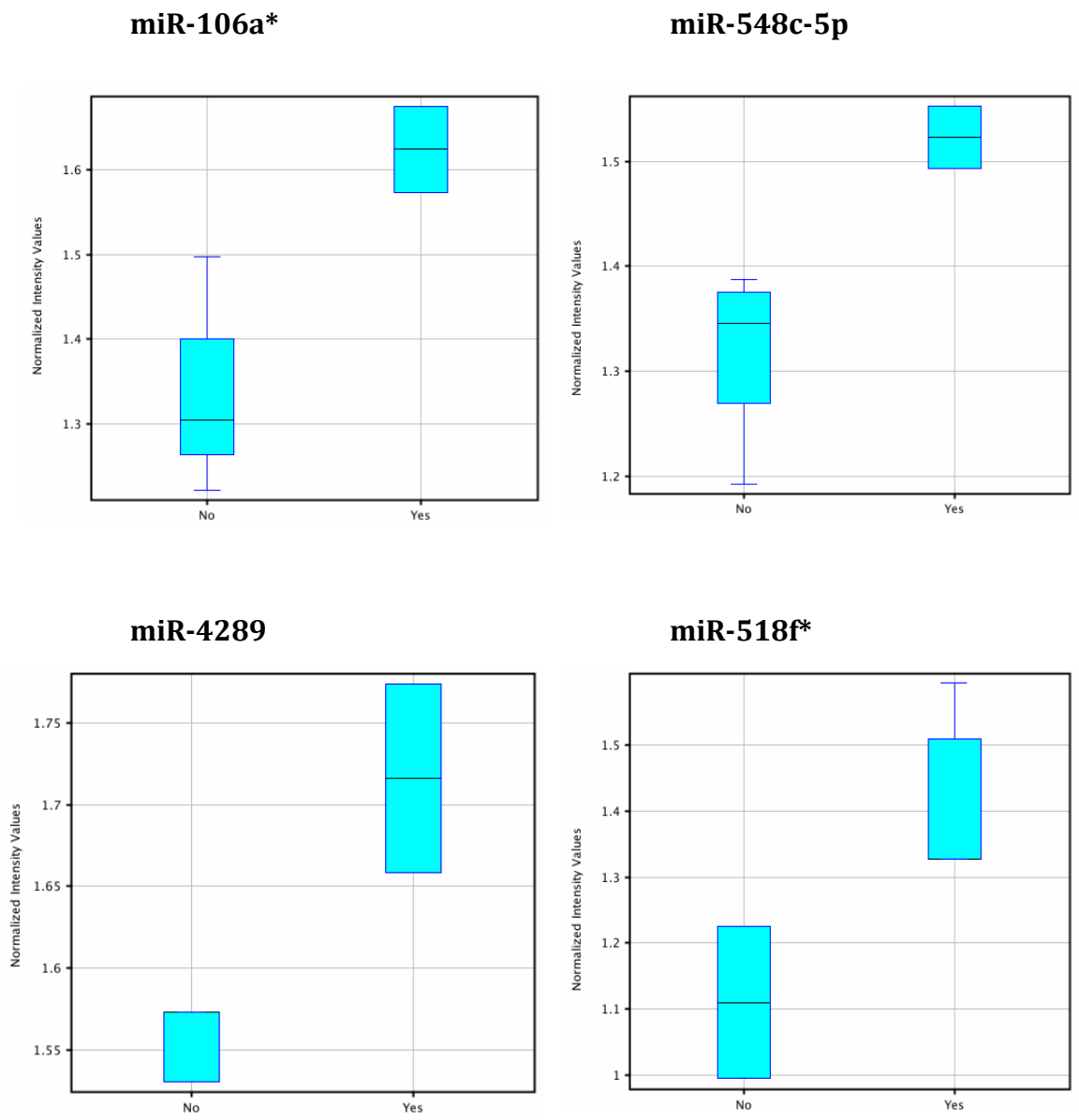

Figure 4.4 miR-106a*, miR-548c-5p, miR-4289 and miR-518f* are upregulated in prostate cells showing changes to CD9 3'UTR activity. Global miRNA expression in all the prostate cell lines was assessed using miRNA microarrays. Prostate cells were then grouped based on their level CD9 3'UTR activity. Analysis 1 grouped cells based on less activity vs no change/increased activity and analysis 2 grouped cell lines based on any change in 3'UTR activity versus no change. GeneSpring software was used to analyse miRNA expression patterns, with miR-106a*, miR-548c-5p, miR-4289 and miR-518f* showing increased expression in prostate cell lines with repression of CD9 3'UTR activity and are predicted to bind to the CD9 3'UTR. All results are shown as arbitrary units.

Table 4.2 miRNAs with expression levels that are upregulated in prostate cell lines with reduced CD9 3'UTR activity

Analysis 1	Upregulated miRNA	CD9 / miRNA alignment[1]
DU145 & PC3 (Targeting) Vs. PrEC, RWPE1, WPE1-NB26, BPH-1 & LNCaP (No change or increase)	miR-106a* [1] miR-589* miR-548c-5p[1] miR-628-3p miR-542-5p miR-766 miR-1302 miR-4289[1]	3' cauucuucacgaAUGUAACGuc 5' hsa-miR-106a* |:|||||| 113:5' uaguauucauucUGCAUUGCua 3' CD9 3' ccGUUUUUGGCGUUAAUGAAAa 5' hsa-miR-548c-5p :||||:|:| |:||||||| 135:5' gaUAAAAGCUGAAGUUACUUUa 3' CD9 3' acuaucgggacgUGUUACg 5' hsa-miR-4289 |||||| 414:5' uaaaaaauuaugACAAUGu 3' CD9

[1] miRNAs highlighted in yellow are predicted to bind to the CD9 3'UTR based on seed sequence complementarity shown in the next column (www.microrna.org/) [174].

Table 4.3 miRNAs that are upregulated in prostate cells displaying an Increase or decrease in CD9 3'UTR activity

Analysis 2	Upregulated miRNA	CD9 / miRNA alignment[1]
PrEC, RWPE1, WPE1-NB26, DU145 & PC3 (increase or decrease) Vs. LNCaP & BPH-1 (no change)	miR-424* miR-523*[1] miR-518f*[1]	3' gucuuucGCGAAGGGAGAUCUc 5' hsa-miR-523* :||| ||||||| 290:5' cuauauuUGCUAGACUCUAGAc 3' CD9 3' cucuuucACGAAGGGAGAUCUc 5' hsa-miR-518f* |||| ||||||| 290:5' cuauauuUGCUAGACUCUAGAc 3' CD9

[1] miRNAs highlighted in yellow are predicted to bind to the CD9 3'UTR based on seed sequence complementarity shown in the next column (www.microrna.org/) [174].

4.2.4 Many miRNAs that are differentially expressed in prostate cancer bind to the CD9 3'UTR

miRNAs that are deregulated in prostate cancer and predicted to regulate the CD9 3'UTR, together with miRNAs identified in Fig 4.4 were assessed for their ability to bind to the CD9 3'UTR *in vitro*. In order to investigate this, specific miRNA mimics were co-transfected with the CD9 3'UTR luciferase construct in HEK293FT cells and luminescence intensity was measured 24 h later. miRNAs with expression changes that followed CD9 3'UTR activity (section 4.2.3) were tested for their ability to bind to the CD9 3'UTR and exert repression of the luciferase reporter. miR-106a* and miR-548c-5p appeared to have no effect on CD9 3'UTR activity, however transfection with miR-4289 and miR-518f* led to a drastic decrease in CD9 3'UTR activity by around 25% and 40% respectively (Fig 4.5).

miR-25 and miR-345 have been previously shown to be overexpressed in prostate cancers and are predicted to regulate CD9 based on results from target prediction databases. Fig 4.3 shows that transfection with miR-25 and miR-345 resulted in a reduction in CD9 3'UTR activity by around 10% and 20% respectively compared to non- targeting control.

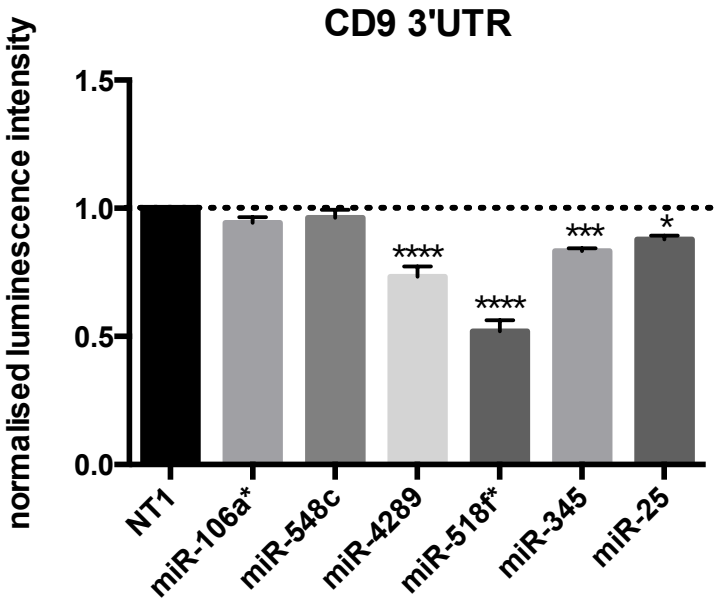

Figure 4.5 Many miRNAs predicted to regulate CD9 bind to the CD9 3'UTR in vitro. A renilla luciferase CD9 3'UTR reporter construct was co-transfected with various miRNA mimics into HEK293FT cells to assess the ability of the miRNAs to modulate the CD9 3'UTR. Cells were transfected for 24 h then lysed and both firefly and renilla luminescence was measured using the Synergy 2 luminometer. Results are shown as luminescence intensity normalised to firefly luminescence (transfection control) and expressed as a fold change relative to a miRNA negative control (NT1). Transfection of miR-106a* and miR-548c-5p mimics led to no change in CD9 3'UTR activity, however miR-4289, miR-518f*, miR-25 and miR-345 resulted in a decrease in CD9 3'UTR activity; *n=3*, results expressed in arbitrary units; *p=0.01**, *p=0.0005****, *p<0.0001*****.

4.2.5 miR-518f* decreases CD9 protein levels in prostate cell lines

The previous experiment showed that miR-4289 and miR-518f* modulate the CD9 3'UTR, however it did not test whether these miRNA can affect endogenous CD9 protein expression in prostate cell lines. Therefore to test this, miR-4289 and miR-518f* mimics were transfected into RWPE1 and DU145 prostate cell lines and CD9 total protein levels were measured at 48h and 72h post-transfection. Transfection of miR-518f* resulted in a significant decrease in CD9 protein expression in RWPE1 at 72h post-transfection and in DU145 cells at both time points (Fig 4.6 & 4.7). On the other hand, miR-4289 had no effect on CD9 protein expression in RWPE1 cells or DU145 prostate cancer cells at 48h post-transfection, however there was a slight decrease in CD9 protein levels at 72h post-transfection compared to mock transfected and cells transfected with the negative control miRNA mimic (Fig 4.6 & Fig 4.7).

4.2.6 miR-518f* is predicted to target a range of genes involved in cancer associated pathways

Based on target prediction software such as microrna.org, miR-518f* is predicted to regulate over 2000 genes, many of which are implicated in cancer progression [174]. This list includes genes such as CD9, ELAV1 (HuR), CELF1 (CUGBP1) and other tetraspanins (e.g. TSPAN8). The full list of genes were analysed using PANTHER [199] to discern which pathways miR-518f* may be involved in regulating. miR-518f* target genes appear to be involved in a variety of cancer associated pathways such as angiogenesis, Wnt signalling, cadherin signalling, cell cycle, p53 signalling, androgen/estrogen/progesterone signalling as well as regulatory pathways which are

commonly deregulated in cancer e.g. proteasome pathway, mRNA splicing and transcriptional control (Table 4.4; for a full list see Table 8.6 Appendix). Another database, miRPath [198] was used to determine which pathways predicted miR-518f* target genes are likely to be involved in. This analysis used the DIANA algorithm, which only found 28 potential gene targets that are known to function in calcium signalling (CALM1 & GNA14), photo transduction (CALM1) and glioma (CALM1).

Table 4.4 Summary of pathways that miR-518f* predicted gene targets are involved in using PANTHER.

Pathway[2]	***Number of genes[1]***
Wnt signalling	38
Integrin signalling	30
Angiogenesis	22
PDGF	19
EGF signalling	18
Cytoskeletal regulation by GTPase	17
Cadherin signalling	17
Ras signalling	15
FGF signalling	14
p53 signalling	13
TGF-beta signalling	12
VEGF signalling	10
PI3K signalling	9
p53 pathway feedback loop 2	9
Ubiquitin proteasome pathway	8
FAS signalling	8
Hypoxia response via HIF activation	7
Notch signalling pathway	6

[1]miR-518f* predicted gene targets were obtained from www.microrna.org/ [174] and
[2]functional pathways were found using PANTHER [199].

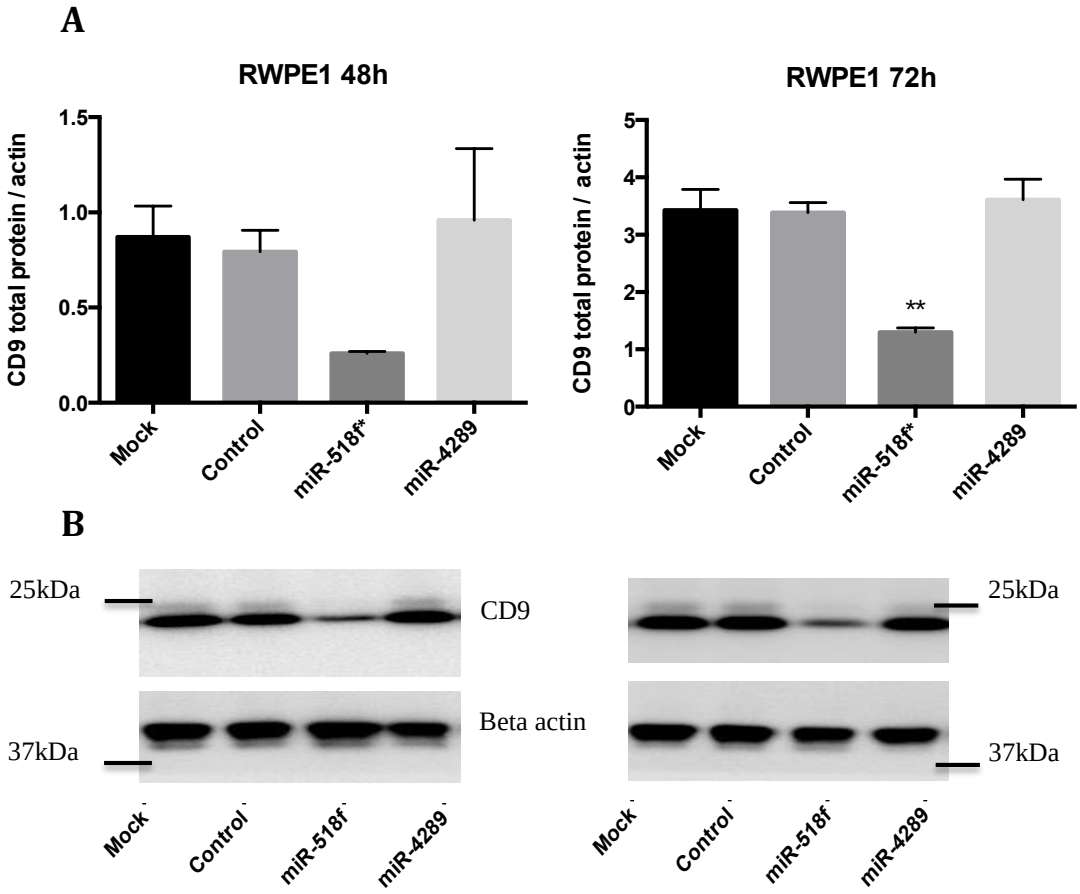

Figure 4.6. miR-518f* decreases CD9 protein expression in RWPE1 prostate cells. A. RWPE1 cells were transfected with miR-518f* and miR-4289 mimics to assess their effect on CD9 protein expression. Protein was lysed with 1% NP-40 lysis buffer 48 h and 72 h post transfection. Protein was loaded onto SDS-PAGE, transferred to nitrocellulose and probed for CD9 using 1AA2 antibody. Protein bands were quantitated using densitometry. Results are shown as CD9 total protein normalised to beta actin protein expression (arbitrary units), which served as the loading control; *n=3*. B A representative western blot is shown below the graphs depicting quantitated protein expression. Transfection with miR-518f* reduced CD9 protein expression in RWPE1 cells at 48 and 72 h post-transfection, whereas miR-4289 had no effect on CD9 protein expression; *p=0.001***.

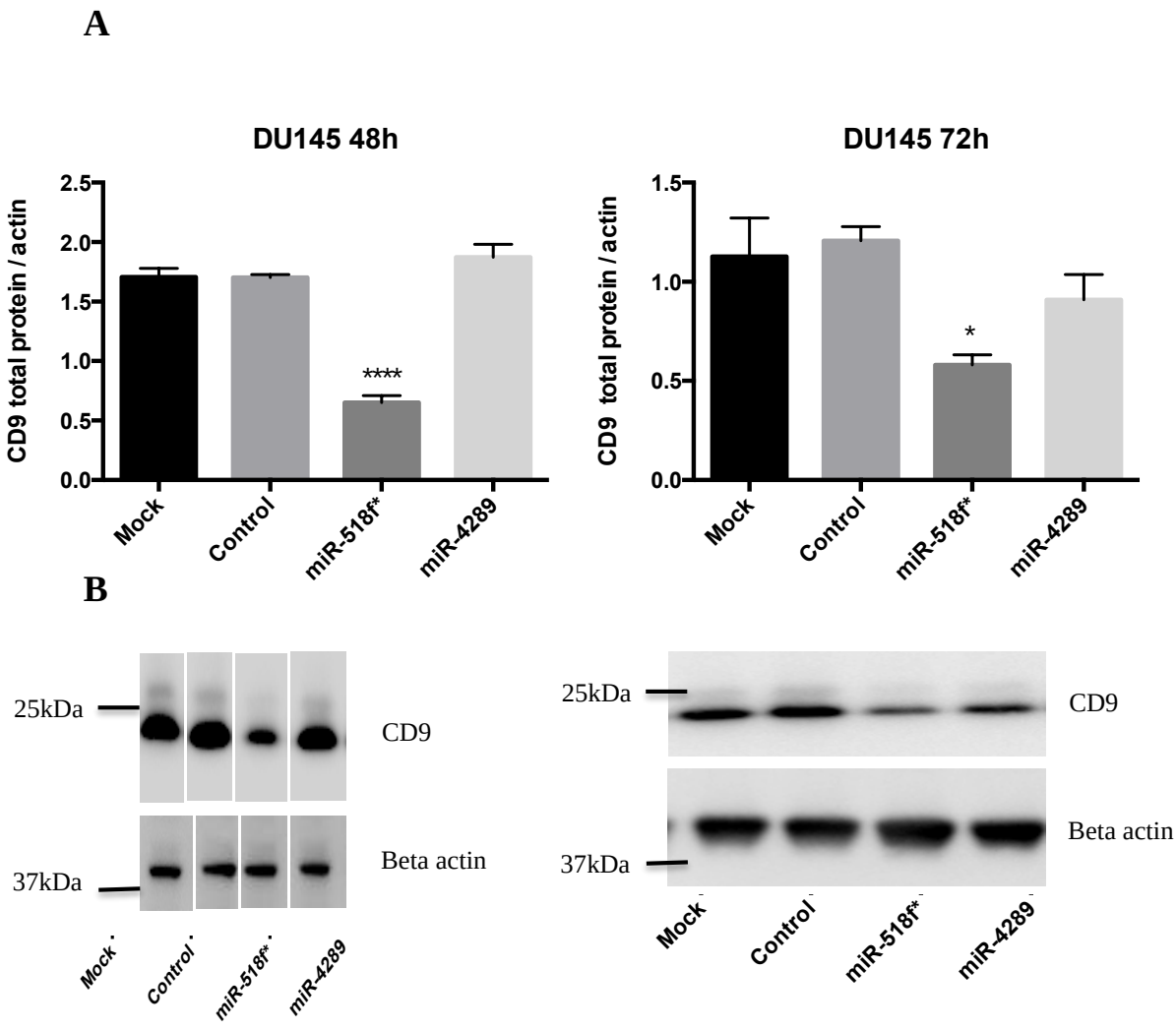

Figure 4.7. miR-518f* decreases CD9 protein expression in DU145 prostate cancer cells. A. DU145 prostate cancer cells were transfected with miR-518f* and miR-4289 mimics to assess their effect on CD9 protein expression. Protein was lysed with 1% NP-40 lysis buffer 48 h and 72 h post-transfection. Protein was electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose and probed for CD9 using 1AA2 antibody. Protein bands were quantitated using densitometry. Results are shown as CD9 total protein normalised to beta actin protein expression (arbitrary units), which served as the loading control, *n=3*. B. Representative western blot depicting quantitated protein expression, bands were cropped to allow order seen however lysates were run on the same gel. Transfection with miR-518f* reduced CD9 protein expression in DU145 prostate cancer cells at 48 and 72 h post-transfection, whereas miR-4289 had no effect on CD9 protein expression at 48h but led to a slight reduction in CD9 protein expression at 72 h post-transfection; *p=0.02*, p<0.0001***.*

4.2.7 miR-518f* influences prostate cell migration and adhesion but not proliferation

The functions of miR-518f* are currently unknown, however based on the functions of the genes it is predicted to regulate, miR-518f* may be involved in cancer processes such as cell proliferation, migration and adhesion to the extracellular matrix. To determine whether miR-518f* is involved in these processes, a miR-518f* mimic was transiently transfected into non-tumourigenic RWPE1 and DU145 prostate cancer cells and their proliferative, migratory and adhesive capacity was assessed 48 h post-transfection. Overexpression of miR-518f* in RWPE1 cells led to no change in cell proliferation over 96 h, significantly decreased initial adhesion to fibronectin (FN) and basement membrane extract (BME) over 1 h and significantly increased migration of RWPE1 cells at both 24 h and 48 h time points compared to control cells (Fig 4.8). In DU145 prostate cancer cells, increased expression of miR-518f* had no effect on cell proliferation or adhesion to fibronectin and BME, however a significant decrease in migration was observed after 48 h (Fig 4.9). Reduction in CD9 expression over the course of the functional assays was confirmed using western blotting at the experimental end point (Fig 4.8 & 4.9).

