INVESTIGATING THE TUMOURIGENIC ASPECTS OF THE BONE MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA

By

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Acknowledgements

Abstract

Citrullination is a calcium-dependent process catalysed by a family of enzymes known as peptidylarginine deiminases (PADIs). PADI type II (PADI2) is strongly linked with the autoimmune disease rheumatoid arthritis, but more recently it has become an attractive research target due to its link with cancer. PADI2 overexpression is found in multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) patients. In this report, we aim to determine the nature of citrullinated proteins in bone marrow stromal cells (BMSCs), the effect this has on their function, and the effect of hypoxia on PADI2 expression. Citrullinated proteins were analysed by immunoblot analysis of HS-5 and HEK293T cell lines after addition of either calcium to stimulate PADI2 activity, or EDTA to inhibit it. HS-5 cells were placed into hypoxic conditions (1% oxygen) for up to 32 hours and analysed by an immunoblot to detect changes in PADI2 expression. The data from this study highlights changes in citrullination between calcium and EDTA treated cells in the HS-5 and HEK293T cell lines. Additionally, PADI2 expression is increased in HS-5 cells after two hours, and maintained after six hours in hypoxia, suggesting hypoxic conditions have an impact on PADI2 activity, and therefore, citrullination. In MM, hypoxia is known to have an effect on the malignant plasma cells, allowing them to migrate and home to new areas of the bone marrow (BM). Although in its initial stages, this study has begun to expand our understanding of the effect citrullination has on BMSC function within the BM microenvironment.

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Abbreviations

Abbreviation	Meaning
aa	Amino Acids
ACPA	Anti-citrullinated protein antibodies
BM	Bone marrow
BMSC	Bone marrow stromal cells
CDK	Cyclin dependent kinase
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal-transition
GFAP	Glial fibrillary acidic protein
HIF	Hypoxia-inducible-factor
IDH1	Isocitrate dehydrogenase 1
IP	Immunoprecipitation
MCF-1	Monocyte chemoattractant protein 1
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
M-protein	Monoclonal protein
MS	Multiple sclerosis
NF-κB	Nuclear factor kappaB
PADI	Peptidyl arginine deiminase
RA	Rheumatoid Arthritis
Rb	Retinoblastoma
SDF-1	Stromal derived factor 1
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
WCL	Whole cell lysate

1. Introduction

1.1.   Multiple myeloma

Multiple myeloma (MM) is a disease predominantly found in patients over 60 years old (Hulin *et al.*, 2009). It is a haematological malignancy characterised by infiltration of malignant plasma cells in the bone marrow (Hideshima *et al.*, 2007) and is associated with an accumulation (>10% increase) of monoclonal protein (M-protein) in the blood. Clinical indicators of MM comprise osteolytic bone lesions, renal failure, hypercalcemia, anemia, peripheral neuropathy (Korde *et al.*, 2011) and an increased susceptibility to infection (Alexander *et al.*, 2007). With around 4,000 new cases every year in the UK, MM accounts for approximately 10% of haematological cancers and contributes to approximately 1% of all cancers (Mutis, 2012).

When a B cell encounters an antigen, it migrates to the germinal centre, known as the primary immune response. Here it matures via somatic hypermutation or antigen selection, followed by a class switch recombination to produce immunoglobulin isotypes (Morgan *et al.*, 2012). The B cell, now a plasmoblast, migrates to the bone marrow (BM) where it finds a receptive niche to support its growth and survival as an antibody-producing plasma cell (Morgan *et al.*, 2012). However, genetic alterations taking place along this route, interfere with the interactions of the plasma cell with the BM microenvironment.

Monoclonal gammopathy of undetermined significance (MGUS) is the asymptomatic, premalignant condition of MM (Manier *et al.*, 2012). The majority of MM cases are preceded by MGUS, however, some cases may arise *de novo* (Manier *et al.*, 2012). For the cell to proceed from a normal plasma cell to MGUS a number of early oncogenic events takes place; namely cyclin D dysregulation (causing cell cycle progression), hyperdiploidy, primary IgH translocations and epigenetic abnormalities (Landgren and Waxman, 2010). Later, the plasma cells undergo further oncogenic events such as RAS mutations (the main genetic difference between MGUS and MM, as RAS mutations occur in only 5% of MGUS patients versus 40-55% in MM patients (Chng *et al.*, 2008)), nuclear factor κappaB (Nf-κB) activating mutations (Bharti *et al.*, 2004), C-MYC rearrangements (Chesi *et al.*, 2008) and tumour suppressor p53 (Neri *et al.*, 1993), PTEN (Chang *et al.*, 2006) and retinoblastoma (Rb) gene (Walker et al. 2010) inactivation, to present MM. Biological transition of MGUS to MM occurs at a rate of approximately 1% each year (Kyle *et al.*, 2010).

It was originally thought that MM was acquired through a linear fashion of genetic mutations, but further research has discovered more recently that this may not be correct. During the later stages of disease progression, some malignant plasma cells gain a clonal advantage allowing them to expand and further evolve into new niches within the BM (Morgan *et al.*, 2012). This eventually creates an environment more suitable for malignant plasma cells, rather than the normal cells. Thus, tumour cell survival is strongly dependent upon the BM microenvironment (Manier *et al.*, 2012). The MM cells lead on to become independent, no longer relying on the BM microenvironment and can be found at extramedullary sites and as circulating leukaemic cells (known as plasma cell leukaemia), which quickly results in patient fatality (Morgan *et al.*, 2012).

1.2.   The bone marrow microenvironment in cancer

To support tumourigenesis, the BM microenvironment is suitably specialised, rich with the resources needed for signalling and growth. Late stage adenocarcinomas, which originate from tumours that include breast, lung, prostate and kidney, metastasise to the BM where the disease becomes incurable and allows cancer cells to avoid chemotherapeutic agents (Olechnowicz and Edwards, 2014). However, changes to constituents of the BM microenvironment have been hypothesised to occur before the arrival of any metastatic cells, as the new microenvironment must be well adapted to support the growth and survival of the tumour (Kaplan *et al.*, 2005). Consequently, novel therapies proposed aim to imbalance the supportive BM microenvironment rather than directly target the cancer cells (Olechnowicz and Edwards, 2014).

Fibroblasts/BM stromal cells (BMSCs), osteoclasts and osteoblasts are all important cellular components of the BM niche (Hideshima *et al.*, 2004). It is important to note that malignant plasma cells are able to home to the BM and adhere to the BMSCs and extracellular matrix (ECM) proteins, such as fibronectin and type I collagen. This adhesion process causes upregulation of cell cycle regulatory proteins and activates an anti-apoptotic cascade (Hideshima *et al.*, 2004). Inflammatory cytokines are key players in modulating mobility of BMSCs to the site of the tumour. Cytokines and chemokines, such as IL-6 (interleukin 6), IL-8, SDF-1 (stromal derived factor 1), and MCF-1 (monocyte chemoattractant protein 1), stimulate tumour growth and maintain an inflammatory response (Spaeth *et al.*, 2008). Furthermore, cytokines are important in epithelial-mesenchymal-transition (EMT) processes by activation of the β-catenin pathway (Giannoni *et al.*, 2012) and also have the ability to activate NF-κB as the result

of decreased oxygen availability within the BM microenvironment (Grivennikov *et al.*, 2010).

1.3.   The bone marrow microenvironment in multiple myeloma

Paracrine and autocrine cytokine production is essential for MM progression. IL-6 causes B-cell differentiation in normal physiology (Pratt, 2002). Moreover, in myeloma, it is the most important inflammatory cytokine, aiding growth and survival of malignant plasma cells (Pratt, 2002). IL-1β is abnormally produced when malignant plasma cells adhere to the BMSCs, causing upregulation of IL-6 release from BMSCs (Dinarello, 2011). IL-6 not only supports the growth and survival of the plasma cells (via the STAT3 and ERK pathways (Honemann *et al.*, 2001)), but also enhances vascular endothelial growth factor (VEGF) production for angiogenesis (Podar and Anderson, 2005) and inhibits antigen presentation of dendritic cells (Manier *et al.*, 2012). IL-6 inhibits the anti-proliferative effects of cyclin-dependent kinase (CDK) inhibitors such as p21, via the PI3K/Akt pathway (Hideshima *et al.*, 2001). As a result, increased serum IL-6 in MM patients reflects an increase in the proliferation rate of malignant plasma cells and represents a poorer prognosis for patients (Manier *et al.*, 2012).

Stromal derived factor-1 (SDF-1) and its chemokine receptor, CXCR4 (present on the surface of the malignant plasma cells), have been shown to induce MM migration *in vitro* and BM homing *in vivo* (Alsayed *et al.*, 2006). CXCR4 inhibitors have been found to prevent migration, suggesting that they play an important role in homing regulation in MM (Alsayed *et al.*, 2006). Consequently, mobilisation of plasma cells out of the BM may be feasible by SDF-1/CXCR4 disruption (Manier *et al.*, 2012).

1.4.   Citrullination

A series of post-translational modifications (PTMs) occur during the progression of MM such as: methylation, acetylation, glycosylation and ubiquitination (Burska *et al.*, 2014). A further interesting change the BMSCs undergo is an irreversible phenotypic alteration known as citrullination. Previous datasets in the Tennant research group have suggested that citrullination may be increased in BMSCs of MGUS and MM patients (unpublished data). Citrullination is a calcium-dependent process (Cordova *et al.*, 2013) that converts a positively charged arginine residue into a neutral citrulline, by loss of the H_2N^+ group (Christophorou *et al.*, 2014), shown in *figure 1.1*. As a result, citrullination often causes an abnormal protein conformation (Shelef *et al.*, 2012), leading to destabilisation of the intramolecular and intermolecular interactions (Foulquier *et al.*, 2007). This encourages protein unfolding, changes to the protein

binding properties and causes alterations to protein function and half-life (Chirivi *et al.*, 2013). For example, partial citrullination of the chemokine CXCL12 has a significant impact on binding to its receptor, CXCR4, whereas complete citrullination of CXCL12 causes complete inactivation (Struyf *et al.*, 2009).
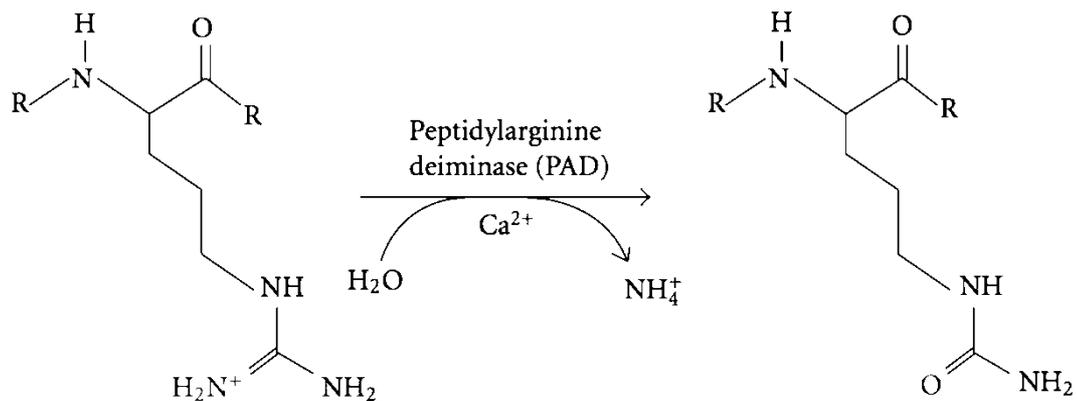
Figure 1.1. – Representation of citrullination, a process that converts a positively charged arginine residue into a neutral citrulline residue, catalysed by PADI enzymes in the presence of calcium (Mohanan *et al.*, 2012).

It has been acknowledged that citrullination plays a role in autoimmune diseases such as multiple sclerosis (MS) and rheumatoid arthritis (RA) (Vossenaar and Robinson, 2005). Citrullinated proteins are recognised as 'non-self' and, therefore, produce an inflammatory response (Tanikawa *et al.*, 2009). Anti-citrullinated protein antibodies are produced before the onset of disease, and may be therapeutically administered to prevent further inflammation or used as a diagnostic biomarker (Chirivi *et al.*, 2013). It is clear citrullination plays a role in inflammatory processes in normal physiology and not only in autoimmune disease. To support this, a study by Coudane *et al.* (2011) demonstrated citrullination in a non-autoimmune inflammatory event Coudane *et al.*, 2011).

