Regulation of cell-cell adhesion by the metastasis suppressor tetraspanin CD82/KAI1

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Abstract

Cell junctions are important sites of intercellular adhesion. They preserve the integrity of epithelial tissue and control cell signalling. Deregulation of genes associated with cellular junctions can result in tumour transformation and invasion. Cell junctions consists of desmosomes, tight junctions, gap junctions and adherens junctions. Cellular transformation, invasion and metastasis is brought about by the dysregulation of cell junction components and are therefore crucial for the suppression of metastases. Preliminary experiments in the lab indicated a potential interaction between the metastasis suppressor CD82 a member of the tetraspanin superfamily pf glycoproteins, and desmosomes. By performing Western blotting and semi-quantitative RT-PCR it showed CD82 regulates the expression of desmosomal proteins plakoglobin and desmoglein2, and the distribution of desmoglein2 within the cell. Despite down regulating plakoglobin and desmoglein2 protein levels, CD82 was found to promote cell-cell adhesion. Plakoglobin and CD82 were found to co-localise by immunofluorescence and an interaction was observed by immunoprecipitation. CD82 did not associate with desmoplakin and was not implicated in the trafficking of desmoplakin to the membrane during desmosome assembly. The significance of these finding remain to be determined, but they provide a useful platform for the development of future work.
Acknowledgments

First and foremost I would like thank my supervisors; Dr Elena Odintsova and Dr Martyn Chidgey for sharing their expert knowledge and providing unwavering support during the course of this project. Without their patience and support this study would not have been possible. I would also like to thank Izabella Bombik, Xiao-Hong Lu and Vera Novitskaya for all their help and guidance in the laboratory.
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Introduction

1.0. Introduction

Cancer is a significant cause of morbidity and mortality globally. Although rates of cancer vary by countries, it is estimated that in 2012, 14.1 million new cases of cancer were diagnosed worldwide (Cancer Research UK, 2014). There have been huge advances in diagnosis and therapeutic modalities for cancer patients, however despite these, metastases are the major cause of cancer related deaths (Fokas et al, 2007). It is suggested that they may account for 90% of all cancer deaths (Fokas et al, 2007). The term “metastasis” was coined in 1829 by Jean Claude Recamier a French gynaecologist who observed that malignant tumours have the ability to colonise and invade distant organs (Paget, 1889, Beavon, 1999, Talmadge and Fidler, 2010). This profound observational study kick started investigations into the mechanisms of metastasis in cancer (Talmadge and Fidler, 2010).

Metastasis is the transfer of disease from one organ or part to another not directly connected to it (Talmadge and Fidler, 2010). Metastasis is a complex phenomenon comprising of a series of biological events (Fokas et al, 2007, Talmadge and Fidler, 2010). A seminal study carried out in 1889 by Stephen Paget, identified the role of the host-tumour interactions. Paget hypothesised that tumour cells colonise selectively distant organs which have an environment that is suitable for enabling the survival of the tumour cell (Fokas et al, 2007, Talmadge and Fidler, 2010). From his findings he developed the theory of “Seed and Soil,” he stated “When a plant goes to seed, its seeds are carried in all directions but they can only live and grow if they fall on congenial soil” (Fokas et al, 2007, Talmadge and Fidler, 2010). Evidence from
Sugarbaker’s experimental study supported Paget’s theory and has also shown that tumours originating in certain organs favour particular sites for metastases (Sugarbaker, 1952). Studies repeated by Fidler and colleagues have verified that metastasis is dependent on interactions between tumour cells and host interactions (Paget, 1889, Hart and Fidler, 1980). These studies provide the basis for ongoing research.

Metastasis development has been described as a multi-step process with a series of distinct stages called “the invasion –metastasis cascade” (Fidler, 2003, Talmadge and Fidler, 2010). Malignant cells from primary tumours are spread to distant organs by invading through local extracellular matrix (ECM) and stromal cell layers and intravasate into proximate blood and lymphatic vessels. Survival in the circulation followed by escape of these cells to extravasate into distant tissue is the next stage of the process. The survival of the cells in foreign microenvironments in order to form micrometastases which develop into clinically detectable macroscopic tumours is known as colonisation (Talmadge and Fidler, 2010, Valastyan and Weinberg, 2011).

It is suggested that the systemic spread of cancer cells is brought about by a number of biomechanical processes and genetic controls (Kaluri and Weinberg, 2009). Metastasis has been described as an enigmatic aspect of cancer disease and as it remains the cause of most cancer deaths, it is an area of intense research (Kaluri and Weinberg, 2009, Tania et al, 2014). Epithelial mesenchymal transition (EMT) has been proposed as a mechanism crucial for the
development of invasive and metastatic growth characteristics of tumour cells (Kaluri and Weinberg, 2009, Tania et al, 2014).

1.1. Epithelial-mesenchymal transition.

The epithelial to mesenchymal transition (EMT) is a biological process through which polarised epithelial cells that ordinarily interact with basement membrane via their basal surface, undergo numerous biochemical changes that allow them to adopt a mesenchymal phenotype (Tania et al, 2014). This includes enhanced migratory capacity, invasiveness, increased resistance to apoptosis and a significant increase in the production of extracellular matrix (ECM) (Kaluri and Weinberg, 2009, Tania et al, 2014). Epithelial and mesenchymal are terms that refer to the shape of the cells and their adhesive properties.

EMT and its reverse process known as mesenchymal to epithelial transition (MET) are recognised as fundamental biological processes during embryonic development and development processes such as mesoderm formation, neural tube formation, organ fibrosis and wound healing (Tania et al, 2014). EMT and its converse, MET are concepts that were first defined by Elizabeth Hay in 1968 (Hay, 1968). She proposed that epithelial cells can undergo phenotypic changes that demonstrate their transformation to mesenchymal cells (Hay, 1968, Hay, 1995). Epithelial and mesenchymal cells differ both morphologically and functionally. During embryogenesis and organ development, the cells within certain epithelia seem to be plastic and are therefore able to switch back and forth through the processes of EMT and MET into epithelial and mesenchymal states (Kaluri and Weinberg, 2009). Although
EMT and MET are recognised as the process through which cells transition from epithelial and mesenchymal and vice versa, they are not considered to be involved in cell specification or cell fate (Acloque et al, 2009).

