Project 1 - INVESTIGATING THE ROLE OF PBF IN THE NUCLEUS AND NUCLEOLUS

AND

Project 2 - AN INVESTIGATION INTO THE ROLE OF ADIPOSITY ON COLORECTAL TUMOURIGENESIS

By

VIKKI LOUISE POOLE

THIS PROJECT IS SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE AWARD OF THE MRES

College of Medical and Dental Sciences

University of Birmingham

August 2012

Overall Contents

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Abstract

Pituitary tumour transforming gene (PTTG) binding factor (PBF or PTTG1IP), is a poorly characterised protein found to be upregulated in thyroid cancer. The protein has previously been determined to contain several predicted signal sequences within its 180 amino acids, and previous studies have shown the nuclear localisation signal (NLS) to be functional. However, it is unknown whether the predicted nuclear export signal (NES) is functional. Use of nuclear/cytoplasmic fractionation and immunofluorescence, in this study, established that when PBF is C-terminally tagged with haemagglutinin (HA), PBF can no longer translocate to the nucleus, implying that the HA tag interferes with recognition of the NLS. The study also attempted to determine functionality of the predicted NES. Homology data revealed the NES is not conserved among six other mammalian species, suggesting it is not evolutionary important and therefore may not functional; however, when exportin-1 (CRM1) was inhibited/knockdowned *in vitro*, immunofluorescence revealed reduced cytoplasmic and nucleolar PBF staining, suggesting the NES may, in fact, have a role in *Homo sapiens*.

Acknowledgments

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List of Abbreviations

Abbreviation	Definition
ARM	Armadillo
BSA	Bovine serum albumin
CNoB	CRM1 Nucleolar Body
COBALT	Constraint-based Multiple Alignment Tool
CPEB1	Cytoplasmic Polyadenylation Element Binding Protein
CRM1	Exportin 1
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
FBS	Foetal Bovine Serum
GC	Guanine-cytosine
HA	Haemagglutinin
IF	Immunofluorescence
LMB	Leptomycin B
NCS	Newborn Calf Serum
NES	Nuclear Export Signal
NIS	Sodium/Iodide Symporter
NLS	Nuclear Localisation Signal
NoLS	Nucleolus Localisation Signal
NPC	Nuclear Pore Complex
PBF	PTTG Binding Factor
PTTG	Pituitary Tumour Transforming Gene
PTTG1IP	Pituitary Tumour Transforming Gene Interacting Protein 1
RIPA	Radioimmunoprecipitation assay
snoRNP	Small Nucleolar Ribonucleoprotein
T_3	Triiodothyronine
T_4	Thyroxine
TBST	Tris-Buffered Saline with Tween
VO	Vector Only
WT	Wild-type
XPO1	Exportin1

Introduction

The Thyroid Gland

The thyroid gland is a highly vascularised organ, found in the neck, which acts as one of the body's largest endocrine organs (Figure 1). The gland is comprised of large follicles in which iodide is concentrated; this iodide is then used in the production of the hormones thyroxine (T_4) and triiodothyronine (T_3), which are key regulators of metabolism and development.

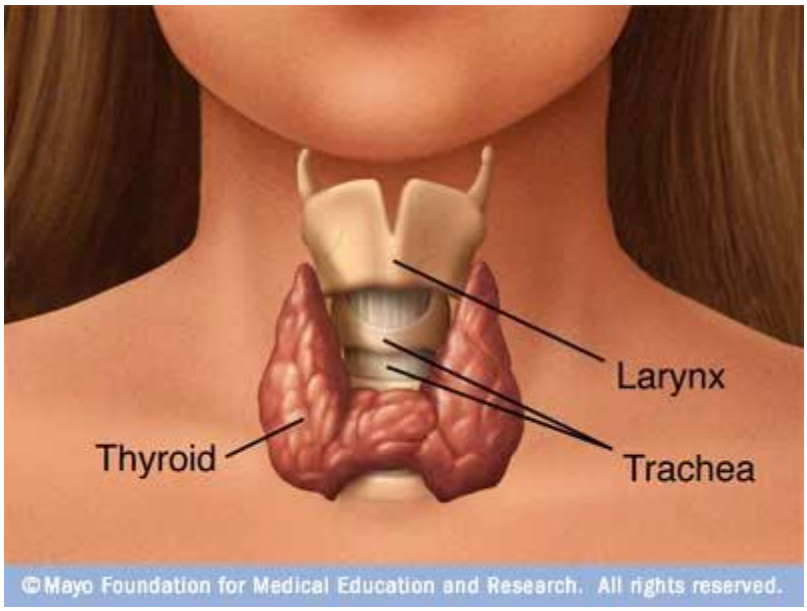


Figure 1– The anatomy of the thyroid gland– Taken from Mayo Clinic 2012

Diseases of the thyroid are relatively common and have been shown to affect approximately 5–10% of the population (Hunt and Wass, 2001). Thyroid cancer is the most common form of endocrine cancer, with an incidence rate that has doubled within the past 30 years; however there has been shown to be a significant correlation between increased incidence and both increased and earlier detection rates (Grodski et al., 2008). There are four major types of thyroid cancers (papillary, follicular, medullary and

anaplastic), which are classified by their different histopathologies (National Cancer Institute, 2012). Papillary thyroid cancer (PTC) is the most common form, representing 60% of all thyroid carcinomas, however the prognosis is relatively good with about 90% of cases having a 10 year cancer-free survival rate (CRUK, 2011). The main treatment for thyroid carcinomas is surgical resection of the thyroid, followed by radioiodide treatment to ablate any residual thyroid tissue (Alford et al., 2011). There are several risk factors for the development of thyroid neoplasms including family history of endocrine malignancy, ionising radiation, and being of the female sex (Grodski et al., 2008). However, despite research into oncogenes such as BRAF and RAS, the mechanisms that lead to the development of thyroid carcinoma are yet to be fully understood. Recent research has indicated that PBF, a little characterised proto-oncogene, may be involved in the aetiology of the disease.

PBF

Pituitary tumor-transforming gene (PTTG) binding factor (PBF), also known as pituitary tumor-transforming gene 1 protein-interacting protein (PTTG1IP), is a 22kDa protein made up of 180 amino-acids. The protein is highly conserved among a variety of species (73% homology to mouse, 67% frog, 60% chicken) yet it shares no homology with other human proteins (Read et al., 2011). The protein was first described in 2000 by Chien & Pei who, through a yeast two-hybrid screen, discovered its capability to interact with PTTG, a human securin (Chien and Pei, 2000). Since its identification, it has only been described in 14 publications. Although PBF was not formally identified until 2000, it had previously been cloned and termed C21orf3 by Yaspo et al in 1998, who located the then unknown protein on chromosome 21q22.3. Initial predictive studies suggested that C21orf3 may be a cell surface glycoprotein important in cell trafficking mechanisms, as it contained a potential N-terminal signal peptide, a transmembrane domain, an endocytosis motif and N-glycosylation sites (Yaspo et al., 1998).

Subsequent studies have since shown that PBF contains a nuclear localisation signal (NLS), suggesting that it has a role in both the cytoplasm and as a nuclear protein (Chien and Pei, 2000) (Figure 2).

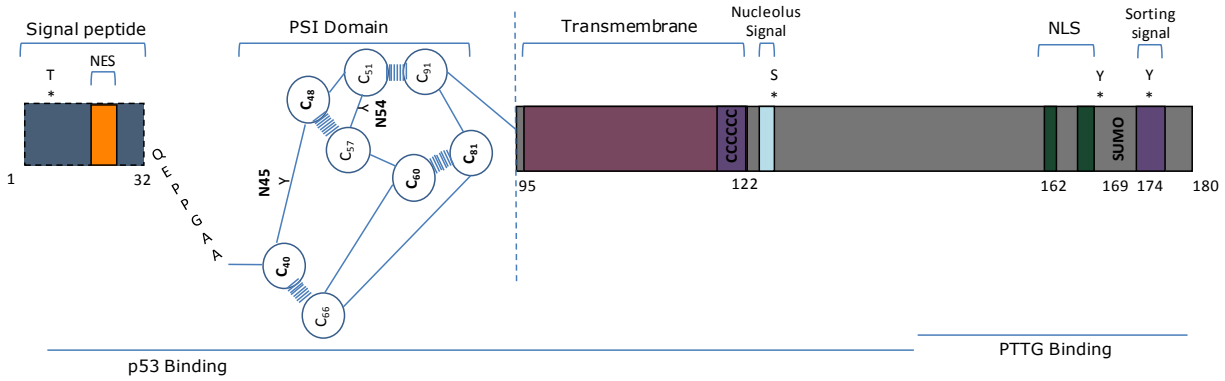


Figure 2 – Structure of Wildtype PBF – PBF contains several different domains and signals, these include an N-terminal signal peptide, containing the proposed nuclear export signal (NES), a PSI (plexin-semaphorin-integrin) domain, a transmembrane region which is then followed by a predicted nucleolus location signal (NoLS), nuclear localisation signal (NLS) and a C-terminal sorting signal. PBF also contains a sumoylation site along with several phosphorylation sites.

Although PBF's function is yet to be fully characterised, studies have shown it is ubiquitously expressed throughout normal human tissue. In 2003, the protein was shown to be upregulated in pituitary adenomas (McCabe et al., 2003); later studies have also shown increased levels in cancers such as breast (Watkins et al., 2010) and thyroid (Stratford et al., 2005). In well differentiated thyroid cancers, such as papillary and follicular, PBF expression has been shown to be significantly increased compared to levels in normal thyroid tissue, and to be an indicator of cancer recurrence. Although PBF is upregulated in these diseases, no associated mutations within the protein have been found (Stratford et al., 2005); however, a recent genomic study of ovarian carcinomas uncovered one patient sample containing a

mutation in PBF, in the protein's transmembrane region, suggesting PBF may have a role in a wider variety of cancers (Network, 2011).

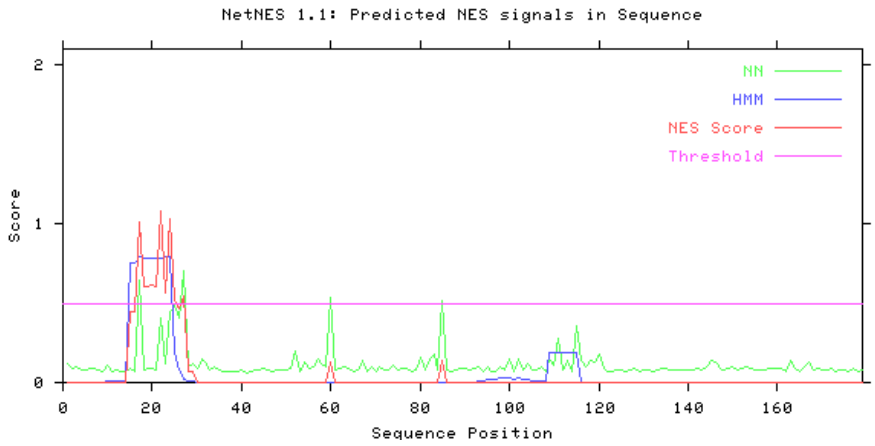
Although PBF's function is generally poorly characterised, a role for it in thyroid cancer, the disease in which PBF has primarily been studied, has been established. In the thyroid, the sodium iodide symporter (NIS) is responsible for transporting iodide into the thyroid follicles, where it accumulates prior to synthesis of thyroid hormones. In thyroid follicular epithelial cells, NIS is usually found at the plasma membrane, allowing the transport of ions into and out of the cell. However, in thyroid cancers, increased PBF levels have correlated with the internalisation of NIS, therefore inhibiting iodide uptake. This is particularly important in the context of radioiodide treatment and imaging, which is used in diagnosing and treating the disease (Smith et al., 2009). PBF has also recently been shown to interact with and regulate p53, a protein associated with 50% of cancers (Read et al., 2012).

As the nature of PBF appears to have different roles in cancer, it is important to establish the protein's function, as this will provide an increased understanding of its therapeutic potential. An insight into function frequently results from establishment of the location of a protein within the cell, as this allows the identification of proteins and organelles with which the protein may interact. Proteins often contain localisation signals within their amino acid sequences, these signals acting like post-codes allowing the protein to be sent to the right 'address' or subcellular compartment and therefore providing a useful approach to predicting the potential location of proteins.

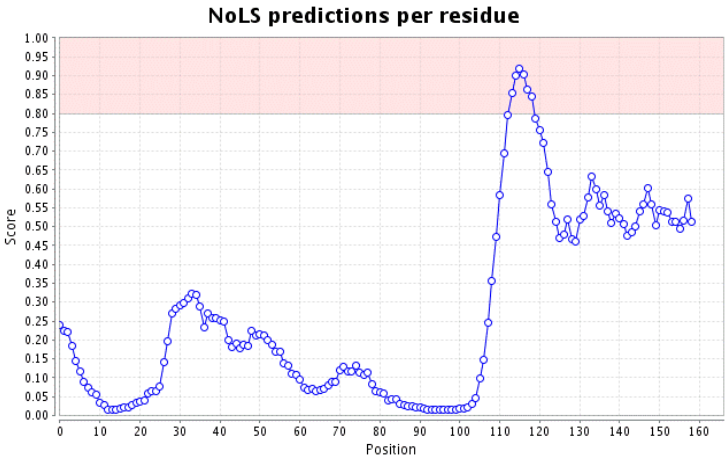
Signal Sequences

First discovered in the early 1970s, localisation signals (known also as signal sequences) have been identified that direct proteins to various cellular compartments such as the nucleus, mitochondria and endoplasmic reticulum. It is accepted within the field that PBF contains a functional C-terminal nuclear localisation signal, which allows the protein to be transported into the nucleus. NLS usually consist of a short sequence of basic amino acids (lysines and arginines) exposed on the protein's surfaces. PBF contains a bipartite NLS between amino acids 149-166, containing several arginines and lysines (Chien and Pei, 2000). Along with signals to enter the nucleus, there is also a sequence known as the nuclear export signal (NES), allowing proteins to be transported out of the nucleus. This signal is usually a series of four hydrophobic residues in a protein that are 'recognised' and bound to by exportins, which then facilitate transport of the protein from the nucleus (La Cour et al., 2004). The amino acid sequence of PBF has previously been submitted to prediction software (CBS Prediction Servers) which showed PBF to have a predicted NES between amino acids 17-27, compromising of a run of hydrophobic leucines (unpublished) (Figure 3A). Although the software predicted the NES to be present, it is unknown whether the sequence is actually functional. PBF has also been shown to have a nucleolus localisation site (NoLS) using prediction software (NOD – Nucleolar localisation sequence detector (University of Dundee) (Figure 3B). These signals are useful tools in both determining the location of PBF, and identifying potential proteins PBF may interact with. For example, if the NES in PBF is truly functional, it is highly likely that PBF interacts with the protein exportin-1.

A



B



C **Sequence of wild-type PBF (NoLS shown in red and NES in green):**

MAPGVARGPTPYWRLRLGGAALLLLLIPVAAAQEPPGAACSQNTNKTCEECL
KNVSCLWCNTNKACLDYPVTSVLPPASLCKLSSARWGVCWVNFEALIITMSV
VGGTLLLGIAICCCCCCRRKRSRKPDRSEEKAMREREERRIRQEERRAEMKT
RHDEIRKKYGLFKEENPYARFENN

Figure 3 – Predicted Signal Sequences

3A – NES Prediction in PBF – The amino acid sequence of PBF was entered into CBS Prediction Server. The software predicted PBF to have an NES between amino acids 17-27. The pink line represents the threshold which, if the red NES score line crosses, suggests there is likely to be an NES.

3B – NoLS Prediction in PBF – The amino acid sequence of PBF was entered into nucleolar localisation sequence detector software. The pink shaded area represents residues that are likely to be involved in a nucleolar location site. There is a predicted NoLS between residues 114 and 138.

3C – Position of predicted NoLS and NES in PBF – The red represents the residues predicted to be involved in the potential NoLS site, whereas the green represent the predicted NES.