1.5. Peptidyl arginine deiminases

A family of enzymes known as peptidyl arginine deiminases (PADI) catalyse citrullination (Shelef *et al.*, 2012). Within the PADI family are five conserved subtypes: PADI type I, II, III, IV and VI (Wang and Wang, 2013), clustered on chromosome 1p35-36 (Chavanas *et al.*, 2004), that each have specific tissue distributions (Wegner *et al.*, 2010[a]) (*Table 1.1.*).

Table 1.1. – An example of protein citrullination in healthy physiology versus the pathological physiology, demonstrating the effect of aberrant citrullination compared to normal citrullination.

Healthy physiology		Pathological physiology		
Citrullinated protein	Function	Protein (Aberrantly citrullinated)	Function	Disease
Keratin and Filaggrin	Proteolytic cleavage and cross-linking in the final developmental stages of keratinocyte differentiation (Wegner *et al.*, 2010) Form a protective matrix (Baka *et al.*, 2012)	**Keratin and Filaggrin**	Decreased keratin 1 deimination within the hyperproliferative epidermis (Baka *et al.*, 2012)	Psoriasis
Myelin Basic Protein (MBP)	Electrical insulation of myelin sheaths	**MBP**	Failure to maintain myelin sheath (Baka *et al.*, 2012)	Multiple Sclerosis (MS)
Histones	Transcriptional regulation and DNA damage response (Wegner *et al.*, 2010)	**Histones**	Disable histone methylation (Baka *et al.*, 2012)	NETosis, tumourigenesis and early stage inflammation (Chirivi *et al.*, 2013)
		Vimentin, fibrinogen, collagen type II and α-enolase	Inflammatory Response (Wegner *et al.*, 2010)	Rheumatoid Arthritis (RA)

PADI type I is found expressed in the epidermis and uterus and specifically targets keratin and filaggrin (Ishida-Yamamoto *et al.*, 2002). Type II, focussed on here, is widely expressed and is detected across a wide range of mammalian tissues (see below). Type III is also found within the epidermis and in hair follicles, targeting filaggrin and trichohyalin (Nachat *et al.*, 2005). Type IV has been found in white blood cells and tumours, targeting nuclear proteins (Nakashima *et al.*, 2002). Type VI is one of the least studied subtypes, although it is known to be present in mammalian ovaries, eggs and embryos (Wright *et al.*, 2003). As demonstrated in *table 1.1*, aberrantly citrullinated proteins are present in a range of diseases. Each of the PADI subtypes, except PADI type III and VI, have been implicated in pathological disease (Wang and Wang, 2013).

Of the five PADI enzymes, PADI type IV (PADI4) has been most widely studied. PADI4 is localised within the nucleus and predominantly citrullinates nuclear proteins such as histones. Other modifications such as histone methylation can alter gene expression. There is evidence to suggest that changes in methylation of histones plays an important role in tumourigenesis (Martinez-Garcia *et al.*, 2011). Methylated histones regulate chromatin structure and function; however, PADI4 has the ability to antagonise histone methylation, converting methyl-arginine to citrulline by targeting sites in the tails of histones H3 and H4 (Denis *et al.*, 2009). Consequently, PADI4 has been increasingly studied and has subsequently been implicated in cancer. PADI4 has been recognised in breast cancer cell lines, such as MCF-7s, where it may play a role in gene transactivation (Zhang *et al.* 2011). Stadler *et al.* (2013), found that in non-invasive MCF-7 cell lines, PADI4 activity reduces EMT and also decreases invasion potential. Nevertheless, increased PADI4 has been found in patients with malignant tumours compared to non-malignant control patients and has, therefore, been implicated in tumourigenesis progression (Chang *et al.*, 2009). PADI4 expression is regulated by oestrogen to mediate the oestrogen response and, additionally, epidermal growth factor receptor (EGFR) signalling in breast cancer (Zhang *et al.*, 2011). Liu *et al.* (2006) demonstrated that reduced or depleted PADI4 causes raised p21 expression, leading to apoptosis via the p53 pathway (Liu *et al.*, 2006). Cherrington *et al.* (2012) established that a sub-population of PADI2 localises to the nucleus of MCF-7 cells where it can bind chromatin (Cherrington *et al.*, 2012). Depletion of PADI2 suppresses MCF-7 proliferation and, therefore, potentially plays a role in breast cancer development (Cherrington *et al.*, 2012). In addition, it has been proposed that PADI2 and PADI4 may function synergistically. Recent findings identified PADI2 overexpression in breast tumours with HER2 mutations (McElwee *et al.,* 2012). This

phenomenon should, therefore, be investigated further to determine the effect these enzymes propose on tumour progression (Monohan *et al.*, 2012).

Although most research has been conducted on PADI4, the recent findings that PADI2 also plays an important role in citrullination of histones (Wang and Wang, 2013) and its association with gene regulation in breast cancer (Cherrington *et al.*, 2012) has made it an attractive target for research.

1.6. PADI type II

PADI2 is widely expressed and found in the following: brain astrocytes, sweat glands, skeletal muscle, spinal cord tissue, macrophages and the epidermis (Foulquier *et al.*, 2007; Wang and Wang, 2013). Substrates known to be citrullinated by PADI2 include: myelin basic protein (Lamensa and Moscarello, 1993), vimentin (Schett and Gravallese, 2012), histones (H3 and H4) (Cherrington *et al.*, 2012), β and γ-actins (Darrah *et al.*, 2012), fibronectin, fibrinogen (Tabushi *et al.*, 2008) and collagen (Yoshida *et al.*, 2006).

Studies, such as that carried out by Cherrington *et al.* (2012), found that PADI2 expression in human mammary gland epithelial cells is able to citrullinate histone H3 at the N-terminus. PADI2 is able to associate with oestrogen receptor alpha to facilitate its translocation from the cytoplasm into the nucleus; however, PADI2 nuclear localisation appears to be reduced as the tumour progresses (Cherrington *et al.*, 2012). It is likely that PADI2 activates target gene transcription when the cell responds to oestrogen stimulation (Zhang *et al.*, 2012) suggesting that PADI2 can act as an epigenetic regulator, controlling gene activity. In addition to the links that have been made with PADI2 and cancer, it has been proposed that PADI2 regulates macrophage cytokine signalling by deimination of IKKγ (forms part of the cytokine responsive IKK complex which regulates NF-κB activity (Rothwarf *et al.*, 1998) and, therefore, cell proliferation and survival) (Lee *et al.*, 2010).

Although little is known about citrullinated fibronectin, it is evident that it has a higher affinity for VEGF and decreases the ability to cause apoptosis in a leukaemic cell line, such as HL-60 (Chang *et al.*, 2005). Mohanan *et al.* (2012) hypothesises that citrullinated vimentin promotes an inflammatory milieu and affects cell migration of tumour cells. It has recently been found that Cl-Amidine is a successful pan PADI inhibitor, allowing inflammatory symptoms to be obstructed (Slack *et al.*, 2011). PADI2 has been further implemented in tumourigenesis by a number of recent studies that

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demonstrate treatment of tumour cells with Cl-Amidine, reduce cancer cell viability separate from normal cell growth (Slack *et al.*, 2011).

Citrullination by PADI2 plays a crucial role in inflammatory processes and is induced in a range of pathologies (Mohanan *et al.*, 2012) including psoriasis (György *et al.*, 2006), alzheimers disease (Ishigami *et al.*, 2005) and multiple sclerosis (MS) (Vossenaar *et al.*, 2003). However, PADI2 expression has most particularly been linked with RA (Wang and Wang, 2013).

1.6.1. PADI type II in rheumatoid arthritis

RA is an autoimmune disease, generating an immune response against self-antigens and is characterised by synovial inflammation of the joints (Shelef, 2012), infiltration of macrophages (Kinne *et al.*, 2000), hyperplasia (McInnes and Schett, 2011) and the presence of autoantibodies (specifically anti-citrullinated protein antibodies (ACPAs)) (Song and Kang, 2010). Inflammation causes the joints to become painful and eventually leads to cartilage and bone destruction (Shelef, 2012). Leukocytes infiltrate the synovial tissue of RA patients and release PADI2 and PADI4 in the synovial fluid, causing an influx of citrullinated proteins (Kinloch *et al.*, 2008). The presence of citrullinated proteins within the synovial fluid of the joint initiates a 'false' immune response causing immune cells to generate ACPAs (Wegner *et al.*, 2010).

In a study by Fan *et al.* (2012), it has been recognised that citrullination of vimentin has significant biological effects in RA patients. This is due to evidence of stimulation of proliferation, pro-inflammatory cytokine secretion (TNF-α and IL-1) and upregulation of PADI4 and RANKL (promotes osteoclast maturation and activation (McInnes and Schett, 2011)) expression in fibroblast-like synoviocytes (Fan *et al.*, 2012), leading to bone erosion (McQueen and Issa, 2013). Furthermore, fibronectin is an important protein that regulates many cell behaviours including migration, adhesion, invasion and survival (Shelef *et al.*, 2012). Fibronectin, an ECM protein known to be citrullinated by PADI2, also plays an important role in synovial fibroblast behaviour, as it increases synovial fibroblast adhesion to cartilage (Shiozawa *et al.*, 1992). Shelef *et al.* (2012) concluded from their study that citrullination of fibronectin leads to impaired migration, adhesion, spreading and integrin-mediated signaling in synovial fibroblasts (Shelef *et al.*, 2012). It is possible that the citrullination of proteins causes aberrant adhesion, leading to the metastasis of cells, increased RA spread and further joint destruction (Shelef *et al.*, 2012).

The weight of evidence suggests protein citrullination has a pathogenic role (Shelef *et al.*, 2012). The level of citrullination appears to be affected by inflammation, as demonstrated by Makrygiannakis *et al.* (2006), who showed that glucocorticoids, administered locally, reduced citrullination in the joints of patients with rheumatoid arthritis. To support this, Foulquier *et al.* (2007) demonstrated that PADI expression in the synovial tissue of RA patients is an inflammation-dependent process.

Patients with autoimmune diseases are thought to be at an increased risk of cancer, compared to the general population. This may be due to the dysregulation of the immune system (Hemminki *et al.*, 2012) in autoimmune diseased patients. More specifically, Abu-Shakra *et al.* (2001) discuss how there is an increased risk of haematological malignancies in RA patients. RA has been strongly associated to other inflammatory diseases (McInnes and Schett, 2011), particularly cancer; nonetheless, this remains extremely controversial (Shen *et al.*, 2014).

1.6.2. PADI type II in multiple myeloma

Although links have been made between PADI2, cancer and other inflammatory pathologies, in terms of MM, little is known about the activity of PADI2 although, it has been suggested that RA patients may be at a higher risk of MM development. This is, however, inconclusive (Shen *et al.*, 2014). Due to the similarities between RA and MM physiology, by applying the substantial knowledge we currently have of the pathophysiology and role PADI2 plays in RA, and transferring it to the pathophysiology of MM, this could potentially aid our understanding of the role it plays in the BM microenvironment of MM patients.