EMTs have been classified into three biological subtypes based upon functional distinctions (Kaluri and Weinberg, 2009). Type 1 EMTs are associated with implantation, embryo formation and organ development (Kaluri and Weinberg, 2009). They do not cause fibrosis or induce an invasive phenotype that can lead to systemic spread through the circulation (Kaluri and Weinberg, 2009). Type 2 EMTs are associated with inflammation and cease when the inflammation is decreased, as can be seen in tissue regeneration and wound healing (Kaluri and Weinberg, 2009). Type 3 EMTs are associated with neoplastic cells that have already undergone genetic and epigenetic changes, particularly in genes that support clonal expansion and growth of localised tumours (Kaluri and Weinberg, 2009). Cancer cells which are generated by a type 3 EMT can form the first step in the metastatic cascade (Acloque et al, 2009). These cells can invade and metastasise and thereby produce the life-threatening symptoms of cancer progression (Kaluri and Weinberg, 2009).

1.2. Tetraspanins

The tetraspanins are a group of transmembrane proteins with 33 mammalian members (Helmer, 2014). They are located on the plasma membrane and in a variety of intracellular organelles and granules within almost all cell and tissue types (Helmer, 2014). Tetraspanins share low sequence homology but have conserved secondary and tertiary structures
composed of four transmembrane domains, two extracellular domains and three relatively short cytoplasmic regions (Le Nour et al, 2006). In spite of the fact, that these proteins are readily observable in large quantities, they have not been studied comprehensively and therefore their functional significance are unknown. What is understood is that tetraspanins organise laterally with other membrane proteins to create tetraspanin enriched microdomains (Helmer, 2014). In the context of tetraspanin enriched microdomains, tetraspanins can affect cell adhesion, migration, invasion, signalling, cell-cell fusion, infection by cancer causing viruses, morphology and survival (Helmer, 2014). All of these functions are influential during the various stages of cancer development.

Tetraspanin CD151 plays crucial roles in the regulation of adhesion and motility of tumour cells. As well as regulating individual tumour cells, it is also responsible for regulating collective tumour cell migration (Johnson et al, 2009). Loss of CD151 destabilises E-cadherin-dependent cell-cell junctions and augments migration of complete tumour cell sheets. It was demonstrated that the loss of CD151 causes an increase in RhoA activation, loss of actin organisation at cell-cell junctions and amplified actin stress fibers at the basal cell surface. Cell-cell contacts were observed to exhibit a 3 fold rise in remodelling rate as well as a reduction in lifespan in CD151 knockout monolayers in comparison with wild type cell-cell contacts. Junctional stability was restored following re expression of CD151 (Johnson et al, 2009).
1.3. KAI1/CD82

KAI1 (CD82) is a member of the tetraspanin superfamily of glycoproteins located on chromosome 11p11.2 (Tsai and Weissman, 2011). CD82 was identified as a metastasis suppressor (Dong et al, 1995) from a screen of genes located on chromosome 11 which suppressed metastasis of rat AT6.1 prostate cancer cells (Tsai and Weissman, 2011). It has been demonstrated that re-expression of CD82 using microcell mediated chromosome transfer suppressed metastatic spread and was down regulated in metastatic prostate cancer (Tsai and Weissman, 2011, Dong et al, 1995). Subsequent research using other metastatic cell lines, comprising of MDA-MB-435, HT1080 sarcoma, lung LLC, liver MHCC97-H, breast LCC6 and prostate LNCaP, further supported the idea that CD82 is a metastasis suppressor (Miranti, 2009). Numerous clinical studies additionally confirmed CD82’s role as a metastasis suppressor as it was demonstrated that loss of CD82 mRNA and protein, strongly correlated with a poorer prognosis in many malignancies such as prostate, colon, ovarian, breast, lung, pancreatic and several others (Miranti, 2009).

CD82 through various mechanism inhibits metastasis. It suppresses cell motility and invasion, promotes cell polarity and in response to extracellular stimuli induces senescence and apoptosis (Tsai and Weissman, 2011). A typical element of these varied features include CD82 regulation of membrane organisation, protein interactions and trafficking, influencing cellular signalling and intercellular communication (Tsai and Weissman, 2011). CD82 associates with cell migration proteins including cell adhesion molecules, growth factor receptor and signalling molecules in tetraspanin-enriched microdomains (Liu and Zhang, 2006) as well as localising to intracellular late endosomes and lysosomes. It is increasingly recognised that
CD82 is in a position to modulate endocytic trafficking of related molecules such as the epidermal growth factor receptor (EGFR) (Helmer, 2014). It has been demonstrated that CD82 regulates compartmentalisation, ligand-induced dimerization and ubiquitylation of EGFR (Odintsova et al, 2013).

Further studies have demonstrated that CD82 limits tumour progression and metastasis in a variety of ways. Primarily CD82 down regulated Frizzled (FZD) proteins which act as WNT co-receptors and therefore prevented the canonical signalling through the WNT-β catenin pathway (Chigita et al, 2012). Secondly CD82 expression was related to reduced β-catenin degradation, by down regulation of GSK-3β and CK1α, leading to accumulation and stabilisation of E-Cadherin-β-catenin complexes at the cell membrane thereby promoting cell-cell adhesion and inhibiting cancer metastasis (Chigita et al, 2012).