Exportin-1

Exportin-1, often referred to as CRM1 or XPO1, is the main protein responsible for exporting proteins containing leucine rich NES from the nucleus. When CRM1 was initially identified, its exact role was unclear, although it was believed to be essential in the maintenance of chromosome structure in fission yeast (Adachi and Yanagida, 1989). However, when genetic mutations were introduced to the CRM1 locus in yeast, a defect in nuclear export of NES-bearing proteins was observed. Furthermore, when studied *in vitro* CRM1-specific antibodies were shown to prevent nuclear export in mammalian cells. These findings suggest that CRM1 is involved in a universal conserved mechanism for the export of proteins out of the nucleus (Kudo et al., 1999). Studies have shown that in steady state CRM1 is located in the nucleus, but that it also has the ability to shuttle between the nucleus and the cytoplasm (Stade et al., 1997). CRM1 works by binding to its substrate (i.e. the protein being exported from the nucleus) and Ran-GTP, with this complex then being translocated through the nuclear pore complex (NPC) to the cytoplasm. Structural studies have shown that CRM1 is a ring-shaped protein that is capable of binding its substrates by their leucine rich NES, at the central convex surface. The leucine-rich helix of the substrate binds specifically into a hydrophobic groove created by helices of CRM1 HEAT repeats (Dong et al., 2009). Leptomycin B has been shown to inhibit CRM1 function by binding to cysteine 529 (Kudo et al., 1999), which is located in the hydrophobic groove, thereby blocking substrate binding (Dong et al., 2009).

Hypothesis

PBF has been found to contain a predictive NES; this study will try and determine whether the signal is functional and under the regulation of exportin-1. Recent laboratory observations have indicated that PBF is unable to enter the nucleus when it is C-terminally tagged with Human influenza hemagglutinin (HA). Therefore, before nuclear export can be studied, it is essential to formally identify if this tag blocks nuclear entry of PBF.

Aims

- To establish whether HA tagged PBF can enter the nucleus.
- To delete the HA tag from the dual tagged PBF (FLAG-PBF-HA).
- To mutate the predicted NES in PBF
- To knockdown and inhibit CRM1 and observe its effect on the localisation of PBF in the cell.

Materials and Methods

Cells culture and transfection

K1 and TPC-1 cell lines were cultured in RPMI media supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), penicillin (10^5 U/l) and streptomycin (100 mg/l).Whereas Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% (v/v) heat-inactivated (FBS), penicillin (10^5 U/l) and streptomycin (100 mg/l) was used to culture HeLA and COS7 African Green monkey kidney epithelial cell lines. All cultures were incubated at 37^{o}C and 5% CO_2 in humidified conditions, being passaged twice weekly and discarded when the passage number became too high for efficient transfection.

Transfection was carried out using Fugene 6 reagent (Roche, Indianapolis) at a 3:1 Fugene 6:plasmid DNA ratio in Opti-MEM media. For protein extraction and immunofluorescence (IF) staining, cells were seeded in six-well plates (containing sterile coverslips for IF) and transfected with 2µg DNA plasmid after 24 hours. For nuclear/cytoplasmic extracts, cells were seeded in T25 flasks and transfected with 5µg DNA plasmid. The plasmids used in transfection were all in the pcDNA3 backbone and plasmids used included pcDNA3 vector only (VO), wild-type PBF, PBF-HA and mutant 1. Mutant 1 was selected as it lacks the amino acids 150-180, meaning there is no NLS; the protein is also HA-tagged at the C-terminus.

Mutagenesis

Stratagene QuikChange Site-Directed Mutagenesis Kit (Agilent technologies) and Stratagene QuikChange II XL Site-Directed Mutagenesis Kit (Agilent technologies) were used for mutagenesis, following the manufacturer's protocol. Wild-type PBF and PBF-HA plasmids were used to attempt to mutate the NES using the following primers:

Double mutation:

Forward – 5’ GCC GTA CTG GAG GTT GCG C**GC** CGG TGG CGC CGC G**GC** GCT CCT GCT GCT CAT CCC G 3’

Reverse – 5’ C GGG ATG AGC AGC AGG AGC **GC**C GCG GCG CCA C**CG** GCG CGC AAC CTC CAG TAC GGC 3’

Single mutation:

Forward – 5' G TAC TGG AGG TTG CGC **GC**C GGT GGC GCC GCG CTG 3'

Reverse – 5' CAG CGC GGC GCC ACC G**GC** GCG CAA CCT CCA GTA C 3'

The primers were designed following specifications given in the kit by the manufacturer and produced by Alta Bioscience, Birmingham UK.

To remove the HA-tag from dual tagged PBF the following primers were used:

Forward – 5’GCT AGA TTT GAA AAC AAC TAA TCT AGA GTC GAC CCG GGC GGC 3’

Reverse – 5’ GCC GCC CGG GTC GAC TCT AGA TTA GTT GTT TTC AAA TCT AGC 3’

From the colonies grown using the mutagenesis kit, 10 were selected and mini-prepped using Wizard Plus SV miniprep DNA purification system (Promega). The DNA was quantified using a 260/280 NanoDrop-1000 spectrophotometer (NanoDrop). 100µg of DNA and 1µl T7 short primer dissolved in nuclease-free water were sent for sequencing (Functional Genomics, Biosciences, Birmingham).

Western blotting and nuclear extract

Proteins were harvested in 200µl RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% v/v Igepal CA-630, 6 mM sodium deoxycholate, 1mM EDTA) containing protease inhibitor cocktail (Sigma, Dorset, UK). Protein concentration was measured using BCA assay using Bovine serum albumin (BSA) as protein standards.

Samples were prepared for loading by combining with 4x Laemmli buffer at a ratio of 3:1 protein: laemmli buffer and denatured for 5 minutes at 95°C. Equal masses of proteins were loaded into

wells along with one well containing 5µl of protein ladder (Precision Plus protein standards dual colour-BioRad). Proteins were separated by electrophoresis at 70 V through 4.5% stacking gels and then 140 V through 12% resolving gels. Proteins were transferred to polyvinylidene fluoride membranes (PVDF), followed by blocking in 5% non-fat milk in Tris-Buffered Saline with Tween (TBST) overnight at 4°C or for 1 hour at room temperature. Membranes were then incubated with primary antibodies in 5% non-fat milk in TBST overnight at 4°C or for 1 hour at room temperature. After washing in TBST (three washes of 10 minutes) blots were incubated with appropriate horseradish peroxidise conjugated secondary antibodies for 1 hour at room temperature. After further washes (three washes of 10 minutes), protein bands were visualised by the ECL plus chemiluminescence detection system on Kodak film for 30 seconds to 20 minutes. Primary antibodies for Western blotting include rabbit anti-PBF (1:500) (Smith et al., 2009), mouse anti-HA (1:1000) (Cambridge biosciences), mouse anti-lamin (1:250), mouse anti-tubulin (1:250), rabbit anti-CRM1 (1:500) (Santa Cruz biotechnology) and mouse anti-β-actin (1:10,000). Secondary antibodies were rabbit anti-mouse (1:2000) and goat anti-rabbit (1:2000).

For nuclear/cytoplasmics extracts, cells were seeded from T25s with a cell count of approximately 3.2×10^6 cells. Protein was extracted using a Nuclear Extract Kit (Active Motif) as per the manufacturer's instructions. Protein concentration for each fraction was calculated using the BCA assay with BSA dissolved in the lysis buffer for the nuclear extract and hypotonic buffer for the cytoplasmic fraction. Western blotting of each fraction was then carried out as described above.

Immunofluorescence

24 hours after transfection, medium was removed from the coverslips and cells were briefly washed in phosphate-buffered saline (PBS). Cells were incubated in 800µl fixing solution (0.1 M phosphate buffer (pH 7.4) containing 2% paraformaldehyde, 2% glucose and 0.2% sodium azide) for 20 minutes at room

temperature. After washing twice in PBS, the cells were permeabilised in ice-cold absolute methanol for 10 minutes at -20°C. Cells were rinsed twice in PBS and blocked in 10% newborn calf serum (NCS) in PBS at room temperature for 30 minutes, followed by incubation with primary antibody at room temperature for 1 hour in 1% BSA in PBS. Primary antibodies used were rabbit anti-PBF (1:150), mouse anti-HA (1:200), rabbit anti-CRM1 (1:150) and mouse anti-fibrillarin (1:150) (Abcam). Following three rinses in PBS, cells were incubated for another hour in secondary antibodies in 1% BSA, 1% NCS in PBS. Secondary antibodies were as follows: Alexa-Fluor 488-conjugated goat anti-mouse IgG and Alexa-Fluor-594-conjugated goat anti-rabbit IgG (Invitrogen) both used at 1:250 along with Hoechst 33342 stain for nuclei (1:1000). Coverslips were rinsed a further three times in PBS before being mounted onto slides with Dako Fluorescent Mounting Solution (Dako, Denmark). After at least 5 hours drying, cells were then visualised using 40x and 100x objective on Zeiss Axioplan fluorescent microscope (Zeiss, Germany).

Disrupting CRM1

siRNA was the first method used to disrupt the CRM1. siRNA transfection was carried out using Lipofectamine reagent (Invitrogen) at 6µl per 1ml of Opti-MEM media. For protein extraction and immunofluorescence (IF) staining, cells were seeded in six-well plates (containing sterile coverslips for IF) and transfected with 1µl of relevant siRNA (40nmol) after 24 hours. For nuclear/cytoplasmic extracts cells, were seeded in T25 flasks and transfected with 5µl of relevant siRNA (40nmol). siRNAs used were CRM1-specific siRNA (Ambion) and the negative control scrambled siRNA silencer (Ambion). The second method utilised the CRM-1 specific inhibitor, Leptomycin B solution (L2913, Sigma Aldrich). The stock Leptomycin B solution was at 2µg/ml dissolved in 70% methanol and was used at a range of concentrations from 1ng/ml – 5ng/ml. For every Leptomycin B experiment, a vehicle only control using the same volume of 70% methanol was carried out in parallel.

Results

Location of HA-tagged PBF

To establish whether HA-tagged PBF can localise to the nucleus two different approaches were taken. A nuclear extract kit was used to compartmentalise the proteins found in the nucleus from those in the cytoplasm, followed by Western blotting to identify which compartment the protein was located in. The second approach was immunofluorescence, which allowed direct visualisation of the protein's location within cells.

Initially the nuclear extract experiment was carried out on non-transfected cells to show that an efficient split between the different fractions was achievable in the cell-lines. Tubulin was used as a marker for the cytoplasm whereas lamin was used as the nuclear marker. The nuclear extract experiment was very variable and only worked with larger cells such as COS7 and HeLAs rather than the smaller TPC-1s and K1s (Figure 4A).

After showing that COS7 cells could be successfully fractionated, cells were then transfected with PBF, PBF-HA and mutant 1 along with a pcDNA3 vector only control. Mutant 1 is also C-terminal HA-tagged and is missing the last 30 amino acid residues, including PBF's NLS and sorting signal. The blot suggests that PBF is mainly a nuclear protein, with very little being found in the cytoplasmic fraction (Figure 4B). When probed with PBF antibody, PBF was present in the nucleus in all cases. However in the case of PBF-HA and mutant 1, it is likely the PBF shown is endogenous rather than transfected form. When probed with HA antibody, as predicted, the majority of the protein was located in the cytoplasmic fractions. However, there appeared to be a small amount present in the nuclear fraction, which may be due to the fact that the nuclear membrane can often be pulled down into the nuclear fraction and if PBF-HA was accumulating there, there is potential for a small amount of carry over. The Western blots

also revealed that PBF appears to form dimers in nucleus, whereas the predominant isoform in the cytoplasm was monomeric. PBF is a glycoprotein, and variously glycoslyated forms normally range between 28 and 37kDa.

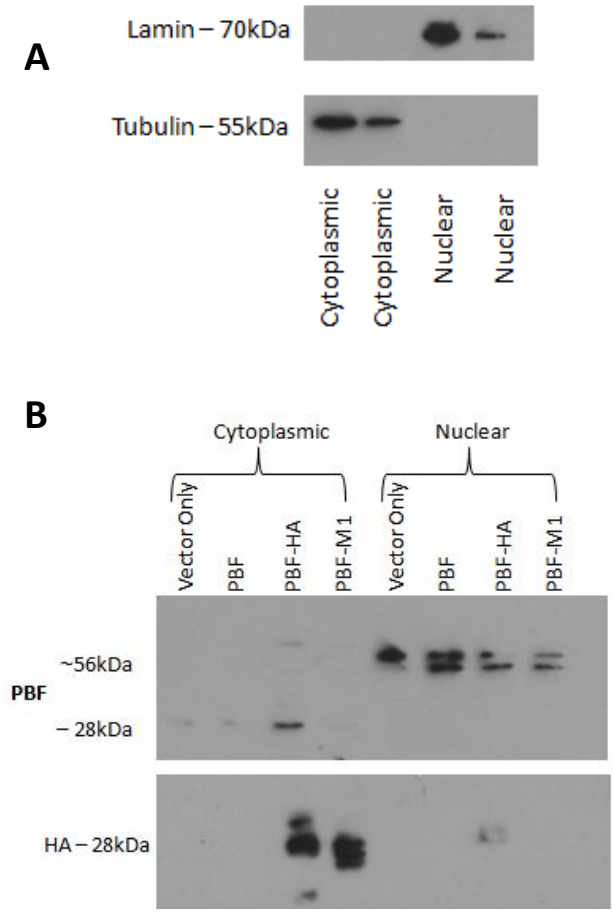


Figure 4 – Nuclear/Cytoplasmic Fractionation

4A – Nuclear/Cytoplasmic Fractions –COS7 cells were fractionated using the Nuclear Extract Kit (see methods and materials). Antibodies to Lamin A/C and tubulin were used to show successful division, as lamin is exclusively a nuclear protein and tubulin is exclusively found in the cytoplasm.

4B – PBF is primarily a nuclear protein, yet HA-tagged PBF is not present in the nucleus – COS7 cells were transfected with pcDNA3 vector only, PBF, PBF-HA and mutant 1 plasmids for 24 hours, before being harvested and separated into nuclear and cytoplasmic fractions using the Nuclear Extract Kit (see methods and materials). The top panel shows the membrane being probed with a PBF specific antibody whereas the bottom panel has been probed using a HA specific antibody. Probing with PBF antibody shows PBF to be mainly located in the nucleus. When probed with HA antibody there is evidence that HA-tagged PBF is mainly a cytoplasmic protein with little to none being located in the nucleus.

Immunofluorescence in all four cell lines reinforced the hypothesis that HA-tagged PBF is unable to enter the nucleus, confirming the Western blot findings. As can be seen in Figure 5, when PBF-HA was transfected into the cell, there appeared to be a 'halo'-like green ring around the nucleus, suggesting that nuclear entry is blocked for the protein. The slight green staining that appeared to be in the nucleus in some of the images is likely to be tagged PBF that is present in the cytoplasm above the nucleus, as the image is a 2D shot of a 3D structure. However confocal microscopy would be needed to verify this.

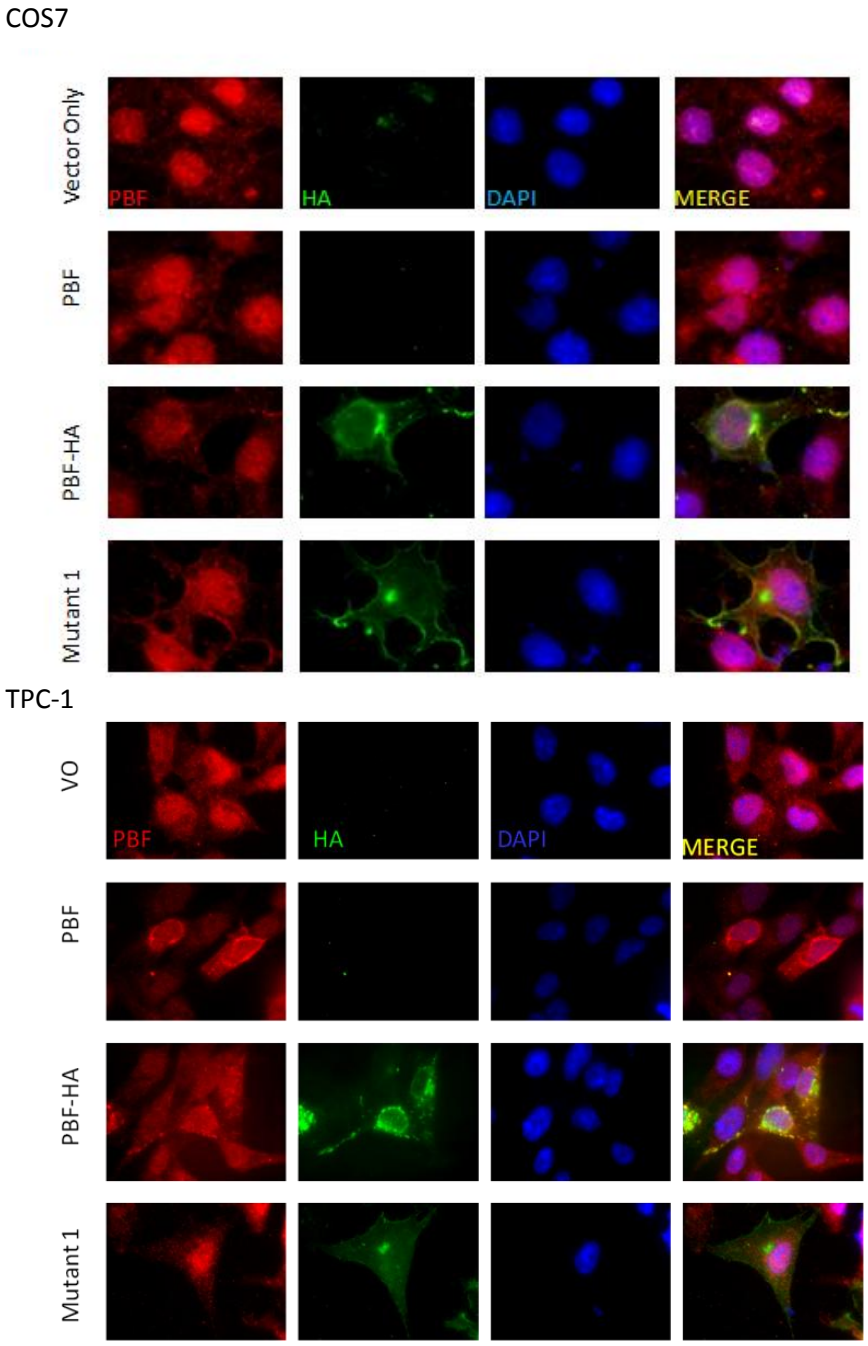


Figure 5 – HA-tagged PBF is not present in the nucleus – COS7 and TPC-1 cell-lines were seeded on coverslips and transfected with pcDNA3 vector only, PBF, PBF-HA or Mutant 1 for 24 hours. Antibodies specific to PBF (red) and HA (green) were used to probe the coverslips, followed by fluorescently tagged secondary antibodies to allow visualisation of the proteins using fluorescence microscopy. Hoechst stain (blue) was used to visualise nucleoli. The green staining suggests that HA-tagged PBF accumulates at the nuclear membrane and is not present in the nucleus; whereas WT PBF is both a cytoplasmic and nuclear protein. Images were taken at 100x magnification.