To support this, a preliminary data from studies carried out in the Tennant research group showed PADI2 expression was elevated in MGUS and MM patients compared to that seen in control patients, suggesting that it may have a role in the pathogenesis of MM. During the transition of an arginine residue to form citrulline by PADI2, an ammonia group is released and converted into urea. It was found that urea concentration was significantly increased in the BM of patients with MGUS and MM compared to control patients (represented in *figure 1.2*), suggestive of PADI activity.
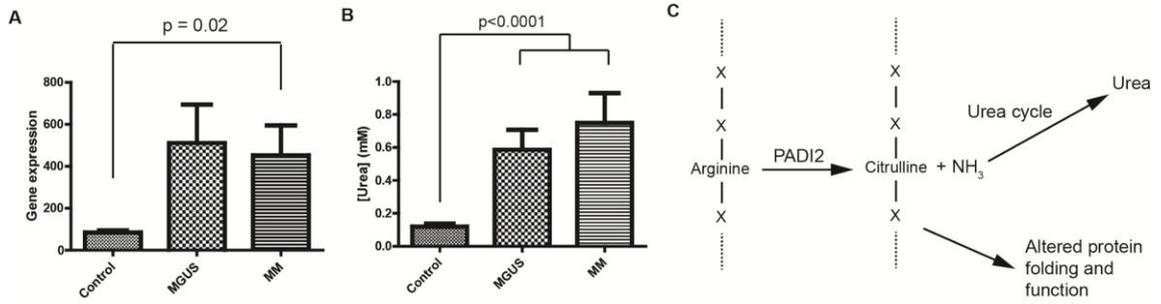
Figure 1.2. – Citrullination of proteins in control, MGUS and MM patients. A: expression of PADI2 in control, MGUS and MM. B: Urea concentration within the BM of control, MGUS and MM. C: Representation of the citrullination mechanism. (Tennant *et al.*, unpublished data)

SLC14A1 is a urea transporter gene, important for regulating osmosis (Garcia-Closas *et al.*, 2011). Further preliminary data also found that, as demonstrated in *figure 1.3*, SLC14A1 is upregulated in the BM plasma of MGUS and MM patients, significant to 0.0001 (calculated by a Kruskall-Wallis statistical test). This additionally supports that there is PADI activity, as SLC14A1 is upregulated to transport urea out, eliminating it from the plasma of the BM.
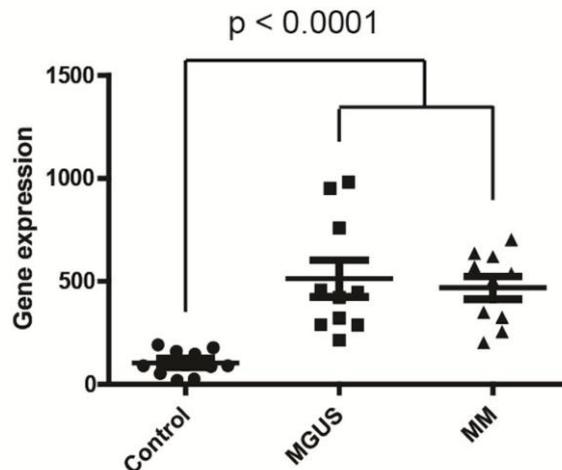
Figure 1.3. – Representation of the changes in SLC14A1 (urea transporter) gene expression in control, MGUS and MM fibroblasts (Adapted from Tennant *et al.*, unpublished data).

1.7. Hypoxia

In normal conditions, the supply and metabolic consumption of oxygen is balanced to meet the demands of the tissue. Hypoxia is the term given when the balance is disturbed, causing the supply of oxygen unable to meet the consumption needed for metabolic processes. Hypoxia is a characteristic feature of solid tumours and can aid in tumour progression and drug resistance (Vaupel and Mayer, 2007). HIF 1-alpha (HIF1-α) and 2-alpha (HIF2-α) regulate gene expression in glycolysis and angiogenesis. Under normal conditions, HIF concentration is maintained at low levels, whereas; under hypoxic conditions, HIFs are upregulated due to degradation inhibition and accumulate within the cell (Giatromanolaki *et al.*, 2010).

1.7.1. Hypoxia and multiple myeloma

Hypoxia promotes metastasis via regulation of proteins involved in EMT. When these proteins are activated, E-cadherin (CDH1) is lost, resulting in decreased adhesion of malignant plasma cells in MM (Azab *et al.*, 2012) enabling them to metastasise. Additionally, hypoxia increases CXCR4 expression allowing the plasma cells to migrate and home to new areas of the BM. This allows the MM cells to recover (Azab *et al.*, 2012).

1.7.2. Hypoxia and PADI type II

In a study carried out by Sambandam and colleagues (2004), it was discovered that in astrocytes, PADI2 expression is increased during progressively longer hypoxic conditions. PADI2 mRNA expression is increased and maintained throughout a 16 hour time window after 2 hours under hypoxic conditions. This later resulted in glial fibrillary acidic protein (GFAP) citrullination. Therefore, in relation to MM, it would be interesting to investigate whether PADI2 expression is increased in a fibroblast cell line (such as HS-5), causing increased citrullination during hypoxic conditions. Although, it must be noted that in some cell lines, citrullination has been seen to cause apoptosis (Gong *et al.*, 1999).

1.8. Objective and aim

Investigations into the role of PADI2 in MM permit a clearer understanding of the interactions that take place between MM and BMSCs. Determining the effect that citrullination by PADI2 has on the interactions in the BM microenvironment will allow further preventative or therapeutic approaches to be identified. Taking what is known about PADI2 in RA and applying it to MM may allow us to determine whether PADI2

plays an important role within the BM microenvironment, as it does within the synovial joint in RA.

Therefore, the objective of this study is to investigate the phenomenon of citrullination as a method by which BMSC's dictate their BM microenvironment to determine disease progression. The aim of the study is to determine the nature of proteins that are citrullinated, to examine the effect of this citrullination on their function and to determine the effect of PADI2 in BMSC's under hypoxic conditions.

2. Materials and Methods

All materials were obtained from Sigma-Aldrich, unless otherwise stated.

2.1. Antibodies

Primary antibodies were incubated at 4°C overnight at the stated concentrations:

Polyclonal anti-PADI2, 1:500 (Abcam®), monoclonal Z-22 anti-PADI2, 1:100 (Santa Cruz Biotechnology®), polyclonal anti-PADI2, 1:500 (Proteintech™), polyclonal anti-citrulline 1:1000 (Abcam®) and clone 54 anti-HIF1-α 1:500 (BD Biosciences). For determining equal loading, membranes that used the same secondary antibody were stripped gently with 0.1% sodium azide and probed with monoclonal AC-40 anti-β-actin 1:4000 (Sigma-Aldrich).

Secondary antibodies used include anti-rabbit horseradish peroxidase (HRP)-linked, 1:4000 (Cell Signalling) and anti-mouse HRP-linked, 1:4000 (Cell Signalling).

2.2. Cell culture

2.2.1. Cell lines

Cell lines used include BMSCs (HS-5) (ATCC®), astrocytes (U87 MG) (CLS Cell Lines Service), U87 isocitrate dehydrogenase 1 (IDH1) mutant (Pim French, Bralten *et al.* 2011), breast tissue cells (MCF10A's, MCF7's (ATCC®), BT474 (Clare Davies, University of Birmingham)) and human embryonic kidney cells (HEK293T) (Clare Davies, University of Birmingham).

2.2.2. Transfections

DMEM (Hyclone™, Healthcare Life Sciences, UK) containing L-glutamine (Hyclone™, Healthcare Life Sciences, UK) and fetal bovine serum (FBS) (Hyclone™, Healthcare Life Sciences, UK) (10%) were used to culture the HEK293T cell line and RPMI medium containing sodium pyruvate for the HS-5 cell line. Cells were seeded 24 hours prior to transfection. As shown in *table 2.1*, jetPEI® DNA transfection reagent (polyplus-transfection™) was use to transfect cells. Media was replaced immediately before each transfection.

Table 2.1 – Cell culture preparations for transfection, in 6 well and 10 cm culture vessels.

Plate/Dish	Media Volume (mls)	Number of cells seeded	JetPEI® transfection reagent (µl)	150mM NaCl (µl)	Plasmid DNA (µg)
6 well	3	2×10^5	6	100	3
10cm	7	2×10^6	30	250	10

Sodium chloride (NaCl) was added together with jetPEI® and separately with the plasmid DNA, each vortexed for 10 seconds. The tubes were combined, always adding jetPEI® to the DNA, and vortexed for a further 10 seconds, followed by a 30 minute incubation period at room temperature. The transfection mix was added to the cells and incubated at 37°C overnight.

2.2.3. Harvesting cells

Media was removed from seeded cells before 1ml 1x phosphate buffered saline (PBS) was added. Cells were scraped with a sterile cell lifter (Fisher Scientific UK) into PBS using a sterile cell scraper, and transferred into an eppendorf. These were centrifuged at 1,500 x g for ~8 minutes and the supernatant discarded. Some transfected cells (to check for PADI2 expression and citrullination) were resuspended in 1x Laemmli (50µL) and boiled for 5 minutes before freezing, whereas others were flash frozen, using dry ice and methanol. Non-transfected cells for other assays were flash frozen and stored at -20°C.

2.3. Immunoblotting

2.3.1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Each sample was lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% triton and 1:100 protease cocktail inhibitor (PIC)) and centrifuged at 13,000 x g for 5 minutes. The supernatant (soluble) was separated and 1x Laemmli buffer (SDS-PAGE sample buffer) added before boiling for 5-10 minutes to denature the soluble proteins. The samples were separated by SDS-PAGE, consisting of a 10% resolving gel and 4% stacking gel. Samples were loaded alongside a molecular weight marker Rainbow™ (GE Healthcare Life Sciences). Each gel was run at 150V for ~55 minutes.

2.3.2. Protein transfer

The SDS-PAGE gel was then transferred onto nitrocellulose membrane (Amersham™Hybond™) at 100V for 1.15 hours. The membrane was blocked with 5%

milk before the appropriate antibody was added. This was left for ~2-3 hours or overnight at 4°C. The membrane was washed quickly 3 times, followed by 3 further washes for 10 minutes each, before adding the appropriate secondary antibody. The wash steps were repeated after 1 hour. Once washed, the immuno-signals were visualised by adding enhanced chemiluminescence (ECL) (GE Healthcare) at a 1:1 ratio and processed using a Konica Minolta developing unit.

2.4.   Endogenous PADI2 expression across different cell lines

1mL of neutralised cell suspension was centrifuged at 13,000 x g for 5 minutes. Excess media was removed and 1x Laemmli buffer added before each sample was boiled for 5 minutes for immunoblot analysis. Cell lines include: BT474 (positive control (Clare Davies, University of Birmingham)), HS-5, HEK293T, MCF10A, MCF7, U87 and U87 IDH1 mutant.

2.5.   Immunoprecipitation of whole cell lysates

25µL of Protein G Sepharose 4 Fast Flow beads (GE Healthcare Life Sciences) were added to an eppendorf and washed using 1mL wash buffer (50 mM Tris/HCl pH 7.5 and 150 mM NaCl). The beads were centrifuged at 1,500 x g and the supernatant removed, followed by 2 further wash steps. Once washed thoroughly, 1mL wash buffer and the appropriate antibody was added (4µg/µL) to the beads and incubated at 4°C overnight on a rotating wheel. The beads were centrifuged at 1,000 x g for 2 minutes and the supernatant removed. 1mL wash buffer was added and the beads centrifuged again. Once the supernatant was removed, the soluble lysate was added and incubated with the beads at 4°C for 2-4 hours on a rotating wheel. 3 wash steps were repeated and 1x Laemmli added, before boiling for 5 minutes and freezing at -20°C until immunoblot analysis.

2.6.   Immunoprecipitation using covalently linked dynabeads®

Harvested transfected cells were placed on ice to thaw then lysed with lysis buffer (20% triton, 50 mM Tris/HCl pH7.5 and 1:100 PIC) by resuspending and incubating on ice for 20 minutes. Cells were centrifuged at 13,000 x g for 10 minutes at 4°C. 40µL dynabeads® (Life Technologies™) were washed with wash buffer (50 mM Tris/HCl pH 7.5 and 150 mM NaCl). The soluble lysed cells were added to the beads and incubated at 4°C for two hours on a rotating wheel. A further 4 washes were carried out before adding 2x Laemmli buffer, boiling for 5 minutes and freezing at -20°C for later immunoblot analysis.

2.7. Calcium/EDTA assay

2.7.1. HEK293T cell line

2 x 15 cm dishes of HEK293T cells were seeded at 1 x 10^6 and 1 x 15 cm dish transfected before harvesting (*as previous*). Non-transfected (control) and transfected (PADI2) cells were thawed on ice for 1 hour before being resuspended in 1mL 1x PBS. A 100µL fraction was removed from PADI2 transfected cells and placed into a separate eppendorf to check for PADI2 expression. Each tube was centrifuged at 1, 500 x g for ~8 minutes, the supernatant was removed and 1x Laemmli buffer added to the 100µL fraction. The remaining 900µL PADI2 transfected cells and control cells were placed on ice for 20 minutes in lysis buffer (20 mM Tris/HCL pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM dithioerythritol (DTE), 10 mM β-mercaptoethanol, 1% triton and 1:100 PIC). Cells were spun back down at 13, 000 x g for 10 minutes and a 50µL fraction of the supernatant, from each PADI2 transfected and control cells were split between two tubes (~400µL) to provide the following:

Control (untransfected cells): + Calcium (10 mM)

                                       + EDTA (Fisher Scientific UK) (1 mM)

PADI2 (transfected cells):   + Calcium (10 mM)

                                       + EDTA (1 mM)

Each tube was left at 37°C for 3 hours, with a 50µL fraction removed at 30 minute intervals. Once removed, 50µL 2x Laemmli buffer was added to stop the reaction and the sample boiled for 5 minutes. Samples were frozen at -20°C until immunoblot analysis (adapted from Assohou-Luty *et al.*, 2014).