Overexpression of CD82 was shown to inhibit cell motility and invasion via CD82 association with α6 integrin reducing laminin adhesion and migration. Decreased laminin adhesion and motility was a result of CD82 decreasing α6 integrin via its internalization (He et al, 2005). Overexpression of CD82 in lung H1299 cells resulted in reduced cell surface expression of β1 integrin through interfering with maturation and glycosylation of β1 integrin. Urokinase plasminogen activator surface receptor (uPAR) activity is decreased 50 fold by expression of CD82. Expression of CD82 resulted in redistribution of uPAR to focal adhesions where it associates with α5β1 (Bass et al, 2005). This interaction interrupts uPAR binding with its ligand uPA, preventing extracellular matrix proteolysis. Cell invasion is regulated by CD82 by regulating the localisation of extracellular proteases (Bass et al, 2005).
Current evidence does not verify the processes that involve gene mutations, loss of heterozygosity, promoter hypermethylation or mutation to explain the loss of CD82. There is however some evidence to suggest that loss of function of CD82 during cancer progression is a complex process that involves several mechanisms including: altered transcriptional regulation, production of a splice variant which produces a dominant negative protein and post translational modification of CD82 (Tonoli and Barrett, 2005). Expression of CD82 is positively regulated by p53, AP2 and junB, loss of expression of these transcription activators corresponds with the loss of CD82 (Stafford et al, 2008).

1.4. Cell-cell junctions

Cell-cell adhesion is a fundamental requisite for the correct functioning of cells, tissues and entire organisms (Brooke et al, 2012). The mechanical and communication functions imperative for tissue morphogenesis and homeostasis in multicellular organisms depends on four major cell-cell junctions: tight junctions, adherens junctions, gap junctions and desmosomes (Nekrasova and Green, 2013). These cell junctions promote adherence and communication between cells and preserve the integrity of the larger tissue (Brooke, et al, 2012). As well as acting as physical bridges between cells, cell junctions play a wider role in signalling cascades, regulating pathways imperative for controlling cell shape and motility, forming cell polarity, establishing a balance between proliferating and differentiation (Nekrasova and Green, 2013). Dysfunction of intracellular junctions are associated with a variety of diseases of genetic, auto-immune, cancerous and infectious aetiology (Wells et al, 2011).
Tight junctions are cellular structures that are located at the apico-basal region of epithelial cell membranes (Wells et al, 2011). Maintenance of the epithelial barrier is considered to be the primary role of tight junctions (Brooke et al, 2012). They regulate the permeability of the paracellular pathway among epithelial cells and maintain tissue homeostasis (Brooke et al, 2012). The apical distribution of tight junctions have been described as “gates” and “fences”. They act as a gate to limit the diffusion of pathogens, small molecules and ions and also act as a fence to confine lipid and membrane proteins along the apico-basolateral axis (Wells et al, 2011). Although the barrier and fence function of tight junctions has been well recognised, the understanding of tight junctions as a complex, multiprotein structure with functions in other cell processes like cell polarity, proliferation and differentiation is more recent (Martin et al, 2014). Increasing evidence shows that tight junctions play a crucial role in the maintenance of cell to cell adhesions and that loss of cohesion of the structure can lead to invasion and metastasis (Martin et al, 2014).

Adherens junctions are evolutionarily conserved dynamic plasma membrane structures which facilitate cell-cell adhesions (Ivanov and Naydenov, 2013). Their primary features are organising and binding the cytoskeleton to the plasma membrane and maintaining close communication between cells (Brooke et al, 2012). Adherens junctions are assembled from a number of proteins, particularly cadherins and catenins which are grouped and stabilised by a variety of cytoplasmic scaffolds (Ivanov and Naydenov, 2013). The key roles of adherens junctions are to regulate tissue architecture via regulation of cell shape, polarity, motility, proliferation and survival (Ivanov and Naydenov, 2013). They are vital to the normal process of tissue morphogenesis and therefore any disruptions can lead to abnormalities in diverse
tissues (Ivanov and Naydenov, 2013). Dysfunction of adherens junctions has been associated with epithelial diseases, however more recently their role in the suppression of cancer development has been recognised (Dusek and Attardi, 2011).

Gap junctions are cell adhesion complexes which facilitate intracellular communication through the exchange of ions and small molecules between cells (Wells et al, 2011). This key function is crucial for homeostasis and responses to external stimuli (Brooke et al, 2012). The loss of gap junctional intracellular communication and alteration of the structural protein of gap junctions, connexins have been observed in tumour cells and are regarded as a hallmark of cancer (Defamie et al, 2014).

Desmosomes are also one of the main cell to cell junctions. They are intercellular adhesion molecules which are anchored through the intermediate filaments to plasma membranes creating a supracellular scaffold, providing mechanical resilience to tissues (Nekrasova and Green, 2013). In tissue types which experience mechanical stress, such as intestinal mucosa, uterus, oviduct, gall-baldder, stomach, thyroid, salivary glands, pancreas and liver, desmosomal structures provide strength to tissues, in particular the skin and heart (Brooke et al, 2012, Nekrasova and Green, 2013). As well as providing mechanical stability, they also enable cell-cell communication through signal transmission (Brooke et al, 2012). The structure of desmosomes has been analysed by electron microscopy and reveals complex organisation and structure (Brooke et al, 2012).
Similar to adherens junctions, desmosomes are built up of three main protein families: cadherins, armadillo proteins and plakins (Nekrasova and Green, 2013, Dusek and Attardi, 2011). The desmosomal cadherins, desmogleins (Dsgs) and desmocollins (Dscs) are transmembrane molecules which facilitate adhesion through their extracellular domains and thereby act as a scaffold for the building of the desmosomal plaque through their cytoplasmic domains (Nekrasova and Green, 2013). Intracellularly, cadherins bind to the armadillo proteins, plakoglobin (Pg) and plakophilins (Pkp1-3) and these then associate with desmoplakin (Dsp) (Dusek and Attardi, 2011, Nekrasova and Green, 2013). The loss of plaque proteins plakophilin 1 and 3 increase the motility of cultured cells (Wells et al, 2011). Desmoplakin interacts with junction plakoglobin and intermediate filaments through its C terminus to create the final link in the chain from the plasma membrane to the cytoskeleton (Dusek and Attardi, 2011, Nekrasova and Green, 2013). Interaction of intermediate filaments with desmosomes raises the strength given by the intermediate filament cytoskeleton through all of the tissue and is therefore crucial for tissue integrity (Nekrasova and Green, 2013).