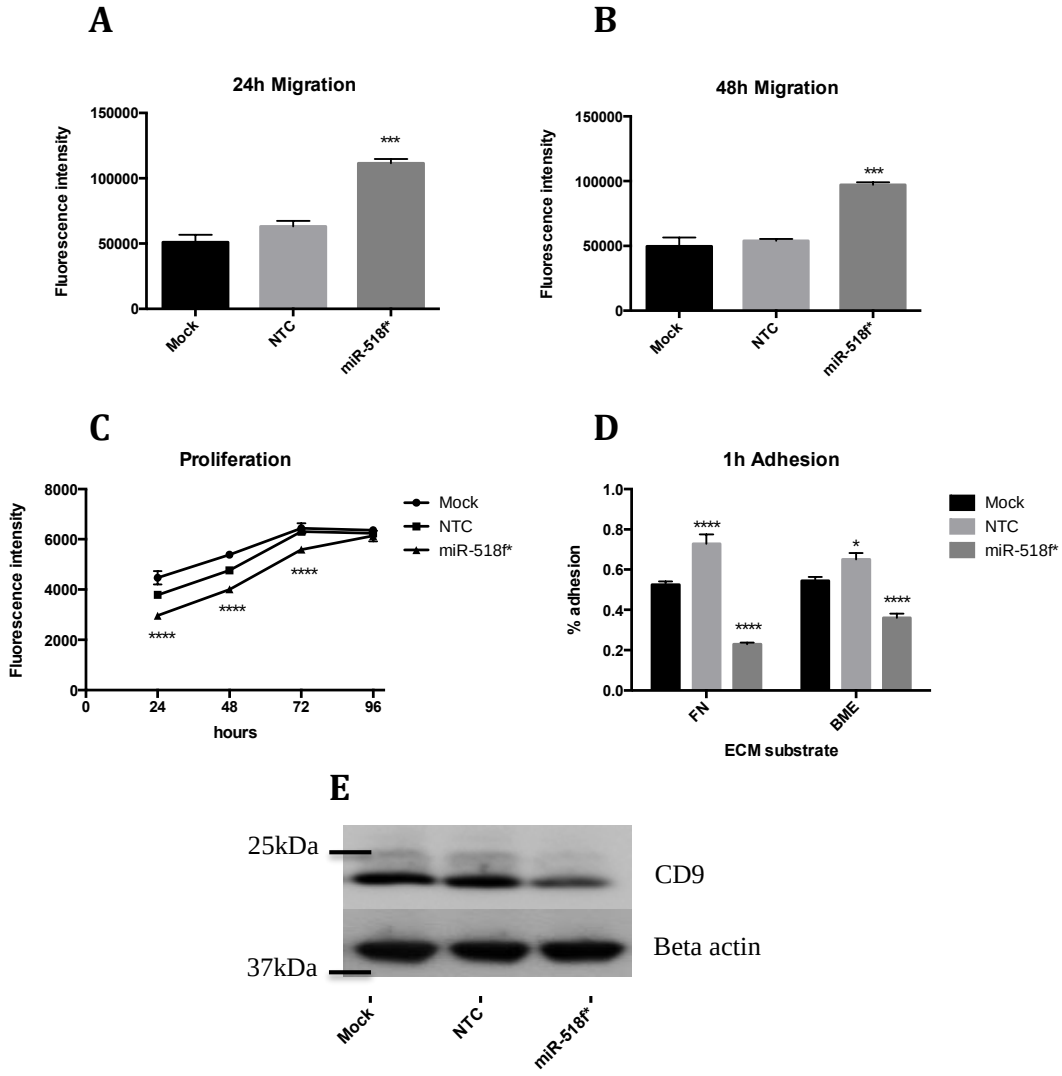

Figure 4.8. miR-518f* increased RWPE1 migration and decreased initial adhesion to ECM substrates. A & B. RWPE1 prostate cells were transiently transfected with a miR-518f* mimic for 48 h, loaded with a fluorescence stain (Calcein AM) and seeded into a transwell migration insert and incubated at 37°C 5% CO_2 for 18 h (A) or 24 h (B). The lower chamber contained media with 10% FBS as a chemo-attractant. Following migration assay, migratory cells were labelled with Calcein AM and the fluorescence intensity was measured. Results are shown as fluorescence intensity of migratory cells normalised to fluorescence intensity of RWPE1 cells prior to commencement of the assay. C. 48 h post-transfection of miR-518f* mimic, RWPE1 cells were seeded into 96 well plates and cell proliferation was assessed by incubating cells in media containing resazurin for 5 h and measuring fluorescence intensity each day up til 72 h. D. 48h post-transfection, RWPE1 cells were loaded with Calcein AM and seeded into 96 well plates coated with fibronectin and BME and incubated at 37°C 5% CO_2 for 1 h. Total cell fluorescence was measured, after which non-adherent cells were washed away with PBS and the fluorescence of adherent cells was measured. Results are expressed as fluorescence intensity of adherent cells / fluorescence intensity

of total cells to give % adhesion. E. 72 h post-transfection of miR-518f* mimic, RWPE1 cells were lysed in 1% NP40 buffer, protein was separated using SDS-PAGE and western blotting with the 1AA2 (CD9) antibody was used to detect CD9 total protein levels. All experiments consisted of mock cells (cells with transfection reagent only) and control cells (cells transfected with a scrambled non-targeting miRNA mimic negative control); *n=3* (except for E which is *n=1*) and all y-axes are shown as arbitrary units. Migration graphs were analysed using ANOVA with bonferroni multiple testing, adhesion and proliferation was analysed using 2-way ANOVA with bonferroni testing; *p=0.01*, p=0.0005***, p<0.0001****.*

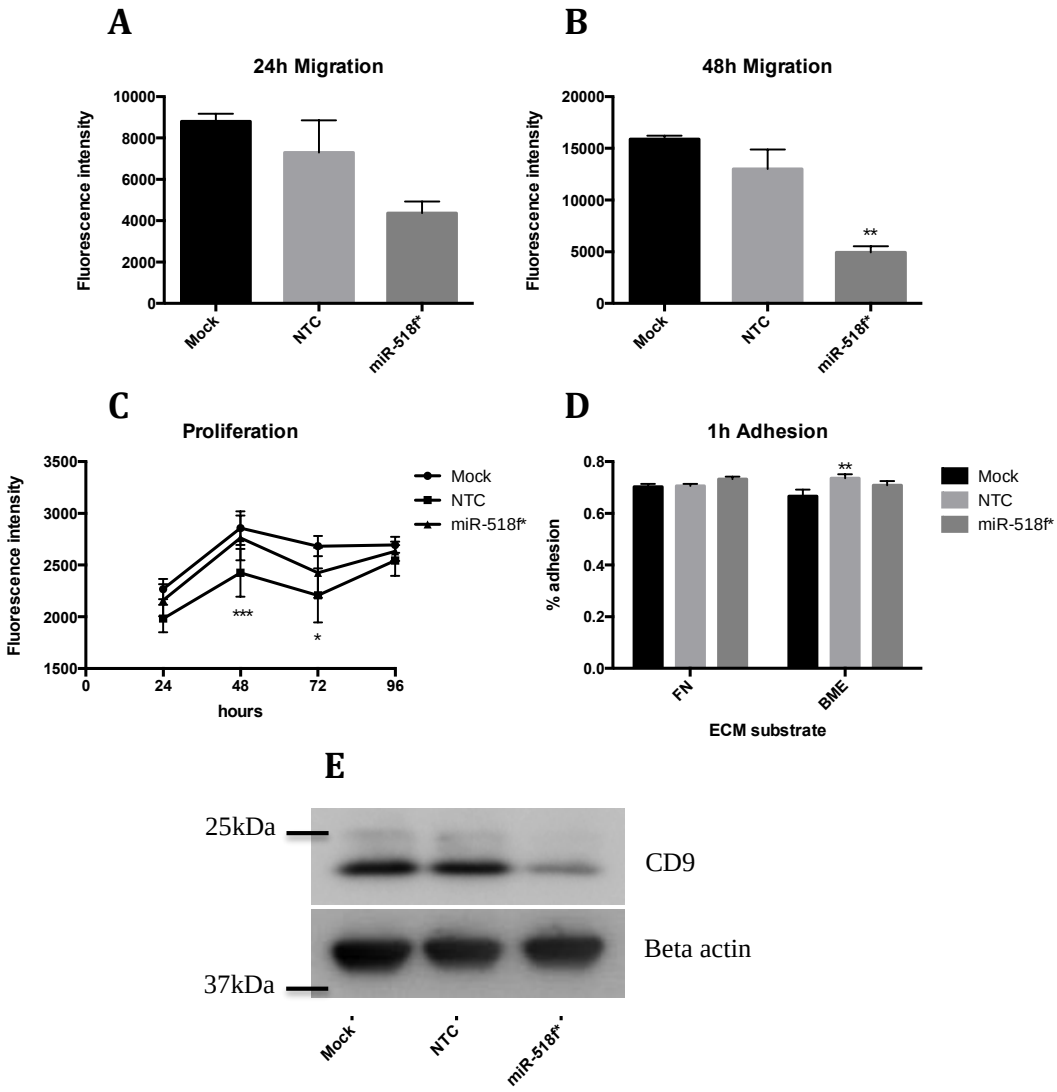

Figure 4.9 miR-518f* significantly decreases DU145 prostate cancer cell migration. A & B. DU145 prostate cancer cells were transiently transfected with a miR-518f* mimic for 48 h, loaded with a fluorescence stain (Calcein AM) and seeded into a transwell migration insert and incubated at 37°C 5% CO_2 for 24 h (A) or 48 h (B). The lower chamber contained media with 10% FBS as a chemoattractant. Following migration assay, migratory cells were labelled with Calcein AM and the fluorescence intensity was measured. Results are shown as fluorescence intensity of migratory cells normalised to fluorescence intensity of DU145 cells prior to commencement of the assay. C. 48 h post-transfection of miR-518f* mimic, DU145 cells were seeded into 96 well plates and cell proliferation was assessed by incubating cells in media containing resazurin for 5 h and measuring fluorescence intensity each day up til 72 h. D. 48 h post-transfection, DU145 cells were loaded with Calcein AM and seeded into 96 well plates coated with fibronectin and BME and incubated at 37°C 5% CO_2 for 1 h. Total cell fluorescence was measured, after which non-adherent cells were washed away with PBS and the fluorescence of adherent cells was measured. Results are expressed as fluorescence intensity of adherent cells / fluorescence intensity of total cells to give % adhesion. E. 72 h post-transfection of miR-518f* mimic, DU145 cells were lysed in 1%

NP40 buffer, protein was separated using SDS-PAGE and western blotting with the 1AA2 (CD9) antibody was used to detect CD9 total protein levels. All experiments consisted of mock cells (cells with transfection reagent only) and control cells (cells transfected with a scrambled non-targeting miRNA mimic negative control); *n=3* (except for E which is *n=1*) and all y-axes are shown as arbitrary units; *p=0.01*, p=0.05**, p=0.0001***.*

4.3 Discussion

Tetraspanin CD9 expression is dysregulated in many types of cancers. CD9 is often referred to as a metastasis suppressor, however it has been found to enhance progression in some tumour types such as melanoma, fibrosarcoma and some types of breast cancer [217-219]. To date only 2 studies have considered the *in vivo* effects of CD9 in prostate cancer progression showing conflicting results [94, 220]. Overexpression of CD9 in a metastatic prostate cancer cell lines increased its invasiveness *in vitro*, but had no effect on tumourigenicity or metastasis *in vivo* [94]. In contrast, CD9 knockdown mice crossed onto a prostate cancer mouse model led to a significant decrease in spontaneous metastasis to the liver [220], thereby implicating CD9 in prostate cancer progression and metastasis.

Given that CD151 is deregulated in prostate cancers and is at least partially regulated by miRNA (Chapter 3), it was hypothesised that CD9 may also be under the control of miRNA in prostate cancer. This chapter aimed to investigate whether miRNAs regulate CD9 and if specific miRNAs are responsible for decreased CD9 protein expression in prostate cancers. In addition, the functional relevance of these miRNAs to prostate cancer progression was also assessed. CD9 mRNA, total protein and cell surface protein

expression levels were firstly characterised in a panel of prostate cell lines. Non-tumourigenic and prostate cancer cell lines displayed similar levels of CD9 mRNA and total protein, however highly tumourigenic prostate cancer cell lines appeared to have lower CD9 cell surface expression. In most solid malignancies a reduction in CD9 protein expression is commonly seen, however decreased cell surface CD9 protein expression may be more relevant as it this population of CD9 that forms TEMs with other tetraspanins and proteins. Decreased CD9 on the cell surface would disrupt the organisation of tetraspanin enriched microdomains and hence signalling from the membrane into the cell. This may have a significant impact on modulation of cancer related processes such as cell adhesion, migration and invasion, which play a crucial role in cancer progression and metastasis. This is highly likely based on recent findings from our laboratory, which showed CD9 knock out TRAMP mice had a significant reduction in metastasis to the liver [220].

In contrast, to what is typically observed in cancer, LNCaP cells, which are derived from a lymph node metastasis of prostate cancer, had high levels of CD9 mRNA, CD9 total and cell surface protein. There is some literature to suggest that up-regulation of CD9, especially on the cell surface, may serve an important role in communication between tumour cells and the microenvironment, allowing transendothelial migration which promotes metastasis [224]. Therefore, LNCaP cells may have increased expression of CD9 in order to traverse into the circulation from the lymphatic system.

This is the first study to characterise CD9 mRNA, total protein and cell surface expression in a range of non-tumourigenic and prostate cancer cell lines. It was expected that the more aggressive prostate cancer cell lines would have a dramatic

reduction in CD9 total protein and cell surface expression however only a modest decrease was observed. The prostate cell line panel is an *in vitro* model that is not perfect and therefore factors such as cell confluency and a lack of interactions with other cell types (e.g. stroma) could all affect CD9 expression levels and may be possible reasons for observing what was expected. However the utility and ease of manipulation of *in vitro* systems makes them worthwhile for studying downstream functional effects. In addition, CD9 mRNA and protein levels did not correlate, but an inverse trend was observed. CD9 total protein and CD9 cell surface protein levels showed a positive correlation that was nearing significance. Therefore, prostate cell lines with high CD9 mRNA typically displayed low levels of CD9 protein, suggesting that CD9 expression may be post-transcriptionally silenced by miRNAs.

Post-transcriptional regulation of CD9 by miRNAs was investigated using the same approach that was utilised for CD151 outlined in chapter 3. Briefly, bioinformatics analysis revealed many miRNAs are predicted to bind to CD9, and a CD9 3'UTR luciferase reporter assay showed that PC3 and DU145 prostate cancer cells endogenously express miRNAs that are capable of binding to the CD9 3'UTR as these cell lines had reduced luciferase expression compared to non-tumorigenic prostate cells. Therefore, a lack of correlation between mRNA and protein levels for CD9 implies that in these cell lines more mRNA does not lead to more protein. This coupled with clear differences in how the CD9 3'UTR is regulated in prostate cancer versus non-cancerous prostate cell lines suggested that miRNAs may regulate CD9 expression and may be one of the mechanisms responsible for decreased CD9 protein levels in some prostate cancers.

miR-25 and miR-345 had previously been implicated in prostate cancer, however in this study they were not found to be significantly overexpressed in prostate cancer cell lines compared to non-cancerous prostate cells. This may be due to a lack of statistical power in this experiment, or miR-25 and miR-345 may not be overexpressed in these particular prostate cancer cell lines compared to human prostate cancer tissues. It is possible that miR-25 and miR-345 have variable expression in prostate cancer cell lines derived from different metastatic sites. Co-transfection of miR-25 and miR-345 mimics with the CD9 3'UTR only led to modest changes in luciferase reporter expression and therefore, as this thesis is focused on the effects of miRNA on tetraspanins rather than prostate cancer in general, the effect of these miRNAs was not further explored.

CD9 3'UTR luciferase assay results combined with bioinformatics analysis and miRNA profiling of the prostate cell lines identified miR-518f* as a potential CD9 regulator. Transfection of a miR-518f* mimic into RWPE1 (non-tumourigenic) and DU145 (prostate cancer) cells resulted in a significant decrease in CD9 total protein expression. RWPE1 cells displayed no endogenous repression / stabilisation of the CD9 3'UTR whereas DU145 cells showed repression of the luciferase reporter. This suggests that miR-518f* is either not endogenously expressed or expressed at low levels in RWPE1 cells or predominately regulates other mRNA transcripts. Interrogation of the signal values from the microarray suggests that the former is most likely. However, transfection of miR-518f* into RWPE1 cells, led to a decrease in CD9 protein levels suggesting that the lack of endogenous repression observed in RWPE1 cells is likely due to minimal expression of miR-518f*. In comparison, DU145 cells showed endogenous repression of CD9 and higher levels of miR-518f* (microarray signal

values) suggesting that miR-518f* may be partly responsible for the observed repression in this cell line.