2.7.2. HS-5 cell line and recombinant PADI2

Cells were plated on a 10 cm cell culture dish, seeded at 2.5 x 10^6 and left overnight at 37°C before being scraped into 1mL 1x PBS and spun down at 1,500 x g for 8 minutes. The supernatant was removed and cell pellet aliquots frozen at -80°C. Cells were thawed on ice, before 200µL sonication buffer (20 mM Tris/HCL pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM dithioerythritol (DTE), 10 mM β-mercaptoethanol and 1:100 PIC) was added. The cells were sonicated for 5 seconds (25%), repeating 3 times, and cooling on ice between repetitions. A fraction (50µL) of the whole cell lysate (WCL) sample was removed as a control on an immunoblot analysis. EDTA (1 mM) was added to one aliquot of cell lysates and calcium chloride (10 mM) and 130 nM recombinant PADI2 protein added to another. The samples were incubated at 37°C for 2 hours and another 50µL fraction of the sample removed for immunoblotting. While

incubating, the protein G sepharose beads were washed in wash buffer (50 mM Tris/HCl pH 7.5) twice and once in sonication buffer. After washes, 1mL fresh sonication buffer was added before adding the α-citrulline antibody (1:100) and incubating at 4°C for 2 hours. The protein G sepharose beads were re-washed 3 times in wash buffer to remove excess/unbound antibody. Cell lysates were added to the protein G beads and the volume made up to 1mL with sonication buffer before incubating at 4°C for 2 hours. The sample was washed 3 times in sonication buffer (centrifuging at 1,000 x g for 1 minute at 4°C after each wash). 1x Laemmli was added and boiled for 5 minutes before immunoblot analysis or freezing at -20°C.

2.7.3. HS-5 cell line and recombinant PADI2 with glycine elution

The above experiment (2.7.3.) was repeated, however, Laemmli buffer was not added and an additional glycine elution step inserted to remove the protein from the beads. 50µL 100 mM glycine (pH 2.3) was added to each eppendorf (+ Calcium/PADI2 and + EDTA) and left at room temperature for 10 minutes, agitating gently. The beads were spun down and the supernatant placed into a new eppendorf to produce the eluent. The eluent was neutralised with 10µL 1M Tris-HCl, pH 8. A 10µL aliquot of each sample was removed for immunoblot analysis (2x Laemmli and boiled for 5 minutes) and the remaining sample frozen at -20°C.

2.8.   HEK293T cell line transfected with recombinant PADI2

Cells were plated at 2 x 10^6 on 3 x 10 cm cell culture dishes and used as follows:

- Control (HEK293T cells)
- Wild type recombinant PADI transfection
- Inactive mutant C645S recombinant PADI2 transfection

Transfections were carried out as indicated in *section 2.2.2*. Cells were harvested 24 hours later, centrifuged at 1, 500 x g for 8 minutes and the supernatant discarded before adding 1x Laemmli buffer and boiling for 5 minutes. WCL's were frozen at -20°C until immunoblot analysis.

2.9.   PADI2 expression in HS-5 cell line in hypoxia

Cells were seeded at 2.5 x 10^6 onto 6 x 10 cm dishes and incubated overnight at 37°C. Once placed into the hypoxia chamber, at each time interval (normoxia (control), 2hr,

4hr, 6hr, 24hr, 32hr) cells were scraped into 1x Laemmli buffer (~60µL), boiled for 5 minutes and frozen at -20°C until immunoblot analysis.

3. Results

3.1. Endogenous expression of PADI2 varies between different cell lines

To determine endogenous PADI2 expression between cell lines, WCL of BT474, HS-5, HEK293T, MCF7, MCF10A, U87 and U87 isocitrate dehydrogenase 1 (IDH1) mutant were analysed by immunoblotting. Samples were separated by a 10% SDS-PAGE gel and probed with anti-PADI2 (Abcam), anti-PADI2 (Proteintech) and anti-β-actin as a loading control (figure 3.1).
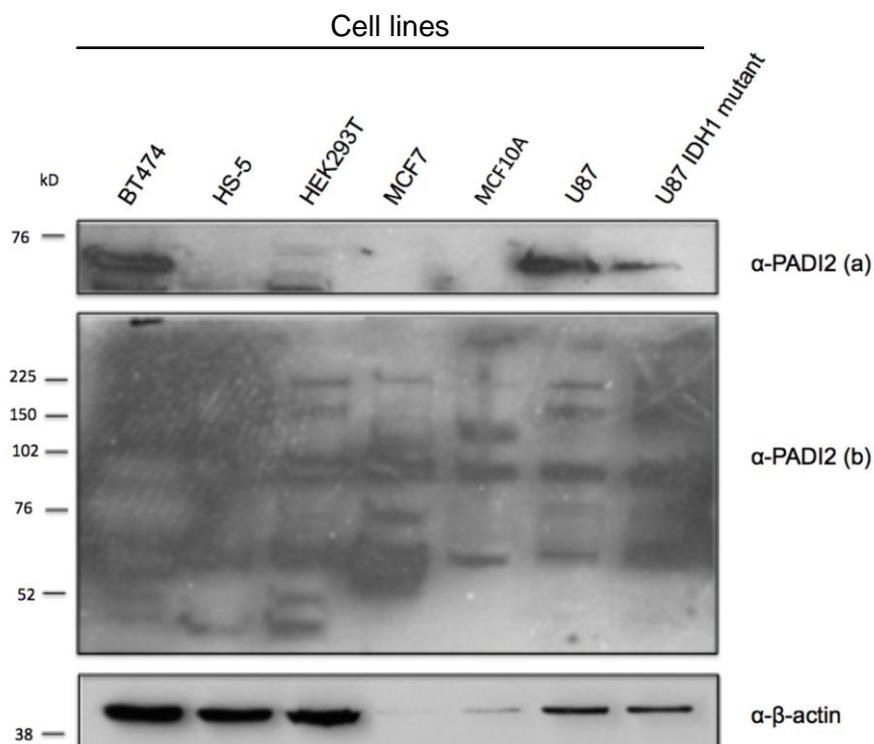
Figure 3.1. – Endogenous PADI2 expression varies between different cell lines. Immunoblot analysis of whole cell lysate of BT474, HS-5, HEK293T, MCF7, MCF10A, U87 and U87 isocitrate dehydrogenase 1 (IDH1) mutant samples. Samples were separated by a 10% SDS-PAGE gel and probed with (a) anti-PADI2 (Abcam), (b) anti-PADI2 (Proteintech) and anti-β-actin as a loading control.

The BT474 cell line was used as a positive control as it is known to have increased endogenous PADI2 expression (McElwee *et al.*, 2012). Both the normal U87 cell line, and one expressing the tumourigenic IDH1 R132H mutation also express endogenous PADI2 whereas HS-5, HEK293T, MCF7 and MCF10A's have little or no expression. However, the MCF7 and MCF10A cell lines in comparison to the others have decreased loading as demonstrated by the anti-β-actin loading control. Therefore, it is possible that MCF7's and MCF10A's do express PADI2 endogenously, but further tests

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are required to determine this. It is clear by the BT474 and U87 lines that the anti-PADI2 (Abcam) was specific, whereas, anti-PADI2 (Proteintech) appeared to be very unspecific making it difficult to detect where PADI2 is present.

3.2. Transfection of PADI2 into HEK293T cells

To ensure PADI2 could be transfected, cells were treated with jetPEI® transfection reagent and WCL were analysed by immunoblotting, probed with anti-PADI2 (Santa Cruz Biotechnology®) and anti-β-actin as a loading control (figure 3.2).
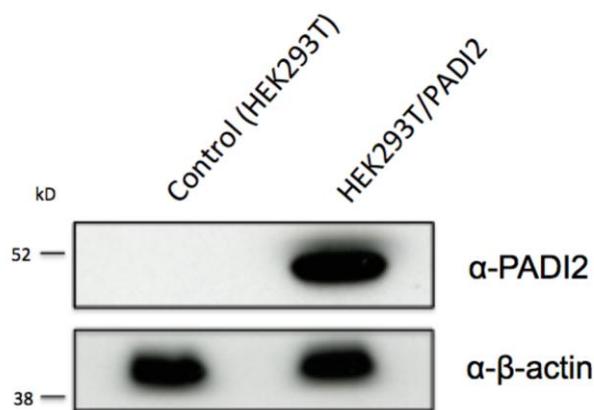
Figure 3.2. – PADI2 transfection into HEK293T cells was confirmed by immunoblot analysis. Cells were treated with jetPEI® transfection reagent, harvested, separated by a 10% SDS-PAGE gel and probed with anti-PADI2 (Santa Cruz Biotechnology®) and loading control anti-β-actin.

PADI2 was successfully transfected into HEK293T cells, although it appeared smaller (~50 kD) than the known PADI2 size (75 kD).

3.3. Immunoprecipitation in HEK293T cells

3.3.1. Immunoprecipitation of PADI2

To find whether PADI2 could be immunoprecipitated from HEK293T transfected cells, cells were treated with 1% triton to lyse cells, the soluble and insoluble fractions divided and the soluble fraction added to protein G sepharose beads with anti-PADI2 (Santa Cruz Biotechnology®) bound. Samples were separated using a 10% SDS-PAGE gel and immunoblotted (3.3 A).

PADI2 appears to have been successfully immunoprecipitated from transfected HEK293T cells; however, it is unclear which band is PADI2. Two bands were present in

the immunoprecipitation with molecular weights or 75 kDa (band 1) and 50 kDa (band 2). Figure 3.3 A, band 1 most likely represents endogenous PADI2 (75 kDa), whilst band 2 represents the transfected DNA (50 kDa).
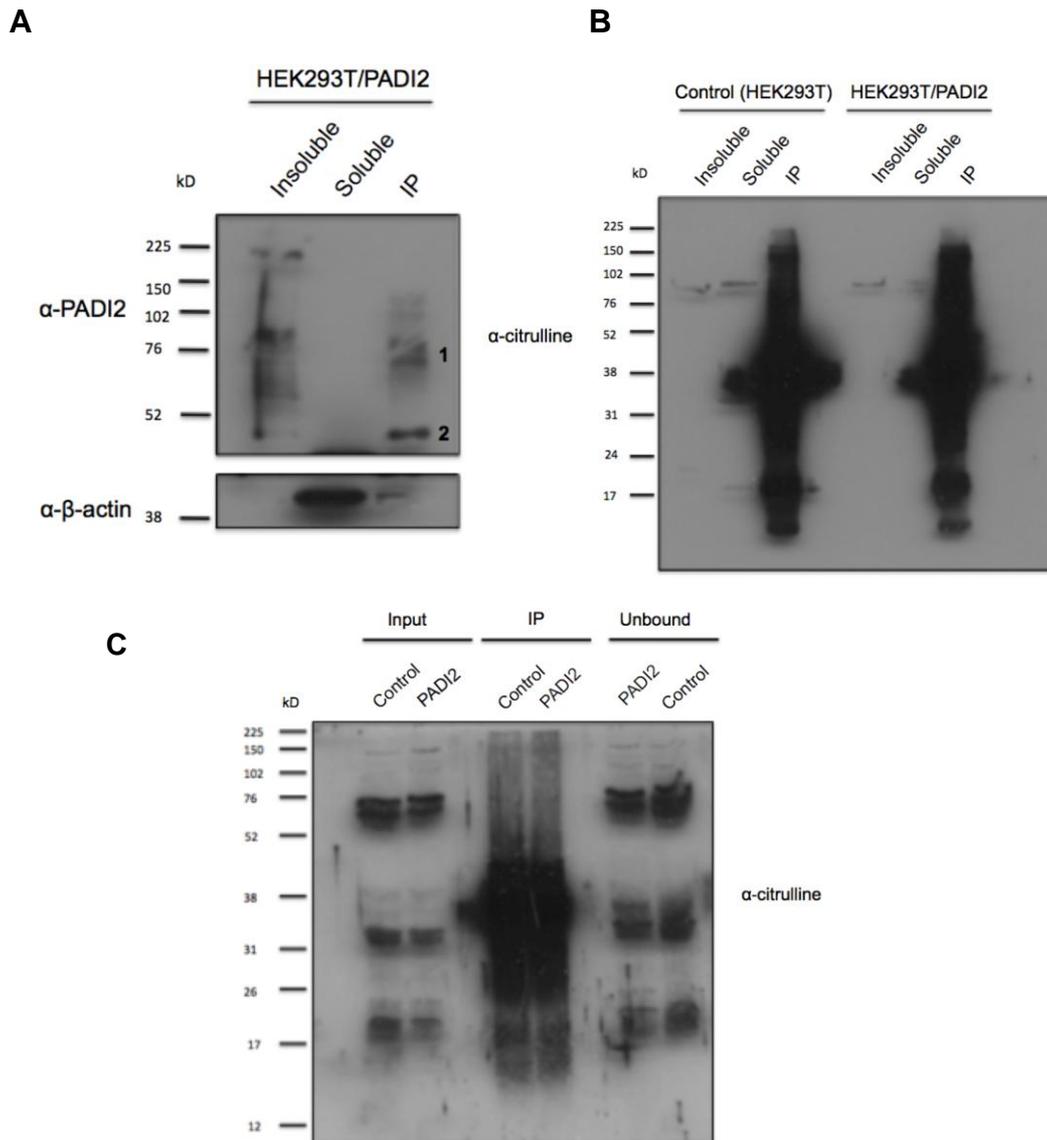
Figure 3.3. – PADI2 has been successfully immunoprecipitated (IP) from HEK293T cell lysate (soluble fraction), although citrullinated proteins are difficult to identify. Cells were treated with 1% triton to lyse cells, and the soluble and insoluble fractions divided. Each fraction of the samples were separated by a 10% SDS-PAGE gel and immunoblotted. (A) Anti-PADI2 (Santa Cruz Biotechnology®) IP, *1* = correct PADI2 size, *2* = the same size band seen in figure 3.2. (B) Anti-citrulline IP between HEK293T cells (control) and PADI2 transfected HEK293T cells (PADI2). (C) Anti-citrulline IP, carried out as above, however, using covalently linked dynabeads® to help reduce the heavy and light chain.