1.5. Project aims

Preliminary experiments and published data indicates a correlation between the expression of CD82 and desmosomal components. It is postulated that CD82 could potentially be implicated in either desmosome assembly/ disassembly or in the regulation of desmosomal adhesive strength. The aim of the study is to investigate the functional importance of the effect of CD82 on the desmosome complex.
Methods and Materials

2.0. Cell culture and transient transfections

The human mammary epithelial cells HB2 and 2.5.2A and their transfectant derivatives HB2CD82 and 2.5.2A shCD82 (Odintsova et al, 2013) were maintained in Dulbecco’s Modified Eagle medium (DMEM) (Gibco) supplemented with 1% Penicillin Streptomycin (Gibco) 10% heat inactivated foetal calf serum (Gibco), 10 µg/ml of insulin and 10 µg/ml of hydrocortisone at 37°C with 5% CO₂. 2.5.2A shCD82 cells were propagated in the same media supplemented with puromycin 1µg/ml.

For the initial evaluation of transfection reagents, cell were grown to 50% confluence on Lab-Tek Chamber Slides (Thermo Scientific) and transfected using GeneJammer (Agilent technologies), FuGENE (Promega) and Lipofectamine2000 (Life technologies) according to the manufacturer’s protocol.

2.1. Western blotting

Whole cell extracts were prepared from cells grown until confluence in 6cm² dishes in 1x Laemmli buffer (10% Glycerol, 2% SDS, Tris-HCl, (pH 6.8) and H₂O) containing protease inhibitors (2mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin) and phosphatase inhibitors (sodium orthovanadate, sodium fluoride and sodium pyrophosphate) and boiled for 5 minutes. 20-30µg of protein was separated by SDS-PAGE (8% or 10% gel) under reducing and non-reducing conditions and transferred to nitrocellulose membranes.
Membranes were washed in Tris-Buffered Saline with 1% Tween 20 (TBS-T) and then blocked with 5% non-fat milk extract in TBS-T for 1h. Membranes were then rinsed with TBS-T before incubation with primary antibody in 5% Bovine serum albumin (BSA) in TBS-T for either 2h at room temperature or overnight at 4°C. Membranes were washed with TBS-T three times for 20 min. The membrane was then exposed to appropriate secondary antibody (diluted 1:20000 in TBS-T containing 5% milk) either coupled to horse radish peroxidase (Dako) or IRDye680/800 secondary antibody for 1h at room temperature. After washing the membranes three times for 20 min, signals were visualized using chemiluminescence regent or LI-COR Odyssey Imaging system. Densitometric analysis was performed using Image J (Schneider et al, 2012).

2.2. Immunoprecipitation

Cells were grown until confluence and washed with ice cold Phosphate buffered saline (PBS) and lysed in buffer containing 1% Triton X-100 (150mM Tris-Cl pH 7.4, 150mM NaCl) or 1% Brij 98 (150mM Tris-Cl pH 7.4, 150mM NaCl) or a mixture of the two, protease and phosphatase inhibitors (as above) were added to all buffers, for 2 h at 4°C on a rotating wheel. Insoluble material was removed by centrifugation (12000rpm for 10 min). The cell lysates were then precleared by incubation with 20µl Anti protein G sepharose beads (Sigma) for 2h at 4°C. Appropriate antibodies were incubated with the lysate for 2h. To collect immunoprecipitated protein complexes 20µl protein G sepharose beads (Sigma) was applied to the lysates on a rotating wheel for 1h. The beads were then washed 3 times in lysis buffer
and the complexes were eluted by boiling the samples with 40µl Laemmli buffer. Proteins were resolved and detected by SDS-PAGE/ western blot as described above.

2.3. Immunofluorescence

For immunofluorescence, cells (5x10^4) were plated on glass coverslips and grown in complete media for 24-36h. Cells were then fixed with 2% paraformaldehyde for 10 min, cover slips were rinsed 3 times with PBS and permeabilised with 0.1% Triton X100/PBS for 2 min. After fixation and permeabilisation cells were blocked with 150µl blocking buffer (PBS, goat serum, human serum and sodium azide) for 60 min. Primary antibody in blocking buffer was applied to the cells for 60 min at room temperature or overnight at 4°C followed by 3 washes with PBS for 10 min. Coverslips were incubated with appropriate alexa fluor conjugated secondary antibody (1:1000) (Molecular probes) for 60 min. Subsequently coverslips were washed 3 times for 20 min and left to air dry. Finally dry coverslips were mounted onto slides with mounting media (Life technologies) and sealed with nail varnish. Images were captured using a LSM 510 META confocal system with 63x objective (NA1.4).

2.4. Dispase assay

Confluent cell cultures grown on 12 well dishes in triplicates were rinsed with PBS and incubated with 2.4 U/ml dispase (PBS) (Roche) for 30 min at 37°C. Released monolayers were rotated on a linear shaker (350rpm) for 20 min before imaging.
2.5. RNA extraction, RT-PCR and semi quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesised from 1µg RNA using high capacity cDNA reverse transcription kits (Applied Biosystems) according to the manufacturer’s instructions. PCR was performed using 1µl cDNA, 2.5µl Buffer 2 (Roche), 0.5µl dNTPs, 1µl primers, 0.2µl High fidelity Taq polymerase (Roche) and 19.8µl H2O. Amplified products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

Table 1. Primer sequences used for semi quantitative PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (BP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
<td>AGAAGCCTGGATAGAGGAG</td>
<td>53</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCACCGCAATCCTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsc2</td>
<td>Forward</td>
<td>GCACTCCTGCAGCCAAT</td>
<td>53</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGACTGAGTTGGATGAGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsc3</td>
<td>Forward</td>
<td>CCATCACTGGCAGGTCTCT</td>
<td>53</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGAGTCACTGGCTGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg2</td>
<td>Forward</td>
<td>CAATCACAGGTAGGTCTCTG</td>
<td>53</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGTGTAAGCTGGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dsg3</td>
<td>Forward</td>
<td>AGATGCTGCATGTTGAGG</td>
<td>56</td>
<td>460</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGTGTAAGCTGGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pkp2</td>
<td>Forward</td>
<td>AGATGCTGCATGTTGAGG</td>
<td>56</td>
<td>460</td>
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2.6. DNA manipulation