In DU145 prostate cancer cells, miR-518f* had no effect on cell proliferation, however decreased adhesion to fibronectin and BME was observed with RWPE1 cells. Moreover, miR-518f* appeared to decrease RWPE1 proliferation, however due to technical issues this result is unreliable. Manipulation of CD9 expression has been typically shown to have little effect on prostate cell proliferation [220], which is consistent with what was observed in this study, however this has not been extensively studied. CD9 is usually important for adhesion to the ECM, however a change in adhesion was only observed in RWPE1 cells not DU145 prostate cancer cells. CD9 has been shown to bind to fibronectin [225], therefore the decrease in CD9 protein levels from miR-518f* may disrupt this interaction resulting in reduced RWPE1 cell adhesion to fibronectin. This has also been observed in RWPE1 cells with stable knockdown of CD9 (Bond *et al* unpublished). Moreover, BME is used to recapitulate the ECM *in vitro*, with decreased levels of CD9 resulting in decreased cell adhesion to BME, suggesting that CD9 interacts with integrins and other proteins known to be involved in adhesion. This was expected as CD9 is known to interact with integrins and other proteins in TEMs, which modulate cell adhesion [34]. It is unclear however why miR-518f* specifically had an effect on adhesion of RWPE1 prostate cells but not DU145 prostate cancer cells. In addition, miR-518f* may affect adhesion of prostate cells to other ECM substrates such as collagens or laminins and/or may have a longer lasting effect on adhesion, not just initial adhesion which was tested in this study.

Transfection of miR-518f* did however lead to enhanced migration of RWPE1 cells, but inhibited migration of DU145 prostate cancer cells. The differential effect of miR-

518f* on migration of non-tumourigenic prostate cells compared to a prostate cancer cell line is interesting. This is likely to be influenced by differences in the expression of miR-518f* target mRNAs including CD9, as well as the expression of other miRNAs that can work co-operatively with or against the actions of miR-518f*. Moreover, CD9 may be involved in different pathways associated with migration in normal compared to prostate cancer cells. There is very little known about the functions of miR-518f*. It has been reported to be a placental specific miRNA, however has also been detected in peripheral blood of healthy non-pregnant women [226, 227]. There have been no studies investigating the function of miR-518f* and its mRNA targets are unknown. Publically available target prediction databases infer that miR-518f* regulates genes involved in cancer related pathways such as Wnt signalling, integrin signalling and angiogenesis. Interestingly, many proteins from these pathways are also CD9 or tetraspanin protein partners and are all involved in cancer progression. Therefore, the results from this chapter suggest that miR-518f* may play an important role in prostate cancer progression via modulation of migration through regulation of CD9 expression and potentially a range of other cancer related proteins. This would need to be confirmed by stable overexpression and knockdown of miR-518f* in normal prostate and prostate cancer cell lines with assessment of functional effects *in vitro* and in mouse models of prostate cancer *in vivo*.

From the approach taken it is unclear whether miR-518f* is having a direct effect on CD9 mRNA / protein levels or whether it is regulating CD9 expression indirectly. A dual luciferase CD9 3'UTR reporter assay showed that miR-518f* binds to the CD9 3'UTR and lowered luciferase expression within 24h of transfection. Ideally, this should be complemented through introducing mutations in the region that miR-518f* is

predicted to bind to the CD9 3'UTR to provide further evidence of direct binding. It is unlikely that within 24h miR-518f* could have a substantial indirect effect on the CD9 3'UTR given the concentration of miR-518f* that is transfected. However, miR-518f*could be functioning synergistically with other miRNAs or RNA binding proteins (RBP) which bind in the same region or nearby, and this could be amplifying the effect on CD9 expression [228]. Moreover, transfection of miR-518f* into cell lines may be exerting an indirect effect on endogenous CD9 expression via modulation of transcription factors (e.g. SP1), RBPs (e.g. HuR) or other proteins which play a critical role in regulating CD9 mRNA or protein stability. Considering the predicted targets of miR-158f* it is quite plausible that an interaction is occurring as the SP1 transcription factor, which is known to modulate tetraspanins [229], and is a predicted miR-518f* target. Furthermore, miR-518f* is also predicted to regulate expression of the RBP HuR which has been shown to differentially influence CD9 mRNA stability in breast cancer cell lines [125].

In this chapter, CD9 mRNA and protein levels were found to be similar in non-tumourigenic and prostate cancer cell lines. However, most prostate cancer cells had decreased CD9 cell surface expression compared to non-tumourigenic prostate cells. Bioinformatics, miRNA profiling and a CD9 3'UTR luciferase reporter assay inferred that miRNAs regulate CD9 expression. miR-518f* was predicted to regulate CD9, was found to bind to the CD9 3'UTR *in vitro* and transfection of miR-518f* significantly reduced CD9 protein expression. Moreover, transfection of miR-518f* led to a differential effect on migration, promoting migration and decreasing adhesion of a non-tumourigenic prostate cells line and reduced migration of a prostate cancer cell line. miR-518f* is also predicted to regulate a range of mRNAs/proteins that are commonly

involved in cancer and therefore, miR-518f* could potentially serve an important role in prostate cancer progression.

Chapter 5: Post-transcriptional Regulation of CD151 & CD9 in breast cancers

5.1 General Introduction

Following on from chapters 3 & 4, it was of interest to elucidate if miRNAs also regulate CD151 and CD9 in breast cancers, since as stated previously, there is very little known about how CD151 and CD9 levels are regulated in normal cells or cancer cells. Similarly to prostate cancer, CD151 protein levels are typically upregulated and CD9 protein expression is commonly downregulated in breast cancers. Previous work from our laboratory has shown that CD151 deletion with the MMTV/PyMT mouse model of breast cancer results in reduced numbers and size of primary tumours with no change to metastases, suggesting that CD151 is involved in primary breast tumour growth [77]. Other groups have shown that depletion of CD151 in a basal breast cancer line led to delayed tumour progression in a mouse xenograft model [74] and $CD151^{-/-}$ MDA-MB-231 xenografts showed less vascularisation and impaired communication with endothelial cells *in vitro* compared to $CD151^{+/+}$ controls [76]. Therefore, CD151 appears to have a multi-faceted role in animal models of breast cancer tumourigenesis and progression, particularly in basal breast cancers.

In human clinical studies, CD151 protein expression is elevated in breast cancers, particularly high grade and triple negative breast cancers [74]. Moreover, high CD151 protein expression is associated with high grade DCIS [230] and decreased overall survival of invasive breast cancers [75, 76]. Therefore, high CD151 expression is typically seen in more advanced stage breast cancers and predicts poor prognosis, suggesting that CD151 is involved in breast cancer progression and metastasis.

CD151 has also been shown to modulate breast cancer cell adhesion, migration and invasion *in vitro,* typically via interactions with various integrins and other signalling molecules. CD151 interactions with laminin-binding integrins (alpha3beta1 & alpha6beta4) are important for integrin signalling [76], ERBB2-dependent signalling [78, 80] and GTPase signalling in breast cancer cells, promoting breast cancer invasion and progression. In fact, CD151 knockdown has been shown to sensitize breast cancer cells to anti-ERBB2 therapies [79]. Knockdown of CD151 also reduced migration, invasion and spreading of basal-like MDA-MB-231 cells due to a reduction in signalling and disruption of interactions between alpha6 integrin and EGFR [74]. Moreover, CD151 has been shown to influence HGF/c-met signalling in breast cancer cells [231], and TGF-beta1 signalling leading to TGF-beta1 induced breast cancer metastasis [232]. In addition, CD151 promotes associations between alpha3 integrin and CD9 and is needed for cell adhesion and cell spreading of MDA-MB-231 cells on laminin-332 [233] and interactions between CD151 and alpha6 integrin are important for breast cancer cell adhesion [234]. Depletion of CD151 has also been shown to inhibit the growth of non-tumourigenic breast cells in 3D culture [230]. Therefore, CD151 is critically involved in many aspects of breast cancer progression and metastasis through modulating various signalling pathways commonly via interactions with integrins.

CD9 is overexpressed in bone metastases compared to primary tumours and visceral metastases and is overexpressed in osteotropic breast cancer cells. An antibody against CD9 was shown to delay homing of a subpopulation of MDA-MB-231 cells which metastasize to bone (B02 cell line) and inhibit bone destruction, suggesting that CD9 expression plays a specific role in enhancing bone metastasis of breast cancer [219].

However, the majority of the literature shows a reduction in CD9 expression in breast tumours, with a more pronounced decrease with breast cancer progression, particularly in lymph node metastases [98-100, 102, 103]. Loss of CD9 with expression of CD31 or VEGF, which are markers of angiogenesis, in breast cancer showed a slight positive correlation with lymph node metastasis [235]. These studies collectively show that high expression of CD9 may promote bone metastasis, but low expression of CD9 appears to enhance lymph node metastasis, suggesting a dual role for CD9 in breast cancer progression.

Typically, a reduction in CD9 protein expression predicts poor patient prognosis in breast cancer. An early study showed that tumours with high expression of CD9 responded better to therapy and patients experienced prolonged survival following diagnosis of metastatic disease [96]. Moreover, patients with breast cancer that are positive for CD9 expression have significantly higher overall survival [103], disease-free survival [98], 5-year survival [98] and relapse-free survival [100] rates compared to patients who have tumours with low CD9 expression or are CD9 negative. However, one study has shown that CD9 protein expression is unlikely to provide any useful further information about breast cancer prognosis [97].

Most in vitro studies concerning CD9 functions in breast cancer have revolved around its modulation of motility/migration. The first study investigating CD9 function showed that tumour cells transfected with CD9 cDNA have low motility and low metastatic potential [103]. Since then, CD9 knock down breast cancer cells were shown to have decreased motility [104, 233] and cell spreading [233], often involving impaired integrin signalling [233]. CD9 has also been shown to associate with and activate beta1

integrins and in conjunction with MMP8 appears to influence cancer cell extravasation *in vivo* [236]. Moreover, native type IV collagen transiently increased CD9 cell surface expression in MDA-MB-231 breast cancer cells resulting in increased migration via a DDR1/CD9-dependent pathway [105]. More recently, a nuclear pool of CD9 protein was observed in breast cancer cell lines and invasive ductal carcinomas and it has been shown to be involved in the mitotic process [237]. Therefore, CD9 appears to play a complex role in cancer cell function, particularly in breast cancer cell migration.

The lack of knowledge about CD151 and CD9 regulation was highlighted in chapters 3 and 4, however recently there has been new evidence that miRNAs regulate CD151 expression in breast cancer cell lines. Transfection of miR-124 and miR-506, which share the same seed sequence, into breast cancer cell lines resulted in a decrease in CD151 mRNA and protein expression [170, 171]. Transfection of these miRNAs induced EMT, resulting in changes to breast cancer cell adhesion, migration, proliferation and invasion [170, 171]. Therefore, given that CD151 and CD9 are also important to breast cancer and its progression to metastastic disease and following on from chapters 3 & 4, it was also of interest to elucidate if miRNAs also regulate CD151 and CD9 in breast cancer cell lines.

5.2 Results

5.2.1 Characterisation of CD151 expression in breast cell lines

CD151 mRNA expression was characterised using real time PCR to assess differences in CD151 mRNA levels in non-tumourigenic breast cells compared to breast cancer cell lines. Non-tumourigenic basal breast epithelial cells appeared to have the highest levels of CD151 mRNA (HMECs & 184A1) compared to breast cancer cell lines (MCF7, T47D, SKBR3 & MDA-MB-231) (Fig 5.1A). MCF7 and T47D which are ER+ breast cancer cell lines displayed the lowest levels of CD151 mRNA compared to HER2+ SKBR3 and triple negative MDA-MB-231 breast cancer cells which had similar levels to non-tumourigenic breast cells (Fig 5.1A). MCF7, T47D and SKBR3 breast cancer cells had very low levels of CD151 total protein and cell surface CD151 protein compared to MDA-MB-231 breast cancer cells and non-tumourigenic breast cells which showed high CD151 protein expression (Fig 5.1B-D). In addition, linear regression analysis revealed that CD151 total protein expression and CD151 cell surface protein expression displayed a significant positive correlation (R^2 0.77, $p = 0.02$; Fig 5.2C). However, analysing total or cell surface CD9 protein expression with CD9 mRNA levels only showed a trend towards positive correlation (Fig 5.2A & B).

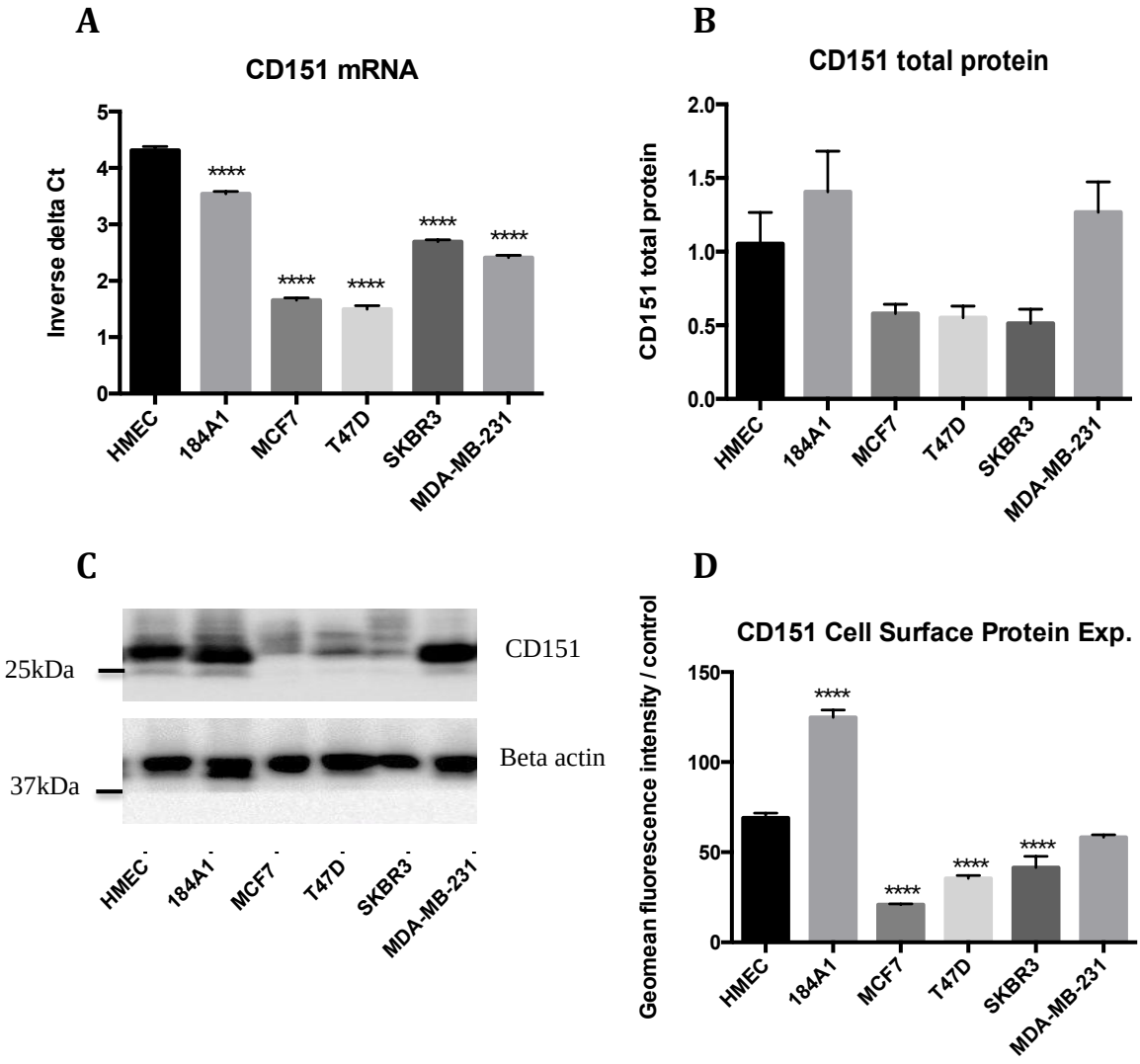

Figure 5.1 CD151 expression in a panel of normal breast and breast cancer cell lines. A. CD151 mRNA expression measured using real time PCR. Total RNA was extracted using TRIzol-chloroform method and reverse transcribed into cDNA using superscript II. Results are shown as inverse delta Ct normalised to the geometric mean of three housekeeping genes. B. CD151 total protein expression across all breast cell lines was measured using SDS-PAGE and western blotting using the IIB1 (CD151) primary antibody. Blots were quantitated using densitometry and are expressed as pixels minus background/mm^2 and are normalised to the loading control beta actin. C. Representative western blot of CD151 total protein expression in the panel of breast cell lines. D. Cell surface CD151 protein expression was measured using flow cytometry with the 11B1 antibody and stained with a FITC conjugated secondary antibody. Results are shown as geometric mean of fluorescence intensity of FITC normalised to isotype matched control antibody; *n=3* for all graphs, with results shown as arbitrary units; *p<0.0001*****.

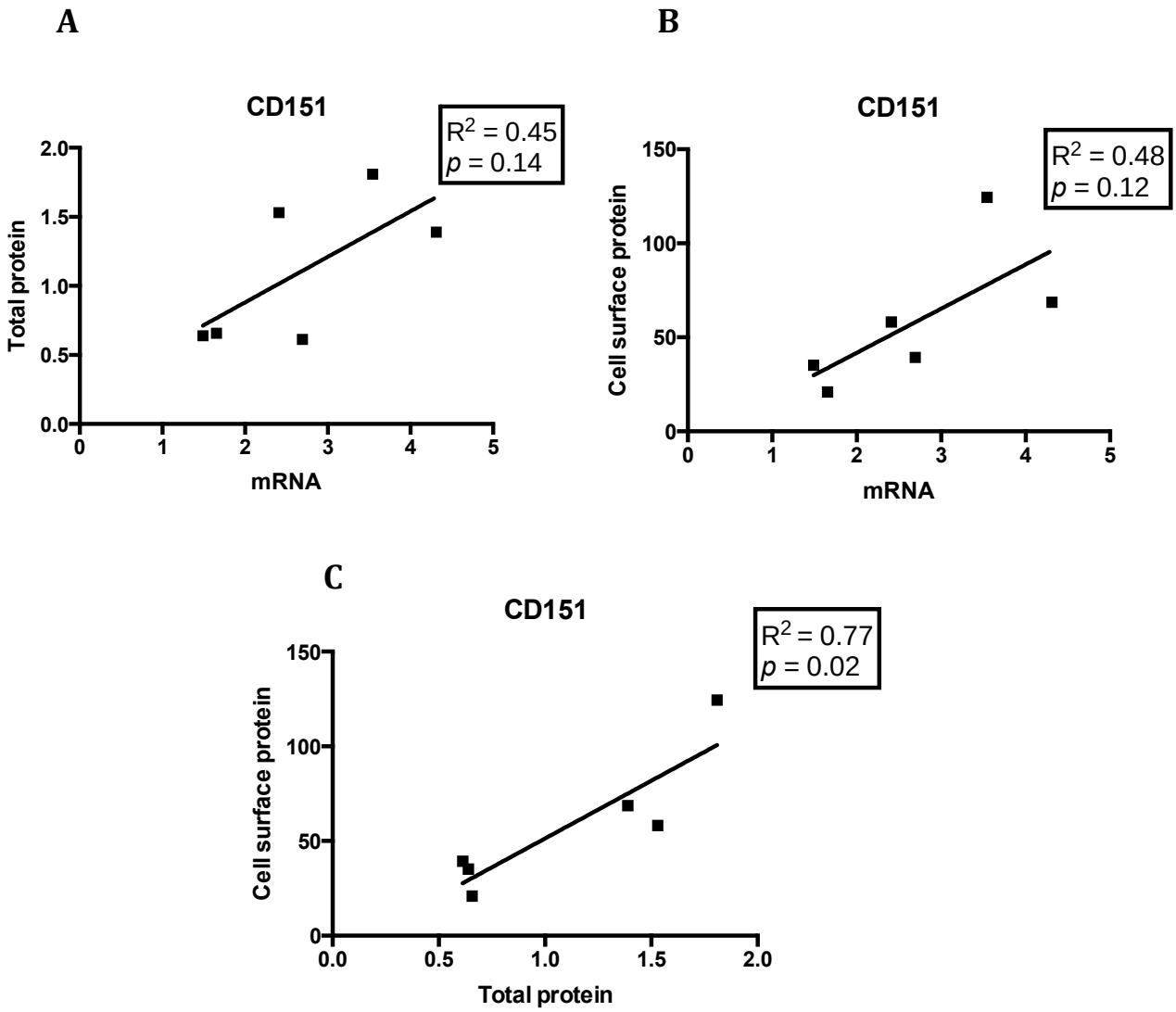

Figure 5.2. Linear regression analysis of CD151 expression in breast cell lines
Linear regression analysis was performed on the geomean of CD151 mRNA, total protein and cell surface protein using GraphPad prism 6 software. A. A Trend towards a positive correlation between CD151 mRNA and CD151 total protein was observed. B. Correlation between CD151 mRNA and CD151 total protein. C. Significant positive correlation between CD151 total protein levels and CD151 cell surface protein expression. Analyses were performed with a *p-value* cut-off of 0.05. Both x and y -axes are shown as arbitrary units.