3.3.2. Immunoprecipitation of citrullinated proteins

Citrullinated proteins present in PADI2 transfected HEK293T cells were also immunoprecipitated, as above except using an anti-citrulline antibody (figure 3.3 B/C). As the result of using a whole antiserum anti-citrulline antibody, it was difficult to predict the best concentration to use for immunoprecipitation and, therefore, the heavy and light chain are too overexposed to visualise the bands (figure 3.3 B). Thus, covalently linked dynabeads® were used (figure 3.3 C), to help visualise the bands by helping to reduce the antibody heavy and light chain, although differences in the bands present between control and PADI2 on this IP are also difficult to analyse due to over-exposure.

3.4. PADI2 DNA is a truncated version

It was surprising that we saw no difference in protein citrullination between control and PADI2 transfected cells. In addition, the PADI2 DNA used to transfect HEK293T cells in previous experiments appeared to be at the wrong size (approximately 50kD). Therefore DNA sequencing analysis was carried out to detect any abnormalities. As shown in figure 3.4, the PADI2 construct previously used is truncated by 223 amino acids. Critically, it is expected these residues contain the C-terminal domain. Studies have shown PADI4 to consist of two domains including the N-terminal and catalytic C-terminal. Furthermore, it has been shown that each PADI shares approximately 50% sequence identity, and even greater similarity within the C-terminal (Arita *et al.*, 2004); hence, it is likely the construct used is catalytically inactive.
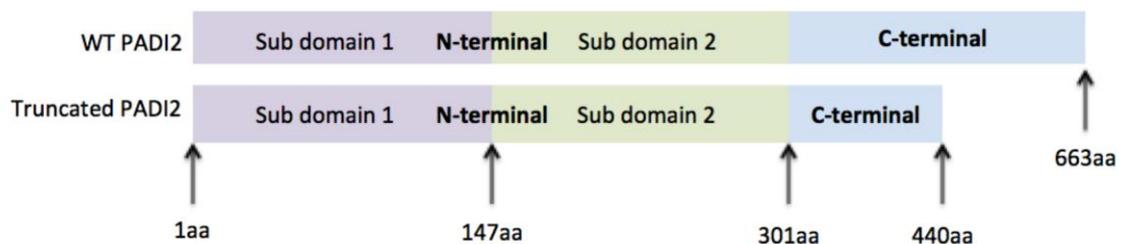

Figure 3.4. – Representation of domain structure of wild type (WT) PADI2 and truncated PADI2. WT PADI2 consists of two main domains; the N-terminal and catalytic C-terminal and is 1-663 amino acids (aa) in length (Arita *et al.* 2004). The truncated version used here is 1-440 aa long and lacks a full catalytic C-terminal domain.

3.5.   Calcium/EDTA assay

3.5.1. The effect of increased calcium/EDTA in HEK293T cell line transfected with truncated PADI2 DNA

Before it became apparent that the PADI2 DNA was truncated, two calcium/EDTA assays were carried out. Although, as previously mentioned, figure 3.3 A suggests that there is endogenous PADI2 present. It is, therefore, important to note that the following results seen are the outcome of endogenous PADI2 as any citrullination can only be the result of the catalytically active, endogenous PADI2.

In an attempt to modulate PADI2 activity in HEK293T cells, cells were treated with 1 mM EDTA, used to chelate calcium ions and inhibit PADI2 activity or 10mM calcium to stimulate PADI2 (due to its calcium-dependent nature) (Assohou-Luty *et al.*, 2014). One aliquot of HEK293T cells (control) and one of PADI2 transfected HEK293T cells (PADI2) was lysed and the supernatant split between two tubes, one for the addition of calcium (control and PADI2) and the other for EDTA (control and PADI2). 20µL of each of the control and PADI2 transfected cells were taken for immunoblot analysis prior to the calcium/EDTA assay to ensure successful transfection (figure 3.5).
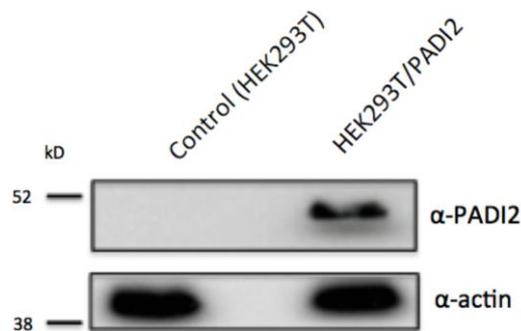
Figure 3.5. – Immunoblot confirming transfection of PADI2 DNA into HEK293T cells. Each sample was separated by a 10% SDS-PAGE gel and probed with anti-PADI2 (Santa Cruz Biotechnology®) and loading control anti-β-actin.
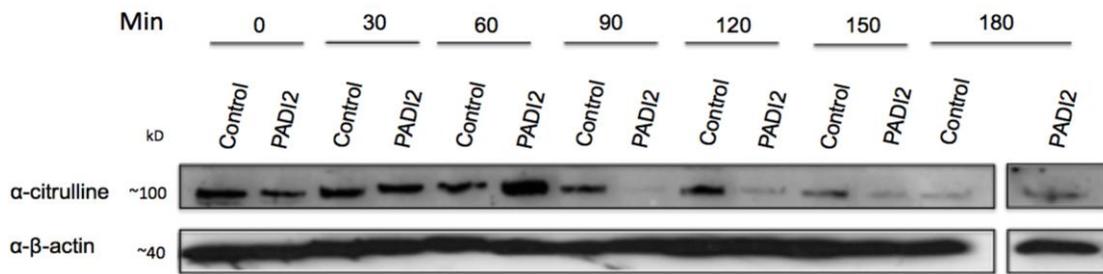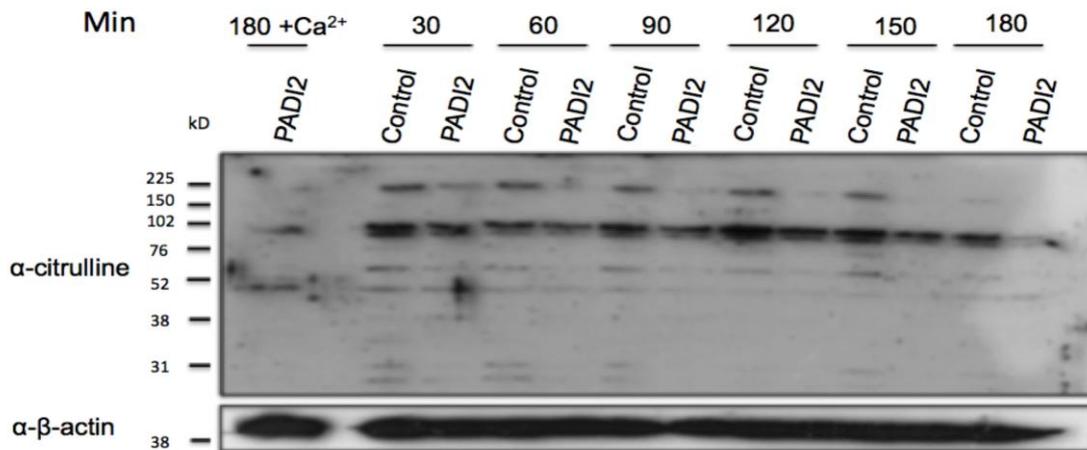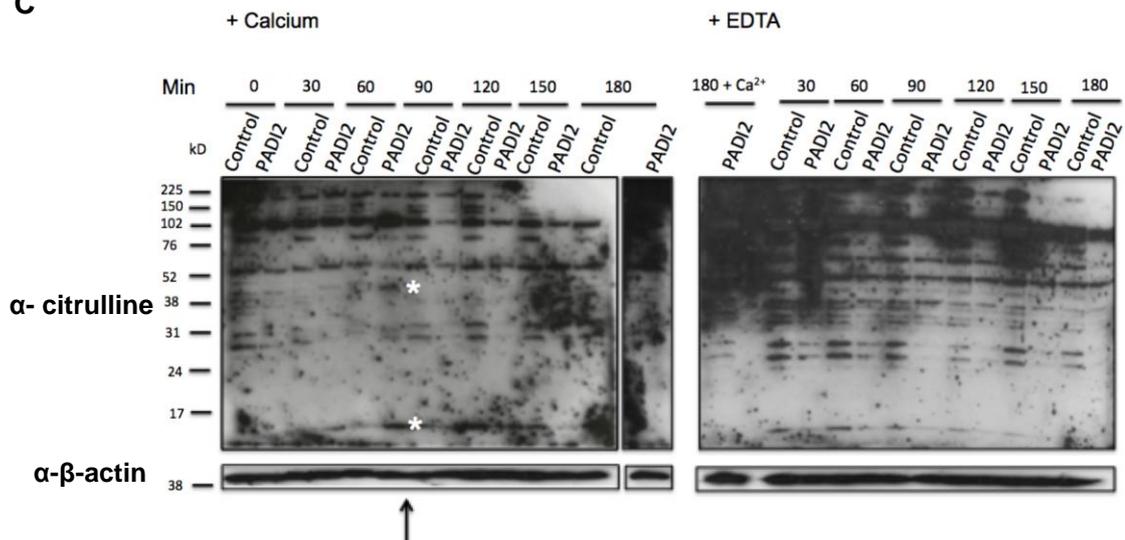
Figure 3.5.1. – Immunoblot analysis of calcium/EDTA assay in PADI2 transfected HEK293T cells (PADI2) and HEK293T cells (control). Adding SDS-PAGE sample buffer stopped each reaction. A 10% SDS-PAGE gel was used to separate the samples. Probes included anti-citrulline and anti-β-actin as a loading control. (A) Short exposure and 10 mM calcium (Ca^{2+}). (B) Short exposure and 1 mM EDTA. (C) Long

exposure of both Ca^{2+} and EDTA. (*) Represent bands that appear significant to PADI2 transfected cells.

Under stimulation of 10 mM calcium, control cells produce increased citrullination in an unknown protein approximately 100 kD (only bands visible under low exposure) up to approximately 150 minutes before decreasing, whereas PADI2 transfected cells only have a citrullinating effect up to 60 minutes.

Control and PADI2 cells treated with 1 mM EDTA did not have the hypothesised effect of preventing citrullination by calcium chelation (hence, + Ca^{2+} 180 minute control); yet, do not follow the same trend as calcium stimulated cells, as the citrullination level, although lower, remains moderately maintained throughout.

Under longer exposure, more bands are exposed. EDTA treated cells appear to have more citrullination occurring in control cells compared with PADI2. However, with added calcium, PADI2 has some significant bands (indicated by * in figure 3.5.1 C) appearing specific at 60 minutes. This time point is also where the protein looks most citrullinated before citrullination is significantly decreased.