2.6.1. Polymerase chain reaction (PCR)

PCR was used to amplify and remove intervening, extraneous DNA between the Dsp and GFP sequences in the plasmid pDsp-GFP-Temp. PCR was carried out using this plasmid as a template for PCR and the primers DP1 (incorporates a BglII site) and DP2 (incorporates a NotI site) (Table 2). A 25μl reaction consisted of 2.5μl buffer 2 (x10) (Roche), 2.5μl DTNPs (2mM) 2.5μl primers (DP1, DP2), 16μl H₂O, 0.5μl high fidelity taq polymerase (Roche) and 1μl template DNA. The thermocycler program consisted of an initial denaturation step at 95°C for 5 min, then 20 cycles of denaturation at 95°C for 30sec, annealing at 63°C for 45 sec, and polymerization at 72°C for 1 min.
Table 2. Primer sequences to amplify and remove intervening, extraneous DNA between the Dsp and GFP sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5’-3’</th>
<th>Annealing temperature (°C)</th>
<th>Product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP1</td>
<td>ATGGGTGAGCAAGGGCGAGGAG</td>
<td>65</td>
<td>700</td>
</tr>
<tr>
<td>DP2</td>
<td>GATGTTCATTTTCGCCGCGCTG</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

2.6.2. Restriction digests

Restriction digest were carried out at 37°C for 90 min. Reaction (20µl) contained 1µl enzyme (10 units/µl) (New England biolabs), 1µl Not I (New England biolabs), 2µl React 3 buffer (x10) (New England biolabs), 2µl BSA (x10) and 14µl of DNA.

2.6.3. Ligation

Ligation reactions were performed using 1.5µl DNA ligase, 1µl T4 DNA ligase (New England biolabs), 8µl insert DNA, 2µl backbone DNA and the volume was made up to 15µl with 2.5µl H₂O. The reaction was carried out at room temperature for 2h.
2.6.4. E.coli transformation

7.5 µl DNA was added to competent XL1 blue E.coli cells thawed on ice and left for 30 min. E.coli cells where then heat shocked at 42°C for 2 min. 1ml of LB medium was added to the cells and incubated at 37°C for 1h. E.coli cells were then precipitated by centrifugation at 6,000rpm for 3 min, the supernatant was removed and cells were resuspended in 100µl LB medium. Cells were spread out on LB agar plates with kanamycin (50µg/ml) and incubated at 37°C overnight.

2.6.5 Preparation of mini and midi prep DNA.

Colonies were picked from LB-Kanamycin agar plates into 5ml (mini) or 25ml (midi) LB-kanamycin (50µg/ml) medium and an overnight culture was produced by incubation at 37°C. Plasmid DNA was isolated and purified using Qiagen mini and midi preps kits according to the manufacturer’s protocol. DNA concentration was determined using Nanodrop (Thermoscientific).

2.6.6 Sequencing

PCR sequencing was carried out using the Big Dye Cycle sequencing kit (Applied Biosystems) according to the manufactures protocol. The sequences of the primers used were: 5’CAAGGACGACGCAACTACA3’, 5’TGTAGTTGCGTCGCCTTG3’, 5’GCAAGTAAAACCTCTACAATG3’. Samples were resolved by the Functional Genomics department based in Biosciences
at the University of Birmingham. Analysis of sequencing data was carried out using Chromas lite software.

2.7. Live cell imaging

Cells grown on Lab-Tek chamber slides were transfected with constructs using lipofectamine2000 as per manufacturer’s instructions. Media was changed after 48h to low calcium medium (0.05mM) (i.e Calcium free DMEM supplemented with 0.05mM calcium chloride) for 18-20 hours. Real time images were acquired at intervals of 1 minute following media change to live cell imaging solution (Gibco) supplemented with 10% Foetal calf serum. Images were analysed using Aim software (Zeiss).
Results

3.0. Changes in the expression levels of CD82 have a selective effect on the expression of the proteins involved in cell-cell adhesion.

Preliminary experiments in the lab and data in published literature (Risinger et al, 2013) indicated a correlation between expression of metastasis suppressor tetraspanin CD82/KAI1 and desmosomal proteins. However, the effect of CD82 expression on desmosomal mediated cell adhesion has not been elucidated. To evaluate the effect of CD82 on desmosomes, we first analysed the protein expression levels of desmosomal proteins along with other cell-cell junction proteins in human mammary epithelial HB2/zeo (transfection control) and HB2/CD82 (transfected with full length CD82) cell lines (Figure 1). Cells were grown until confluence and lysed in 1x laemmli buffer. Results of the western blotting indicated that overexpression of CD82 leads to the down regulation of plakoglobin (Pg) and desmoglein2 (Dsg2) whereas desmoplakin (Dp), desmoglein3 (Dsg3), desmocollin2 (Dsc2), plakophilin2 (Pkp2), tight junction and adherens junction proteins were unaffected.

To provide further evidence that expression levels of Pg and Dsg2 are indeed modulated by CD82, we used breast cancer cell line where CD82 expression is depleted in 2.5.2A/shCD82. The data indicates that the knocking down CD82 results in an increase in Pg and Dsg2 expression (Figure 2). Thus, we concluded that CD82 specifically modulates expression of Pg and Dsg2.
Figure 1. The effect of CD82 overexpression on the expression of cell-cell adhesion proteins.

(a) Western blot analysis for the effect of CD82 on the expression of cell adhesion proteins.

(b) Densitometric analysis of the western blots.

(a)

(b)
Furthermore we performed semi quantitative RT-PCR on RNA extracted from HB2/zeo and HB2/CD82 cells to assess whether there was any effect of CD82 overexpression on the expression of desmosomal proteins at the mRNA level. No difference was observed regarding the expression of desmosomal proteins with expression of CD82 (Figure 3). This suggests that loss of Pg and Dsg2 protein, as an effect of CD82 occurs post translationally.
Figure 3. Semi quantitative RT-PCR analysis for desmosomal proteins. Agarose gel electrophoresis following semi quantitative RT-PCR of 7 desmosomal proteins and the house keeping gene actin (Actb) from RNA extracted from cell lines HB2/zeo and HB2/CD82. Lane 1=HB2/zeo PCR product, lane 2= HB2/CD82 PCR product.