5.2.2 Characterisation of CD9 expression in a panel of breast cell lines

Tetraspanin CD9 expression was characterised in the same panel of breast cell lines. CD9 mRNA as measured by real time PCR, was expressed at a similar level in all breast cell lines except for T47D and MDA-MB-231 breast cancer cells, which displayed low levels of CD9 mRNA (Fig 5.3A). In terms of protein expression, SKBR3, MDA-MB-231 and T47D breast cancer cells had low expression of CD9 total protein compared to low tumourigenic MCF7 and non-tumourigenic HMEC and 184A1 cells (Fig 5.3B & C). Moreover, T47D and MDA-MB-231 breast cancer cells displayed very low levels of CD9 cell surface expression compared to all other breast cell lines (Fig 5.3D). Similar to CD151, CD9 mRNA and total protein expression were found to significantly positively correlate (R^2 = 0.76, p = 0.02; Fig 5.4A). In addition, CD9 mRNA versus CD9 cell surface protein expression, and CD9 total protein versus CD9 cell surface protein levels only showed a trend towards positive correlation (Fig 5.4B & D).

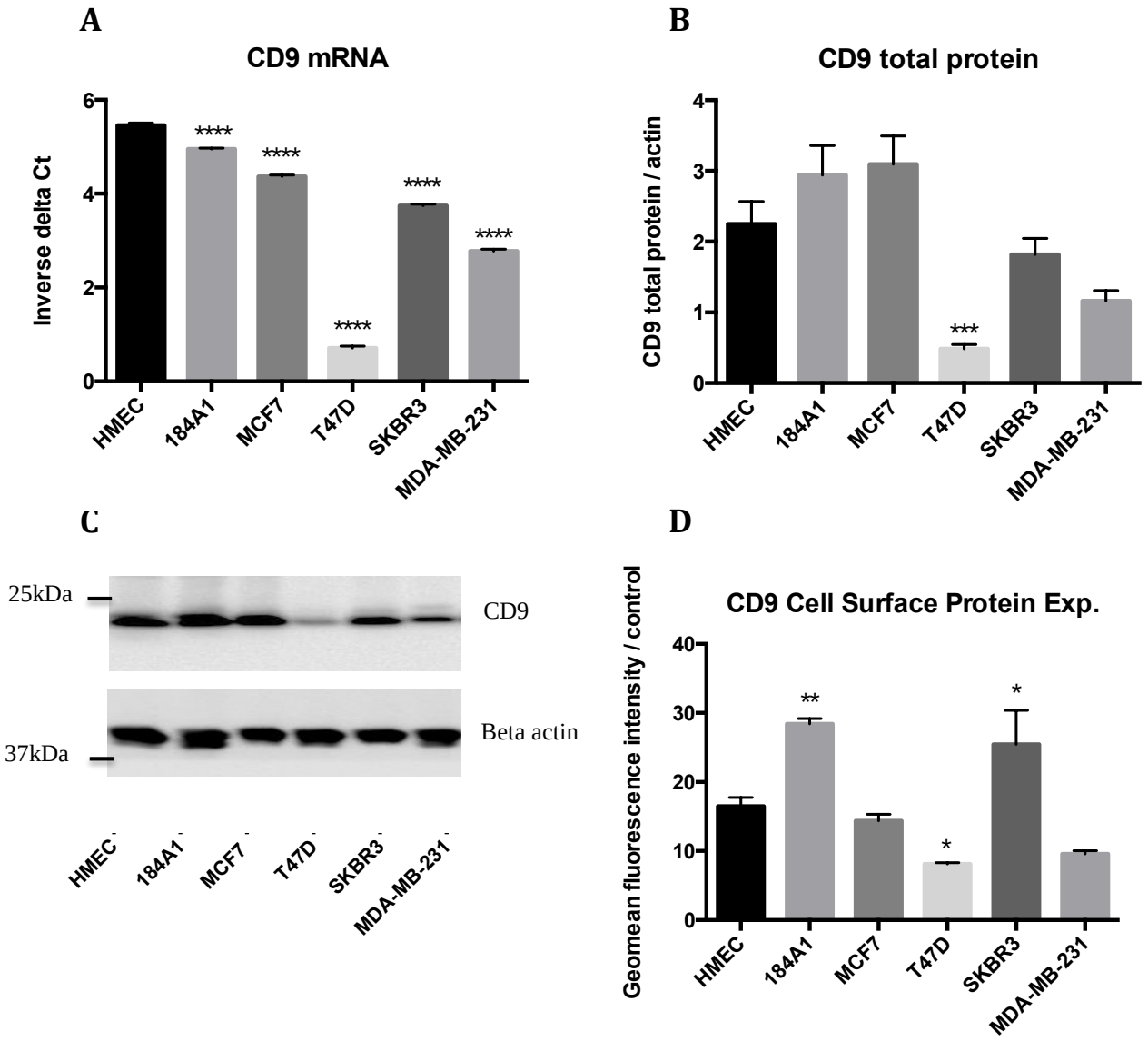

Figure 5.3 Characterisation of CD9 mRNA and protein expression in normal breast and breast cancer cell lines. A. CD9 mRNA expression was quantitated using real time PCR. Briefly, RNA was isolated using TRIzol-chloroform, reverse transcribed into cDNA and quantitated using real time PCR. Results are shown as geometric mean of inverse delta Ct normalised to three housekeeping genes. B. CD9 total protein was assessed by SDS-PAGE and western blotting with the 1AA2 (CD9) antibody. Protein bands were quantitated using densitometry on multi-gauge. Results are shown as pixel count minus background/mm^2 normalised to beta actin loading control. C. Representative western blot of CD151 total protein expression in breast cell lines. D. CD9 cell surface protein expression was measured using flow cytometry with the 1AA2 (CD9) antibody and stained with a FITC conjugated secondary antibody. Results are shown as geomean fluorescence intensity of FITC minus fluorescence intensity of isotype matched antibody control; *n=3* for all graphs with the y-axis shown as arbitrary units; *p<0.05*, p=0.002**, p=0.0009***, p<0.0001****.*

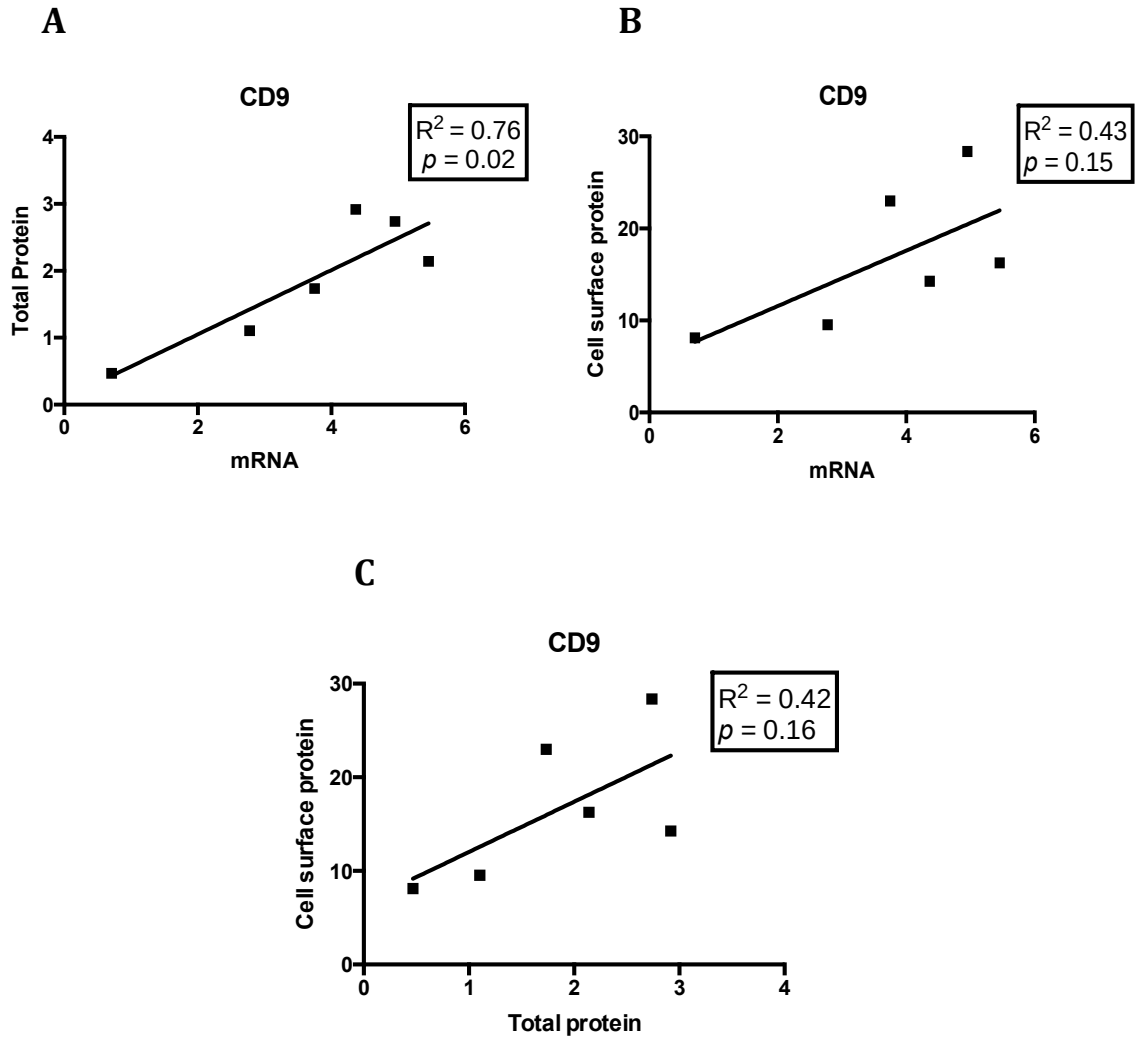

Figure 5.4. Analysis of correlations between CD9 mRNA and protein levels in breast cell lines. Linear regression analyses were performed using the geomean of results from fig 5.3 on GraphPad prism 6. A. Significant positive correlation was found between CD9 mRNA and CD9 total protein expression. B. CD9 mRNA and CD9 cell surface protein expression appeared to show a trend towards a positive correlation. C. CD9 total protein and CD9 cell surface protein expression also appeared to positively correlate. All analyses were performed using a *p-value* cut-off of 0.05 for significance.

5.2.3 Regulation of the CD151 & CD9 3'UTR in breast cell lines

To define the extent to which CD9 and CD151 are controlled by their 3'UTRs in breast cancer, a dual luciferase reporter assay using the CD9 or CD151 3'UTR was undertaken in a panel of normal breast and breast cancer cell lines to determine differences in tetraspanin 3'UTR activity. Non-tumourigenic breast cells displayed more of a decrease in CD151 3'UTR activity by miRNAs compared to breast cancer cell lines which showed little or no repression of CD151 3'UTR activity (Fig 5.5A). Conversely, all breast cells appeared to have increased luciferase output with CD9 3'UTR, with this effect most prominent in non-tumourigenic breast cell lines (Fig 5.5B).

5.2.4 miRNA profiling of human breast cell lines

As miRNA are known to be deregulated in cancer and exert their affects at the 3'UTR, miRNA microarrays were used to determine which miRNAs have altered expression in the panel of breast cancer cells (MCF7, T47D, SKBR3 & MDA-MB-231) compared to non-tumourigenic breast cells (HMEC & 184A1). Cell lines were grouped based on their tumourigenicity in order to define miRNAs with differential expression in non-tumourigenic versus tumourigenic cell lines. GeneSpring software was used to provide statistical analysis.

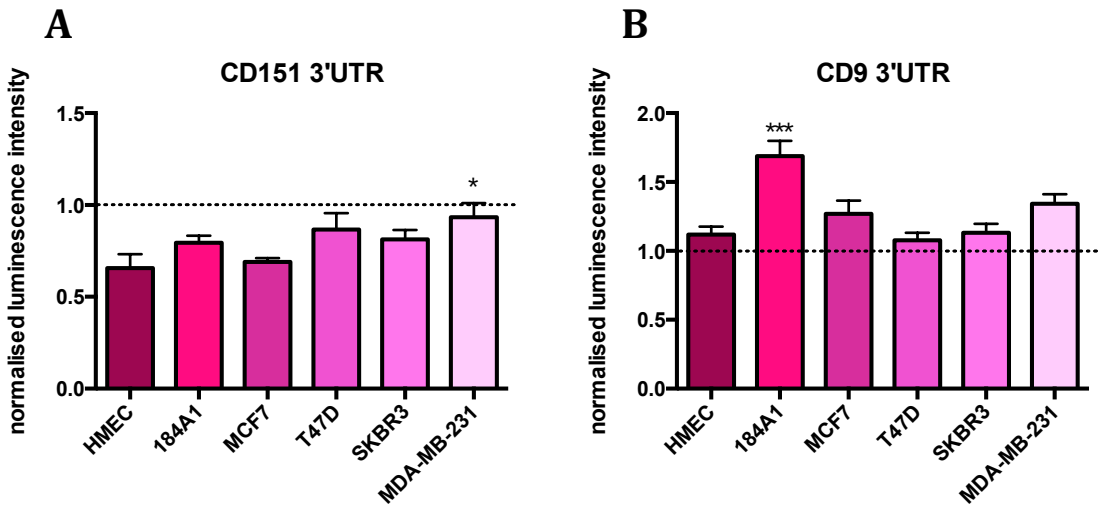

Figure 5.5 CD151 & CD9 3'UTR activity in a panel of normal breast and breast cancer cell lines. Luciferase reporter constructs containing the CD151 or CD9 3'UTR were transfected into a panel of non-tumourigenic and tumourigenic breast cell lines. Luminescence intensity (measure of 3'UTR activity) was determined using a luminometer after 24 h of transfection. A. Non-tumourigenic and lowly tumourigenic breast cancer cells showed the highest level of CD151 3'UTR repression as shown by a decrease in luminescence, compared to more tumourigenic breast cancer cells which displayed very little CD151 3'UTR repression or no change in CD151 3'UTR activity. B. Most breast cell lines displayed an increase in CD9 3'UTR activity (higher than the positive control). Results are expressed as luminescence intensity normalised to firefly luciferase transfection control and empty 3'UTR vector positive control (shown as a dotted line at 1). All graphs depict results (*n=3*) shown as arbitrary units; *p=0.01**, *p=0.0001****.

Many miRNAs were found to be significantly downregulated in breast cancer cell lines compared to non-tumourigenic breast cells, including miR-31* (56 fold), miR-127-3p (110 fold) and miR-1909 (15.5 fold) (Fig 5.6A&B). In addition, several miRNAs were shown to be significantly upregulated in breast cancer such as miR-1226 (8 fold), miR-345 (10 fold), miR-25 (4 fold) and miR-15a (3 fold) (Fig 5.6A & C). Some of the miRNAs that were differentially expressed in breast cancer cell lines compared to non-tumourigenic breast cell lines from the miRNA arrays were further validated using qPCR (Fig 5.7). Two miRNAs, miR-25 (Fig 5.7A) and miR-15a (Fig 5.7B) were

significantly highly expressed in T47D, SKBR3 and MDA-MB-231 breast cancer cells compared to non-tumourigenic HMEC cells. Moreover, SKBR3 and MDA-MB-231 breast cancer cells displayed significantly higher miR-27a (Fig 5.7C) and miR-27b (Fig 5.7D) expression compared to non-tumourigenic HMEC cells.

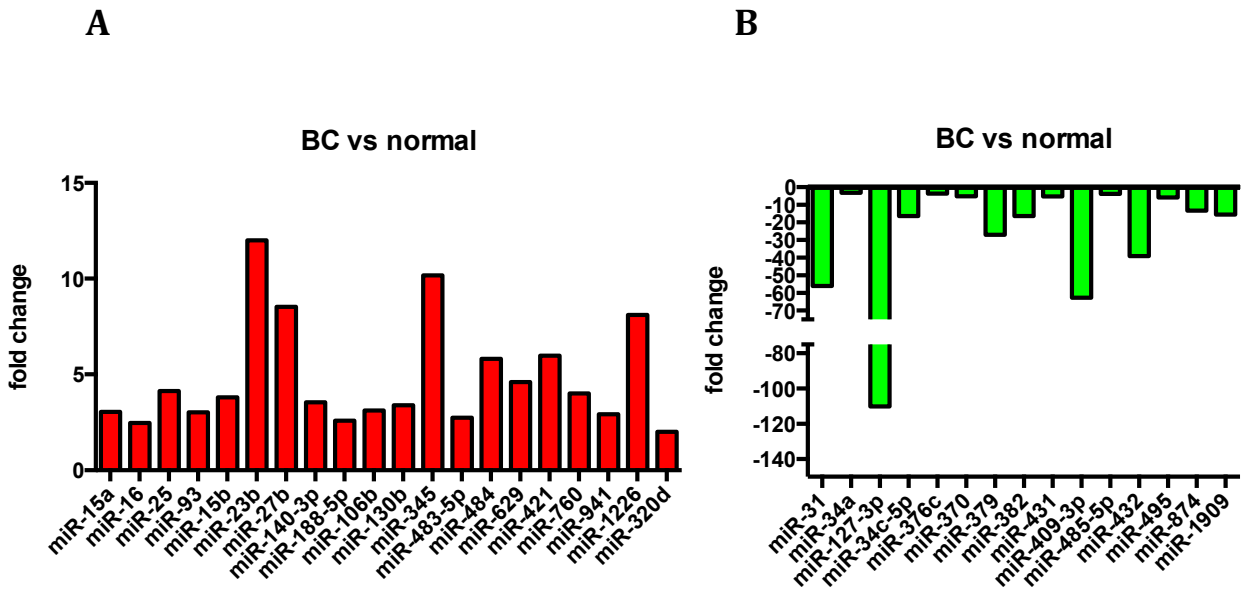

Figure 5.6 Differential expression of miRNAs in breast cancer cells compared to non-tumourigenic breast cells. Total RNA was extracted using TRIzol-chloroform method, amplified, labelled and hybridised to affymterix miRNA microarrays to profile miRNA expression across the panel of breast cell lines. GeneSpring software was used to normalise, filter and statistically analyse the datasets. Breast cell lines were grouped according to tumourigenicity (non-tumourigenic vs tumourigenic) and an unpaired t-test was performed with a *p-value* cut-off of 0.05 for significance and a fold change cut-off of 2. A. miRNA that were significantly upregulated in breast cancer cell lines (MCF7, T47D, SKBR3 & MDA-MB-231) compared to non-tumourigenic breast cells (HMEC & 184A1). B. miRNA that were significantly downregulated in breast cancer cell lines versus non-tumourigenic breast cells (*p-value* 0.05 or lower; fold change of -2 or greater). The y-axes of all graphs are shown as arbitrary units.