Deleting the HA tag from dual tagged PBF

To enable transfected PBF to be discerned endogenous PBF, it needs to be labelled with a fluorescent tag. The HA tag has been shown to block nuclear entry of PBF, therefore interfering with PBF's function, so a different tag would be preferential. The group have a FLAG tagged version of PBF in which the protein is also C-terminally HA tagged; the FLAG tag is inserted after residue 34 in PBF. To remove the HA tag, site-directed mutagenesis was attempted to delete the HA-tag sequence from the dual-tagged plasmid (Figure 6A). Unfortunately the mutagenesis was unsuccessful and the sequencing revealed that all the samples still contained the HA tag (Figure 6B). The process was repeated with an optimisation step of adding PCR enhancer to try and improve the efficiency of the reaction. Again unfortunately the reaction was unsuccessful and the HA tag remained intact.

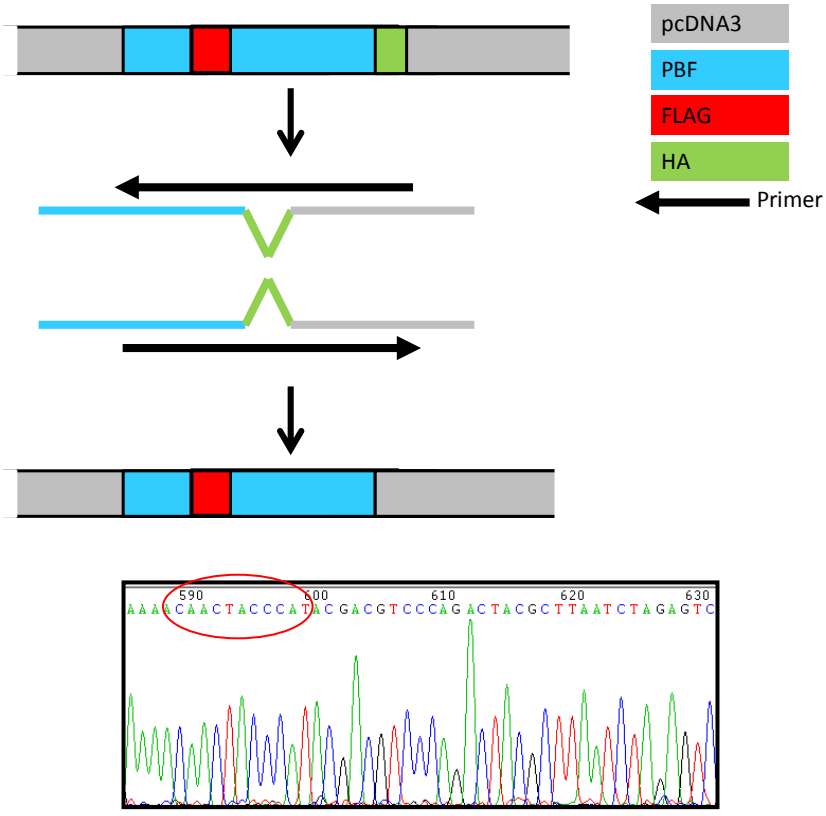


Figure 6 – Deletion of the HA Tag from PBF

A - Mechanism of deleting the HA tag from PBF – Primers are designed to anneal to and extend only PBF and the pcDNA3 backbone, missing out the HA tag, so the HA tag is no longer coded for in newly synthesised DNA.

B –Unsuccessful deletion of the HA tag from PBF – The sequencing shows that the HA tag was not deleted from the PBF plasmid. If the tag had successfully been deleted the sequence would have read ACAACTAATCT rather than the ACAACTACCAT as circled above.

Mutating the NES

To investigate whether the nuclear export signal (NES) is functional in PBF, the signal needs to be disrupted and compared to PBF with a normal NES. Mutagenesis can be used to introduce mutations into plasmid DNA. Mutation of the NES was attempted, with the aim that prediction software would no longer recognise the NES, but the N-terminal signal peptide sequence would not be affected. Various mutations were entered into prediction software to determine which one would be the most efficient at disrupting the NES signal, while leaving the signal peptide intact. Mutating Leucine 17 to an Alanine and Leucine 22 to Alanine provided a total depletion in NES prediction. Site-directed mutagenesis was then attempted using primers designed to induce the dual mutation (see Materials and Methods). However, the mutagenesis failed and no bacterial colonies grew. Attempts to solely mutate Leucine 17 to Alanine were then made using a new set of primers (see Materials and Methods), as this single mutation provided the smallest NES signal from the residues involved; however again these attempts led to failure to grow bacterial colonies. Mutagenesis was repeated, with both primer sets, using a two step protocol as described in Wang and Malcolm (1999) and with the PCR enhancer; again these unfortunately failed to grow colonies. Due to the repeated failure of the mutagenesis, it was decided to take a different approach to interrupt the NES function.

Silencing CRM1

As CRM1 is the main protein involved in nuclear export, particularly with proteins containing a hydrophobic nuclear export signal, like PBF, it was decided to knock this protein down and see if there was any difference in the localisation of PBF. CRM1 was knocked down using a CRM1-specific siRNA. To confirm efficient knockdown of the CRM1, Western blotting and immunofluorescence were used to compare protein levels between scrambled and CRM1 specific siRNAs (Figure 7A). Once confirmed that

knockdown was efficient it was then used in the variety of cell-lines transfected with VO and PBF. Using immunofluorescence, the effect of CRM1 knockdown on the location of PBF was examined in COS7 cells (Figure 7B). Knockdown of CRM1 in COS7 cells led to reduced staining for PBF in the cell cytoplasm and also appears to have blocked PBF's entry to the nucleolus. In the control, with scrambled siRNA, there appeared to be colocalisation between PBF and fibrillarin (a nucleolus marker) depicted by the yellow staining. However when CRM1 levels were depleted there was no colocalisation and PBF appeared to form halo-like structures around the periphery of the fibrillarin. When PBF was solely examined it was evident that there were regions of the cell that PBF was being excluded from, observed by the holes in the staining. A similar effect was observed in K1 cells (Appendix 1). CRM1 was further knocked-down in COS7 cells transfected with PBF-HA, to investigate whether HA-tagged can enter the nucleus or if was entering but then being exported (Figure 7C). There was no difference in the location PBF-HA between control or CRM1 knockdown, suggesting that PBF-HA cannot enter the nucleus to begin with and the lack of presence in nucleus is not due to nuclear export.

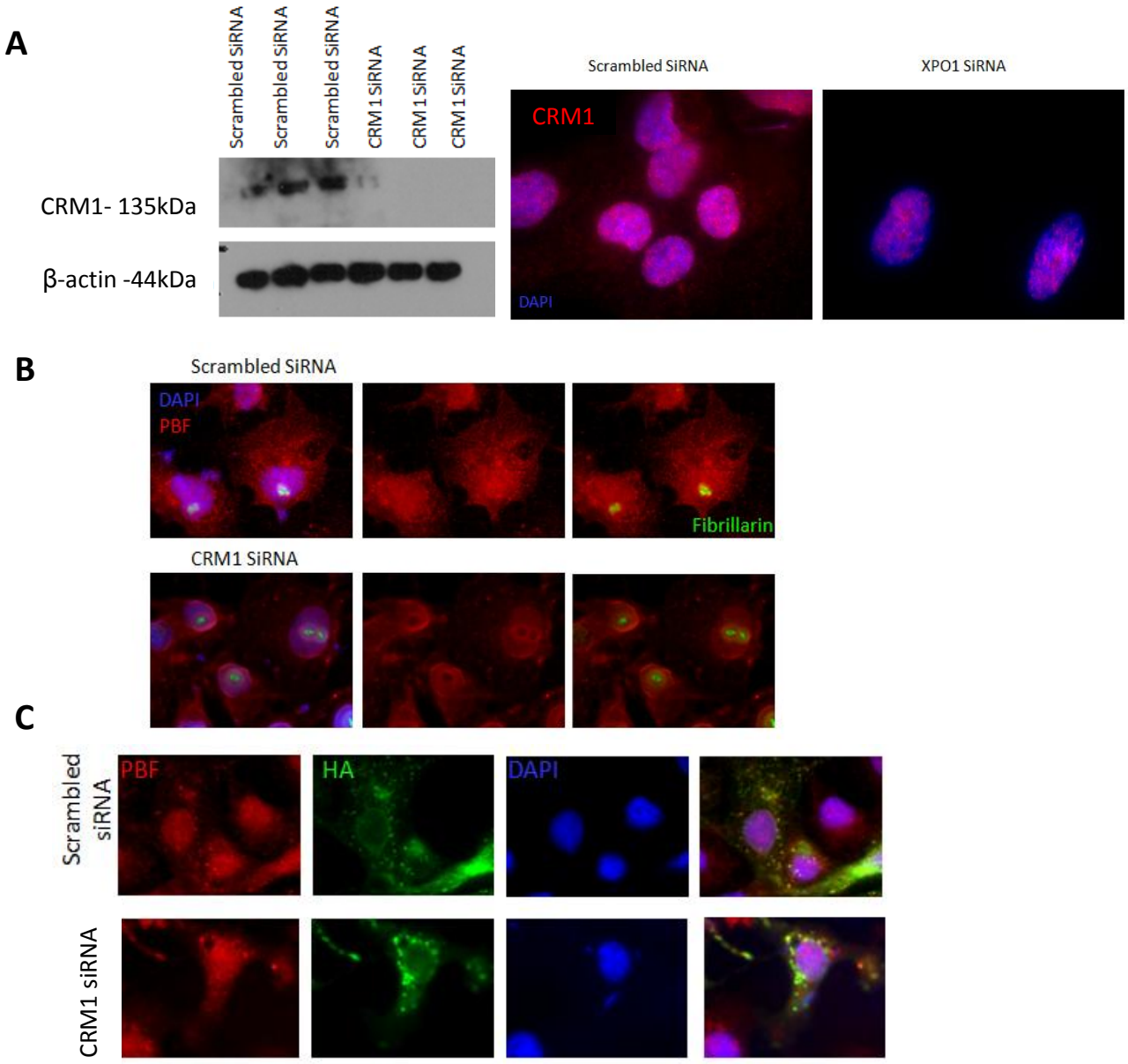


Figure 7 – Knockdown of CRM1

7A – Efficient knockdown by siRNA – Western blotting and immunofluorescence showed efficient knockdown of CRM1 in COS7s. Cells were transfected with scrambled or CRM1 siRNA (40 nmol) for 24 hours before probing with CRM1 specific antibody (red in immunofluorescence). Microscopy images were taken at 100x magnification.

7B – Knockdown of CRM1 decreases cytoplasmic PBF – COS7 cells were transfected with either scrambled (negative control) siRNA or CRM1 siRNA (40 nmol) for 24 hours. Coverslips were probed with PBF (red) and fibrillarin (green) antibodies while nucleoli were stained blue with Hoechst stain. When CRM1 was knocked down there appeared to be less cytoplasmic and nucleolar PBF compared to control. Images were taken at 100x magnification.

7C – CRM1 is not responsible for PBF-HA not being present in the nucleus – COS7 cells transfected with PBF-HA were also transfected with scrambled or CRM1 siRNA (40 nmol) for 24 hours. Coverslips were probed with PBF (red) and HA (green) specific antibodies while nucleoli were stained blue with Hoechst stain. There was little difference between the locations of PBF-HA in either treatment suggesting accumulation of PBF-HA at the nuclear membrane is not due to nuclear export. Images were taken at 100x magnification.

Inhibition of CRM1 using Leptomycin B

Along with CRM1 knockdown, to further study the effect of CRM1 on PBF, Leptomycin B (LMB) was used as a potent inhibitor of CRM1. To optimise the treatment, the inhibitor was used at a variety of different timepoints, at different concentrations, in different cell lines. As observed in K1s (Figure 8), even at the lowest concentration of inhibitor there were visible effects of LMB. Again there appeared to be less cytoplasmic PBF and more unstained regions that correspond with the nucleolus. There were more apparent holes in the staining, but unlike the siRNA treatment there was no accumulation of PBF at the boundary of the nucleolus. At 8 hours with only 1ng/ml, the cells appeared to begin to recover with there being more cytoplasmic PBF present and more distributed staining in the nucleus; in fact there appeared to be an excess of PBF in the nucleolus. This effect was also observed in COS7 and HeLA cells treated in the same manner (Appendix 2 and 3).

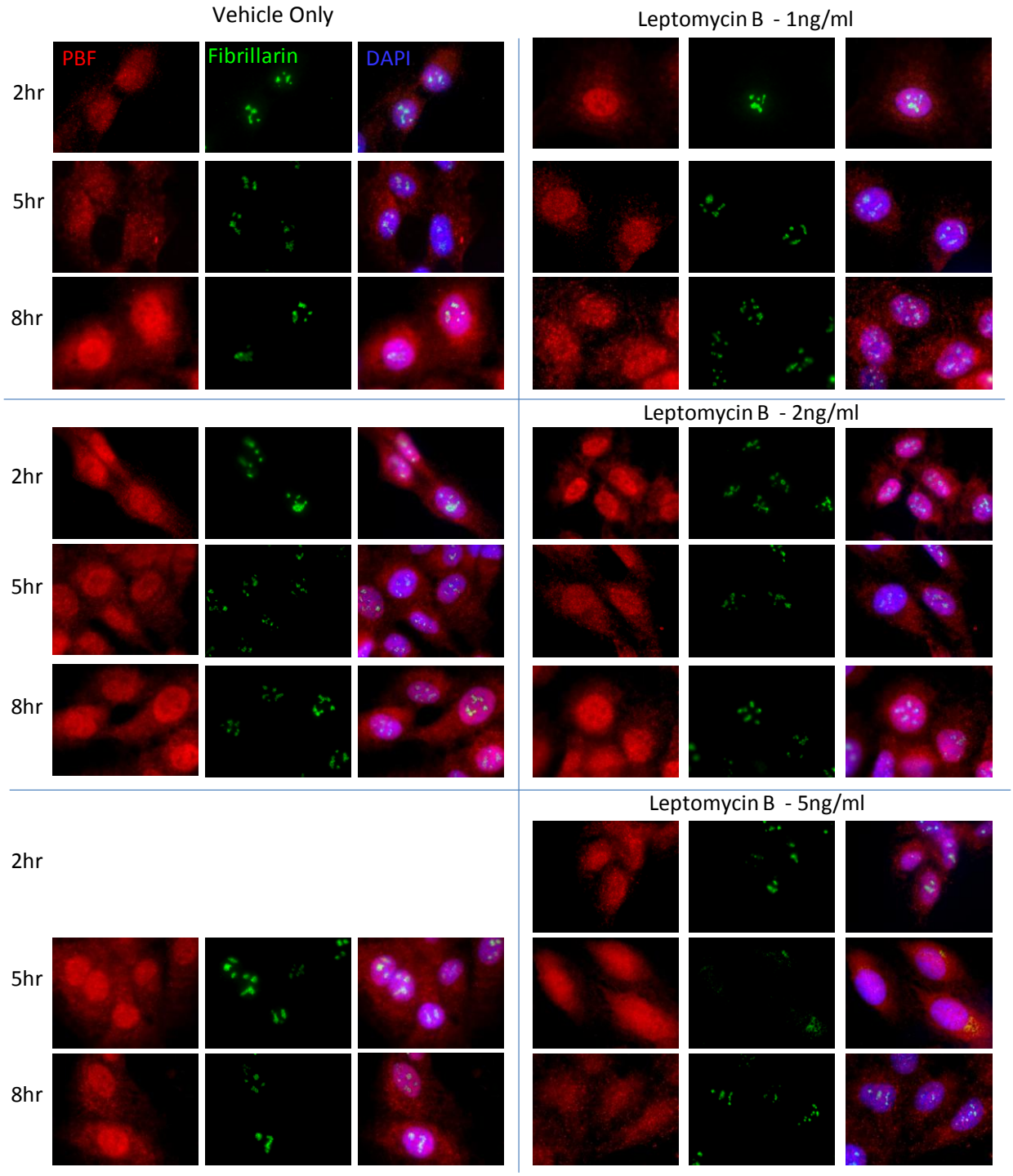


Figure 8 – K1 cells treated with different concentrations of Leptomycin B for different lengths of time - K1s were seeded on coverslips and incubated with either vehicle only (DMSO) or varying concentrations (1,2 or 5ngml) of the CRM1 inhibitor, Leptomycin B, for 2, 5 or 8 hours. Coverslips were probed with PBF (red) and fibrillarin (green) specific antibodies while nucleoli were stained blue with Hoechst stain. Images were taken at 100x magnification.