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3.1. Investigation of the cellular distribution of various adhesion proteins in cells with various levels of CD82 expression by immunofluorescence.

To confirm western blots and assess the cellular distribution of the proteins, all 4 cell lines were stained with a variety of antibodies against cell junction proteins. The cell junction proteins tested were localised to the cell-cell contacts. Images were collected using the same settings to allow comparison of the fluorescence intensity. In confirmation of the data obtained by Western blotting, staining of the cells with anti-plakoglobin and anti-desmoglein antibodies resulted in changes in fluorescence intensity in accordance with the expression levels of CD82 (Figure 4, top panels). Interestingly quantification of the fluorescence intensity of the images revealed a possible increase in the expression of desmoplakin and occludin in the 2.5.2A/shCD82 cells and an increase in E-cad and Zo1 in HB2/CD82 cells. The level of CD82
expression seemed to have no dramatic effect on the cellular distribution of cell junction proteins for the most part. However one dramatic change in the staining pattern was observed for Dsg2 in HB2/CD82 cells, a speckled staining pattern rather than a complete membrane staining.

**Figure 4.** Immunofluorescence staining of cell adhesion proteins. (a) Cells grown on cover slips, fixed and incubated with appropriate primary antibody and either Alexa fluor 488 (green) or Alexa fluor 568 (red) conjugated secondary antibodies. (b) Measurement of fluorescent intensity at cell-cell contacts using Image J.
3.2. Co-localisation and interaction between Pg and CD82.

Distribution of the proteins in the same cellular compartment may indicate their interaction. We used immunofluorescence to assess the level of co-localisation between plakoglobin and CD82. Plakoglobin is mainly found in the cell-cell contacts with minor dot-like distribution in the cytoplasm. On the other hand, CD82 is distributed equally in the intracellular vesicular compartments and in cell-cell contacts. We found that CD82 and plakoglobin partially co-localize in cell-cell contacts indicating possible interaction between these proteins (Figure 5). To investigate interaction between Pg and CD82 further, we carried out immunoprecipitation (IP) experiments. HB2/CD82 cells were grown to confluency and lysed in lysis buffer (0.5% triton X-100 and 0.5% brij 98 containing protease and phosphatase inhibitors). Antibodies against Pg and CD82 were used in the IP reactions. The signal for both proteins was detected in the reciprocal reactions indicating that a subset of each protein could be found in one complex. On the other hand, interaction of desmoplakin and CD82 was not detected in immunoprecipitation experiments in current conditions (Figure 6).

Figure 5. Co-localisation of CD82 and Pg in HB2/CD82 cells. HB2/CD82 cells stained for Pg (Catenin γ) (green) and CD82 (TS82) (red). The white square area marked is shown enlarged on the right side illustrating individual and merged staining.
3.3. The effect of CD82 expression on cell-cell adhesion.

To assess whether the effect of CD82 expression on Pg and Dsg2 alters cell-cell adhesion a dispase assay was performed on all 4 cell lines. Confluent monolayers were released by incubation with dispase and subjected to mechanical stress to induce fragmentation. HB2/CD82 cells displayed stronger cell-cell adhesion despite the loss of Pg and Dsg2 proteins when compared to the parental cell line HB2/zeo. The reverse effect was observed in cells with reduced CD82 expression, 2.5.2A/shCD82 displayed weaker adhesion with increased fragmentation when compared to the parental 2.5.2A cell line, despite the increase in Pg and Dsg2 protein levels. (Figure 7). It was concluded that overexpression of CD82 has a positive
effect on cell-cell adhesion despite the loss of Pg and Dsg2, and reduced expression of CD82 correlates with weaker adhesion.

**Figure 7. Dispase assay for cell-cell adhesion.** (a) Top row represents released monolayers. Bottom row represents respective fragmented monolayers after 20 mins on linear shaker. (b) quantification of fragmented monolayer (the data is representative of experiments performed in triplicate).
3.4. Generation of a GFP-tagged desmoplakin construct

It has been demonstrated that CD82 regulates protein trafficking and cell membrane dynamics of its partner proteins (Tsai and Weissman, 2011). Taken together our data indicated that CD82 was important for the maintenance of cell-cell contacts. To examine if CD82 plays a role in the trafficking of desmosomal proteins to the plasma membrane a Dsp-green fluorescent protein (GFP) fusion protein was produced, consisting of the N-terminal plakin domain (residues 1-1022) of human Dsp fused to full length GFP. The Dsp plakin domain was chosen for this experiment because previous experiments have shown that it is targeted to the membrane in transfection experiments (Godsel et al, 2005). In order to produce a construct encoding the Dsp-GFP fusion protein, DNA encoding Dsp residues 1-1022 was cloned into vector pEGFP-N1 (Addgene), upstream of the GFP coding sequence to produce plasmid pDsp-GFP-Temp. A PCR reaction was then performed using this plasmid as template and PCR primers DP1 (which incorporates a BglII site) and DP2 (which incorporates a NotI site) (Table 2). This was designed to remove intervening, extraneous DNA between the Dsp and GFP sequences and to ensure that the two coding sequences were in frame (Figure 8). The PCR product (Figure 9a) was cut with restriction enzymes BglII and NotI, purified and ligated with purified BglII/NotI cut pDsp-GFP-Temp backbone DNA. The ligated DNA was transformed into competent XL1-Blue bacterial cells and plated out onto LB/kanamycin plates. Individual colonies were grown in liquid LB/kanamycin and plasmid miniprep DNA prepared. Plasmid DNA was digested with BglII and NotI and digested DNA resolved by agarose gel electrophoresis in order to confirm the presence of the insert (Figure 9b). One clone containing the insert was sequenced to confirm that the extraneous intervening DNA between the DP and GFP coding sequences had been successfully removed and to confirm
that no other mutations had been inadvertently included during the PCR amplification step (Figure 10). This plasmid, designated pDsp-GFP, was used in subsequent transfection experiments.