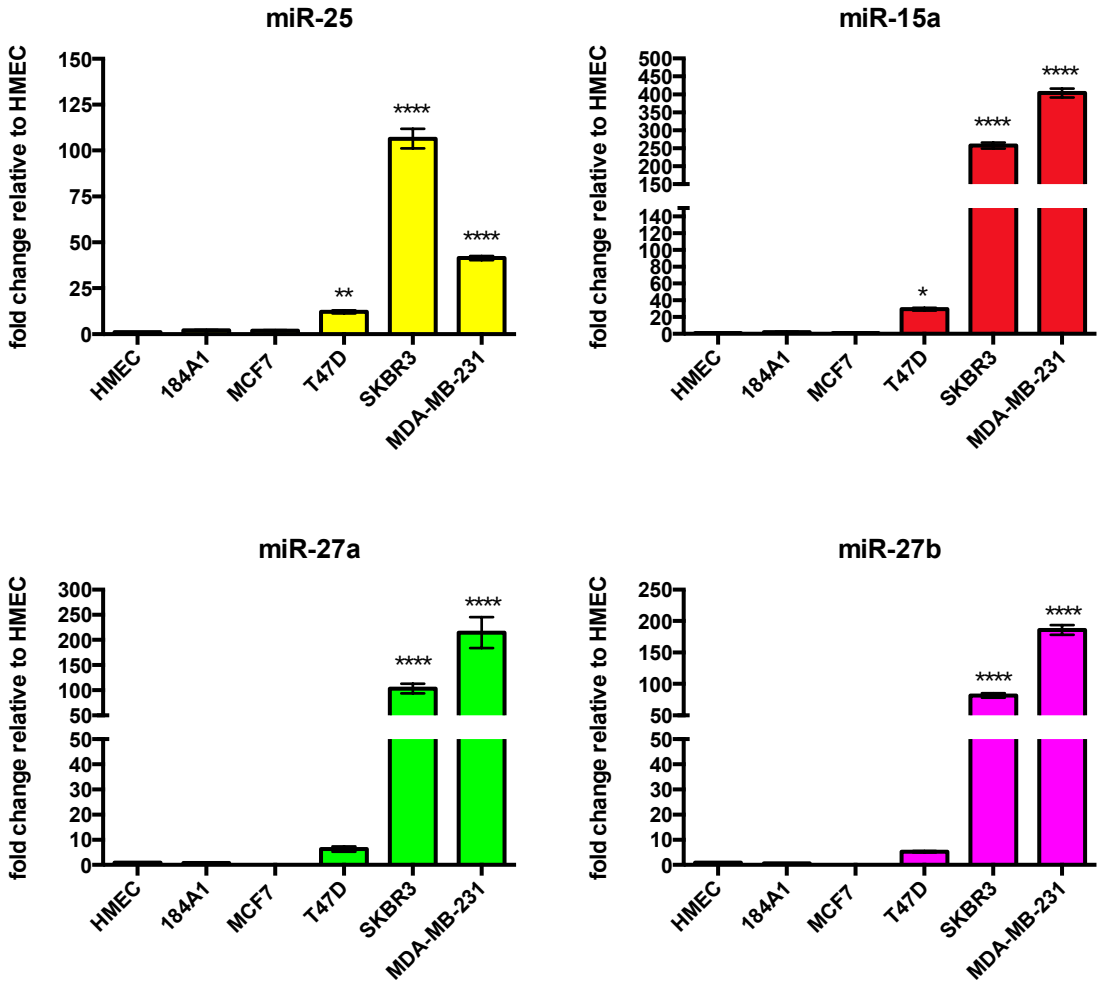

Figure 5.7 PCR validation of miRNA overexpressed in breast cancer cell lines.
Total RNA was extracted from all breast cell lines, reverse transcribed into cDNA using miRNA reverse primers and expression was measured using real time PCR. miR-25 (yellow), miR-15a (red), miR-27a (green) and miR-27b (pink) were all overexpressed in highly tumourigenic breast cancer cell lines (SKBR3 & MDA-MB-231) and T47D breast cancer cells compared to low tumourigenic MCF7 cells and non-tumourigenic breast cells. All results were normalised to the geomean of 3 house-keeping genes and are expressed as a fold change relative to normal primary breast cells (HMEC); *n=3* with the y-axis of all graphs shown as arbitrary units, *p=0.05**, *p=0.01***, *p=0.0001*****

5.2.5 miR-637 and miR-1226 do not modulate CD151 protein expression in breast cells

miR-637 was previously shown to decrease CD151 protein expression in a normal prostate and a prostate cancer cell line (chapter 3.2.7). However, it was unclear whether miR-637 only modulates CD151 expression in prostate cells or whether it could also affect CD151 protein levels in breast cell lines. miR-637 mimic was transfected into 184A1 non-tumourigenic breast and MDA-MB-231 triple negative breast cancer cells and protein lysates were collected 72 h post-transfection. SDS-PAGE and western blotting revealed no change in CD151 protein expression in 184A1 or MDA-MB-231 cells following miR-637 transfection compared to controls (Fig 5.8). Similarly, miR-1226, which was significantly upregulated in the breast cancer cell lines and was shown to increase CD151 3'UTR activity, also had no effect on CD151 protein expression in 184A1 and MDA-MB-231 cells compared to controls (Fig 5.8).

5.2.6 miR-518f decreases CD9 protein expression in non tumourigenic breast and triple negative breast cancer cells*

CD9 3'UTR regulation was found to be quite similar across the breast cell lines (Fig 5.5B). Therefore, miR-518f*, which dramatically decreased CD9 protein levels in RWPE1 and DU145 prostate cancer cells, was tested for its ability to regulate CD9 expression in breast cell lines via transfection of a miR-518f* mimic. Transfection of miR-518f* into 184A1 and triple negative MDA-MB-231 cells led to a significant decline in CD9 total protein expression (Fig 5.9). In addition, miR-4289 which was upregulated in prostate cancer cell lines with CD9 3'UTR repression was tested for its ability to modulate CD9 protein levels in breast cell lines. Transfection of a miR-4289 mimic had no significant effect on CD9 total protein expression in 184A1 or MDA-MB-231 cells over 72h post-transfection (Fig 5.9).

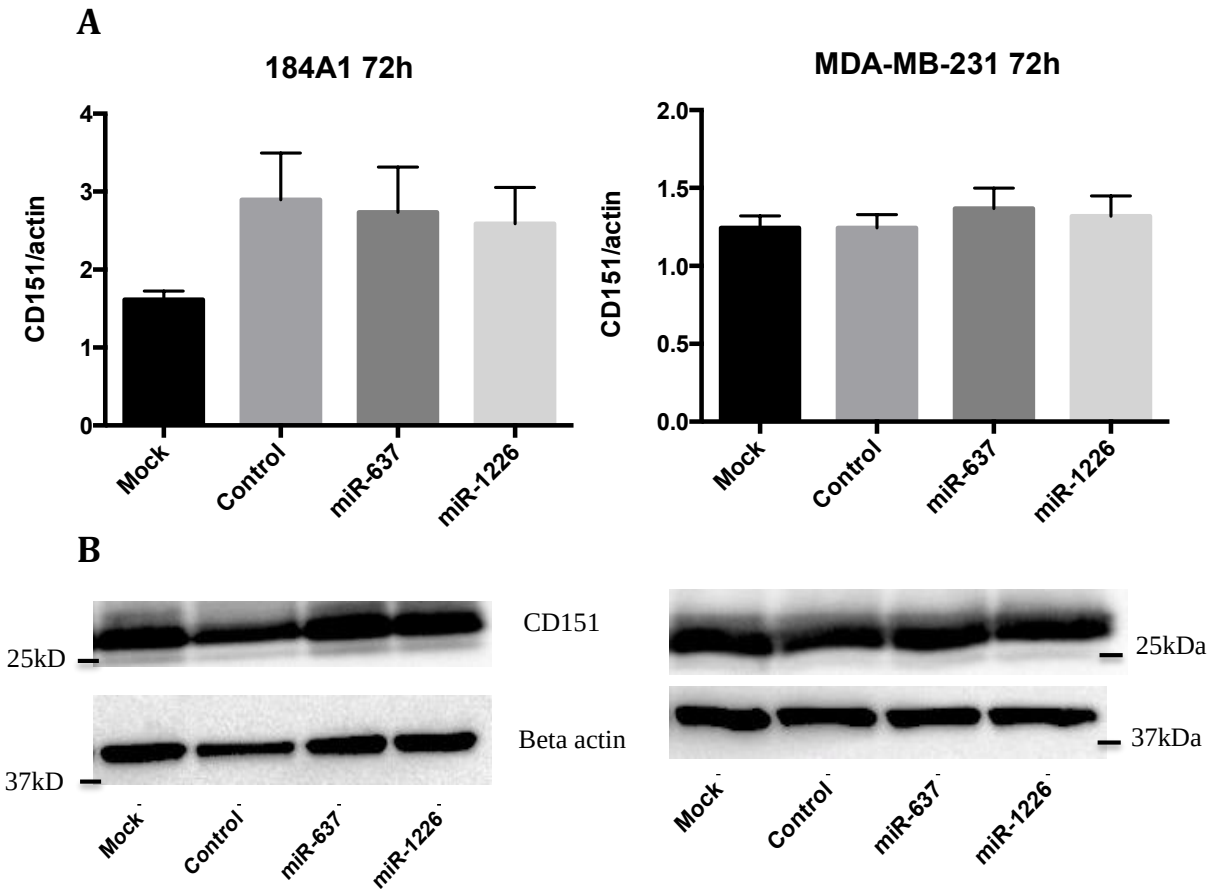

Figure 5.8. miR-637 and miR-1226 do not affect CD151 protein expression in breast cell lines. miR-637 and miR-1226 mimics (50nM) were transfected into 184A1 and MDA-MB-231 cells, protein was lysed in 1% NP40 lysis buffer 72 h post transfection and run on SDS-PAGE. Blots were probed with IIB1 (CD151) antibody and beta actin (loading control) and were quantitated by densitometry using Multi-gauge software. Mock denotes cells transfected with transfection reagent but no miRNA mimic and control cells were transfected with a scrambled miRNA mimic negative control. A. Results shown as CD151 protein normalised to beta actin (arbitrary units); *n =3*. B. A representative western blot of CD151 protein expression in 184A1 (left) and MDA-MB-231 (right) cells following transfection of miRNA mimics.

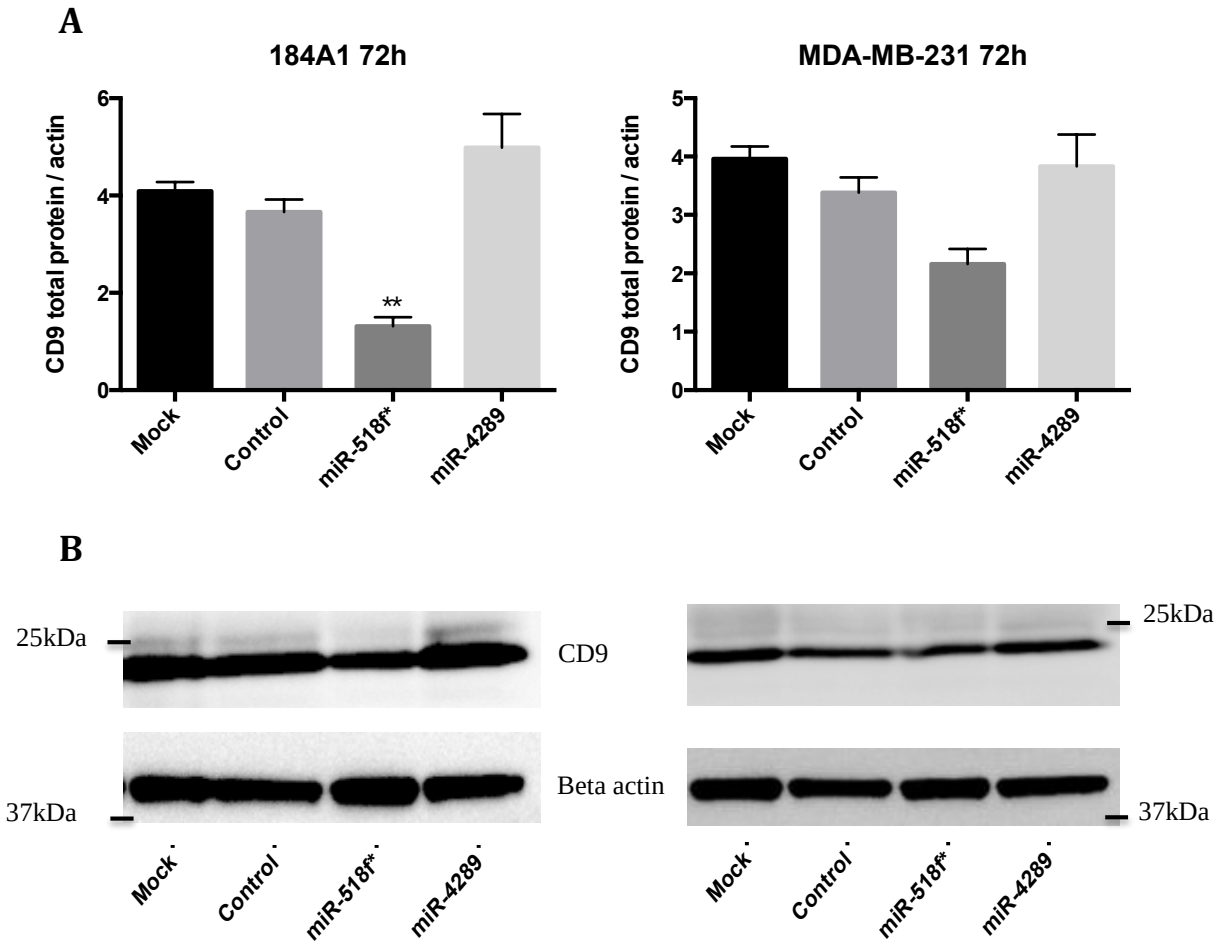

Figure 5.9 Transfection of miR-518f* reduces CD9 protein expression in breast cell lines. miR-518f* and miR-4289 mimics (50nM) were transfected into 184A1 and MDA-MB-231 cells, protein was lysed in 1% NP40 lysis buffer 72 h post transfection and run on SDS-PAGE. Blots were probed with 1AA2 (CD9) antibody and beta actin (loading control) and were quantitated by densitometry using Multi-gauge software. Mock denotes cells transfected with transfection reagent but no miRNA mimic and control cells were transfected with a scrambled miRNA mimic negative control. A. Results shown as CD9 protein normalised to beta actin (arbitrary units); *n =3*. B. A representative western blot of CD9 protein expression in 184A1 (left) and MDA-MB-231 (right) cells following transfection of miRNA mimics; *p=0.008***.

5.2.7 miR-518f modulates migration of non-tumourigenic breast and breast cancer cells*

A miR-518f* mimic was transiently transfected into breast cell lines to determine if miR-518f* modulates the migration, adhesion and proliferation of breast cells. Transfection of miR-518f* into 184A1 non-tumourigenic breast cells led to a significant increase in 24h migration, but did not affect migration at 18 h (Fig 5.10A & B). miR-518f* also increased 184A1 cell proliferation (Fig 5.10C) and adhesion to basement membrane extract (BME) (Fig 5.10D). A substantial knockdown in CD9 protein expression was observed over the duration of the functional assays, as evidenced by a decrease in CD9 total protein levels at the final time point (Fig 5.10).

Similarly, in MDA-MB-231 breast cancer cells, transfection of miR-518f* mimic resulted in increased migration at 6 and 18 h time points compared to controls (Fig 5.11A & B), however this affect was no longer observed beyond the 24 h time point (Fig 5.11C). miR-518f* had no substantial effect on MDA-MB-231 cell proliferation (Fig 5.11D) or adhesion to BME or fibronectin (Fig 5.11E). Assessment of CD9 total protein expression using western blotting provided evidence that CD9 protein levels were decreased during the extent of the functional assays (Fig 5.11F).

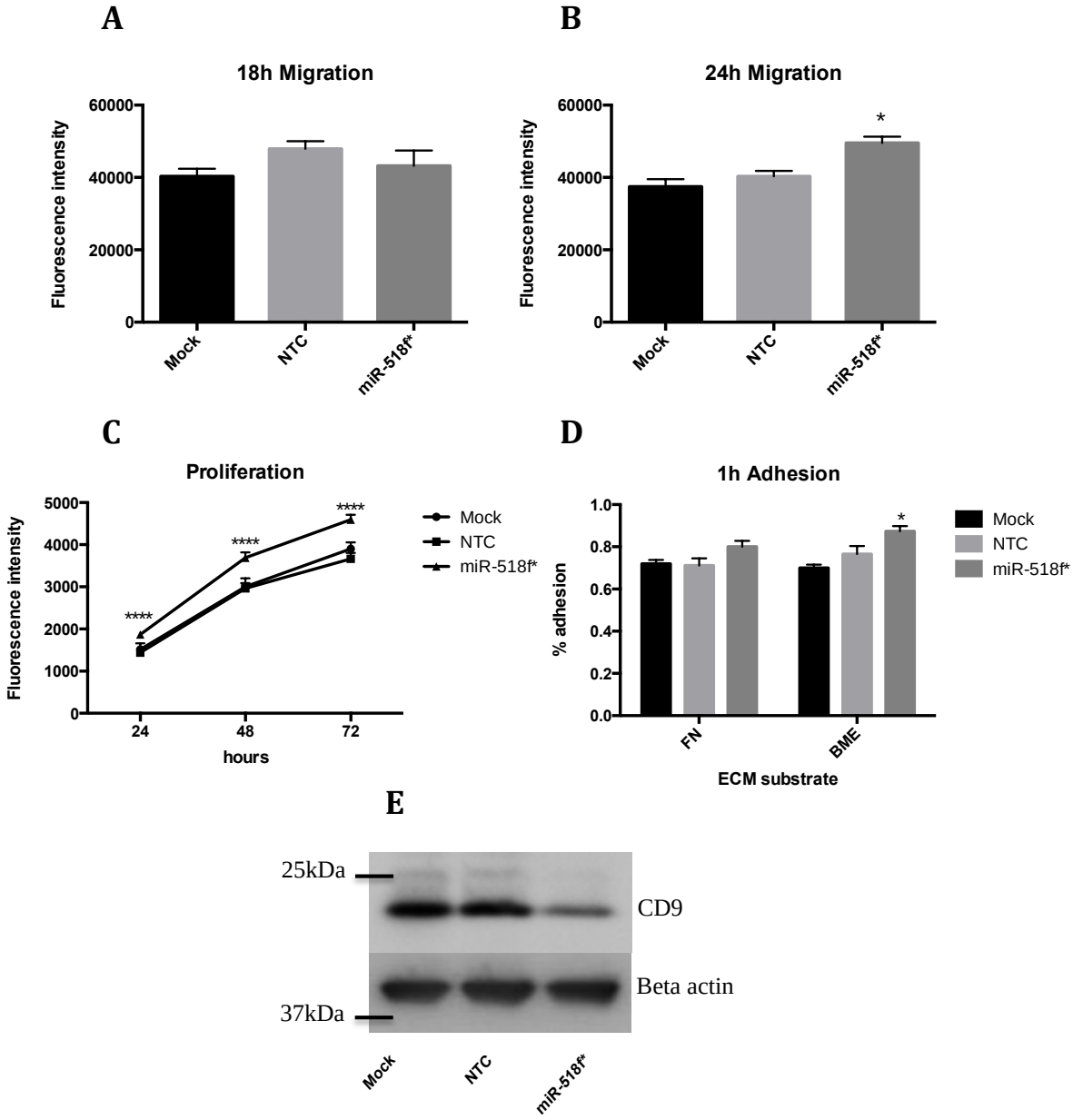

Figure 5.10 miR-518f* increases 184A1 cell migration, adhesion and proliferation. A & B. 184A1 breast cells were transiently transfected with a miR-518f* mimic for 48h, loaded with a fluorescence stain (Calcein AM) and seeded into a transwell migration insert and incubated at 37°C 5% CO_2 for 18 h (A) or 24 h (B). The lower chamber contained media with 10% FBS as a chemoattractant. Following migration assay, migratory cells were labelled with Calcein AM and the fluorescence intensity was measured. Results are shown as fluorescence intensity of migratory cells normalised to fluorescence intensity of 184A1 cells prior to commencement of the assay. C. 48 h post-transfection of miR-518f* mimic, 184A1 cells were seeded into 96 well plates and cell proliferation was assessed by incubating cells in media containing resazurin for 5 h and measuring fluorescence intensity each day up til 72 h. D. 48 h post-transfection, 184A1 cells were loaded with Calcein AM and seeded into 96 well

plates coated with fibronectin and BME and incubated at 37°C 5% CO_2 for 1 h. Total cell fluorescence was measured, after which non-adherent cells were washed away with PBS and the fluorescence of adherent cells was measured. Results are expressed as fluorescence intensity of adherent cells / fluorescence intensity of total cells to give % adhesion. E. 72 h post-transfection of miR-518f* mimic, 184A1 cells were lysed in 1% NP40 buffer, protein was separated using SDS-PAGE and western blotting with the 1AA2 (CD9) antibody was used to detect CD9 total protein levels. All experiments consisted of mock cells (cells with transfection reagent only) and control cells (cells transfected with a scrambled non-targeting miRNA mimic negative control); *n=3* (except for E which is *n=1*) and all y-axes are shown as arbitrary units; *p=0.01**, *p<0.0001*****.