Homology

A localisation signal is more likely to be functional if the signal is conserved within other species. The sequences of PBF in four different species and the predicted sequences of PBF in another three species were obtained from the National Center for Biotechnology Information (NCBI) Homologene tool (http://www.ncbi.nlm.nih.gov/homologene). The sequences were aligned to allow easy comparison using the NCBI Constraint-based Multiple Alignment Tool (COBALT). The peptide sequences were also entered into the CBS prediction server to see if they also contain a predicted nuclear export sequence. All of the species, except *Gallus gallus*, had sequences that contained an NES score that exceeded the threshold of 0.5 on the NES score (Figure 9). The positioning of the potentially involved amino acids, shows the high level of homology between PBF sequences among different species. However, although most the sequences contain amino acids predicted to participate in a nuclear export signal, most of the species contain only one or two residues expected to be involved, suggesting the signal is unlikely to be functional in most of the species.

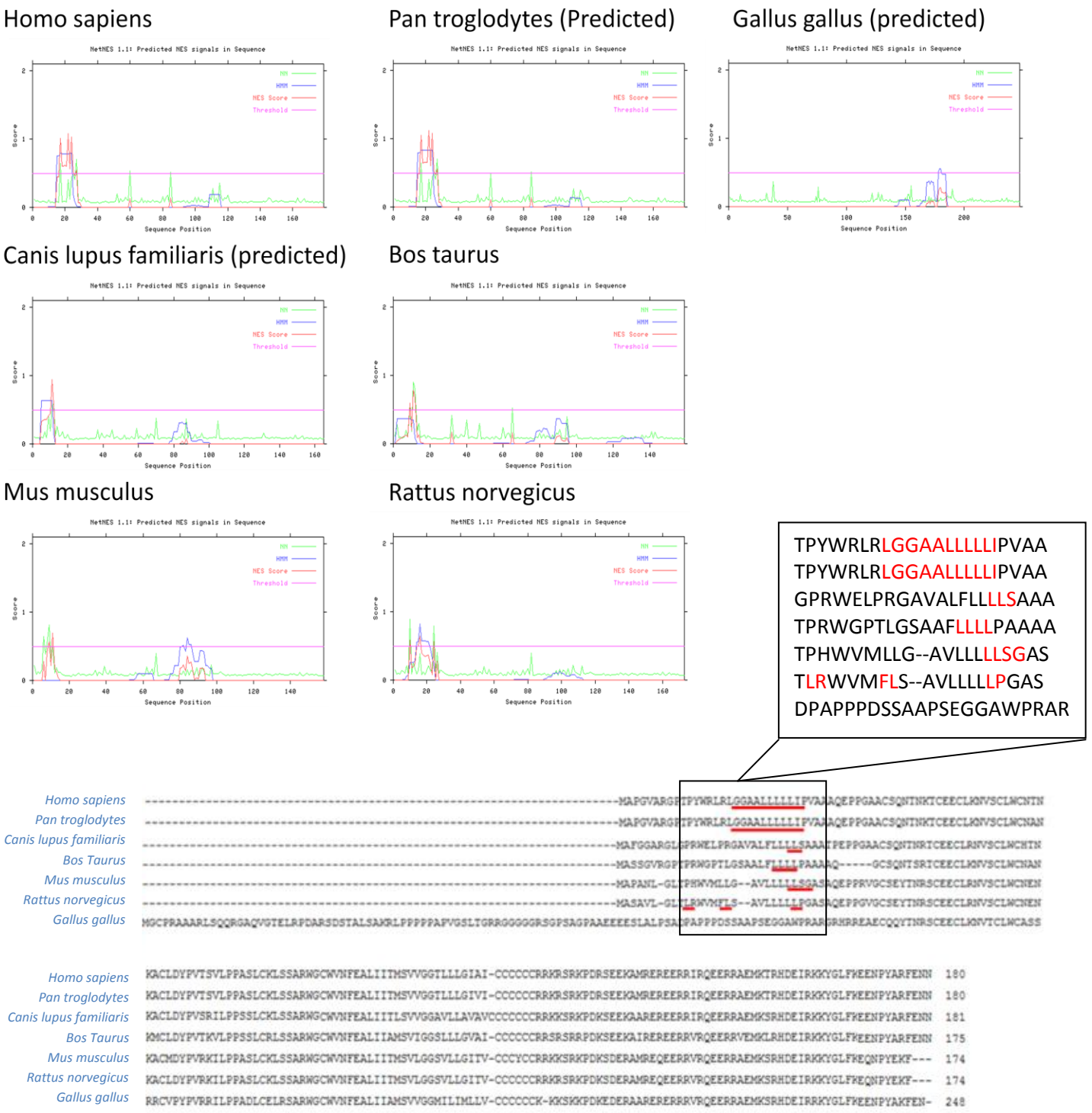


Figure 9 – NES positions and scores for PBF in different species – The top panel shows the NES score of PBF in 7 different species including *Homo sapiens*. Where the red line (NES Score) crosses the pink threshold line, residues are predicted to be involved in NES. The bottom panel shows the positioning of the NES scoring residues in PBF of the same seven species. The NES consensus sequence is $L_{xxx}L_{xx}L_xL$, where "L" is a hydrophobic residue (often leucine) and "x" is any other amino acid.

Discussion

Location of HA-tagged PBF

As was observed by immunofluorescence and cell fractionation, HA-tagged PBF is unable to translocate to the nucleus unlike endogenous WT PBF. This is likely to be due to the HA tag being added to the C-terminus of PBF close to the NLS. Nuclear import is a regulated process that involves importins binding to cargo proteins, transporting them through the nuclear envelope as a complex and releasing them on the other side. The most commonly used pathway for a cargo protein with a bipartite NLS, such as PBF, is through the use of importin α as an adaptor protein. Importin α contains two binding sites for NLS in the form of Armadillo (ARM) tandem repeats; the repeats form a banana-shaped structure with the crucial NLS binding residues tryptophan, asparagine and acidic amino acids lining the inner concave of the molecule (Fontes, Teh and Kobe, 2000). The basic residues that form NLS are recognised by these molecules and make specific interactions with them. The primary binding site, ARM repeats 1-4 is occupied by the larger part of the bipartite NLS, with repeats 6-8 accommodating the second smaller part. The location of NLS are usually in unstructured extended regions of the protein, so the protein can adapt to bind the importin. Once bound to importin α, the complex further binds to importin β in the cytoplasm and then translocates to the nucleus through nuclear pore complexes (NPCs). Once in the nucleus, both importins dissociate and the cargo protein is released (Stewart, 2007). With the HA tag being within 15 residues from the end of the bipartite NLS, it is probable that when PBF is folded as it would be in the cell, the HA is looping round and concealing the residues involved in the NLS so importin α can no longer recognise or interact with them, meaning that PBF is denied entry into the nucleus. Blocking entry into the nucleus may be interfering with a significant amount of PBF's role, as PBF has been shown to interact with an array of DNA damage proteins, such as p53, Rad6 and mdm2 (Read et al., 2012), so a N-terminally tagged version of PBF may be more appropriate, as it has already seen to be able to enter the nucleus in the previous study by Chien and Pei (2000).

Mutagenesis

As described earlier, mutating the NES in PBF and studying the effect was a major aim of this investigation but led to repeated failures. Unfortunately this is probably due to limitations within PBF itself; the region coding for the NES is extremely GC rich (>70%). The mutagensis kit used recommends using primers with a GC content between 40-60%, however, such a low percentage in this region of PBF is unattainable. Having such a high GC content leads to the primers forming complex secondary structures and mispriming, as guanine repeats form intra and interstrand folding by hydogen bonding with neigbouring guanines (Jensen, Fukushima and Davis, 2010). As such a high GC primer content is inevitable with this mutation, unfortunately oligonucleotide-directed mutagenesis appears to be unsuitable and a new method needs to be sought. One such method is to delete a fragment of PBF using restriction enzymes and reintoduce the fragment as an oligonucleotide containing the desired mutation. This method of restriction fragment deletion may also provide more successful results for removing the HA tag from PBF.

Functional NES?

The fact that there is very little homology in the NES between species suggests the signal is likely not be functional. However, data from experiments with CRM1 suggests there may be a role for the NES in *Homo sapiens* at least. In many immunofluorescence images where CRM1 was inhibited or depleted, there appeared to be reduced levels of cytoplasmic PBF, suggesting less PBF is being exported from the nucleus. Nuclear/Cytoplasmic fractionation +/- LMB or CRM1 siRNA would be ideal methods to confirm this observation, but unfortunately due to constraints of the kit, the fractionation was unsuccessful on treated cells. With the homology data showing that the NES has not been conserved through evolution and the images between different cell lines not being fully consistent, it is likely that CRM1 does have a role in the nuclear export of PBF, yet it may not be PBF's only way out of the nucleus.

One thing that is clearly evident though, is the increased number of unstained regions that correspond with the nucleolus, marked using fibrillarin, especially when CRM1 is knocked down. The nucleolus is a subnuclear organelle that is not bound by a membrane, and is fundamental for rRNA processing and ribosome production, but also has non-traditional roles such as protein degradation, cell cycle regulation and cell stress responses. The nucleolus has also been shown to be a major regulator of proliferation and cell growth by being able to modulate the compartmentalization of p53 and mdm2 (Chennupati et al., 2011). CRM1 was first reported to transport molecules from the nucleoplasm to the nucleolus by Boulon, et al (2004), where they observed that CRM1 was responsible for transporting U3 small nucleolar ribonucleoprotein (snoRNP) from cajal bodies to the nucleolus. In 2009, the transport of the cytoplasmic polyadenylation element-binding protein 1 (CPEB1) to the nucleolus was studied by Ernoult-Lange, et al; they reported that when CRM1 was inhibited by LMB, CPEB1 was excluded from the nucleolus producing immunofluorscence images similar to those observed with PBF. They went on to describe how CRM1 formed bodies in the nucleolus which they termed CNoBs (CRM1 Nucleolar Bodies). CPEB1 colocalised with these CNoBs when tranfected into HeLA cells, but when treated with LMB, the CNoBs vanished both in tranfected and untransfected cells (Ernoult-Lange et al., 2009). If PBF is interacting with CRM1 in the cell as suspected, perhaps it is also found in these CNoBS, and when CRM1 is inhibited with LMB, there appeared to be unstained PBF regions in the nucleolus as the CNoBs no longer exist.

If PBF is truly found in CNoBS, it potentially opens up a wider variety of roles for the largely uncharacterised protein. Both p53 and mdm2 have been shown to interact with CRM1 in the role of nuclear export, and have roles in the nucleolus, so perhaps these CNoBs act as a platform for their interactions with PBF. The CNoBs have also been hypothesised to be involved in ribosomal transcription (Ernoult-Lange et al., 2009). If proven, PBF could prospectively have a role in the process. Unfortunately

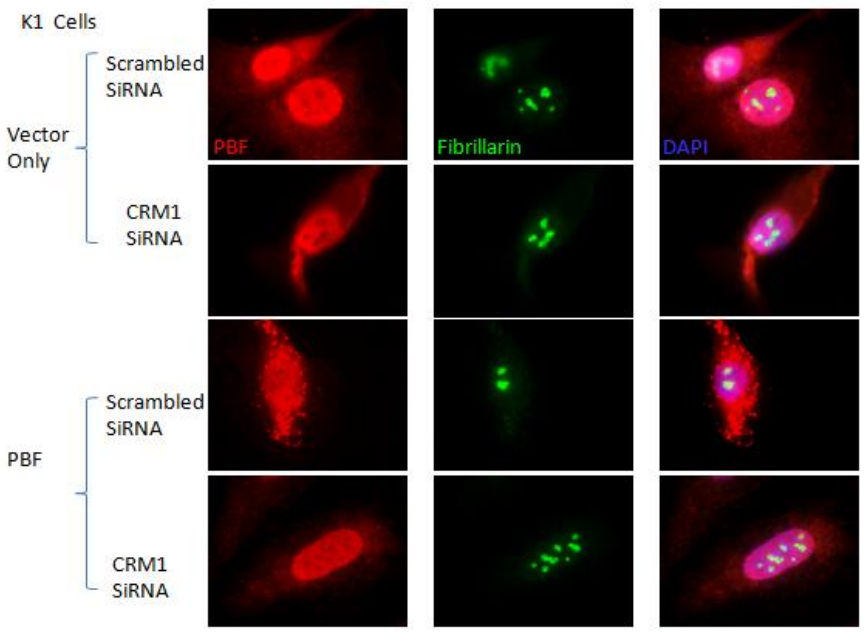
all these roles are purely speculative and a considerable amount of further investigation would be needed to confirm these hypothesises.