**Figure 8. Creating a Dsp-green fluorescent protein fusion protein.** A schematic of the PCR reaction which was designed to remove intervening, extraneous DNA between the Dsp and GFP sequences.

**Figure 9. Creating a Dsp-green fluorescent protein fusion protein.** (a) PCR product obtained following amplification of DNA with primers DP1 and DP2. Lane 1 markers, lane 2 amplified PCR product. (b) Screening of transformed colonies by Bgl II and Not I restriction digests for the presence of the insert. Lane 1 markers, lanes 2-7 represent colonies screened for the insert. The insert is highlighted in lane 7 restriction digest.
Figure 10. Sequencing to confirm that the intervening DNA between the DP and GPF coding sequences had been successfully removed and to confirm that no other mutations had been introduced during the PCR reaction.

3.5. Effect of CD82 on Dsp trafficking to the membrane.

In order to optimise the efficiency of transfection of HB2zeo cells various transfection reagents were tested. Gene jammer, Fugene and Lipofectamine were used to transfect HB2/zeo cells with pDsp-GFP and CD82-RFP. Lipofectamine was found to have the highest transfection efficiency (Figure 11) and was used in the following experiment.
To assess if CD82 plays a role in trafficking of Dsp to the plasma membrane and desmosome assembly, HB2/zeo cells were transfected with pDsp-GFP and full length CD82-RFP constructs. To induce the formation of desmosomes cell media was changed to calcium free media for 20h (48h after transfection) and then restored to normal calcium media. Real time images were acquired at 1 minute intervals following the calcium switch. 10 min post calcium switch CD82 and Dsp were observed to move and accumulate at the cell-cell contacts and this was also shown by changes in fluorescent intensity at cell-cell contact measured using Aim software (Zeiss). Despite localising at the cell-cell contact CD82 did not appear to be involved in the trafficking of Dsp to the membrane (Figures 12, 13 and 14).
Figure 12. Time interval imaging for the localisation of transfected proteins during cell contact assembly. Live cell imaging for the assembly of cell-cell contacts in HB2 cells following media change to full calcium media from low calcium media. Images before media change (0) and 20min and 60min post calcium switch.
Figure 13. Increase in fluorescence at the cell-cell contact following calcium switch. (Time interval recording of 1 min for 40 min post calcium switch). (a) HB2/zeo cells transfected with Dsp-GFP and CD82-RFP. (b) Increase in fluorescent intensity of Dsp-GFP and CD82-RFP at the cell-cell contact following calcium switch. This increase in intensity was specific for the cell-cell border and not detected in other parts of the cells.
Figure 14. Fluorescence analysis showing plateauing of Dsp-GFP and CD82-RPF intensity at the cell-cell contact recording commencing 40 min (1 min interval) post media change. (a) HB2/zeo cells transfected with Dsp-GFP and CD82-RFP. (b) Fluorescence analysis showing plateauing in Dsp-GFP and CD82-RPF intensity at the cell-cell contact 40 min post calcium switch. The measurement of intensity was specific for the cell-cell border and not detected in other parts of the cells.

(a)                                                                                                 (b)
Discussion

Cell junctions are important sites of intercellular adhesion. They preserve the integrity of epithelial tissue and control cell signalling (Knights et al, 2013). Deregulation of genes associated with cellular junctions can result in tumour transformation and invasion. Desmosomes, tight junctions, gap junctions and adherens junctions are a network of intercellular junctions which provide intercellular anchoring and signalling contributing to the maintenance and integrity of epithelial tissues. (Knights et al, 2013). Cellular transformation from an epithelial to mesenchymal nature is brought about by the dysregulation of desmosomal components and is therefore a crucial step in the metastatic potential of cells (Knights et al, 2013).

Tetraspanin proteins are a superfamily of transmembrane proteins which are associated with a wide range of cellular activities in many biological systems and are involved in health and disease (Romanska and Berditchevski, 2010). It been established that the tetraspanin CD151 plays crucial roles in the regulation of adhesion and motility of tumour cells (Johnson et al, 2009). It was shown that loss of CD151 destabilises E-cadherin-dependant cell-cell junctions and augments migration of complete tumour cell sheets. The loss of CD151 causes an increase in RhoA activation, loss of actin organisation and perturbed localisation of E-cadherin and associated proteins and amplified actin stress fibers at the basal cell surface. Cell-cell contacts were observed to exhibit a 3 fold rise in remodelling rate as well as a reduction in lifespan in CD151 knockout monolayers in comparison with wild type cell-cell, this effect was reversed.
with re expression of CD151. In another study it was shown that CD151 association with α3β1 integrin regulates the gene expression of PTPμ, which in turn interacts with several cadherins, such as E-cadherin, and regulates E-cadherin mediated cell–cell adhesion by organizing a multimolecular complex containing PKCβII, RACK1, PTPμ, β-catenin, and E-cadherin strengthening cell adhesion (Chattopadhyay et al, 2003).

CD82 a member of tetraspanin superfamily, is classified as a metastasis suppressor. Through various mechanisms CD82 inhibits metastasis. It has been shown to suppresses cell motility and invasion, promote cell polarity and in response to extracellular stimuli induce senescence and apoptosis (Tsai and Weissman, 2011). The recent availability of CD82–knockout mice showed desmoplakin, cadherin 10 and desmin to be significantly upregulated in CD82 null cells in comparison with wild type cells (Risinger et al, 2013). Our analysis conducted by Western blotting revealed Pg and Dsg2 protein expression to be significantly reduced in the mammary epithelial cell line HB2 transfected to overexpress CD82 (HB2/CD82) (Figure 1). Examining the effects of reducing CD82 expression by shRNA in the breast cancer cell line 2.5.2A on cell-adhesion proteins, we observed an increase in plakoglobin and desmoglein2 proteins level (Figure 2). This alteration in the expression of plakoglobin and desmoglein2 as an effect of CD82 occurs post translationally as no difference in the expression was observed at the mRNA level by semi quantitative RT-PCR (Figure 3). The level of CD82 expression did not have as significant an effect on the expression of desmoplakin as shown by the CD82 knockout study where the cells analysed were mouse embryonic fibroblasts whereas our analysis was conducted on a human mammary epithelial cell line. Despite differences in the findings of the studies relating to the regulation of the expression of different desmosomal
proteins in comparison, a consistent role for CD82 in the expression of desmosome components can be established.