As stated above, it is important to note that the transfected PADI2 is a truncated version and, therefore, the effects seen may be the result of full-length endogenous PADI2 present in the cells. Furthermore, the truncated version of PADI2 could possibly be having a dominant negative effect, which is discussed later.

Another example of this experiment is shown in figure 3.5.2.
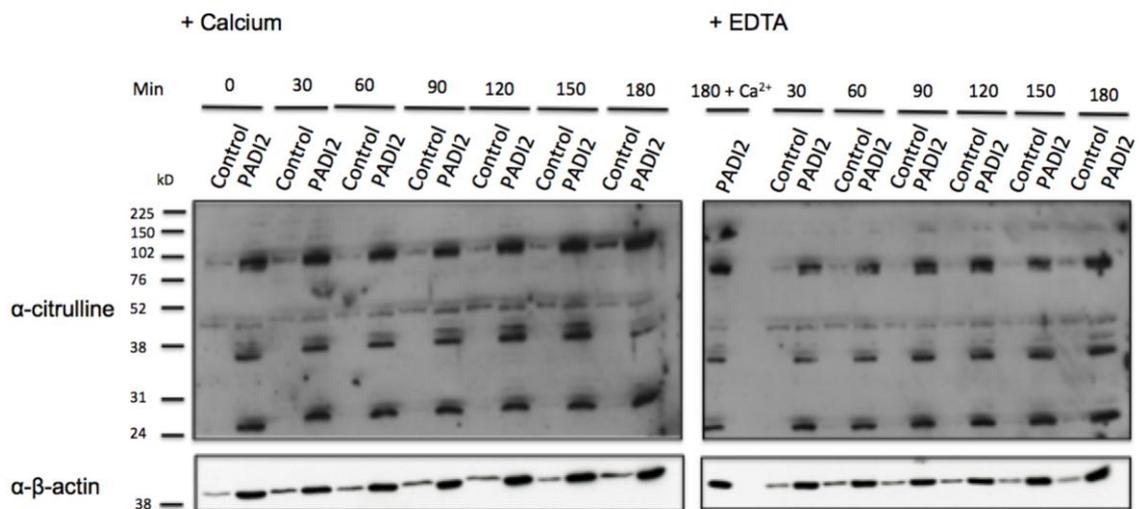
Figure 3.5.2. – Immunoblot analysis of repeated calcium/EDTA assay in PADI2 transfected HEK293T cells (PADI2) and HEK293T cells (control), separated by a 10% SDS-PAGE gel, representing the difference in citrullination of proteins (anti-citrulline) and loading control (anti-β-actin).

With regard to the changes observed in the citrullination of the unknown protein of approximately 100 kD, citrullination appears to remain constant with calcium stimulation in PADI2 transfected cells, whereas it slowly increases within the control. Although the loading control shows unequal loading between the two groups, they appear moderately even within each group. Therefore, it is unfortunately only possible to compare them to one another. As a result of this, it is difficult to conclude whether the bands that appear around 38 kD and 24 kD in both the calcium and EDTA are specific to PADI2 or if it was due to loading error. Citrullination within the PADI2 transfected cells appear to increase slightly over time in both calcium and EDTA groups. Due to the little amount of control sample loaded, it is possible that the citrullination is failing to be detected by the antibody. This experiment should be repeated with equal loading in order to observe differences between the two groups (+ calcium and + EDTA).

3.5.2. Differences in citrullination between HS-5 and HEK293T cells lines treated with recombinant PADI2

As the result of using a truncated version of PADI2, full-length baculovirus expressed recombinant PADI2 was obtained whilst a full-length active and inactive mutant (C645S) version of PADI2 were cloned.

For each of the HS-5 and HEK293T cell lines, cells were lysed using a sonicator and the soluble fraction split between three tubes and left untreated (lysate), treated with 10 mM calcium alone or with 10 mM calcium + 130 nM recombinant PADI2.
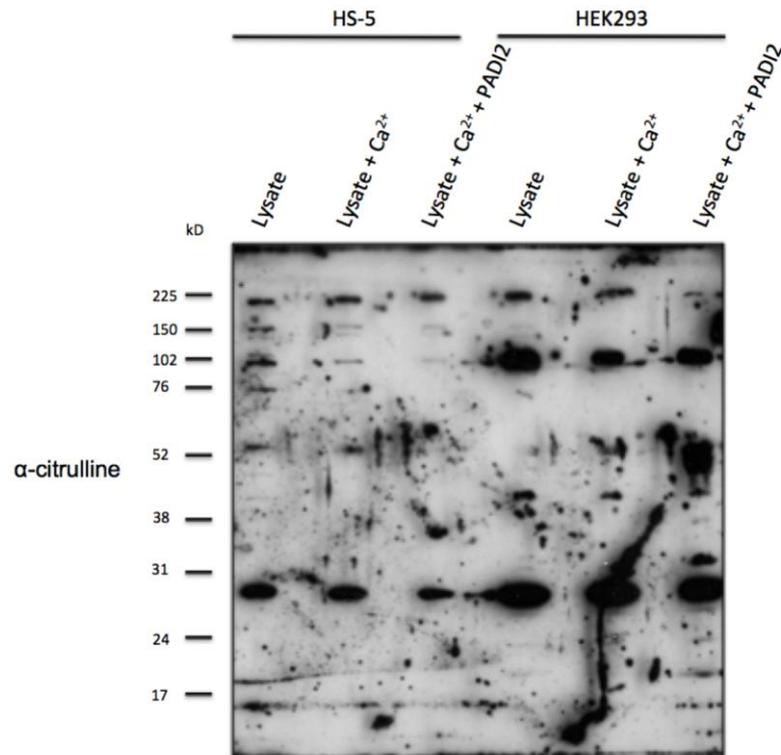
Figure 3.5.3. – Full-length recombinant PADI2 has little effect on citrullination in HS-5 and HEK293T cell lines. Immunoblot analysis of cell lysates, cell lysates treated with calcium alone (10 mM) and cell lysates with calcium (10 mM) and recombinant PADI2 (130 nM). Each reaction was stopped with SDS-PAGE sample buffer. Samples were separated using a 10% SDS-PAGE gel and probed with anti-citrulline.

Both HS-5 and HEK293T cell lines continue to present obvious bands at a size of ~102 kD and ~30 kD as seen in previous experiments. Due to no anti-β-actin loading control, it is difficult to conclude the findings, but it is likely the case that HEK293T cells show increased citrullination due to a larger amount of sample loaded. Furthermore, HS-5 cells appear to produce bands at approximately 150 kD and 17 kD, whereas none appear to be visible at this size in HEK293T cells. It is possible this could be unique to HS-5 cells due to the assumption of more HEK293T sample loading. On the other hand, from this immunoblot, it is difficult to infer whether there is any change within each group alone, as citrullination appears to remain the same and produce no significantly different bands with recombinant PADI2 added. Therefore, an

immunoprecipitation was carried out to enrich for citrullinated proteins from the HS-5 cell line treated with recombinant PADI2.

3.5.3. Citrullination changes after anti-citrulline immunoprecipitation of calcium/EDTA assay in HS-5 cell line when treated with recombinant PADI2

To analyse the effect of citrullination caused by recombinant PADI2 in HS-5 cells, a calcium/EDTA assay was carried out (as described in section 3.5.1), followed by an anti-citrulline immunoprecipitation (figure 3.5.4).
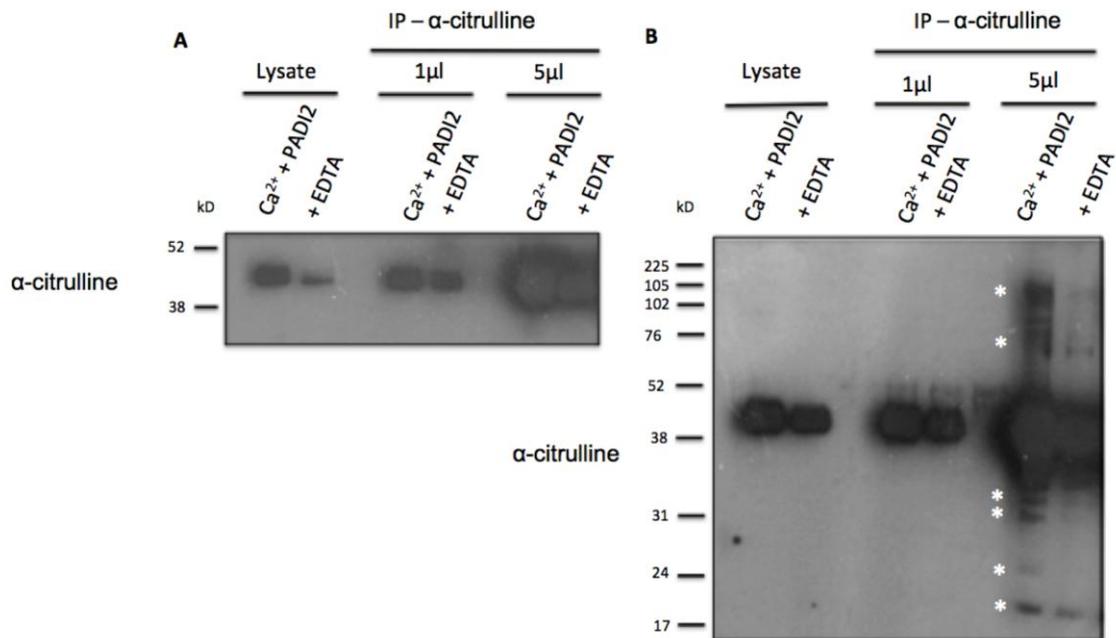
Figure 3.5.4. – Calcium and recombinant PADI2 treated HS-5 cells have increased citrullination compared to those treated with EDTA. Immunoblot analysis of HS-5 soluble lysate, split between two eppendorfs and treated with 10 mM calcium (Ca^{2+})/130 nM PADI2 and 1 mM EDTA and soluble lysate anti-citrulline immunoprecipitation (IP) treated with 10mM Ca^{2+}/130 nM PADI2 and 1 mM EDTA, each incubated for 2 hours at 37°C. Each reaction was stopped with SDS-PAGE sample buffer. Samples were separated using SDS-PAGE gel (10%) analysis and probed with anti-citrulline. (A) Short exposure. (B) Long exposure. (*) Represents bands significant to added calcium and PADI2 in comparison to EDTA.

Here, it is important to note that although there is not an anti-β-actin loading control, the same lysate was split between two tubes equally (400µL); therefore, we expect loading to be equal between samples.

Within the lysate, it appears that there is increased citrullination within the calcium and PADI2 treated cells, where it is decreased in the presence of EDTA (as seen in figure 3.5.4 A). This trend continues to be the case after immunoprecipitation. It is visible in figure 3.5.4 B that there are bands unique to the soluble lysate immunoprecipitation treated with calcium and PADI2, as indicated (*). Nonetheless, the band seen with short exposure at approximately 45 kD is over exposed with long exposure, suggesting the presence of a strongly citrullinated protein. This makes it difficult to analyse other citrullinated proteins around 35-52 kD in size. However, long exposure is necessary to detect differences in other citrullinated proteins outside of this size region.