Further Work

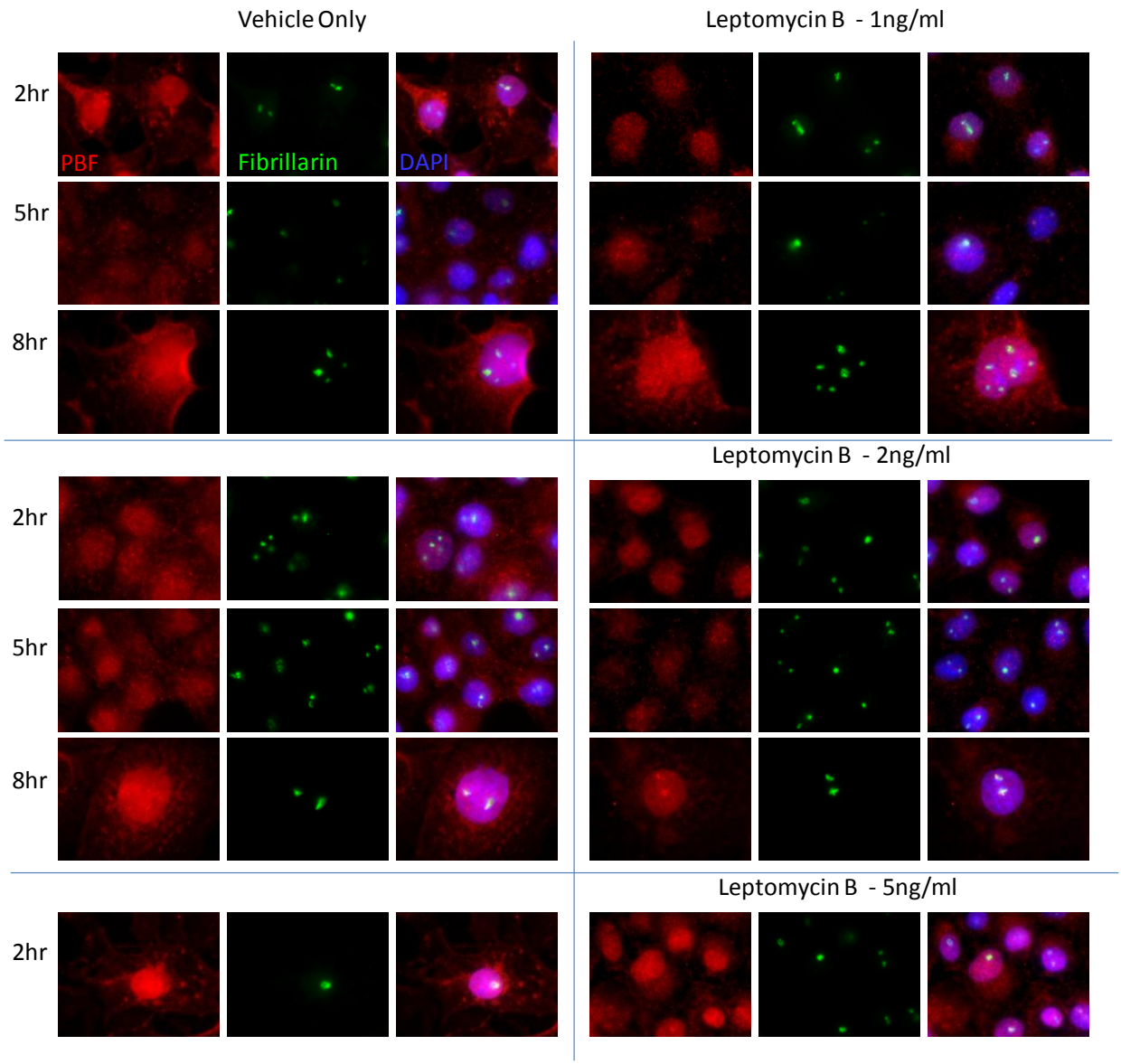
With regards to the nuclear export of PBF, there are two main experiments that would be required to establish whether the NES is functional and if CRM1 is responsible for nuclear export. The prime task is to create a PBF plasmid containing the NES mutation. This would hopefully be possible through gene cloning as described above. Once a mutant is generated, immunofluorscence could be used to establish any differences in the location of WT PBF and ΔNES PBF; the location of the mutant could also be verfied by nuclear/cytoplasmic fractioning. To confirm whether CRM1 is involved in the nuclear export of PBF, optimisation of the nuclear/cytoplasmic fractionation protocol with LMB treated and CRM1 knocked down cells would be advantangeous to quantify differences in the levels of PBF between fractions using densitometry.

Another key task for furthering this work would be the development of a tagged form of PBF that is capable of nuclear entry. This PBF could be created in one of two ways, either by deleting the HA-tag from the dual tagged PBF by restriction fragment deletion, depending on locations of restriction sites, or by N-terminally inserting a HA-/FLAG-tag instead. After establishing that this newly tagged form of PBF is capable of nuclear entry, interactions between CRM1 and PBF could be truly determined. Co-immunopreciptiation and confocal microscopy would be essential techniques for studying the proteins interactions and confirming PBF is colocalising in CNoBs. If this is observed, as hypothesised, further work could then focus on whether CNoBs are acting as a platform for PBF's interactions with other proteins or for undetermined roles of PBF.

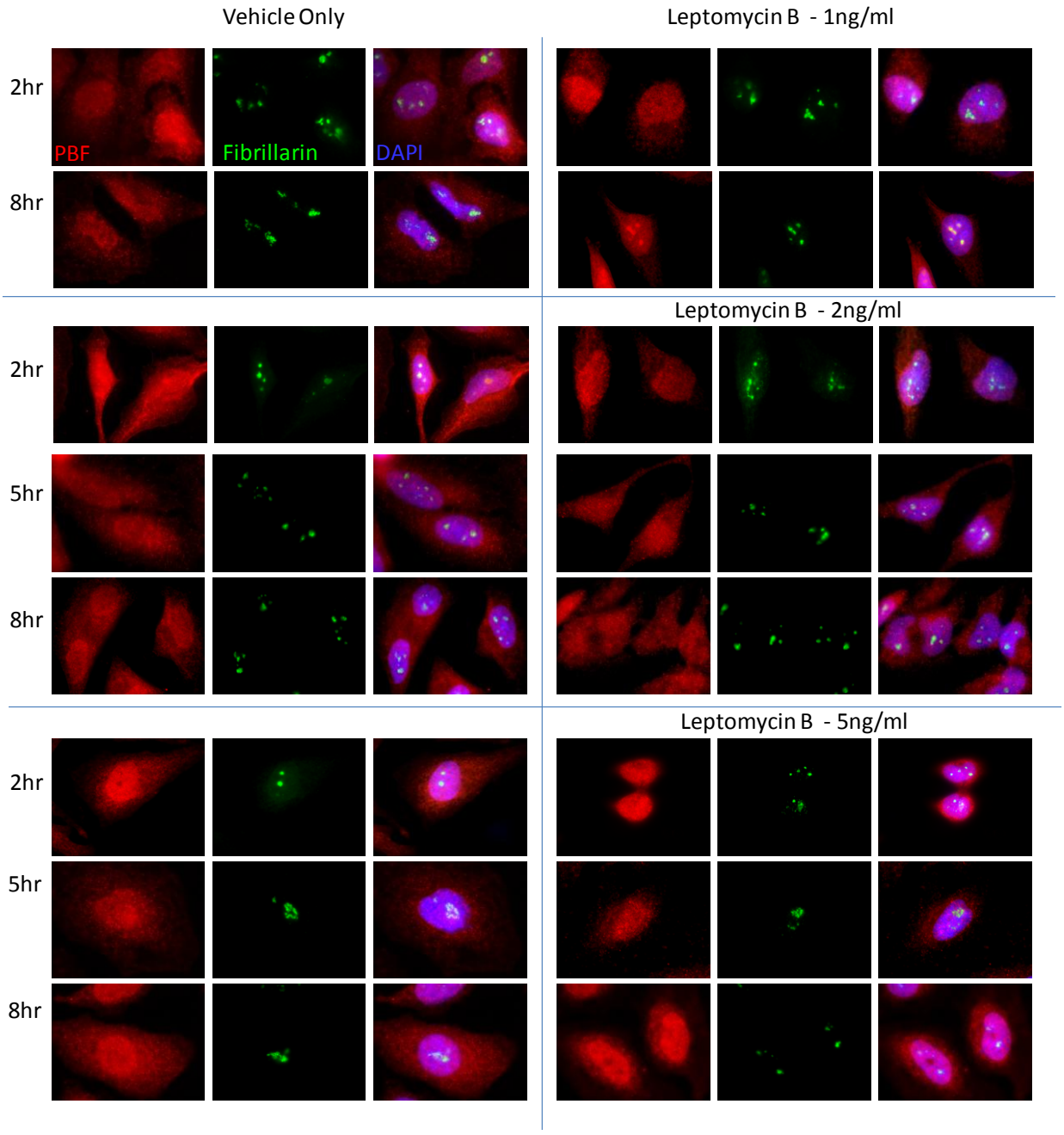
Appendices



Appendix 1 - Knockdown of CRM1 decreases cytoplasmic PBF –K1 cells were transfected with either vector only pcDNA3 or PBF plasmids for 24 hours. The differently transfected cells were then further transfected with either scrambled (negative control) siRNA or CRM1 siRNA (40 nmol) for 24 hours. Coverslips were probed with PBF (red) and fibrillarin (green) antibodies while nucleoli were stained blue with Hoechst stain. The top two rows of images show the effect of CRM1 siRNA on endogenous PBF, whereas the lower two on cells with transfected PBF. When CRM1 was knocked down, there appeared to be less cytoplasmic and nucleolar PBF compared to control, in both endogenous and overexpressed situations. Images were taken at 100x magnification.



Appendix 2 – COS7 cells treated with different concentrations of Leptomycin B for different lengths of time – COS7s were seeded on coverslips and incubated with either vehicle only or the CRM1 inhibitor, Leptomycin B, at either 1, 2 or 5 ng/ml for 2, 5 or 8 hours. Coverslips were probed with PBF (red) and fibrillarin (green) specific antibodies while nucleoli were stained blue with Hoechst stain. Images were taken at 100x magnification.



Appendix 3 – HeLA cells treated with different concentrations of Leptomycin B for different lengths of time – HeLAs were seeded on coverslips and incubated with either vehicle only or the CRM1 inhibitor, Leptomycin B, at either 1, 2 or 5 ng/ml for 2, 5 or 8 hours. Coverslips were probed with PBF (red) and fibrillarin (green) specific antibodies while nucleoli were stained blue with Hoechst stain. Images were taken at 100x magnification.

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AN INVESTIGATION INTO THE ROLE OF ADIPOSITY ON COLORECTAL TUMOURIGENESIS

By

VIKKI LOUISE POOLE

THIS PROJECT IS SUBMITTED IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE AWARD OF THE MRES

College of Medical and Dental Sciences

University of Birmingham

August 2012

Abstract

Colorectal cancer (CRC) is a leading cause of cancer death, causing 16,000 deaths each year in the UK alone. Obesity is a major risk factor for CRC, and with a quarter of the UK being classified as clinically obese, it is important to establish how obesity affects CRC. Recently, adipose tissue has been described as an endocrine organ, secreting hormones that may be mediators of CRC proliferation. In this study, human adipocytes were cultured and their secretomes collected prior to and after adiopogenesis. The adipocyte conditioned media was analysed to identify the components and their effect on CRC cells. The study showed that differentiated ACM was capable of increasing both the viability and proliferation of CRC cells. Antibody arrays and ELISA detected the presence of the adipokines, leptin, IL-6 and hepcidin, in differentiated ACM. All three adipokines were individually observed to increase proliferation and viability of CRC cells. Mechanistic analysis, using hepcidin inhibition and reporter assays, suggest that there is a trend towards leptin and IL-6 functioning by increasing hepcidin expression in CRC cells. There is also emerging evidence to suggest that IL-6 and leptin are capable of functioning through the Wnt signalling pathway.

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Table of Abbreviations

Abbreviation	Definition
ACM	Adipocyte Conditioned Media
APC	Adenomatous Polyposis Coli
BMI	Body Mass Index
BrdU	5-Bromo-2'-Deoxyuridine
CRC	Colorectal Cancer
C_T	Cycle Threshold
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
Dsh	Dishevelled
ELISA	Enzyme-Linked Immunosorbent Assay
ENA-78	Epithelial cell-derived Neutrophil-Activating Peptide 78
FAP	Familial Adenomatous Polyposis
FCS	Foetal Calf Serum
gp130	Glycoprotein 130
GSK3	Glycogen Synthase Kinase 3
HBRC	Human Biology Research Council
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IBMX	3-Isobutyl-1-Methylxanthine
IL-11	Inter-Leukin 11
IL-6	Inter-Leukin 6
IL-6 R α	Inter-Leukin 6 receptor alpha
IL-8	Inter-Leukin 8
JAK	Janus kinase
LARII	Luciferase *Assay* Reagent II
LC MS/MS	Liquid Chromotography Mass Spectrometry/Mass Spectrometry
Lgr5	Leucine-Rich Repeat-containing G-protein coupled Receptor 5
LIF	Leukemia Inhibitory Factor
LRP5/6	Low-density Lipoprotein Receptor-related Protein 5/6
MAPK	Mitogen-Activated Protein Kinases
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
No Stim	Stimulation media alone – DMEM 0.5% FCS, 1% P/S
P/S	Penicillin and Streptomycin
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PI3K/Akt	Phosphoinositide 3-Kinase/Protein Kinase B

qRT-PCR	Quantitative Reverse Transcriptase – Polymerase Chain Reaction
SEM	Standard Error of the Mean
STAT	Signal Transducer and Activator of Transcription
TCF	T cell factor/lymphoid Enhancer Factor
TIMP-2	Tissue Inhibitor of Metalloproteinases 2
TMB	Tetramethyl-Benzidien

Introduction

Colorectal Cancer

The mammalian colon is compromised of four major sections (the ascending, the transverse, the descending and the sigmoid colon) and with the rectum makes up the large bowel (Figure 10). The main function of the colon is to store faecal matter and regulate its release while absorbing electrolytes and water (CRUK, 2011).

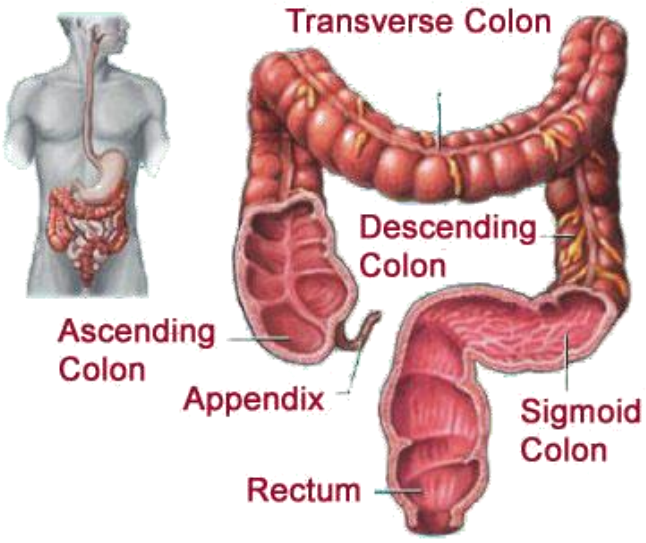


Figure 10 - Anatomy of the Colon - Taken from Colonic Clinic, 2011

Carcinoma of the colon or rectum is referred to as colorectal cancer (CRC) or bowel cancer, and is the third most common cancer in the UK, being responsible for around 16,000 deaths a year in the UK alone (Statistics, 2010). CRC usually originates in the epithelial cells lining the colon, starting out as a benign polyp before becoming malignant. Treatment varies depending on how advanced the cancer is, but the principal aim is total resection of the tumour; surgery can be curative in cases caught early, but adjuvant chemotherapy and radiation may be given if the cancer is more advanced. If the disease has become

metastatic, however, palliative care is often the only option (Cunningham et al., 2010). The major contributing factors for CRC are diet, lifestyle and increasing age, with minority factors including family history of CRC, hereditary conditions, such as familial adenomatour polyposis (FAP) and hereditary non polyposis colorectal cancer (HNPCC), and chronic bowel diseases, such as ulcerative colitis and Crohn's disease (CRUK, 2011). Diet is a major contributor to CRC, particularly in the western world where diets consist of high-fat iron-loaded meats and very little fibre; along with diet, obesity has been shown to significantly correlate with colon cancer risk in both men and women (Larsson and Wolk, 2007).

Obesity

Obesity is defined by the World Health Organisation (WHO) as abnormal or excessive fat accumulation that may impair health, giving the definition a body mass index (BMI) value of above 30 (WHO, 2012). In 2009, almost a quarter of adults in England were classified as clinically obese with this percentage steadily rising (The NHS Information Centre, 2011). A study in 2001 identified that the cancers most associated with obesity include breast, endometrium, colon and kidney (Bergstorn et al., 2001). Together with its association with cancer, obesity is also a leading cause of other conditions including diabetes, hypertension, infertility, ischemic heart disease and stroke.

Adipose tissue, or fat, is not only vital for cushioning major organs but it is essential for the storage of lipids, however, having too much is problematic. There are two major depots of fat, visceral or subcutaneous, which are classified by their locations in the body and each produces a unique spectrum of adipokines (Samaras et al., 2010). The greater omentum is a large visceral fat pad, mainly comprised of adipocytes, that functions as the 'policeman' of the abdomen and is a storage site for energy-dense lipids. Adipose tissue is increasingly being described as an endocrine organ, due to its ability to produce adipokines; these adipokines are being found to have new roles outside the

traditional energy homeostasis and are being implicated as mediators of obesity's link with cancer. Adipokines are cytokines secreted by adipose tissue; over 200 have been identified, with at least 50 being exclusive to adipocytes (Paz-Filho et al., 2011). Adipose tissue is comprised of mainly mature differentiated adipocytes, with a small percentage of immature preadipocytes; these preadipocytes undergo adipogenesis, a series of morphological changes along with cessation of cell growth, expression of many lipogenic enzymes and extensive lipid accumulation to become fully differentiated (Rosen and Spiegelman, 2000). Differentiation also leads to changes in their secretomes (Rosenow et al., 2010). This study primarily concentrates on the adipokines leptin, inter-leukin 6 (IL-6) and hepcidin.