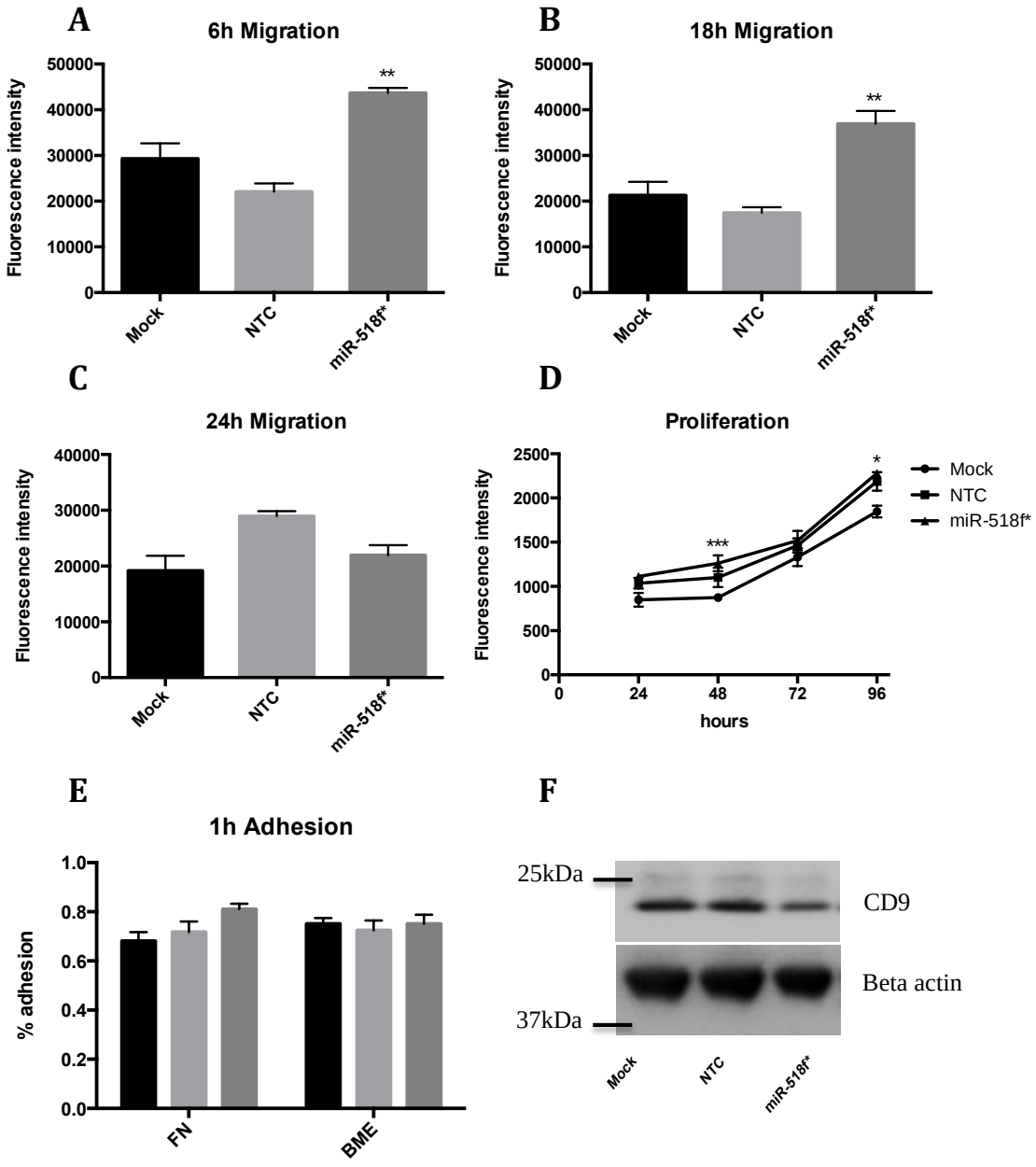

Figure 5.11 miR-518f* increases MDA-MB-231 breast cancer cell migration.

A, B & C. MDA-MB-231 breast cancer cells were transiently transfected with a miR-518f* mimic for 48h, loaded with a fluorescence stain (Calcein AM) and seeded into a transwell migration insert and incubated at 37°C 5% CO_2 for 6 h (A) or 18 h (B) or 24 h (C). The lower chamber contained media with 10% FBS as a chemo-attractant. Following migration assay, migratory cells were labelled with Calcein AM and the fluorescence intensity was measured. Results are shown as fluorescence intensity of migratory cells normalised to fluorescence intensity of MDA-MB-231 cells prior to commencement of the assay. D. 48 h post-transfection of miR-518f* mimic, cells were seeded into 96 well plates and cell proliferation was assessed by incubating cells in media containing resazurin for 5 h and measuring fluorescence intensity each day up til 72 h. E. 48 h post-transfection, cells were loaded with Calcein AM and seeded into 96

well plates coated with fibronectin (FN) and basement membrane extract (BME) and incubated at 37°C 5% CO_2 for 1 h. Total cell fluorescence was measured, after which non-adherent cells were washed away with PBS and the fluorescence of adherent cells was measured. Results are expressed as fluorescence intensity of adherent cells / fluorescence intensity of total cells to give % adhesion. F. 72 h post-transfection of miR-518f* mimic, cells were lysed in 1% NP40 buffer, protein was separated using SDS-PAGE and western blotting with the 1AA2 (CD9) antibody was used to detect CD9 total protein levels. All experiments consisted of mock cells (cells with transfection reagent only) and control cells (cells transfected with a scrambled non-targeting miRNA mimic negative control); *n=3* (except for E which is *n=1*) and all y-axes are shown as arbitrary units; *p=0.03*, p=0.001**, p=0.0001***.*

5.3 Discussion

As discussed in chapter 3, there is very little information known about regulation of CD151 in normal or cancer cells. CD151 protein stability has previously shown to be modulated by GRAIL [130] and SP1 transcription factor [129] was found to influence transcription of CD151, however at commencement of this project there was no knowledge about CD151 post-transcriptional regulation, particularly with regard to breast cancer. Therefore, the aim of this chapter was to determine whether miRNAs from chapter 3 and other miRNAs that are deregulated in breast cancer could modulate CD151 protein expression in breast cell lines.

Firstly, CD151 mRNA, total protein and cell surface protein expression was characterised in a panel of breast cell lines. Breast cancer cell lines had significantly less CD151 mRNA and showed a trend towards lower CD9 total protein compared to non-tumourigenic breast cells. However, MDA-MB-231 cells had CD151 total and cell surface protein expression that was comparable with normal breast cells. In addition, only CD151 cell surface protein and CD151 total protein levels positively correlated and reached statistical significance, suggesting that CD151 total protein and cell surface protein expression is very similar in breast cell lines. The fact that breast cancer cell lines did not express higher levels of CD151 protein compared to non-tumourgenic cells was unexpected, as CD151 protein expression is typically upregulated in breast cancer and breast cancer cell lines [74-76]. However, previous studies have typically determined CD151 expression in one breast cancer cell line such as MDA-MB-231 and have not made the comparison to non-tumourigenic breast cells. Without characterising CD151 protein expression in normal breast cells, breast cancer cell lines such as MDA-MB-231 and SKBR3 would appear to have enhanced CD151 protein expression

compared to other breast cancer cell lines with low tumourigenicity. In addition, the normal breast cells used in this study were primary and immortalised breast epithelial cells which express basal cell markers [238], and basal breast epithelial cells are known to express high levels of CD151 protein compared to luminal breast epithelial cells [77].

Even though CD151 mRNA and total protein levels were shown to correlate, it is still likely that regulatory mechanisms are responsible for the levels of CD151 mRNA and protein observed at any given time. For example, CD151 3'UTR regulation by miRNA may influence CD151 mRNA stability or directly affect translation of CD151 mRNA into CD151 protein. Therefore, a dual luciferase CD151 3'UTR reporter assay was used to investigate whether miRNAs regulate CD151 expression in breast cell lines. Highly tumourigenic breast cancer cell lines (MDA-MB-231 & SKBR3) showed slightly less repression of the luciferase reporter compared to non-tumourigenic breast cells, suggesting that the CD151 3'UTR was not being targeted by miRNAs. This implies that highly tumourigenic breast cancer cells do not express or have low levels of miRNAs that can regulate CD151 or express other factors which can antagonise miRNAs (see Ch6 General Discussion), and hence CD151 expression is stabilised and remains high.

It was of interest to determine which miRNAs have differential expression in breast cancer cells compared to normal breast cells. Whole genome miRNA profiling showed that a vast number of miRNAs are up and downregulated in breast cancer versus normal cells, many of which are predicted to bind the CD151 3'UTR based on bioinformatic analysis (from Ch3). Some of these miRNAs were validated using real time PCR, such as miR-27a, miR-27b, miR-15a and miR-25, which were upregulated in most breast cancer cells compared to non-tumourigenic breast cells. All of these miRNAs are

known to be involved in regulating breast cancer and its progression. miR-25 has been found to be part of a serum miRNA signature that can non-invasively discriminate between breast cancer cases and controls [239] and miR-25 is upregulated in the serum of breast cancer patients [240]. miR-25 expression also correlates with breast cancer prognosis [241] is known to promote drug resistance to doxorubicin [242] and tends to regulate triple negative breast cancers [243]. miR-27a has also been reported to be upregulated in invasive breast cancer [244, 245], is known to be involved in breast cancer progression [246, 247] and is predictive of overall and disease-free survival [244]. miR-27b is upregulated in breast cancers and knockdown of miR-27b suppresses breast cancer cell growth [248], and is also upregulated in metastatic triple negative breast cancers [249]. In contrast, miR-15a, which was found in this study to be upregulated in most breast cancer cell lines, is commonly downregulated in breast cancer [250, 251]. Therefore, further validation into the prognostic value of these miRNAs is needed to determine whether they would be useful as prognostic markers in breast cancer. A similar analysis was attempted with the miRNA expression profiles as for the prostate lines but as this analysis did not show significant variability it was not possible, and as such the miRNAs used for further testing were the same as for the prostate cancer study in order to see if these miRNAs also affect breast cancer cells.

Next it was of interest to test whether miR-637, which decreased CD151 protein expression in DU145 prostate cancer cells could also decrease CD151 in a normal breast and a breast cancer cell line. Moreover, miR-1226, which was found to bind to the CD151 3'UTR and increase luciferase expression (Ch3) and has increased expression in breast cancer cells (miRNA microarrays) was tested for its ability to

increase CD151 protein expression in breast cell lines. miR-637 and miR-1226 were chosen to investigate further because they had already been shown to influence CD151 3'UTR regulation (Ch3) and miRNAs that were differentially expressed in breast cancer cell lines compared to non-tumourigenic breast cell lines only showed minor changes to CD151 3'UTR regulation.

Over expression of miR-637 did not decrease or change CD151 total protein levels in normal breast cells or in an aggressive breast cancer cell line compared to controls. Likewise, transfection of miR-1226 did not increase or influence CD151 total protein levels in normal breast or in an aggressive breast cancer cell line compared to controls. Expression of CD151 was only assessed at 72h post transfection, therefore there is the possibility that both miRNAs may affect CD151 protein levels at an earlier time point and the effect may be diminished by 72h. However, CD151 protein is known to have a long half-life so this is unlikely to be the case (Weidenhofer et al 2015 unpublished; [128]).

Despite no effects on CD151 expression in 184A1 and MDA-MB-231 cells, it is possible that miR-637 and miR-1226 may affect CD151 protein expression in other types of breast cancer, as the function of miRNAs is likely to be dependent on the availability of its target mRNA transcripts. Therefore, the role of miR-637 and miR-1226 in modulating CD151 expression would need to be elucidated in the future in a range of breast cancer cell lines such as SKBR3, MCF7 and T47D cell lines. miR-637 has been reported to inhibit HER2 signalling and cell growth in HER2-amplified breast cancer cell lines [187], therefore miR-637 may play an important role in interfering with CD151 and HER2 signalling in SKBR3 breast cancer cells which are HER2 positive.

miR-1226 is a mirtron, which means it is produced from intronic splicing, bypassing miRNA processing and is physiologically active [252]. Therefore, miR-1226 may affect CD151 expression at a much earlier time point than 72 h post-transfection. Moreover, miR-1226 has been shown to induce cell death in other breast cell lines such as MCF7 and MCF10A via downregulation of mucin-1 oncoprotein [183]. This highlights that miR-1226 may have an effect on CD151 expression in other normal breast and breast cancer cell lines, and this would need to be clarified with future experiments. Recently, miR-506 and miR-124 were shown to downregulate CD151 expression in breast cancer cells, resulting in a reduction in breast cancer cell proliferation, migration, invasion and adhesion [170, 171], suggesting that miRNAs are important for regulation of CD151 in breast cancer.

The second aim of this chapter was to investigate if miRNAs regulate another tetraspanin, CD9, in breast cancers. CD9 is commonly referred to as a metastasis suppressor and low CD9 protein expression usually predicts poor prognosis in many types of cancers. CD9 clearly has important functions in breast cancer progression and metastasis in which CD9 protein expression is commonly downregulated in advanced stage breast cancer and metastasis. However, as discussed in Ch4, the mechanism/s responsible for the reduction in CD9 expression are currently unknown. There is a striking lack of knowledge about regulation of CD9 expression. In breast cancer cell lines, an RNA-binding protein HuR was shown to stabilise CD9 mRNA levels in one breast cancer cell line but destabilise CD9 mRNA in another breast cancer cell line [125]. This suggests that CD9 is regulated by RNA-binding proteins in breast cancer, but also that other post-transcriptional forms of regulation such as miRNAs may influence CD9 expression in breast cancers. Therefore, this chapter also aimed to

determine if increased expression of miRNAs in breast cancer are partly responsible for the decreased CD9 expression observed.

Firstly, CD9 expression was characterised across a panel of normal breast and breast cancer cell lines. Normal breast and a breast cancer cell line with low tumourigenic potential displayed similar levels of CD9 mRNA, however most breast cancer cell lines had lower CD9 mRNA expression. The latter, also showed a trend towards reduced CD9 total protein and cell surface protein expression compared to normal breast cells. This was expected as the majority of the literature shows decreased CD9 expression in breast cancers, particularly with progression and in lymph node metastases of breast cancer [98-100, 102, 103]. CD9 mRNA and total protein levels were also shown to significantly positively correlate. This does not necessarily mean that CD9 is not regulated at the transcriptional or post-transcriptional levels, as changes in CD9 3'UTR regulation may influence CD9 transcript levels which in turn affect CD9 protein levels. Therefore, CD9 3'UTR regulation was assessed in the breast cell lines by transfecting CD9 3'UTR luciferase construct into the panel of breast cell lines. CD9 3'UTR activity was similar across all the breast cell lines except for non-tumourigenic 184A1 breast cells which expressed more luciferase protein, suggesting the CD9 expression is stabilised via its 3'UTR in normal breast cells. Therefore, normal breast cells may express miRNAs or other factors that are capable of binding to the CD9 3'UTR and stabilising CD9 transcripts.

As discussed in chapter 4, there are many miRNAs that are predicted to regulate CD9 expression. miRNAs that displayed differential expression in breast cancer cell lines compared to non-tumourigenic breast cell lines only showed a modest effect on CD9

3’UTR regulation. However, overexpression of miR-518f* was shown to significantly decrease CD9 protein expression in RWPE1 non-tumourigenic prostate and DU145 prostate cancer cells (Ch4). Therefore, it was of interest to determine whether miRNAs such as miR-518f* are capable of manipulating CD9 expression in breast cell lines. Transfection of miR-518f* mimic in non-tumoruigenic 184A1 breast cells led to a significant reduction in CD9 protein expression and showed a trend towards lower CD9 levels following transfection in MDA-MB-231 breast cancer cells. Another miRNA, miR-4289 which was found to bind to the CD9 3’UTR had no effect on CD9 protein levels in breast cell lines. Transfection of miR-518f* into 184A1 breast cells resulted in a significant increase in adhesion to BME, 24 h migration and proliferation. miR-518f* also increased migration of MDA-MB-231 breast cancer cells but did not change cell adhesion or proliferation. These results show that miR-518f* mediated knockdown of CD9 increases migration, proliferation and adhesion of breast cell lines. This is in contrast to literature, which shows that CD9 knockdown in breast cancer cell lines results in decreased motility and cell spreading [104, 233]. However, the effects seen on adhesion and proliferation may be due to other mRNA targets of miR-518f* or may be a result of disruption of interactions between CD9 and partner proteins specifically occurring in the cell lines analysed, that are involved in these pathways. As discussed in chapter 4, there are no papers published on the function or mRNA targets of miR-518f*, however using bioinformatics it is predicted to regulate many genes that are involved in pathways that play a prominent role in breast tumourigenesis and metastasis such as calcium signalling, Wnt signalling, integrin signalling and angiogenesis. Therefore, miR-518f* decreases CD9 protein levels in breast cell lines, resulting in an increase in 24 h migration, increased adhesion to BME and increased proliferation. miR-518f* also

increased breast cancer cell migration and is predicted to regulate other genes involved in breast cancer, which suggests that it may influence breast cancer progression.