3.5.4. The differences in citrullination after anti-citrulline immunoprecipitation of calcium/EDTA assay in HS-5 cell line treated with recombinant PADI2 and eluted with glycine

The same assay was repeated with an additional glycine elution step to remove the protein from the beads after immunoprecipitation in an attempt to remove the heavy and light chain and present a clearer result.
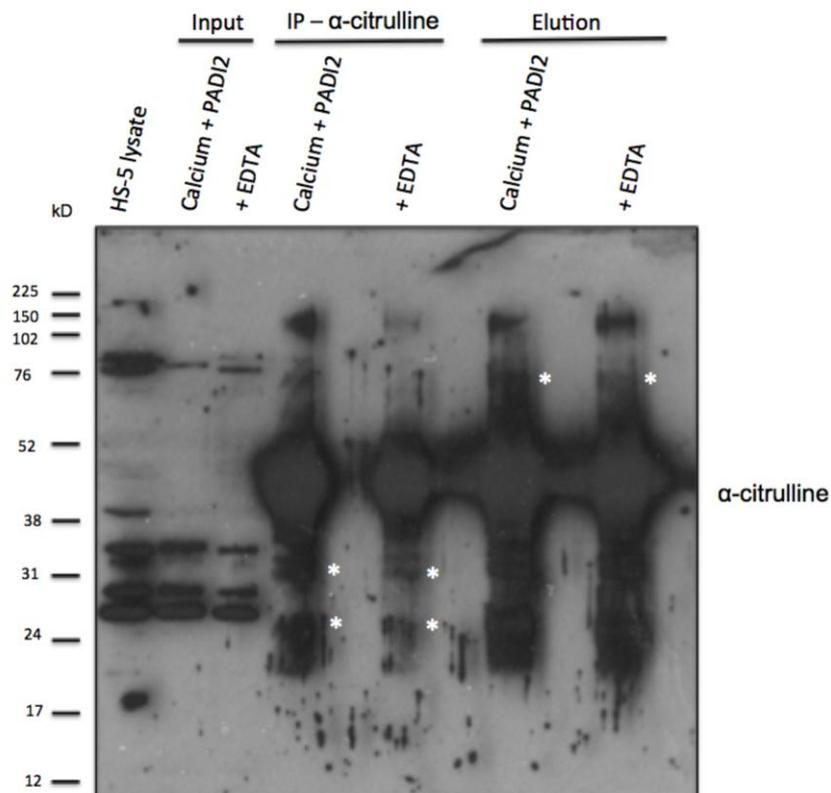

Figure 3.5.5. – Calcium and recombinant PADI2 treated HS-5 cells have increased citrullination compared to those treated with EDTA. Immunoblot analysis, treated as described in figure 3.5.4, with an additional glycine elution step after

immunoprecipitation (IP). Each reaction was stopped with SDS-PAGE sample buffer. Samples were separated using SDS-PAGE gel (10%) analysis and probed with anti-citrulline. (*) Represents bands significant to added calcium and PADI2 in comparison to EDTA.

Due to over-exposure, it is difficult to analyse the differences between bands thoroughly. It is clear that, as seen previously (figure 3.5.4), although there is some citrullination occurring with added EDTA after immunoprecipitation, there are obvious differences when compared to cells treated with calcium and PADI2, as indicated (*) in figure 3.5.5. The glycine elution step unfortunately does not help make the immunoblot clearer. It is possible that the strong band, as seen formerly in figure 3.5.4 B, is a strongly citrullinated protein. Therefore, this experiment needs to be repeated, loading less of each sample to avoid over exposure, as there may be some differences visually blocked by this band.

3.6. HEK293T cell line transfected with PADI2 wild type and PADI2 C645S mutant

To understand citrullination changes in PADI2 WT compared to the C645S mutant (inactive version), both were cloned into a mammalian expression vector, pCMV-Tag2B, so that a FLAG-tag sequence was added to the N-terminus of PADI2. Both PADI2 WT and mutant were successfully transfected into HEK293T cells using jetPEI® transfection reagent. After separating using a 10% SDS-PAGE gel, samples were analysed by immunoblotting (figure 3.6).

First, the immunoblot was probed with anti-PADI2 to check for a successful transfection. Once it was evident both WT and mutant transfected cells were expressing PADI2, the blot was probed with anti-citrulline. Citrullinated proteins appear to be present in each sample, although it is noticeable that the PADI2 mutant has less citrullination. In support of this, from the loading control, there is possibly less PADI2 WT loaded, strengthening the possibility that PADI2 mutant produces less citrullination. Although, it is difficult to confirm that citrullination changes from figure 3.6, therefore, further tests including the use of a PADI inhibitor such as Cl-Amidine should be performed before conclusion.
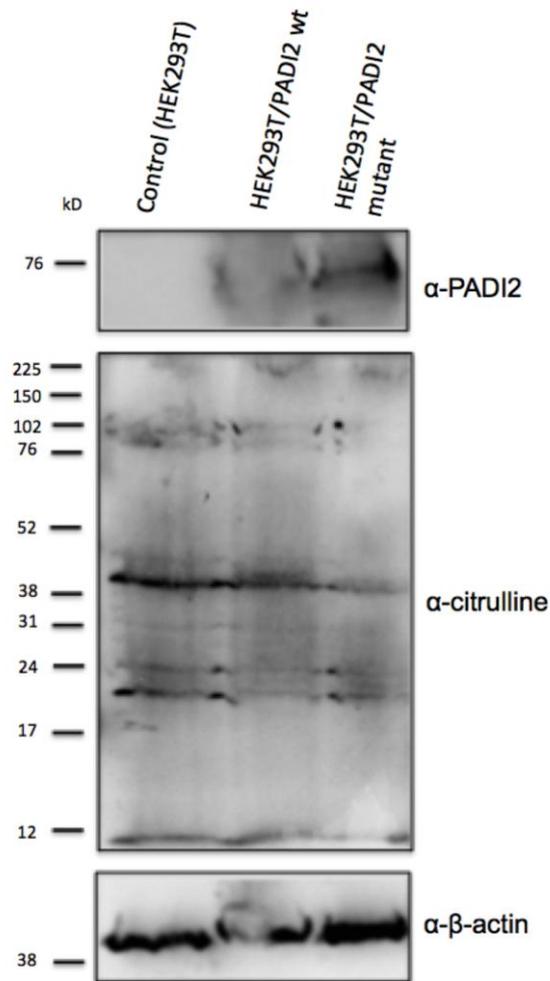
Figure 3.6. – HEK293T cell line lacks endogenous expression of PADI2, although successfully expresses transfected PADI2 wild type (WT) and inactive mutant (C645S). When probed for citrullination, it is visible that there is less citrullination present in the inactive mutant version of PADI2, as predicted. Immunoblot analysis of HEK293T cell line transfected with PADI2 wild type and PADI2 inactive mutant, using jetPEI® transfection reagent. Samples were separated by a 10% SDS-PAGE gel and probed with anti-PADI2 (Abcam), anti-citrulline and loading control anti-β-actin.

3.7. Hypoxia

3.7.1. PADI2 expression is induced in HS-5 cell line in hypoxic conditions

As Sambandam and colleagues (2004) found that astrocytes have increased PADI2 expression in hypoxic conditions, we investigated if this too was the case in HS-5 cells.
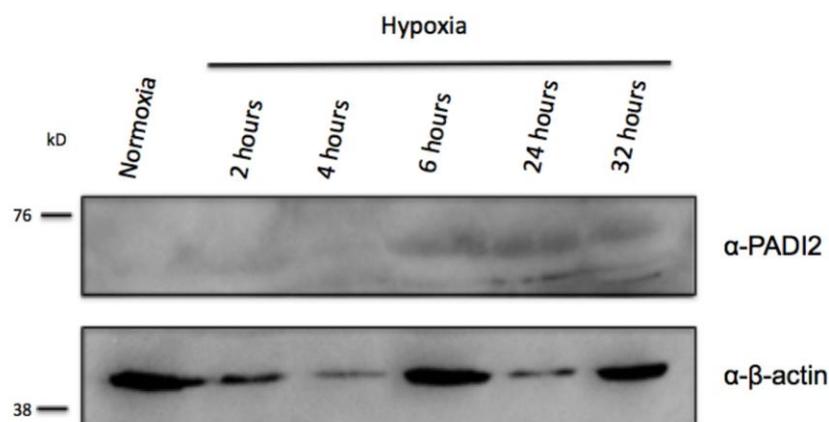
Figure 3.7. – PADI2 expression is increased after, at most, 6 hours and maintained in hypoxia (1% oxygen). Immunoblot analysis of HS-5 cell line seeded at 2.5 x 10^6 and placed in hypoxia for 2, 4, 6, 24 and 32 hours. Cells were scraped into 1x Laemmli buffer (SDS-PAGE sample buffer) in hypoxic conditions, separated by a 10% SDS-PAGE gel and probed with anti-PADI2 (Abcam) and loading control anti-β-actin.

Figure 3.7 and figure 3.7.1 show two examples of changes to PADI2 expression after incubating HS-5 cells in hypoxia (1% oxygen) for different lengths of time. In figure 3.7, it appears that PADI2 expression is increased after six hours in hypoxic conditions; however, as demonstrated in 3.7.1, in separate experiments PADI2 expression is increased after two hours in hypoxic conditions. This is probably due to the uneven loading, represented by the loading control (anti-β-actin), as the two hour and four hour samples in figure 3.7 both have less protein loaded. Overall, it appears that hypoxia does have an effect on PADI2 expression in the HS-5 cell line.
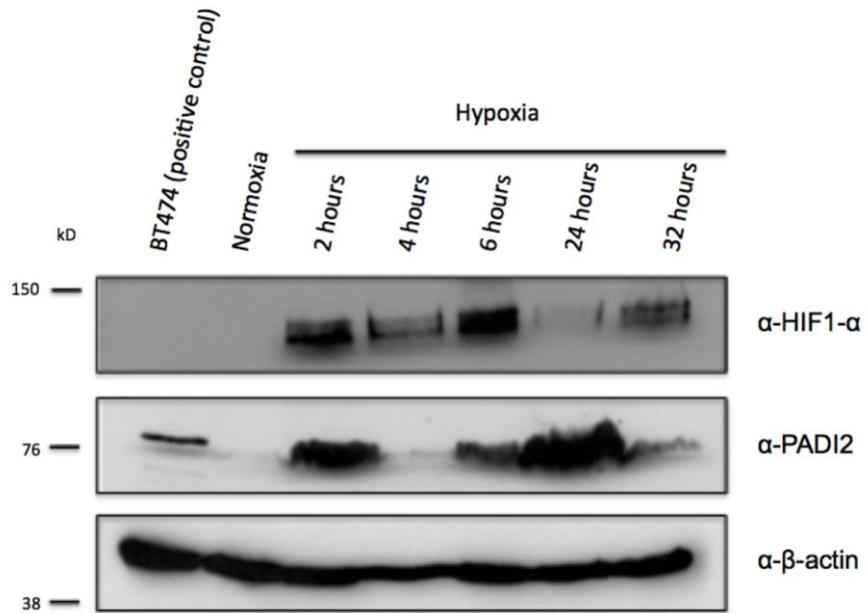
Figure 3.7.1. – PADI2 expression is induced after only 2 hours in hypoxia conditions. Immunoblot analysis of HS-5 cell line seeded at 2.5 x 10^6 and placed in hypoxia (1% oxygen) for 2, 4, 6, 24 and 32 hours. BT474 is a positive control, known to express PADI2 under normal conditions. Cells were scraped into 1x Laemmli buffer (SDS-PAGE sample buffer) in hypoxic conditions and separated by a 10% SDS-PAGE gel. Probed with anti-HIF1α to ensure cells were hypoxic, anti-PADI2 (Abcam) and loading control anti-β-actin.

4. Discussion

In addition to its strong implication in the autoimmune disease RA, PADI2 has also been linked to cancer and other inflammatory diseases including psoriasis (György *et al.*, 2006), alzheimers disease (Ishigami *et al.*, 2005) and MS (Vossenaar *et al.*, 2003). In fact, it has been suggested that RA patients may be at a higher risk of MM development (Shen *et al.,* 2014). Given the similarities in the physiology of RA and MM, what is known about the role PADI2 plays in RA could be transferred to MM to help identify the effect citrullination has on BMSCs within the BM microenvironment. This will ultimately aid our understanding of the disease and potentially yield future novel therapeutic targets. Studies investigating PADI activation, their substrate specificities and therefore their citrullinating effect, are currently of high priority (Darrah *et* al., 2012). In this study, the aim was to determine the nature of proteins being citrullinated in BMSCs, the potential effect that these proteins have within the BM microenvironment, and to determine the changes that occur in PADI2 expression in BMSCs under hypoxic conditions.

This study revealed changes in citrullination as the result of analysing HS-5 (BMSC cell line) cell lysates treated with recombinant PADI2 and excess calcium or with EDTA. The PADI2 DNA used in the initial calcium/EDTA assays was a truncated version and, therefore, cells ectopically expressing PADI2 did not produce accurate results. It was found that endogenous PADI2 expression is upregulated after just two hours in hypoxia and this expression is maintained for 32 hours (figure 3.7.1).