Leptin

Leptin is a 16kDa hormone that is comprised of 167 amino acids and is predominantly, but not exclusively, produced by adipocytes (Hardwick et al., 2001). Circulating leptin levels have been shown to positively correlate with adipose tissue mass, linking obesity with increased leptin concentrations (Paz-Filho et al., 2011). Leptin was first characterised in mice as a product of the *obese* (*ob*) gene, a homologue of the human *LEP* gene (Gunter and Leitzmann, 2006). When energy levels are high, in healthy individuals, leptin is produced to reduce appetite; however in an obese state, adipose tissue overproduces leptin leading to the body becoming desensitised to the signal and appetite is no longer reduced (Harvey, Lashinger and Hursting, 2011). Along with being involved in controlling appetite, leptin has also been shown to be a growth factor for many cell types, including colon epithelial cells (Hardwick et al., 2001). Leptin is detected at the cell surface by the leptin receptor, a single membrane spanning protein, which is ubiquitously expressed in most tissues (Sun et al., 2010); RNA splicing causes the expression of several isoforms of the receptor. Different isoforms of the receptor have been shown to interact with different signalling pathways, for example, the full-length isoform signals through the

Janus Kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway, whereas the short isoform is involved in the mitogen-activated protein kinase (MAPK) signal transduction pathway (Hardwick et al., 2001).

IL-6

Inter-leukin 6 (IL-6) is a pleiotropic cytokine heavily involved in inflammatory responses; it was first identified as a B cell differentiation factor (Akira et al., 1990). Serum IL-6 levels have been shown to be significantly increased in obesity (Roytblat et al., 2000), suggesting that obesity is a chronic pro-inflammatory state (Fenton and Birmingham, 2010). A variety of different cell types are capable of excreting IL-6 in both homeostatic and inflammatory circumstances, including adipocytes (Vicennati et al., 2002). IL-6 functions through two different receptors, primarily glycoprotein 130 (gp130), the less specific receptor that is also able to interact with molecules such as leukaemia inhibitory factor (LIF) and IL-11, and IL-6 receptor α (IL-6R α)(Meager and Wadhwa, 2007). IL-6 has also been associated with colon cancer, with both protein and mRNA levels being elevated in both serum and tumour samples (Bromberg and Wang, 2009).

Hepcidin

Hepcidin is a 22 amino acid long peptide that is known for being the 'master regulator' of iron. When it was first discovered in 2001, it was identified as being secreted by the liver to act as a urinary antimicrobial peptide (Lago et al., 2007), but subsequently since then other roles for the peptide have been established. The most studied role of hepcidin is its role in systemic iron haemostasis, where it has been shown to inhibit both the absorption of iron by enterocytes and the efflux of recycled iron from

macrophages. Hepcidin has also been shown to be upregulated in chronic inflammation (Peyssonnaux et al., 2007) and to be an adipokine, therefore increased levels have been shown to correlate with obesity (Bekri et al., 2006). In hepatocytes, hepcidin has been observed to be upregulated by both leptin (Chung et al., 2007) and IL-6 (Wrighting and Andrews, 2006).

Adipokine targets

Although adipokines have been identified, it has not yet fully established how they have their localised effects. Leptin and IL-6 have been shown to be mediators of the JAK/STAT signalling pathway and to induce hepcidin, but this may not be their only method of action. Several potential targets of the adipokines will be looked at in this study, these include:

- Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) – also known as GRPR49, is a downstream target of Wnt signalling. Lgr5 has no known ligand nor mode of intracellular signalling, so is often referred to as an 'orphan' receptor (McClanahan et al., 2006). Increased expression in Lgr5 is due to Wnt signalling and in many colorectal cancers, where APC (adenomatous polyposis coli) is mutated, Lgr5 is overexpressed (Walker et al., 2011).
- c-myc – cellular myc is a transcription factor that regulates cell proliferation. In many forms of cancer (e.g. lung and breast carcinoma), c-myc is found to be upregulated. Upregulation of c-myc leads to multiple genes no longer being regulated, resulting in uncontrolled progression through the cell cycle and increased proliferation. c-myc is also a target gene of Wnt signalling (He et al., 1998), yet can be also regulated by MAPK and PI3K/Akt (phosphoinositide 3-kinase/protein kinase B) (Zhu, Blenis and Yuan, 2008) pathways. c-myc has also been shown to regulate hepcidin levels (Bayele, McArdle and Srai, 2006) and have a role in apoptosis (Prendergast, 1999).

- Cyclin D1 – cyclin D1 is a key regulator in the cell cycle, promoting the transition from G_1 to S phase; it has also been shown to have roles in cell metabolism and migration along with adipocyte differentiation (Fu et al., 2004) and to be induced by a variety of pathways including the MAPK, Wnt and JAK/STAT (Klein and Assoian, 2008).

Wnt Signalling

The Wnt signalling pathway induces the expression of target genes, such as β-catenin, Lgr5 and c-Myc, by allowing β-catenin to translocate to the nucleus and interact with T cell factor/lymphoid enhancer factor (TCF), activating it's transcriptional properties (Figure 11); when there is no Wnt ligand present, the pathway is switched off as APC complexes with β-catenin, leading to its phosphorylation and degradation (Schneikert and Behrens, 2007). The Wnt family of proteins are key mediators in cell signalling, particularly in situations such as embryogenesis, but also in homeostasis and tissue maintenance, with perturbations leading to the development of cancer and degenerative diseases (Logan and Nusse, 2004). Wnt signalling is often deregulated in CRC, as mutations arise in APC or, less often, in β-catenin. Around 60% of mutations in APC, observed in CRC, are due to a point mutation, leading to the production of a stop codon which truncates the protein; this truncation means APC can no longer regulate β-catenin degradation (Schneikert and Behrens, 2007). The RKO cell-line used in this study is derived from a CRC that contains wild-type APC, whereas SW480 cells are APC null, so have constitutive Wnt signalling.

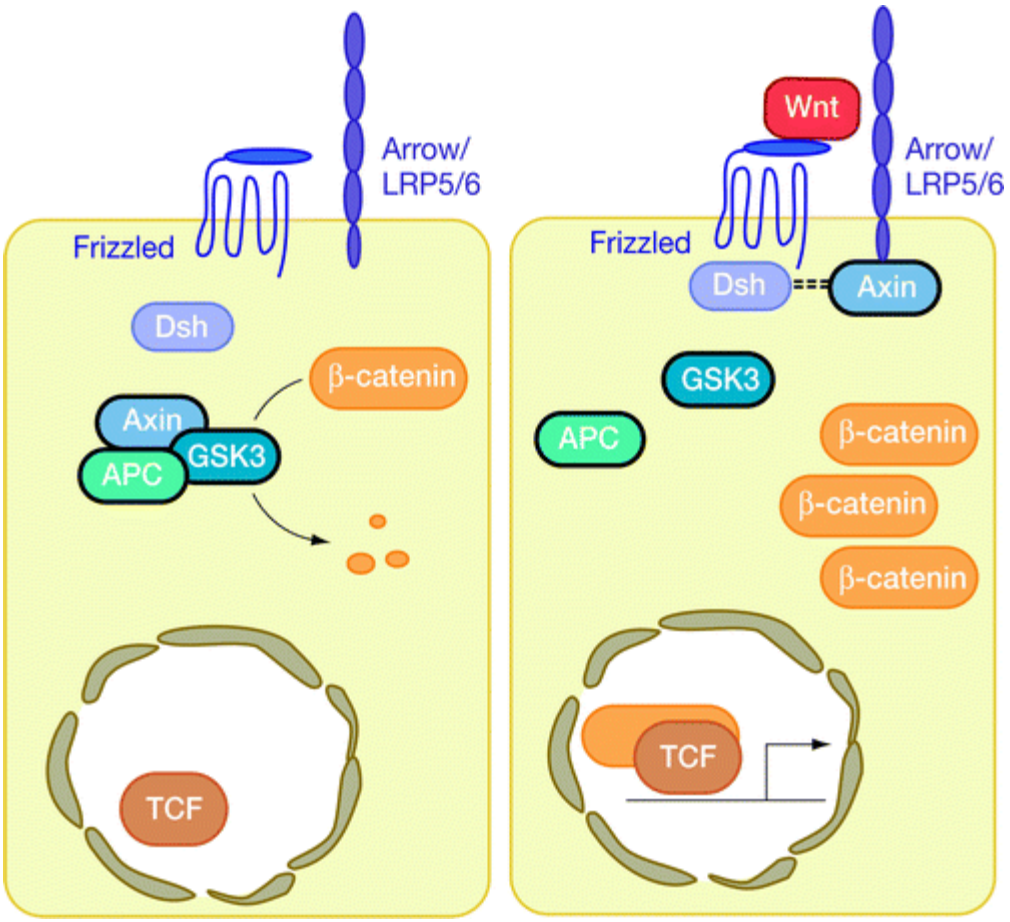


Figure 11 - Wnt Signalling - Taken from Logan and Nusse, 2004 – When there is no wnt ligand for the Frizzled receptor, as shown on the left, there is no signalling and therefore no transcription of target genes such as Lgr5. This is due to the fact β-catenin is inhibited by being phosphorylated and held in complex with a range of molecules including APC; this complex is then targeted for degradation. However when Wnt signalling is activated by a Wnt ligand binding to Frizzled, β-catenin is no longer held in complex, or degraded, and is free to act as a transcription factor for target genes.

Hypothesis

Colorectal adenocarcinoma progression is accelerated by the cocktail of adipokines secreted from adipocytes. The adipokines involved are leptin and IL-6, which induce hepcidin production, stimulating the Wnt signalling. Hepcidin is also hypothesised to be an adipokine itself, being locally secreted by greater omental adipose tissue.

Aims

- To establish whether greater omentum adipose tissue secretes adipokines that accelerate colorectal cancer cell growth and proliferation.
- To establish which adipokines are responsible for this phenotype.
- To assess whether the adipokines are functioning through the Wnt signalling pathway.

Materials and Methods

Cell Culture

RKO and SW480 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 1% penicillin (10^5 U/l)/streptomycin (100 mg/l) (P/S). All cultures were incubated at 37^{o}C and 5% CO_2 in humidified conditions and being passaged biweekly.

Adipocyte Culture

Samples were obtained from the BioBank, who had collected the samples from the greater omentum of consenting patients undergoing colorectal surgery. Following the removal of blood vessels and connective tissue samples were homogenised and incubated at 37^{o}C for 1hour in 2mg/ml Collagenase II (C-6885 sigma) in DMEM F-12 (~5g sample per 10ml collagenase solution). After digestion, 10ml of serum-free DMEM F-12 media was added before the sample was filtered through sterile muslin gauze and spun for 5 minutes at 1500rpm. Supernatant was discarded and the pellet resuspended in serum-free media, prior to being filtered through a falcon sieve and being centrifuged for 5 minutes at 1500rpm. The supernatant was discarded and the pre-adipocyte pellet was resuspended in growth media (DMEM F-12 with 10% FCS and 1% P/S). The pre-adipocytes were plated onto 12-well Corning cell bind plates (Appleton woods) with ~6g of fat per plate. The cultures were incubated at 37°C and 5% CO_2 in humidified conditions.

After overnight incubation, the cells were washed with serum-free media until there was no longer visible evidence of red blood cells. Cells were incubated in fresh growth media until deemed about 70% confluent; once confluent, growth media was removed and 1ml serum-free DMEM F-12

added overnight. The serum-free media was collected the following day and frozen for pre-differentiated adipocyte secretome analysis. Cells were then given 1ml differentiation media (DMEM-F12 with L glutamine (PAA) containing 1% P/S, 10% FCS, 33uM Biotin (B4639, Sigma), 17µM pantothenate (17018, Sigma) and 0.2nM T3 (T2877, Sigma)) which had 167nm insulin (19278, Sigma), 1µM cortisol and 0.5µM 1-methyl-isobutylxanthine (IBMX) (17018, Sigma) added prior to use. After 4 days, the media was removed and fresh differentiation media was added, but this time without IBMX; cells were cultured in this media until the adipocytes had fully differentiated (~14 days). Once differentiated, cells were placed in serum free DMEM F-12 media overnight and the media collected so the secretome from differentiated adipocytes could be analysed. A cycle of differentiation media and serum-free media was then carried out until the cells were no longer viable. After sufficient secretome was collected, samples were pooled according to their stage, pre-differentiated and differentiated adipocyte conditioned media (ACM). These pools were subsequently used in all further ACM experiments.

Ethical Considerations

Ethical approval was given by the Human Biology Research Centre, under the HBRC application code 11-068. All patients consented to the resection of adipose tissue prior to surgery.

Cell Stimulation and Inhibition Treatments

ACM Media – Used on cells in viability and proliferation assays and RNA extraction, cells were stimulated with ACM, collected and pooled as described above. For ACM stimulation, differentiated

ACM was compared to pre-differentiated ACM (collected from immature cells) but also to serum-free DMEM F-12 media.

No Stimulation – referred to as no 'stim' throughout, this control was of stim media (0.5% FCS, 1% P/S in DMEM) alone. As with all treatments, different volumes were used for different experiments as described per method.

Leptin Stimulation – Leptin (stock 1mg/ml, Peprotech) was diluted in stim media to give a final concentration of 60ng/ml.

IL-6 Stimulation – IL-6 (stock 100µg/ml, Peprotech) was diluted in stim media to give a final concentration of 10ng/ml.

Hepcidin Stimulation – Hepcidin (stock 100µM, Alta Biosciences) was diluted in stim media to give a final concentration of 1µM.

Leptin Inhibition – Anti-human leptin/OB Antibody (stock 500µg/ml, MAB398 R&D Systems) was diluted in stim media to give a final concentration of 3.2ng/ml.

IL-6 Inhibition – Anti-human IL-6 antibody (stock 50µg/ml, 500-P26G Peprotech) was diluted in stim media to give a final concentration of 0.1ng/ml.

Hepcidin Inhibition – Hepcidin antagonist peptide (Alta Biosciences) was dissolved in water to create a 100µM stock solution, this was then diluted in stim media to give a final concentration of 1µM.

Viability and Proliferation Assays

Viability was assessed by using a MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. MTT is a pale yellow tetrazole, which can be cleaved by mitochondrial dehydrogenase in cells that are viable and metabolically active. The cleavage products are dark blue formazan crystals that can be solubilised upon cell lysis; once lysed the colour and therefore absorbance are proportional to the

number of viable cells in each condition. RKO and SW480 cells were seeded at 7,500 per well in a 96 well plated and allowed to grow until 80% confluent. Media was then removed and samples were incubated with the appropriate stimulation/blocker (100µl per well for chemical stims, 50µl for ACM stimulation). After 24 hours with the appropriate stimulation, 10µl of MTT (5mg/ml in PBS) was added to each well and incubated at 37°C for 3 hours. Media was removed and 100µl of Dimethyl sulfoxide (DMSO) was added to each well to solubilise the crystals, before briefly shaking and reading the absorbance at 450Å using the Wallac Victor plate reader.

Proliferation was assessed using a cell proliferation ELISA BrdU (5-bromo-2'-deoxyuridine) (colorimetric) assay (Roche). BrdU is a synthetic pyrimidine analogue, which is incorporated in place of thymidine into DNA during replication. This analogue can be detected by a peroxidise conjugated BrdU antibody, which cleaves tetramethyl-benzidien (TMB) substrate producing a blue colouring, therefore providing a method to quantify proliferation. Cells were seeded and treated with different stimulations as in the viability assay above. After incubating for 24 hours, 10µl of 10µM BrdU labelling solution was added to each well before returning the plates to the incubator for 4 hours. Labelling solution was removed; 200µl FixDenat was added to each well for 30 minutes at room temperature before being removed. Once removed, 50µl of anti-BrdU-POD (10µM) was added to each well and left for 90 minutes. Antibody solution was then removed and the wells washed 3 times with 200µl BrdU wash solution before 100µl of BrdU substrate solution was added to each well. After brief shaking, the absorbance was then read 405Å using the Wallac Victor plate reader.

Antibody-Array

Pre-differentiated and differentiated secretomes were compared using the Human Adipokine Antibody Array 1 (RayBiotech), which was carried out as per manufacturer's instructions. The film was then

scanned to create an electronic version using Biorad GS-800 Densiometry scanner and analysed using QuantityOne 4.6.7 (basic).

Cellular Secretome Preparation

RKO and SW480s were seeded into three Corning T75 vented flasks (per treatment) and cells were grown to 70% confluence in DMEM containing 10% FCS and 1% P/S. Once confluent, cells were then stimulated with no stim, leptin, hepcidin or IL-6. After 18 hours with the appropriate stimulation, cells were washed 4 times in sterile PBS, before 8ml of completely serum-free DMEM was added; 18 hours later the media was collected, sterile filtered and pooled per treatment and cell-line. Secretomes were stored at -20°C until required.