In conclusion, CD151 mRNA, total protein and cell surface protein levels were found to be low in most breast cancer cell lines compared to non-tumourigenic breast cells, however triple negative MDA-MB-231 breast cancer cells showed similar CD151 expression to non-tumourigenic breast cells. Moreover, CD151 total protein and cell surface protein expression levels significantly positively correlated. Most breast cancer cell lines showed less repression of CD151 3'UTR activity, suggesting that maybe breast cancer cells lack expression of miRNA regulating CD151. However, miR-637 and miR-1226, which are predicted to regulate CD151 (Ch3) and were shown to bind to the CD151 3'UTR (Ch3) did not modulate CD151 protein levels in 184A1 or MDA-MB-231 breast cancer cells. CD9 mRNA, total protein and cell surface protein expression was also lower in breast cancer cell lines compared to non-tumorigenic breast cells, with CD9 mRNA and total protein levels significantly positively correlating. CD9 3'UTR activity across the breast cell lines was similar, and transfection of miR-518f* (decreased CD9 protein levels in prostate cell lines; Ch4) into 184A1 and MDA-MB-231 cells resulted in a significant decrease in CD9 protein expression. miR-518f* significantly increased migration of 184A1 and MDA-MB-231 cells and increased 184A1 cell proliferation and adhesion to basement membrane extract. Therefore, miR-518f* is capable of regulating CD9 expression and modulates normal breast and breast cancer cell migration *in vitro*.

Chapter 6: General Discussion

Tetraspanin CD151 and CD9 expression levels are commonly altered in many types of cancers. CD151 protein levels are typically overexpressed in cancers and it is considered a metastasis enhancer, whereas CD9 protein expression is commonly reduced in cancers and is usually considered to be a metastasis suppressor [34]. One of the main gaps in tetraspanin literature is that very little is known about tetraspanin regulation and the mechanisms responsible for tetraspanin deregulation in cancers such as prostate and breast cancer. Therefore, this thesis aimed to elucidate if CD151 and CD9 are post-transcriptionally regulated my miRNAs in prostate and breast cancers.

This thesis is the first study to characterise CD151 and CD9 mRNA, total protein and cell surface protein expression in a range of non-tumourigenic breast and prostate cells as well as breast and prostate cancer cell lines. Highly tumourigenic prostate cancer cell lines had similar levels of CD151 mRNA but showed a trend towards higher levels of CD151 total protein compared to non-tumourigenic prostate cells. In fact, a significant inverse correlation was found between CD151 mRNA and total protein levels. Therefore, CD151 mRNA expression in prostate cancer does not positively predict CD151 protein expression. As a result, prognostic studies which only measure CD151 mRNA levels in prostate cancer may not yield useful information or may be useful in predicting an inverse relationship with CD151 protein expression and prostate cancer progression and/or other clinicopathological parameters. Although CD151 protein expression did not reach significance as expected based on studies showing increased CD151 protein levels in prostate cancer [69, 72], a trend towards higher expression was still observed.

Analysis of a panel of breast cell lines showed no correlation between CD151 protein and mRNA levels. Furthermore apart from the triple negative breast cancer cell line MDA-MB-231, which showed similar levels of CD151 total protein and cell surface protein to non-tumourigenic breast cells, the breast cancer cell lines displayed lower levels of CD151 mRNA and total protein compared to non-tumourigenic breast cells. This was unexpected, as a number of studies have shown increased CD151 protein expression in human breast tumours [74, 75]. However, the majority of *in vitro* studies have only assessed CD151 expression in MDA-MB-231 or MCF7 cell lines and have not made the comparison with normal breast cells as this study has. The normal breast cell lines used in this thesis were normal mammary epithelial cells with a basal phenotype, whereas the majority of breast cancer cell lines used are of luminal epithelial origin. Therefore, it is difficult to compare normal basal-like cells to breast cancer cell lines, especially when basal breast epithelial cells are known to express high levels of CD151 [77].

Prostate and breast cancer cells showed differential relationships between CD9 mRNA, total protein and cell surface protein expression. Prostate cancer cells displayed higher levels of CD9 mRNA, similar CD9 total protein expression and lower CD9 cell surface protein levels compared to non-tumourigenic cells. The majority of the literature shows decreased CD9 protein expression in prostate cancer [82], however in the present study only CD9 cell surface protein levels were lower than non-tumourigenic prostate cells. This suggests that low cell surface CD9 expression may play an important role in prostate cancer, not total protein levels. Cell surface CD9 binds to tetraspanins, integrins and other signalling proteins to form TEMs, which are known to be involved in cancer cell adhesion, migration, invasion and signalling [34]. Therefore, a lack of CD9

expression on the cell surface may disrupt the organisation of TEMs and therefore signalling into the cell resulting in a more aggressive prostate cancer phenotype governed by increased migration and invasion. Moreover, CD9 mRNA and total protein levels did not correlate, however a trend towards an inverse relationship was observed. Therefore, in prostate cancer, CD9 mRNA levels cannot be used to predict CD9 protein expression, suggesting that future studies need to measure CD9 protein expression directly and particularly focus on plasma membrane levels, or assess both mRNA and protein levels to gain functional information about CD9. However, most breast cancer cell lines had low levels of CD9 mRNA and low CD9 protein expression compared to normal breast cells. In addition, MDA-MB-231 and T47D breast cancer cell lines displayed much lower levels of CD9 cell surface expression compared to other breast cancer cell lines and normal breast cells. This was expected as most of the CD9 breast cancer literature has found decreased CD9 protein expression in breast cancer cell lines such as MDA-MB-231 and in triple negative breast tumours [100, 102]. Moreover, CD9 mRNA and total protein expression showed a positive correlation. Therefore, in breast cancer, CD9 mRNA levels are predictive of CD9 protein expression, which has been suggested as an indicator for better patient prognosis [96, 98, 103].

The relationship between CD151 and CD9 mRNA and protein levels appeared to be different in prostate cancer compared to breast cancer, which suggests that these tetraspanins are predominately regulated by different mechanisms. For example in prostate cancer cell lines, CD151 mRNA is low however CD151 protein expression is high. Therefore, prostate cancer cells must stabilise CD151 mRNA transcripts or limit CD151 protein degradation in order to maintain high levels of CD151 protein compared to normal prostate cells. In contrast, breast cancer cells express low levels of CD151

mRNA and protein, except for MDA-MB-231 cells, which have similar CD151 protein expression to normal breast cells. In breast cell lines, CD151 mRNA and protein levels showed a positive trend towards correlating, however only total protein and cell surface protein expression significantly correlated. Therefore, in breast cancer, CD151 expression does not appear to be heavily post-transcriptionally regulated when compared to what is observed with CD151 expression in prostate cancers. A similar relationship was seen with CD9 expression in prostate and breast cell lines, where CD9 mRNA is high in prostate cancer cells but protein levels are similar or lower compared to normal prostate cells suggesting that CD9 expression in inhibited by regulatory factors such as miRNAs. However, most breast cancer cells displayed low CD9 mRNA and low CD9 protein levels suggesting that reduced levels of CD9 protein are most likely due to lower CD9 mRNA levels compared to normal breast cells. This does not negate a role for miRNAs and other regulatory factors in regulating CD9 mRNA transcript levels, which may be at least partially responsible for the low CD9 mRNA levels in these cell lines.

In line with these results, this thesis was also the first study to assess tetraspanin 3'UTR activity in breast and prostate cell lines using a dual luciferase 3'UTR reporter assay. Alternative CD151 and CD9 transcripts exist however the 3'UTR is the same in all transcripts [44, 81]. Therefore, any results from utilising the CD9 or CD151 3'UTR are applicable to all CD9 and CD151 transcripts respectively. T47D and MDA-MB-231 breast cancer cell lines showed less luciferase repression when transfected with the CD151 3'UTR luciferase construct. Therefore, in these breast cancer cells, miRNAs or other regulatory proteins are not binding and degrading the mRNA or translationally repressing protein expression compared to other breast cell lines. This was expected, as

MDA-MB-231 breast cancer cells express high levels of CD151 protein. In comparison, highly tumourigenic prostate cancer cells had repression of luciferase protein. This suggests that in prostate cancer cell lines CD151 is regulated by more than just its 3'UTR as these cell lines actually had higher levels of CD151 protein expression compared to normal prostate cells. Whilst the luciferase assay is unable to identify what mechanism this may be due to only the 3'UTR of CD151 included in the assay, it is likely that post-transcriptional or translational/post-translational regulation is altered leading to increased CD151 protein expression in prostate cancer. For example, GRAIL which is an ubiquitin E3 ligase known to affect CD151 stability [130], may be differentially expressed in prostate cancer cells compared to normal cells, resulting in more stable CD151 protein expression. This notion would need to be tested in the future to validate whether this is the mechanism responsible or not.

CD9 3'UTR luciferase constructs were also transfected into breast and prostate cells, with the results fitting with protein expression data from these cell lines. All breast cell lines showed similar levels of CD9 3'UTR activity, which is in line with the fact that CD9 mRNA and protein levels significantly correlated. Prostate cancer cell lines (DU145 and PC3) with similar total CD9 protein but higher CD9 cell surface protein expression, showed more repression of luciferase compared to other prostate cells. Therefore, highly tumourigenic prostate cancer cell lines appeared to endogenously express miRNAs or other regulatory factors that are capable of binding to the CD9 3'UTR and can modulate mRNA transcript or protein levels. As a consequence, miRNAs may be responsible for the decreased CD9 protein expression observed in many prostate cancer cell lines and human prostate tumours.

Intriguingly the non-tumourigenic cell lines showed an increase in luciferase activity when regulated by the CD9 3'UTR compared to luciferase activity from the control vector without a 3'UTR. This may be an artefact of the assay, however, in some cases the extent of the increase in luciferase was substantial. If true, this implies that some factor can bind to the CD9 3'UTR in normal cells and stabilise CD9 mRNA transcripts. This could be due to miRNAs binding to the 3'UTR and stabilising mRNA transcripts instead of degrading mRNA. This has been previously observed in other studies which show that the cell cycle can influence the actions of miRNAs, resulting in some mRNA transcript levels going up and others down [253, 254]. In addition, RNA binding proteins are also capable of binding to 3'UTRs and are known to influence stability of mRNA transcripts [255]. Bioinformatic analysis of the CD151 and CD9 3'UTRs using RBPmap [256] showed that many RNA binding proteins are predicted to bind to the CD151 and CD9 3'UTRs (see Table 8.2 & 8.5 Appendix). Most of the RNA binding proteins were predicted to bind at multiple sites and are known to play a role in many types of cancer. Furthermore, the RNA binding protein HuR has been shown to differentially modulate the stability of CD9 mRNA levels in MCF7 and MDA-MB-231 breast cancer cell lines [125]. Moreover, another RNA binding protein, CUGBP1 has been found to interact with CD9 mRNA [126]. Therefore, RNA binding proteins could be playing a vital role in the regulation of tetraspanins, particularly CD9 in normal cells and cancer cells.

Normal cells tend to express higher levels of CD9 protein, this may be due to RNA binding proteins stabilising CD9 mRNA transcripts resulting in higher protein levels compared to cancer cells. Cancer cells may or may not express these RNA binding proteins or they may serve a different purpose. For example, the subcellular localisation

of HuR has been linked to disease free survival of cancer patients, with normal cells showing predominately nuclear expression and cancer tissues and cancer cell lines having strong cytoplasmic expression of HuR which is commonly predictive of decreased survival rates [257]. HuR mRNA and protein levels have been assessed in the breast and prostate cell line panels used in the current study. Most cell lines showed similar levels of HuR mRNA and protein except some prostate cancer cell lines displayed slightly higher levels of HuR total protein compared to non-tumourigenic cells (Weidenhofer *et al* unpublished 2015). Given the importance of subcellular localisation of HuR to its cellular function, further experiments are needed to determine the role of HuR in breast and prostate cancers and how important it is to CD9 expression.

Another point to consider is the interplay between miRNAs and RNA binding proteins. There are a couple of reviews highlighting the notion that miRNAs and RNA binding proteins can influence the expression and function of each other by a range of mechanisms in cancer [228]. miRNAs can decrease the expression of RNA binding proteins, however RNA binding proteins can affect the levels of miRNAs either directly or by modulating the expression of key components of the miRNA biogenesis and maturation pathway (e.g. Dicer). In addition, miRNAs and RNA binding proteins that regulate the same mRNA 3'UTRs can act cooperatively to destabilise mRNA transcripts or can compete with each other for binding. Therefore, we need to understand more about the complex nature of miRNA and RNA binding protein interactions involving tetraspanins and whether they are vital to cancer initiation and progression.

In chapters 3, 4 and 5, miRNAs that were predicted to bind the CD151 or CD9 3'UTR were found to influence protein expression regulated at the 3'UTR. miR-637 only showed modest effects on CD151 protein expression levels when transfected into RWPE1 and DU145 prostate cells, and had no effect on CD151 protein levels in 184A1 and MDA-MB-231 breast cells. As a result of this, the functional role of miR-637 was not further investigated in this study, however future experiments are required to ascertain the importance of this miRNA to prostate cancer. In terms of CD9, there was a wide range of evidence supporting a role for miR-518f* in regulating the CD9 3'UTR in breast and prostate cell lines. miR-518f* is predicted to bind to the CD9 3'UTR based on sequence complementarity, its expression followed changes in CD9 3'UTR activity across a panel of prostate cell lines and was shown to bind to the CD9 3'UTR. In addition, transfection of miR-518f* into breast and prostate cell lines resulted in a significant reduction in CD9 protein expression, which was further shown to differentially affect cell migration. In non-tumourigenic 184A1 breast cells and MDA-MB-231 breast cancer cells, overexpression of miR-518f* increased cell migration and in 184A1 and an increase in proliferation and adhesion to BME was also observed. However, when transfected into prostate cell lines, miR-518f* increased the migration and decreased initial adhesion of non-tumourigenic RWPE1 prostate cells but decreased the migration of DU145 prostate cancer cells.

A reduction in CD9 protein expression typically increases the migration of cancer cells *in vitro* [104], therefore transfection of miR-518f*, which decreases CD9 protein levels, was predicted to increase migration of breast and prostate cell lines. However, it is unknown why transfection of miR-518f* decreased the migration of DU145 prostate cancer cells. The unexpected finding of miR-518f* decreasing migration could be

explained by the other predicted mRNA targets, many of which are involved in cancer related processes such as migration. Therefore, in DU145 prostate cancer cells the effect of miR-518f* on other mRNA targets may predominate over the effect on CD9. Another possible explanation is that reduced CD9 expression in DU145 cells may disrupt TEMs by incorporating different tetraspanins and other proteins into TEMs resulting in changes to various signalling pathways such as those involved in cell migration. In DU145 prostate cancer cells, CD9 may interact with different proteins and hence this may help to explain why a miRNA could increase migration in some cell lines but decrease migration in other cell lines, however this would need to be further investigated in the future. This is plausible as other studies have shown that overexpression of CD9 in human melanoma cell lines and PC-3M-LN4 prostate cancer cells increased their migration and invasion *in vitro* and therefore the functions of CD9 in different cell lines are not clear [94, 217].

miRNA profiling of prostate and breast cell lines in the present study has shed light on miRNAs that are differentially expressed in prostate and breast cancer cell lines compared to non-tumourigenic cells. This combined with current literature gives more information on which miRNAs may be useful as biomarkers and/or prognostic markers for breast and prostate cancers. However, further investigations into the expression and function of these miRNAs are needed. Circulating miRNAs [258, 259] and miRNAs in exosomes [260, 261] circulating in the blood are currently being assessed for use as biomarkers and/or prognostic markers in cancer, thus it would be of interest to determine if miRNAs found in the current study are also found within blood or other fluids and whether they could be used as a non-invasive test to diagnose or predict progression of cancers.

Based on the results from this thesis (Ch4 & 5), miR-518f* clearly significantly decreases CD9 protein expression in breast and prostate cell lines. Therefore, further experiments investigating the role of miR-518f* in breast and prostate cancer animal models are required to determine if miR-518f* mimics and inhibitors may be useful new therapeutics/therapeutic targets for inhibiting cancer progression. Given that CD9 is expressed on the surface of exosomes [34, 262], it would also be of interest to test whether miR-518f* is expressed in exosomes and whether overexpression of miR-518f* in cancer cells disrupts the organisation, release and/or function of cancer exosomes and hence cancer progression. Human clinical trials are underway in the US involving liposomal or nanovesicle formulations with miRNA mimics and inhibitors administered intravenously into advanced stage cancer patients to assess dosing and toxicity, with very few side effects being reported [263, 264]. This is following a number of preclinical animal trials, which showed great safety profiles and provides hope towards manipulating the expression of miRNAs as a novel therapy for cancer patients. Perhaps, manipulating the levels of miR-518f* in cancer patients with tumours that have particular CD9 expression profiles may be efficacious. For example patients with tumours expressing low levels of CD9 but high levels of miR-518f* may benefit from a therapeutic that introduces a miR-518f* inhibitor into the tumour, in order to inhibit cancer progression. Therefore, if this technology is well tolerated and shows efficacy against cancer in humans, miRNAs such as miR-518f* may be effective candidates for use as novel therapies for inhibiting cancer progression, especially in patients with particular CD9 profiles, and may be used as biomarkers for cancer in the future.