It is presently unclear why CD82 a metastasis suppressor would down regulate the expression of Pg and Dsg2 which are principally considered to act as tumour suppressors and therefore the mechanisms remain to be elucidated. Data from a significant number of research studies examining the general role of CD82 in normal cells have proposed two key modes for its action. The first mode of action is that CD82 depends directly on its physical interaction with other proteins. The second proposed mode is that CD82 affects vital signalling pathways, indirectly, via phosphorylation-mediated activation of proteins (Tonoli et al, 2005). He at al (2005) proposed that CD82 regulates action of other transmembrane proteins through a “touch and down” mechanism, through which the protein contacts and subsequently inhibits other proteins. This was demonstrated by reduced laminin-1 adhesion in the prostate cell line Du145 made to re-express to CD82, this observed effect resulted from CD82 associating with the α6 integrin (laminin-1 receptor) and driving its internalisation (He at al, 2005). This could possibly apply and explain the effect of the level of CD82 expression on the cell surface expression levels of Pg and Dsg2 and especially the disrupted staining pattern observed by immunofluorescence by associating and driving the internalisation of the proteins (Figure 3). However the mechanism for the down regulation of overall cellular protein level of Pg and Dsg2 remains unresolved. Interestingly quantification of the fluorescence intensity of the images revealed a possible increase in the expression of desmoplakin and occludin in the 2.5.2A/shCD82 cells and an increase in the adherens junction protein E-cad and the tight junction proteins Zo1 in HB2/CD82 cells.
It has been previously shown that CD82 promotes homophilic cell adhesion by inhibiting β-catenin nuclear translocation, preventing degradation by down regulation of GSK-3β and CK1α, resulting increase in the accumulation and stabilization of β-catenin at the cell membrane strengthening the E-cadherin-β-catenin complex (Chigita et al, 2012). It was expected that the reduced expression of Pg and Dsg2 would result in weaker cell-cell adhesion; to assess this a dispase assay was performed. We observed that the cells overexpressing CD82 (HB2/CD82), had stronger cell-cell adhesion and formed less fragments when compared to parental cell line HB2. The reverse effect was observed in cells with reduced CD82 expression, 2.5.2A/shCD82 displayed weaker adhesion with increased fragmentation when compared to the parental 2.5.2A cell line, despite the increase in Pg and Dsg2 protein levels.

Pg and β-catenin are homologous armadillo repeat proteins (Choi et al, 2009). Plakoglobin is the only protein that the desmosomes and adherens junctions have in common. It has been established in instances of β-catenin knockout that adherens junctions are unaffected and remain intact due to Pg compensation (Wickline et al, 2013). Despite appropriately compensating at the adherens junction Pg was unable to recover β-catenin’s TCF/LEF and transcription activity and functions of Wnt signalling (Wickline et al, 2013). A study of plakoglobin null- mutant mice showed β-catenin localised to desmosomes and interacted with desmoglein, however it was not able to completely compensate for the lack of Pg as there was reduced cell adhesion (Bierkamp et al, 1997). It is possible that reduced Dsg2 and especially Pg expression did not have a dramatic effect on reducing cell-cell adhesion due to
β-catenin compensation and as well as a natural increase in the membrane bound β-catenin and stabilisation of the E-cadherin-β-catenin complex as an effect of CD82.

A prevalent component of these versatile effects of CD82 include regulation of membrane organization as well as protein interactions and trafficking, coordinating cellular signalling and intercellular communication. We believed CD82 plays a role in the trafficking of desmosomal proteins to the plasma membrane. Therefore we created an N-terminal (amino acids 1-1022) Dsp-Green fluorescent protein. HB2/zeo cell were transiently transfected with DSP-GFP and CD82-RFP and 48h post transfection cell media was changed to low calcium media for 20h to induce disassociation of calcium dependent cell junctions. This was followed by switch to full calcium media to induce reformation of cell-cell contacts. Live cell imaging showed the Dsp-GFP move and accumulate at the cell-cell border 10 min after the media change. Fluorescent intensity at the cell-cell border was observed to increase over the next 30min, after which the fluorescent intensity plateaued. CD82-RFP intensity was also observed to increase at the cell-cell contact. However it is unlikely to play a role in the trafficking of Dsp as no co-localisation was observed during the initial movement and accumulation of Dsp-GFP at the cell-cell contact. Around 60 min post calcium switch once Dsp-GFP and CD82-RFP intensity had stabilized co-location was observed. We concluded that CD82 was not involved in the trafficking of Dsp to the membrane.
Limitations of this study and directions for future work

The most immediate question that arises from this project is how does CD82 regulate, protein levels of Dsg2 and Pg? Firstly, the effect should be confirmed by analysing expression levels in other cell lines. Secondly, the interaction should be confirmed by using purified recombinant proteins in vitro or alternatively by using a yeast two-hybrid system. The interaction can be further studied and classified using mutant proteins lacking either the N or C terminus with the aim of identifying the interacting regions. In hindsight it would have been more relevant to have constructed a Pg-GFP construct and performed live cell imaging with this. Analysis of the cellular distribution of proteins can be improved and quantified by performing western blot analysis of subcellular fractioned proteins. Quantifying gene expression can be enhanced by using more sensitive approaches such as Real time PCR to overcome the limitations of semi quantitative PCR.

Conclusion

A number of important observations have been made during the course of this study. Thus changes in CD82 expression had significant effects on the expression of desmosomal proteins Dsg2 and Pg, and overexpression of CD82 results in a dramatic redistribution of Dsg2. Importantly, CD82 was found to co-localise with Pg, and a physical interaction between the two proteins was observed. The significance of these remain to be determined, but they provide a useful platform for the development of future work.
References


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