Leptin, Hepcidin and IL-6 ELISAs

Secretomes from RKOs and SW480s treated with no stim, Leptin, Hepcidin and IL-6 along with pre-differentiated and differentiated ACM were assessed for Leptin, Hepcidin and IL-6 using ELISA (Enzyme-linked immunosorbent assay). Human IL-6 Quantikine ELISA and human leptin Quantikine ELISA (R&D systems) were carried out as per manufacturer's instructions. For the Hepcidin ELISA, secretomes were extracted using the S-500 extraction kit (Peninsula Laboratories) and then hepcidin levels were quantified using Hepcidin-25 (human) peptide enzyme immunoassay (Peninsula Laboratories) using protocol VI.

TOPFLASH reporter assay

The TOPFLASH reporter is a dual-luciferase assay (Promega) that detects Wnt/β-catenin signalling activity. Cells were seeded 40,000 cells per well in 12 well plates and maintained until 60% confluent. Transfection master mixes were made up from the appropriate number of wells, that contained 0.8µg FOP or TOP plasmid (a kind gift from Bertovalstien et al), 0.1µg renilla (Promega), 3µl of Extremegene (Roche) and made up to 50µl with Optim-MEM media (Gibco, Invitrogen) per well. Media was removed from cells and replaced with fresh 10% FCS DMEM, before half the cells had 50µl of FOP transfection mix added to them and the other half had 50µl TOP mix. The cells were then incubated at 37°C for 18-24 hours. Media was removed and 1ml of the appropriate stimulation media (either no stim, Leptin or IL-6) was added for 24hours. Media was then removed and the cells washed 3 times in non-sterile PBS, 200µl of passive lysis buffer (Promega), was added to each well and left on a rocker for 30 minutes, plates were then frozen at -20°C for at least 24 hours. Samples were thawed and 20µl of each sample was transferred to a white 96 well plate. At the plate reader, 50µl of Luciferase Assay Reagent II (LARII) was added to each well and immediately luminescence was measured on the Wallac Victor plate reader. The reaction was then stopped by the addition of 50µl of Stop and Glo reagent to each well and again luminescence was measured.

Hepcidin reporter assay

The hepcidin reporter is very similar to the TOPFLASH assay but instead identifies expression of hepcidin rather than Wnt targets. Cells are seeded in the same manner but are transfected with different plasmids. The transfection mixes for this are assay are compromised of 0.8µg Hepcidin (a kind gift from Dr Paul Sharp, Kings College London) or pGL3-basic plasmid (Promega), 0.1µg renilla (Promega), 3µl of TransIT-LT1 (Geneflow) and made up to 50µl with Optim-MEM media (Gibco, Invitrogen) per well. Cells

were transfected with these mixes, stimulated and measured in exactly the same way as the TOPFLASH assay above.

RNA extraction

Cells were seeded in 6 well plates at 75,000 per well. After 24 hours, cells were stimulated with either 500µl ACM or 1ml of chemical stimulation. After incubation with the treatment for 24 hours, 200µl TRIzol reagent (Invitrogen) was added to each well and extracted using the TRIzol manufacturer's protocol. After extraction, RNA was then quantified using a Nanodrop ND-1000 Spectrophotometer.

cDNA generation

Reverse transcription system (Eurogentec) was used to synthesise cDNA. Nuclease-free water and 0.4µg of RNA was combined to give a total volume of 8.1µl, which was then combined with 11.9µl of reverse transcription mastermix (4µl 25mM $MgCl_2$, 2µl of reverse transcription buffer (10x), 4µl 2.5mM dNTP mix, 1µl random nonamer, 0.4µl RNAse inhibitor, 0.5µl EuroScript reverse transcriptase). The samples were subjected to a reverse transcription thermal cycle (BioRad My Cycler™ thermal cycler) as follows:- initial step - 10 minutes at 25°C; reverse transcriptase step - 30 minutes at 48°C; inactivation of reverse transcriptase enzyme - 5 minutes at 95°C; finally held at 4°C.

TaqMan quantitative real-time polymerase chain reaction (qRT-PCR)

1µl of cDNA was loaded in either duplicate or triplicate into a 96-well reaction plate, 14µl of the relevant qRT-PCR mastermix (Table 1 for myc, Table 2 for Lgr5, cyclin D1 and hepcidin) was then added. 18S

ribosomal RNA as used as an internal control throughout. The plate was then sealed and frozen at -20°C until needed. Once thawed the plates were pulse centrifuged and loaded into the Applied Biosystems 7500 Fast Real-Time PCR detection system. The standard reaction protocol consisted of 10 minutes at 95°C, 40 cycles of 10 seconds at 95°C and finally 1 minute at 60°C. After amplification, samples were the analysed using SDS software which allowed deduction of cycle threshold (C_T) values.

Reagent	Volume per well (µl)
Rox passive reference	4.8
Sensimix	1200
Myc Forward Primers	240
Myc Reverse Primers	240
Myc Probe	240
18S Probe	12
18S Primers	24
Nuclease-free Water	29.2

Table 1 - qRT-PCR master mix for Myc

Reagent	Volume per well (µl)
Rox passive reference	4.8
Sensimix	1200
Relevant Taqman mix (containing primers and probe)	160
18S Probe	12
18S Primers	24
Nuclease-free Water	839.2

Table 2 - qRT-PCR master mixes for Cyclin D1, Lgr5 and Hepcidin

Statistics

Results are expressed as mean±SEM. Minitab was used to ensure the data was normally distributed before paired, two-tailed student T-tests were performed on Microsoft Excel. A *P* value of less than 0.05 was regarded as significant.

Results

Differentiation of Adipose Cells

Pre-adipocytes were extracted from patient samples and cultured. Two different forms of adipocytes were cultured; the first being the immature pre-differentiated type, and the second the more mature differentiated type. The differentiated form, were the most interesting for this study, as they represent the majority of cells found in adipose tissue and are the cells that, *in situ,* would secrete the majority of adipokines. The pre-differentiated form can be used as an internal control, as they later mature into the differentiated type. To determine whether the cells were fully differentiated, microscopy was used, as there were obvious visible differences between the two cell types. Figure 12 shows the large differences between the two cell types; pre-differentiated adipocytes looked like normal cells, compared to the differentiated cells that were observed to contain large bubbles or vesicles storing triglycerides. Conditioned media was collected from both types of cells to analyse secretomes.

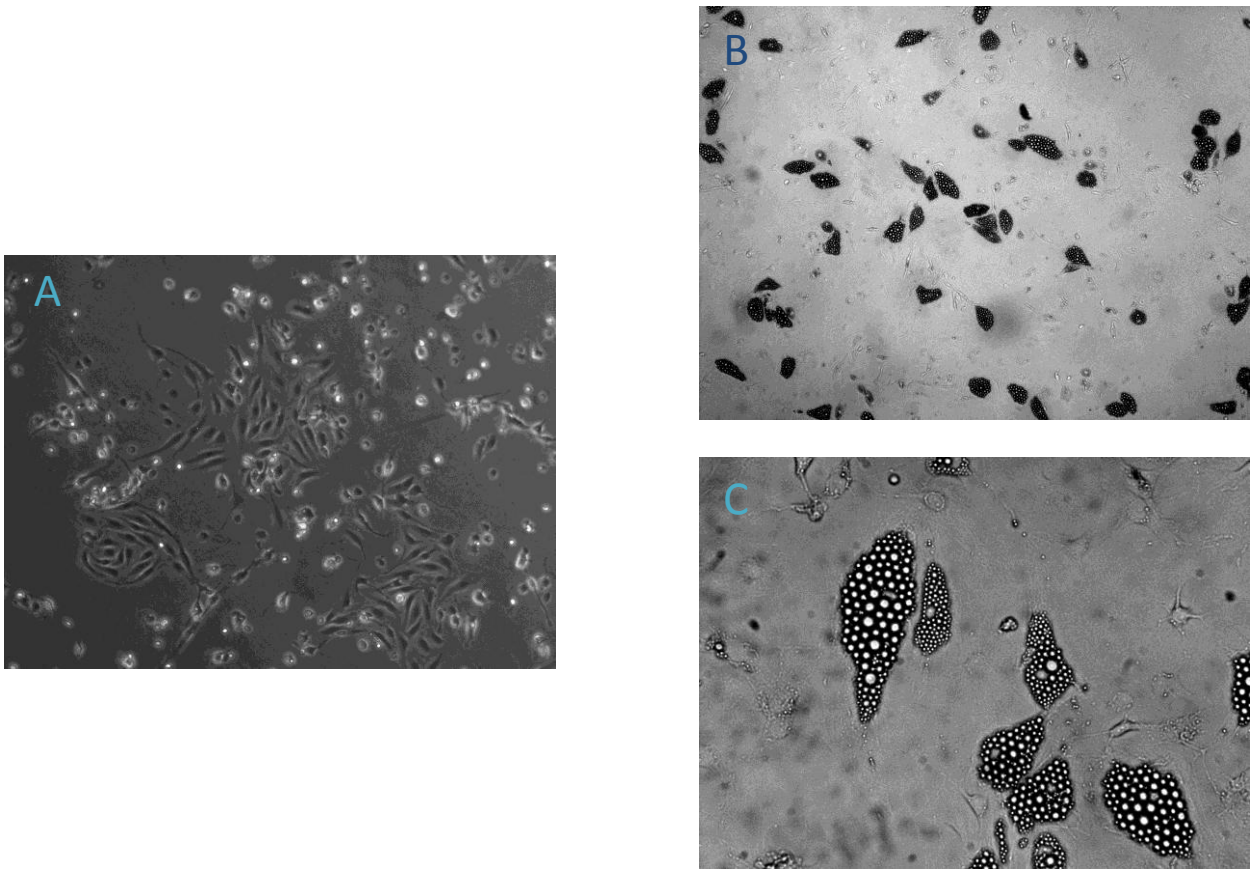


Figure 12 – Adipocytes in culture – Pre-adipocytes were extracted from patient samples and cultured in plates. (A) (magnification x5) shows the immature pre-differentiated adipocytes cultured in growth media (see methods and materials). (B) and (C) (magnification x5 and x40 respectively) show the more mature differentiated adipocytes, cultured in differentiation media (see methods and materials); these differentiated cells can clearly be distinguished due to being mainly comprised of large vesicles filled with triglycerides.

ACM's effect on viability and proliferation

The effect of conditioned media, from pre-differentiated and differentiated adipocytes on the viability and proliferation of two colorectal carcinoma cell-lines, was assessed. RKO and SW480 cell-lines were the primary cells used throughout this investigation as they are robust, easy to maintain and popular lines among colorectal carcinoma literature. Incubation with differentiated ACM led to significant increases in both proliferation and viability of both cell lines (Figure 13). Incubation with pre-differentiated ACM led to a more varied response by increasing viability but decreasing proliferation.

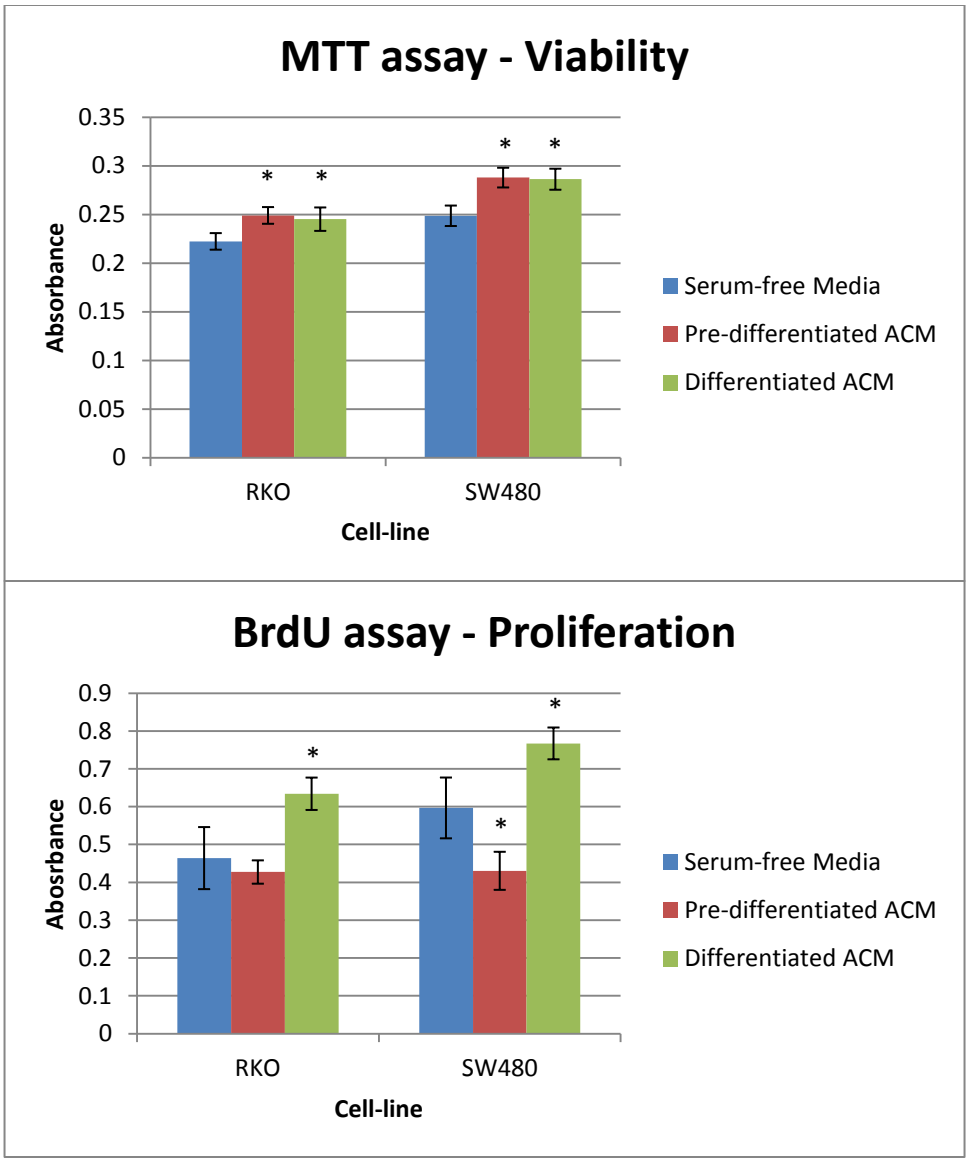
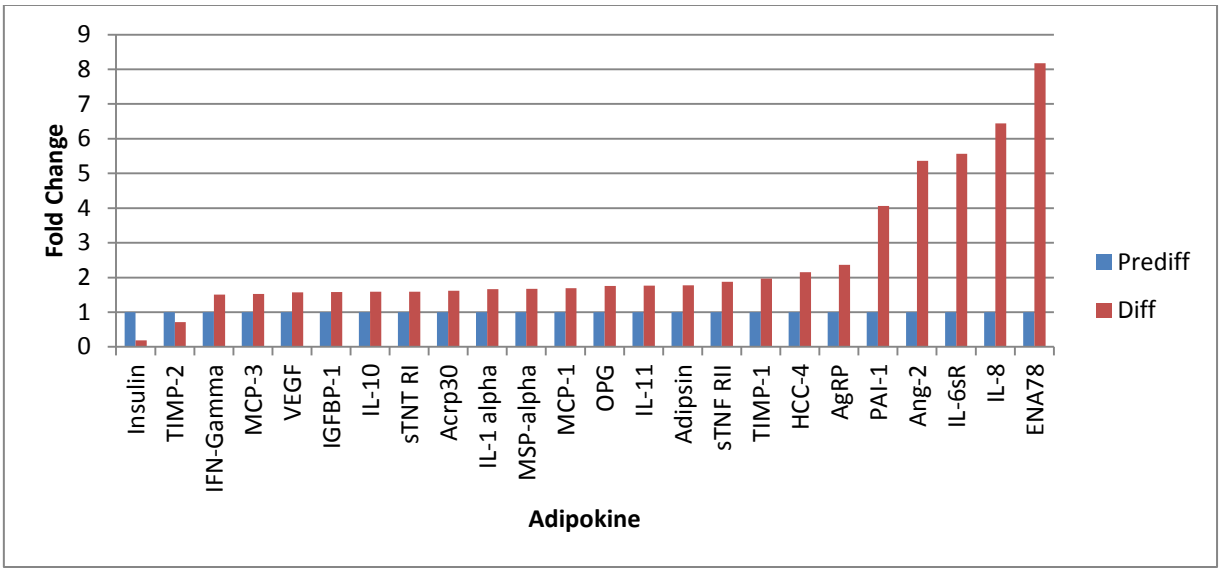


Figure 13 – The effect of ACM on the viability (MTT assay) and proliferation (BrdU assay) of Colorectal Cancer cell-lines – SW480 and RKO cells were incubated with pre-differentiated and differentiated ACM along with serum free media overnight before MTT and BrdU assays were performed as described in materials and methods. Assays were quantified by changes in absorbance (at 450nm and 405nm for MTT and BrdU respectively). Mean data ±SEM is plotted with * representing statistical significance ($p<0.05$), determined by a paired, two-tailed student T-test, compared to the relevant serum-free media control as. n=3 with triplicate results in each n.