4.1. Calcium/EDTA assay

Before it was discovered that the PADI2 DNA was a truncated version, it was difficult to determine why two bands were detected (figure 3.3 A). Endogenous PADI2 (~75 kD) immunoprecipitated from PADI2 transfected HEK293T cells revealed an additional band present at approximately 50 kD. Initially it was thought that the 75 kD band was the transfected PADI2 DNA, however, this became less likely when the previous transfection immunoblot was analysed (figure 3.2). Citrullinated proteins observed until the PADI2 DNA was sequenced, was the result of endogenous PADI2, as the truncated version is missing the important catalytic domain, as demonstrated in figure 3.4. This therefore, would not have a citrullination effect. Although, calcium/EDTA assays conducted before this produced some very interesting results. PADI2 transfected HEK293T cells show increased citrullination over time, until 60 minutes (figure 3.5 A), where it begins to decline. This suggests that transfected PADI2 has a

dominant negative effect, allowing endogenous PADI2 to citrullinate proteins before it begins to change the activity, where the decline becomes noticeable. This hypothesis is supported by the results of the control cells (non-transfected HEK293T cells), as they continue to express citrullinated protein for longer.

Proteins that are most commonly known to be citrullinated include: vimentin (Bang *et al.,* 2007), α-enolase (Kinloch *et al.* 2005), fibrinogenβ (Zhao *et al.,* 2008), fibrinogenγ (van Steendam *et al.,* 2010), fibronectin (Chang *et al.,* 2005), collagen type II (Yoshida *et al.,* 2006), filaggrin (Union *et al.,* 2002), β-actin (Darrah *et al.,* 2012) and histone H3 (Wang *et al.,* 2009). Although this study is yet to identify proteins by mass spectrometry (due to time constraints), similar bands consistently appeared around 230 kD, 100 kD, 50 kD, 40 kD, 25 kD and 17 kD and were observed throughout the different calcium/EDTA assay immunoblot results. After the first calcium/EDTA assay, proteins approximately 230 kD, 100 kD, 50/60 kD and 17 kD were identified with calcium stimulation. However, further bands of around 50 kD, 40 kD and 25 kD were identified with added EDTA, which is surprising given that EDTA should inhibit PADI2 activity. By comparing the results to other studies it is possible to predict the type of proteins likely to have been citrullinated in this study. In a study by Bang *et al.* (2007) citrullinated vimentin was identified at approximately 50 kD, which is also present in the results of this study (figure 3.5 C). Although, citrullinated α-enolase is also a protein of similar size at approximately 47 kD (Kinloch *et al.,* 2005), as is filaggrin at 50 kD (Union *et al.,* 2002). Filaggrin was found in the study by Union and colleagues (2002), to be present at 50 kD yet, also has an additional band at 25 kD. A band seen at 25 kD is present in figure 3.5 C + EDTA. Fibrinogenγ is around 100 kD (demonstrated by van Steendam *et al.,* 2010), as is a band of that size in figures 3.5 and 3.5.1. Lastly, a 17 kD band is visible in figure 3.5 C, suggesting a possible citrullinated histone. Citrullinated histone H3 has been found by Wang *et al.* (2009) to be 17 kD when cells are activated with calcium, as it is here (figure 3.5 C). When the experiment was repeated (figure 3.5.1), the main bands were 100 kD, 40 kD and 25 kD in size with no change between the calcium stimulated and EDTA groups. This suggests that fibrinogenγ, β-actin and filaggrin were potentially key citrullinated proteins. β-actin was found to be citrullinated by PADI2 in cell lysates by Darrah *et al.* (2012), supported by van Beers *et al.* (2013) who found citrullinated β-actin epitopes in RA serum, although these were identified by ELISA as the antibody was unable to detect citrullinated β-actin during immunoprecipitation. Moreover, mass spectrometry analysis must be carried out to confirm which proteins were citrullinated, or an immunoblot analysis, probing

specifically for these potential citrullinated proteins from citrullination immunoprecipitates.

Based on the hypotheses that proteins such as vimentin, fibrinogen and α-enolase are citrullinated in this study, it could indicate changes occurring that cause an inflammatory response (Wegner *et al.*, 2010), directly relevant to what is known about the characteristics of MM. Also, citrullination of histones, possibly present in this study, is interesting, as this has been previously implicated with NETosis, tumourigenesis and early stage inflammation (demonstrated in table 1.1) (Chirivi *et al.*, 2013). Nevertheless, this speculation must be confirmed by mass spectrometry analysis.

Changes in citrullination in the HS-5 cell line (after immunoprecipitation of cell lysates) were clearly visible between cells treated with recombinant PADI2 and calcium compared to EDTA. The trend remains, with bands appearing around 17 kD (histone), 25 kD (filaggrin), 40 kD (β-actin), 100 kD (fibrinogenγ) and 250 kD (fibronectin) as described previously (figure 3.5.3). It was initially thought that the bands were difficult to distinguish as the result of either the heavy and light chains remaining from the antibody. Therefore, a glycine elution was carried out to remove the protein from the protein G sepharose beads to ensure no antibody was remaining. However, strong bands remained in the central region of approximately 38-52 kD in size, suggesting a strongly citrullinated protein was present within this region. In a normal cellular context, PADI2 will only citrullinate specific substrates; however, it has been shown that, if PADIs have access to only purified substrate, most of them have the ability to citrullinate it (Darrah *et al.*, 2012). It is important to note that cells were lysed and thus PADI2 had full access to the cellular content regardless of substrate localisation and may, therefore, citrullinate non-physiologically relevant substrates.

The addition of EDTA to HEK293T cells failed to have the desired effect of preventing protein citrullination unlike in the study by Assohou-Luty *et al.* (2014), (from which the assay was adopted) where citrullination is prevented by the addition of EDTA. However, this may be the result of Assohou-Luty *et al.* using COS-1 cells, where citrullinated proteins cannot be detected under normal calcium conditions, in comparison to the HEK293T cells used in this study. EDTA is a calcium chelator, chelating only free calcium ions. As little is known about PADI2, it is not clear how quickly it binds to available calcium, and so, this may explain why EDTA showed little effect in the first instance. Thus, recombinant PADI2 was a better approach, as it is free of calcium prior to the experiment, helping to eliminate this as a possibility. There was a

clear difference in citrullination, indicated in figure 3.5.3, during the calcium/EDTA assay that used non-transfected cell lysates and alternatively adding in recombinant PADI2 and calcium/EDTA, when compared to the effect cells ectopically expressing PADI2 (although truncated) have on citrullination, after an immunoprecipitation to enrich for citrullinated proteins was performed. Furthermore, rather than transfecting PADI2 into HS-5/HEK293T cells, harvesting and treating the lysate with calcium to activate PADI2, a better approach demonstrated by Darrah *et al.* (2012), may be to activate endogenous PADI2 using ionomycin as it increases the level of calcium intracellularly. In the study by Darrah and colleagues (2012), citrullination was greatly induced after a four-hour incubation period with ionomycin, compared to their control cells (neutrophils) that did not show any signs of citrullination.

4.2. PADI2 expression in hypoxia

In the first instance, it appeared that PADI2 was not expressed in hypoxic conditions until six hours (figure 3.7). Although due to unequal loading it was difficult to conclude from this. Therefore, the experiment was repeated. A BCA assay was not performed, which resulted in less accurate protein quantification, and therefore less accurate loading. The reason for this was to prevent reoxygenation of the cells. Thus, the samples were scraped directly into an equal amount of SDS-PAGE sample buffer (1x Laemmli) and boiled. From the results of the second experiment (figure 3.7.1), it became clear that PADI2 expression was induced by two hours in hypoxia and maintained for at least 32 hours. Thus, our initial investigations suggest that hypoxia does play a role in PADI2 expression. PADI2 expression was expected to increase in hypoxia after Sambandam and colleagues (2004) found that PADI2 was markedly increased in astrocytes after eight hours in hypoxic conditions, with PADI2 mRNA levels increased and maintained after just two hours. This later resulted in increased protein citrullination.

Hypoxic conditions in MM increase expression of the chemokine receptor, CXCR4 present on the surface of MM cells. This allows circulating malignant plasma cells to home to new areas of the BM (Azab *et al.,* 2012). Hypoxia activates EMT machinery by decreasing CDH1 expression allowing metastasis to take place (Azab *et al.,* 2012). Therefore, it is interesting that PADI2, and consequently citrullination, is increased in hypoxic conditions, and may potentially play a role in changing the adhesion process, as it does within the synovial joint of RA patients, demonstrating a crucial role in the homing process. Shelef *et al.* (2012) report that increased citrullination of proteins such as fibronectin lead to changes in synovial fibroblast behaviour, impairing their ability to

adhere and migrate within the rheumatoid joint of a RA patient. From the findings in this study and from knowledge gathered in previous studies, it appears that BMSCs in an increasingly hypoxic environment eventually lead to an increase in PADI2 expression and as a result, aberrant citrullination. In terms of MM, it is possible that when the malignant plasma cells within the BM microenvironment become hypoxic, PADI2 is increased causing aberrant citrullination in the BMSCs, and ultimately affects their ability to spread, migrate and adhere to the BM. To determine whether this hypothesis is correct, further experiments need to be repeated to confirm the findings in this study, not only in HS-5 (BMSC cell line) cells but also in BMSCs obtained from MGUS and MM patients.

4.3.   Conclusions and future work

In this study, differences were observed in citrullination between HS-5 cells treated with recombinant PADI2 and calcium compared to added EDTA. However, added EDTA did not have the desired effect of inhibiting all or most citrullination, as it was still present. This may be due to the concentration (1 mM) added, and therefore, increasing the amount of EDTA added may help in repeat experiments. Additionally, during this assay, significantly less recombinant PADI2 (130 nM) was used compared to the experiment by Darrah *et al.* (2012) who used 700 nM. It would be beneficial to increase the amount of recombinant PADI2 added to each experiment. As discussed above, treating with ionomycin may be a more effective approach to stimulate PADI2 expression, and therefore, citrullination.

The PADI2 DNA in this study was a truncated version, it is therefore important that the calcium/EDTA assays should be carried out using full-length PADI2 in order to determine the true effects of citrullination.

Owing to issues with unequal loading, visualising each immunoblot using a LI-COR Odyssey system would provide a better, quantitative method for imaging than the chemiluminescence technique used in this study. As a BCA assay was not carried out to protein quantify before loading onto an SDS-PAGE gel, a LI-COR Odyssey system, which gives a linear signal, may provide a more accurate approach to obtaining a true result by normalising to the loading control.

The citrullination antibody used in this study was the whole antiserum. Therefore, it has a lot of background present and there is potential that it binds to non-specific proteins. By treating transfected cells with Cl-Amidine, a pan-PADI inhibitor, there should be no

or very little citrullination present, and consequently would indicate the specificity of the antibody. Additionally, an shRNA experiment for the long-term gene knockdown of PADI2 in HS-5 or MGUS/MM patient fibroblast cells would enable the specificity of PADI2 antibodies used in this experiment to be checked. This is an experiment that could be carried out in normal conditions, but also in hypoxia, to identify whether PADI2 expression remains to be increased.

It would also be interesting to measure the mRNA levels of PADI2 in BMSCs during a progressively hypoxic environment, as Darrah *et al.* (2012) established that PADI2 mRNA expression is increased and maintained throughout a sixteen hour time window after two hours under hypoxic conditions. Additionally, treating non-transfected HS-5 cells or MGUS/MM patient cells with ionomycin in hypoxia to encourage endogenous expression of PADI2, and therefore stimulate citrullination may prove interesting, as it will allow citrullinated proteins, produced as the result of hypoxia, to be identified. This would enable the aberrantly citrullinated proteins in the BM microenvironment to be mimicked *in vitro* more closely to the physiological BM microenvironment *in vivo*.

Once specific substrates have been identified as the result of mass spectrometry analysis, it would be attractive to identify if these same proteins are citrullinated in BMSCs of MGUS and MM patients as the result of increased PADI2 expression and determine the significance of citrullinated protein activity in the context of MM. Ultimately, the first step would be to determine if MGUS and MM patient BMSCs show increased citrullination and to carry out calcium/EDTA assays and hypoxia experiments in these cells to determine the proteins aberrantly citrullinated. To investigate this further, co-culture experiments (possibly performed at different oxygen tensions) to partially mimic the BM microenvironment *in vitro*, utilising JJN3 and H929 MM cells, could be performed to determine the effect of these citrullinated proteins on MM cell phenotypes, for example, MM cell proliferation or cell survival.

Overall, further investigation into the role of citrullination in the BM microenvironment in MM remains, but this study has provided the initial steps in understanding the effect that changes in PADI2 expression has on BMSCs. Continuing from this study will be the mass spectrometry confirmation of which citrullinated proteins are present. This will unveil future potential targets for research/therapy and to aid our understanding of the effect aberrant citrullination has on the behaviour of BMSCs within the BM microenvironment of MM patients.

5.   References

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