Chapter 7: References

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Chapter 8: Appendix

Table 8.1 Full list of miRNA predicted to regulate the CD151 3'UTR

hsa-miR-95	hsa-miR-154	hsa-miR-3663-3p	hsa-miR-558
hsa-let-7d	hsa-miR-15a	hsa-miR-370	hsa-miR-564
hsa-let-7i*	hsa-miR-15b	hsa-miR-371-3p	hsa-miR-567
hsa-miR-103	hsa-miR-16	hsa-miR-371-5p	hsa-miR-574-3p
hsa-miR-103a	hsa-miR-1827	hsa-miR-377*	hsa-miR-574-5p
hsa-miR-107	hsa-miR-183	hsa-miR-378*	hsa-miR-582-3p
hsa-miR-1182	hsa-miR-186*	hsa-miR-410	hsa-miR-584
hsa-miR-1184	hsa-miR-188-3p	hsa-miR-412	hsa-miR-587
hsa-miR-1185	hsa-miR-18a*	hsa-miR-423	hsa-miR-588
hsa-miR-1192	hsa-miR-1909	hsa-miR-423-3p	hsa-miR-590-3p
hsa-miR-1193	hsa-miR-1911*	hsa-miR-423-5p	hsa-miR-591
hsa-miR-1200	hsa-miR-1913	hsa-miR-424	hsa-miR-592
hsa-miR-1202	hsa-miR-1914	hsa-miR-425-3p	hsa-miR-593
hsa-miR-1204	hsa-miR-1915*	hsa-miR-4256	hsa-miR-595
hsa-miR-1205	hsa-miR-194	hsa-miR-4257	hsa-miR-596
hsa-miR-1207-3p	hsa-miR-195	hsa-miR-4259	hsa-miR-597
hsa-miR-1207-5p	hsa-miR-1975	hsa-miR-4260	hsa-miR-604
hsa-miR-122	hsa-miR-1976	hsa-miR-4270	hsa-miR-608
hsa-miR-1225-3p	hsa-miR-198	hsa-miR-4271	hsa-miR-609
hsa-miR-1225-5p	hsa-miR-199a-3p	hsa-miR-4277	hsa-miR-612
hsa-miR-1226	hsa-miR-199b-3p	hsa-miR-4290	hsa-miR-615-5p
hsa-miR-1226*	hsa-miR-20a*	hsa-miR-4298	hsa-miR-619
hsa-miR-1227	hsa-miR-2127	hsa-miR-431	hsa-miR-622
hsa-miR-1229	hsa-miR-214	hsa-miR-432	hsa-miR-623
hsa-miR-1231	hsa-miR-218-2*	hsa-miR-4323	hsa-miR-626
hsa-miR-1233	hsa-miR-22	hsa-miR-4441	hsa-miR-629
hsa-miR-1234	hsa-miR-220b	hsa-miR-4455	hsa-miR-632
hsa-miR-1236	hsa-miR-220c	hsa-miR-449	hsa-miR-637
hsa-miR-124	hsa-miR-221*	hsa-miR-449a	hsa-miR-639
hsa-miR-124-3p	hsa-miR-222-5p	hsa-miR-449b	hsa-miR-641
hsa-miR-1244	hsa-miR-2355	hsa-miR-450b-3p	hsa-miR-642
hsa-miR-1245	hsa-miR-2355-5p	hsa-miR-453	hsa-miR-643
hsa-miR-1254	hsa-miR-24	hsa-miR-454	hsa-miR-644
hsa-miR-1256	hsa-miR-27a	hsa-miR-4667-5p	hsa-miR-646
hsa-miR-1261	hsa-miR-27b	hsa-miR-4700-5p	hsa-miR-647
hsa-miR-1262	hsa-miR-296	hsa-miR-4708-3p	hsa-miR-648
hsa-miR-1263	hsa-miR-296-3p	hsa-miR-4725-3p	hsa-miR-649
hsa-miR-1265	hsa-miR-296-5p	hsa-miR-4728-5p	hsa-miR-650
hsa-miR-1267	hsa-miR-297	hsa-miR-4763-3p	hsa-miR-652
hsa-miR-127-3p	hsa-miR-298	hsa-miR-483-3p	hsa-miR-654-5p
hsa-miR-127-5p	hsa-miR-299-3p	hsa-miR-483-5p	hsa-miR-657

hsa-miR-1272	hsa-miR-29a	hsa-miR-484	hsa-miR-658
hsa-miR-1274b	hsa-miR-29b	hsa-miR-485-5p	hsa-miR-661
hsa-miR-1275	hsa-miR-29c	hsa-miR-486-3p	hsa-miR-663
hsa-miR-128	hsa-miR-301a	hsa-miR-486-5p	hsa-miR-664*
hsa-miR-1281	hsa-miR-301b	hsa-miR-490-3p	hsa-miR-665
hsa-miR-1283	hsa-miR-30a	hsa-miR-491-5p	hsa-miR-668
hsa-miR-1285	hsa-miR-30b	hsa-miR-493	hsa-miR-671-3p
hsa-miR-1286	hsa-miR-30c	hsa-miR-495	hsa-miR-672
hsa-miR-1289	hsa-miR-30c-1*	hsa-miR-497	hsa-miR-675
hsa-miR-128a	hsa-miR-30c-2*	hsa-miR-503	hsa-miR-675b
hsa-miR-128b	hsa-miR-30d	hsa-miR-505*	hsa-miR-7
hsa-miR-129-3p	hsa-miR-30e	hsa-miR-506	hsa-miR-7-1*
hsa-miR-1291	hsa-miR-31	hsa-miR-506-3p	hsa-miR-7-2*
hsa-miR-1292	hsa-miR-3118	hsa-miR-508	hsa-miR-720
hsa-miR-1293	hsa-miR-3127	hsa-miR-510	hsa-miR-760
hsa-miR-1301	hsa-miR-3129	hsa-miR-512-5p	hsa-miR-761
hsa-miR-1304	hsa-miR-3129-5p	hsa-miR-513	hsa-miR-762
hsa-miR-1305	hsa-miR-3135	hsa-miR-513-5p	hsa-miR-764
hsa-miR-1307	hsa-miR-3157	hsa-miR-513a-3p	hsa-miR-766
hsa-miR-1308	hsa-miR-3167	hsa-miR-514	hsa-miR-767-3p
hsa-miR-130a	hsa-miR-3194	hsa-miR-514b-3p	hsa-miR-769-3p
hsa-miR-130b	hsa-miR-323-5p	hsa-miR-515-5p	hsa-miR-770-5p
hsa-miR-1321	hsa-miR-324-3p	hsa-miR-516a-3p	hsa-miR-873
hsa-miR-1323	hsa-miR-326	hsa-miR-516b*	hsa-miR-874
hsa-miR-133a	hsa-miR-330-3p	hsa-miR-519e*	hsa-miR-876-3p
hsa-miR-133b	hsa-miR-330-5p	hsa-miR-520a-5p	hsa-miR-876-5p
hsa-miR-134	hsa-miR-331	hsa-miR-524	hsa-miR-886-5p
hsa-miR-135b*	hsa-miR-331-3p	hsa-miR-524-3p	hsa-miR-887
hsa-miR-139-3p	hsa-miR-331-5p	hsa-miR-525-5p	hsa-miR-890
hsa-miR-140	hsa-miR-339	hsa-miR-526b	hsa-miR-920
hsa-miR-140-5p	hsa-miR-339-3p	hsa-miR-541	hsa-miR-922
hsa-miR-143	hsa-miR-339-5p	hsa-miR-542-5p	hsa-miR-92a-1*
hsa-miR-145	hsa-miR-342-3p	hsa-miR-544	hsa-miR-92a-2*
hsa-miR-146a*	hsa-miR-342-5p	hsa-miR-544-3p	hsa-miR-93*
hsa-miR-146b-3p	hsa-miR-344b-1-3p	hsa-miR-544a	hsa-miR-933
hsa-miR-147	hsa-miR-344d	hsa-miR-544b	hsa-miR-936
hsa-miR-148a	hsa-miR-344e	hsa-miR-545	hsa-miR-939
hsa-miR-148b	hsa-miR-34a	hsa-miR-548m	hsa-miR-940
hsa-miR-149	hsa-miR-34b*	hsa-miR-548o	hsa-miR-943
hsa-miR-149*	hsa-miR-34c	hsa-miR-548p	
hsa-miR-150	hsa-miR-34c-5p	hsa-miR-550	
hsa-miR-152	hsa-miR-361-3p	hsa-miR-552	
hsa-miR-153	hsa-miR-3619-5p	hsa-miR-553	
hsa-miR-1538	hsa-miR-363*	hsa-miR-555	

Predicted miRNA were sourced from mirna.org [174], TargetScan [175], miRDB [176] and miRWalk [177].

Table 8.2 Predicted RNA binding motifs in the CD151 3'UTR from RBPmap

RNA binding protein	Number of sites in 3'UTR
BRUNOL4	4
BRUNOL5	4
BRUNOL6	7
CNOT4	3
CUG-BP	29
ESRP2	5
FUS	1
HNRNPA1 / HNRNPA1L2	3
HNRNPA1	1
HNRNPA2B1	3
HNRNPF	10
HNRNPH2	4
HNRNPK	13
HNRNPL	8
HNRNPM	2
HNRPLL	7
HuR	3
IGF2BP2	5
IGF2BP3	3
KHDRBS1	1
KHDRBS2	1
KHDRBS3	1
LIN28A	1
MBNL1	69
NOVA1	10
PABPC1	1
PABPC3	3
PABPC4	1
PABPN1	1
PCBP1	16
PCBP2	12

PCBP3	1
PTBP1	20
RALY	4
RBM24	2
RBM38	4
RBM42	3
RBM6	4
RBMS1	4
SAMD4A	4
SART3	1
SFPQ	2
SNRPA	2
SRSF1	8
SRSF10	1
SRSF2	57
SRSF3	75
SRSF5	23
SRSF7	2
SRSF9	3
TARDBP	10
TIA1	6
TRA2B	1
YBX1	20
YBX2	2
ZC3H10	4
ZC3H14	2

RNA binding proteins predicted to bind to the CD151 3'UTR (bases 880 – 1545) were found using RBPmap [256]

8.3 miR-637 expression is associated with breast cancer patient survival

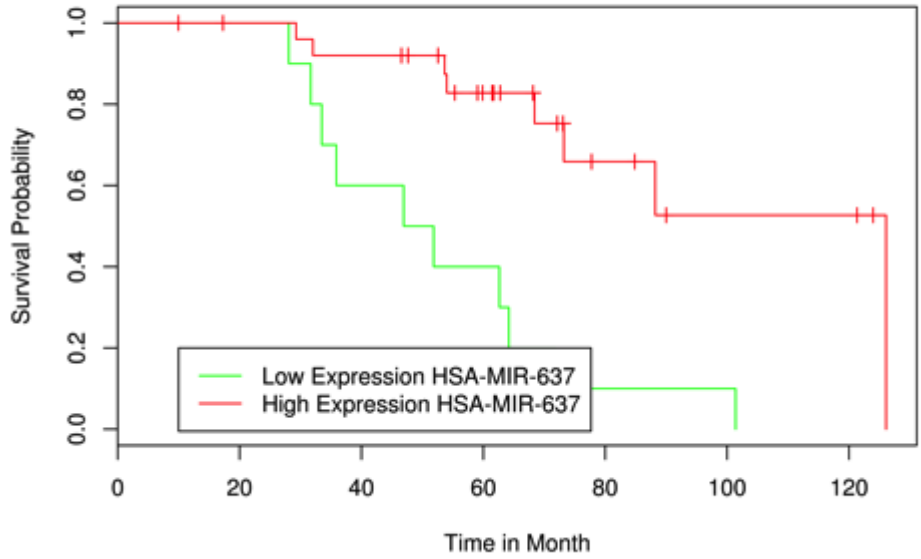

Figure 8.3. Low expression of miR-637 predicts poor survival of breast cancer patients. The GEO dataset (GSE37405) Global microRNA expression profiling of high-risk ER+ breast cancers from patients receiving adjuvant Tamoxifen monotherapy: a DBCG study was analysed using MIRUMIR [204] to determine the effect of miR-637 on breast cancer survival. Group 1 is low expression of miR-637 (*n=10*) and group 2 is high expression of miR-637 (*n=27*); Chisq= 13.8 on 1 degrees of freedom, *p= 2e-04.*

Table 8.4 miRNAs predicted to regulate the CD9 3'UTR using a range of target prediction databases

hsa-let-7a-2*	hsa-miR-3149	hsa-miR-523*
hsa-let-7g*	hsa-miR-3153	hsa-miR-524-5p
hsa-miR-103	hsa-miR-3155	hsa-miR-526a
hsa-miR-105	hsa-miR-3159	hsa-miR-526c
hsa-miR-106a*	hsa-miR-3167	hsa-miR-541*
hsa-miR-107	hsa-miR-3171	hsa-miR-542-5p
hsa-miR-10a*	hsa-miR-3184	hsa-miR-544
hsa-miR-1178	hsa-miR-3185	hsa-miR-545
hsa-miR-1179	hsa-miR-32	hsa-miR-548a-5p
hsa-miR-1182	hsa-miR-325	hsa-miR-548ab
hsa-miR-1207-5p	hsa-miR-330	hsa-miR-548ak
hsa-miR-1224-3p	hsa-miR-330-3p	hsa-miR-548am-5p
hsa-miR-1225-5p	hsa-miR-335	hsa-miR-548ap-5p
hsa-miR-1229	hsa-miR-335*	hsa-miR-548aq-5p
hsa-miR-1231	hsa-miR-338-5p	hsa-miR-548ar-5p
hsa-miR-124	hsa-miR-33b*	hsa-miR-548as-5p
hsa-miR-1243	hsa-miR-340	hsa-miR-548au-5p
hsa-miR-1251	hsa-miR-345	hsa-miR-548b-5p
hsa-miR-1257	hsa-miR-34b*	hsa-miR-548c-3p
hsa-miR-1260	hsa-miR-3529-3p	hsa-miR-548c-5p
hsa-miR-1260a	hsa-miR-361-5p	hsa-miR-548d-3p
hsa-miR-1260b	hsa-miR-3613-3p	hsa-miR-548d-5p
hsa-miR-1262	hsa-miR-363	hsa-miR-548h
hsa-miR-1263	hsa-miR-367	hsa-miR-548h-5p
hsa-miR-1266	hsa-miR-368	hsa-miR-548i
hsa-miR-127-5p	hsa-miR-3688-5p	hsa-miR-548j
hsa-miR-1276	hsa-miR-369-5p	hsa-miR-548k
hsa-miR-1280	hsa-miR-370	hsa-miR-548l
hsa-miR-1283	hsa-miR-371	hsa-miR-548m
hsa-miR-1286	hsa-miR-374a	hsa-miR-548n
hsa-miR-129-5p	hsa-miR-374b	hsa-miR-548o
hsa-miR-1290	hsa-miR-376a*	hsa-miR-548o-5p
hsa-miR-1302	hsa-miR-376c	hsa-miR-548p
hsa-miR-1303	hsa-miR-378*	hsa-miR-548q
hsa-miR-1308	hsa-miR-379	hsa-miR-548u

hsa-miR-1323	hsa-miR-380	hsa-miR-548w
hsa-miR-138-2*	hsa-miR-382	hsa-miR-548x
hsa-miR-141*	hsa-miR-3924	hsa-miR-548y
hsa-miR-142-5p	hsa-miR-409-3p	hsa-miR-549
hsa-miR-144	hsa-miR-410	hsa-miR-551b*
hsa-miR-148a	hsa-miR-411*	hsa-miR-555
hsa-miR-148b	hsa-miR-424	hsa-miR-559
hsa-miR-149*	hsa-miR-4261	hsa-miR-561
hsa-miR-152	hsa-miR-4271	hsa-miR-562
hsa-miR-15a	hsa-miR-4276	hsa-miR-568
hsa-miR-15b	hsa-miR-4281	hsa-miR-5688
hsa-miR-16	hsa-miR-4282	hsa-miR-570
hsa-miR-16-1*	hsa-miR-4289	hsa-miR-572
hsa-miR-16-2*	hsa-miR-429	hsa-miR-577
hsa-miR-181a*	hsa-miR-4292	hsa-miR-578
hsa-miR-182	hsa-miR-4294	hsa-miR-579
hsa-miR-185	hsa-miR-4298	hsa-miR-589
hsa-miR-186	hsa-miR-4299	hsa-miR-589*
hsa-miR-186*	hsa-miR-4305	hsa-miR-590-3p
hsa-miR-187	hsa-miR-4306	hsa-miR-590-5p
hsa-miR-188-3p	hsa-miR-4309	hsa-miR-592
hsa-miR-1909	hsa-miR-4310	hsa-miR-599
hsa-miR-1911	hsa-miR-4317	hsa-miR-601
hsa-miR-192*	hsa-miR-433	hsa-miR-605
hsa-miR-195	hsa-miR-449c	hsa-miR-606
hsa-miR-200b	hsa-miR-454	hsa-miR-607
hsa-miR-200c	hsa-miR-454-3p	hsa-miR-615
hsa-miR-2054	hsa-miR-484	hsa-miR-616
hsa-miR-208	hsa-miR-486-3p	hsa-miR-621
hsa-miR-208b	hsa-miR-491	hsa-miR-624*
hsa-miR-2114	hsa-miR-491-3p	hsa-miR-625
hsa-miR-216a	hsa-miR-493*	hsa-miR-627
hsa-miR-218	hsa-miR-494	hsa-miR-628
hsa-miR-219-1-3p	hsa-miR-495	hsa-miR-628-3p
hsa-miR-219-2-3p	hsa-miR-496	hsa-miR-634
hsa-miR-219-5p	hsa-miR-497	hsa-miR-641
hsa-miR-25	hsa-miR-5007-5p	hsa-miR-642
hsa-miR-2861	hsa-miR-511	hsa-miR-643
hsa-miR-296	hsa-miR-513a-5p	hsa-miR-647
hsa-miR-298	hsa-miR-514	hsa-miR-653
hsa-miR-29c*	hsa-miR-515-3p	hsa-miR-656
hsa-miR-301	hsa-miR-515-5p	hsa-miR-661
hsa-miR-301a	hsa-miR-516b	hsa-miR-664
hsa-miR-301b	hsa-miR-517*	hsa-miR-671-5p
hsa-miR-302a*	hsa-miR-518d-5p	hsa-miR-672

hsa-miR-302f	hsa-miR-518e-5p	hsa-miR-7-1*
hsa-miR-3065-5p	hsa-miR-518e*	hsa-miR-7-2*
hsa-miR-30a	hsa-miR-518f-5p	hsa-miR-766
hsa-miR-30a-3p	hsa-miR-518f*	hsa-miR-767-3p
hsa-miR-30b	hsa-miR-519a-5p	hsa-miR-802
hsa-miR-30c	hsa-miR-519a*	hsa-miR-872
hsa-miR-30c-1*	hsa-miR-519b-5p	hsa-miR-875-3p
hsa-miR-30c-2*	hsa-miR-519c-5p	hsa-miR-876-5p
hsa-miR-30d	hsa-miR-519e	hsa-miR-889
hsa-miR-30e	hsa-miR-520c-5p	hsa-miR-891b
hsa-miR-31	hsa-miR-520d-5p	hsa-miR-924
hsa-miR-3120	hsa-miR-520f	hsa-miR-92a
hsa-miR-3122	hsa-miR-520g	hsa-miR-92b
hsa-miR-3125	hsa-miR-520h	hsa-miR-935
hsa-miR-3128	hsa-miR-522-5p	hsa-miR-944
hsa-miR-3135	hsa-miR-522*	
hsa-miR-3148	hsa-miR-523-5p	

Predicted miRNA were sourced from mirna.org [174], TargetScan [175], miRDB [176] and miRWalk [177].

Table 8.5 Predicted RNA binding motifs in the CD9 3'UTR using RBPmap

RNA binding protein	*Number of predicted sites*
A1CF	4
BRUNOL4/5/6	27/27/4
CPEB4	31
CUG-BP	8
DAZAP1	3
HNRNPC/L1	48/48
HNRNPF/L/M/U	7/3/23/4
HuR	48
KHDRBS1/2/3	5/5/12
MATR3	4
MBNL1	52
PABPC1/3/4/5	6/4/8/6
PABPN1	6
PTBP1	14
PUM2	7
RALY	45
RBM24/38/41/S1/S3	6/6/10/11/15
SART3	6
SFPQ	7
SRSF2/3/7	16/18/3
TARDBP	30
TIA1	48
TRA2B	10
TUT1	8
U2AF2	35

ZC3H14	50
ZCRB1	4
ZNF638	14

RNA binding proteins predicted to bind to the CD9 3'UTR (bases 872 – 1314) were sourced from RBPmap [256]

Table 8.6 miR-518f* is predicted to regulate genes involved in cancer associated pathways

Cancer Associated Pathway	***Number of genes***
Androgen/estrogen/progesterone biosynthesis	2
Angiogenesis	22
Apoptosis signalling	13
Cadherin signalling	17
Cell Cycle	2
Cytoskeletal regulation by Rho GTPase	17
DNA replication	3
EGF receptor signalling pathway	18
FAS signalling pathway	8
FGF signalling pathway	14
General transcription my RNA polymerase I	1
General transcription regulation	3

Hedgehog signalling pathway	4
Hypoxia response via HIF activation	7
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	4
Insulin/IGF pathway-protein kinase B signalling cascade	6
Integrin signalling pathway	30
JAK/STAT signalling pathway	1
Notch signalling pathway	6
Oxidative stress response	3
PDGF signalling	19
PI3 kinase signalling pathway	9
Ras signalling pathway	15
TGF-beta signalling pathway	12
Ubiquitin proteasome pathway	8
VEGF signaling pathway	10
Wnt signaling pathway	38
p38 MAPK pathway	6
p53 pathway by glucose deprivation	3
p53 pathway feedback loops 2	9
p53 pathway	13

Predicted gene targets of miR-518f* were obtained from microrna.org/ and were subjected to a pathways analysis using PANTHER [199].