Determination of ACM constituents

To determine what exactly is in the adipocyte secretome, a human adipokine antibody array was performed to see which of 64 potential molecules were found in the ACM from pre-differentiated and differentiated adipocytes. Of the 64 molecules tested for, there were 23 adipokines that increased by more than a factor of 0.5 from pre-differentiated ACM to differentiated ACM; yet there were only two (insulin and TIMP-2) that decreased by more than a factor of 0.25 (Figure 14A). The largest difference between the two ACMs was the volume of IL-6, with there being over a 50 fold increase in the levels of IL-6 in differentiated ACM compared to pre-differentiated (Figure 14B). Appendix 4 and Appendix 5 show the differences between all 64 adipokines present on the array.

A



B

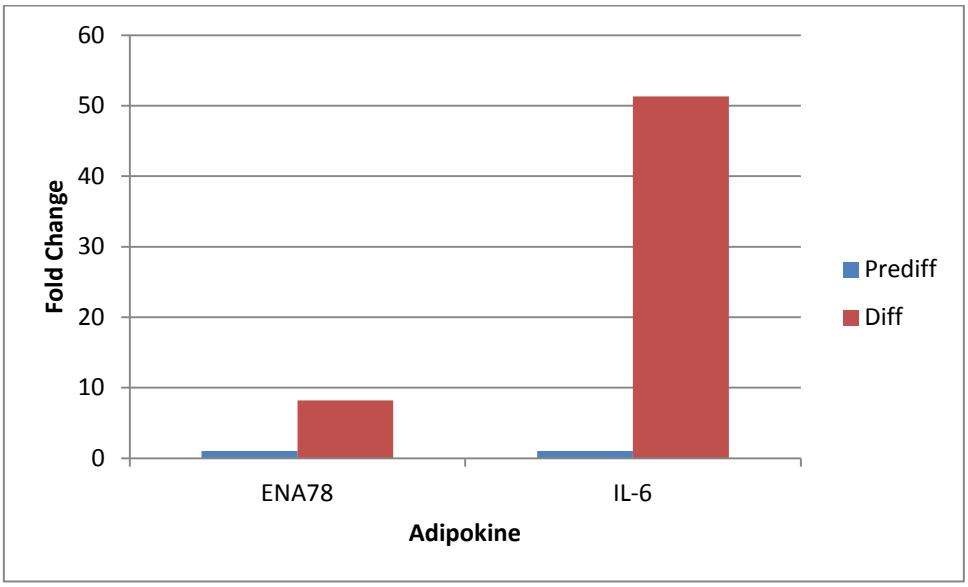


Figure 14 – Human adipokine Array Data – A human adipokine array containing antibodies to 64 adipokines was used to probe pre-differentiated and differentiated ACM to establish which adipokines were present in each ACM. The density of the spots produced from the array was calculated using a densitometer, and the densities for both ACM normalised to pre-differentiated, for each adipokine, to allow comparison between the two ACM. (A) shows the differences in the 24 adipokines (minus IL-6) that either increased by a factor of more than 0.5 or decreased by a factor of 0.25 between pre-differentiated and differentiated ACM. (B) shows the massive increase in levels of IL-6 between pre-differentiated ACM and differentiated ACM compared to the next largest increase, in the adipokine, ENA78.

To confirm the large increase in IL-6, a more specific IL-6 ELISA was used. The ELISA also allowed quantification of levels of IL-6 by the use of pre-determined standards. The use of the ELISA confirmed there was a large increase between IL-6 in differentiated ACM (217pg/ml) compared to pre-differentiated ACM (7pg/ml), but quantified the increase to be a fold change of 29 (Figure 15) rather than 50 observed in the antibody array.

Although there was no real difference in leptin levels on the antibody array (1.18 fold change from pre-differentiated to differentiated), literature suggests that leptin is playing a large part in the adipocyte secretome. To confirm whether leptin has a role, a more sensitive leptin ELISA was utilised with the two forms of ACM. The leptin ELISA showed that there was a considerable difference in the level of leptin between the two secretomes, differentiated ACM was quantified to have a concentration of 18pg/ml compared to the 1.7pg/ml in pre-differentiated, a fold change of 11 (Figure 15).

Hepcidin, the laboratory's main protein of interest, is hypothesised to be present in ACM but unfortunately was not present on the antibody array, so a specific hepcidin ELISA had to be utilised instead. Hepcidin was observed to be increased in differentiated ACM with a concentration of 1782pg/ml compared the 994pg/ml in pre-differentiated ACM; giving a 1.8 fold change (Figure 15). Serum free media, used as a control, has previously been shown to contain 0pg/ml hepcidin, leptin or IL-6.

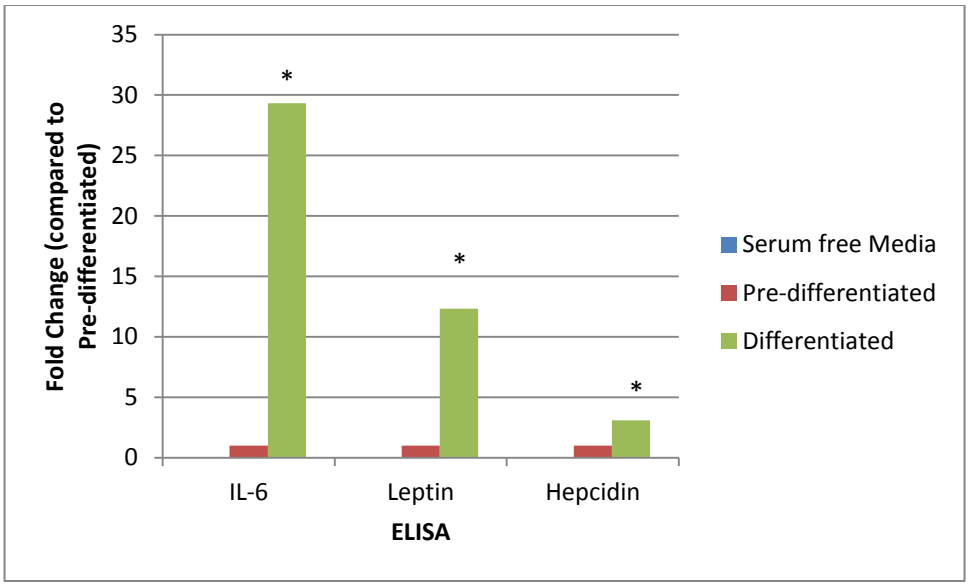
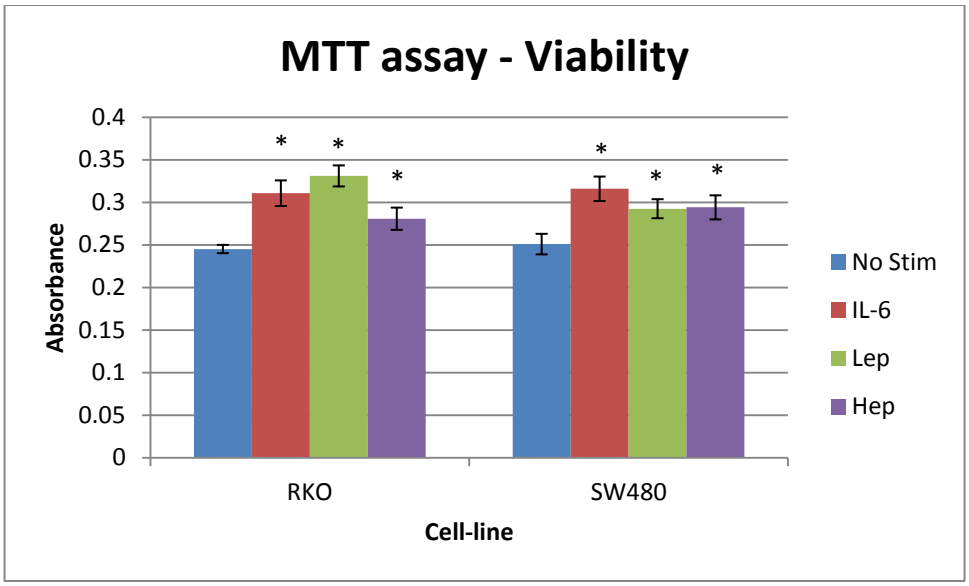


Figure 15 - IL-6, Leptin and Hepcidin ELISAs – Leptin, IL-6 and hepcidin ELISAs were used to compare the levels of the three adipokines in serum-free media, pre-differentiated ACM and differentiated ACM. The graph shows the fold change from pre-differentiated ACM to differentiated ACM in IL-6, leptin and hepcidin levels. Serum-free media contained 0pg/ml of any of the adipokines. * represents statistical significance ($p<0.05$), as determined by a paired, two-tailed student T-test, comparing differentiated ACM to pre-differentiated ACM in that cell-line. n=3 with triplicate results in each n.

Effect of Leptin, IL-6 and Hepcidin on the viability and proliferation of CRC cell-lines

To identify whether the three molecules analysed by ELISA were responsible for the increased viability and proliferation observed in RKO and SW480 cells with differentiated ACM, cells were treated with IL-6, leptin and hepcidin before viability and proliferation assays were carried out. All three molecules significantly increased the viability and proliferation of RKOs and SW480s (Figure 16), mirroring the effect observed when cells were treated with differentiated ACM.



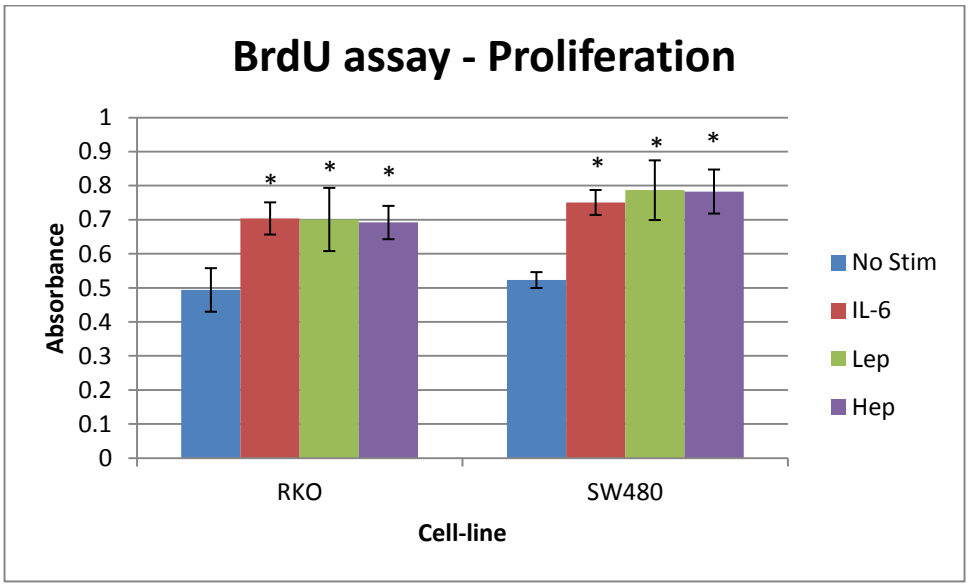


Figure 16 - IL-6, Leptin and Hepcidin all increase the viability and proliferation of colorectal carcinoma cells – RKO and SW480s were stimulated with IL-6 (10ng/ml) , leptin (60ng/ml), hepcidin (1µM) or a no-stim control for 24 hours before MTT and BrdU assays were performed (see materials and methods). Assays were quantified by changes in absorbance (at 450nm and 405nm for MTT and BrdU respectively). Mean data ±SEM is plotted with * representing statistical significance ($p<0.05$), determined by a paired, two-tailed student T-test, compared to the relevant no stim control in that cell-line. n=3 with triplicate results in each n. Treatment with IL-6, leptin and hepcidin significantly increased the proliferation and viability of RKO and SW480 cells compared to a media alone control.

To further establish whether these molecules were involved in responses observed in cell-lines when treated with differentiated ACM, cells were treated with ACM along with IL-6, leptin and hepcidin inhibitors/blockers. Treatment with the inhibitors reversed the increase in proliferation and viability observed when incubated with differentiated ACM alone (Figure 17). Proliferation of both cell-lines was returned to control (serum-free media) levels when treated with inhibitors/blockers of IL-6, leptin and hepcidin; whereas treatment with the inhibitors reduced cell viability below serum-free levels.

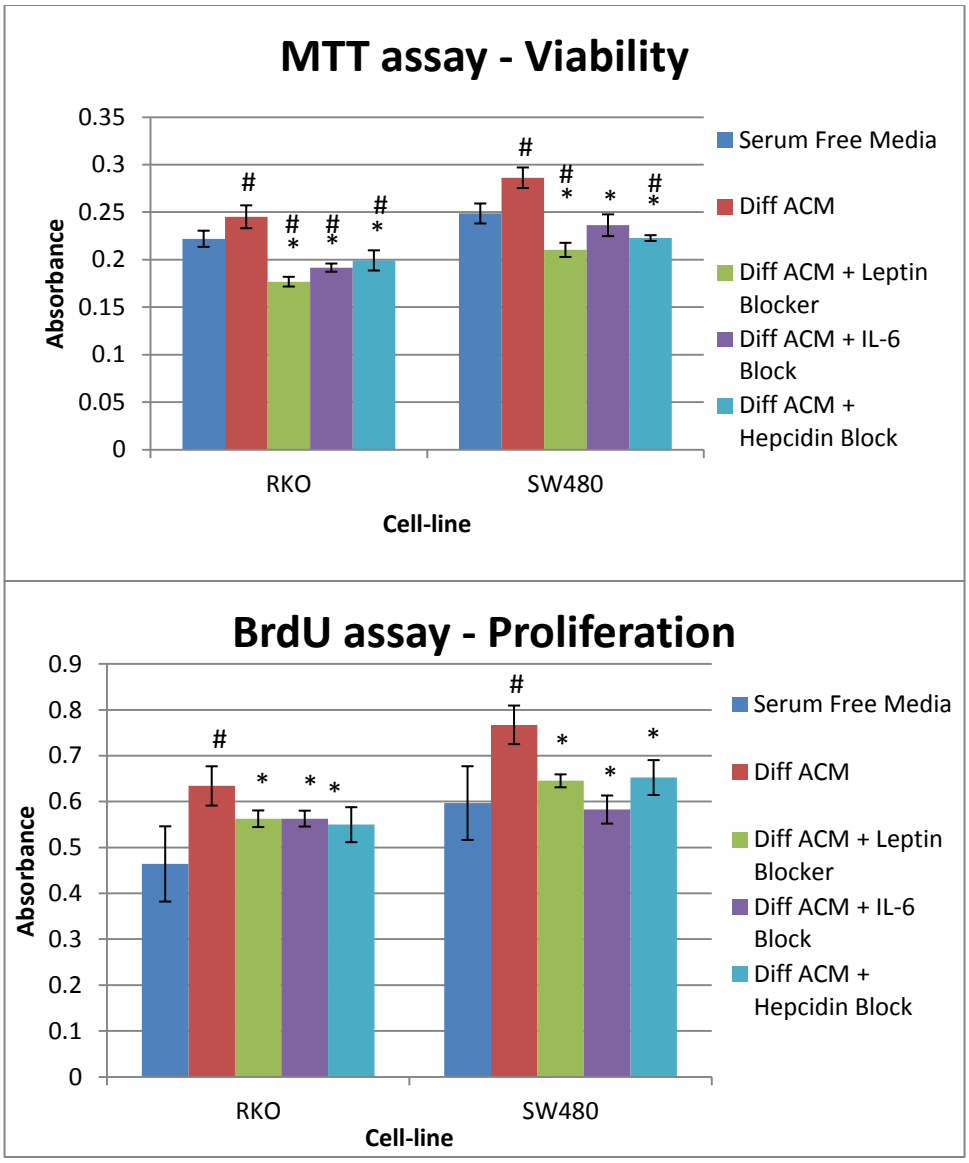


Figure 17 - Colorectal Carcinoma Cells treated with ACM and Leptin, IL-6 or Hepcidin Inhibitors – RKO and SW480s cells were treated with differentiated ACM alone or with leptin (3.2ng/ml), IL-6 (0.1ng/ml) or hepcidin (1µM) antagonists for 24 hours before MTT and BrdU assays were performed. Assays can be quantified by changes in absorbance (at 450nm and 405nm for MTT and BrdU respectively). Mean data ±SEM is plotted, with # representing statistical significance compared to the relevant serum-free media control and * representing statistical significance compared to treatment with differentiated ACM. Statistical significance is determined by a paired, two-tailed student T-test with $p<0.05$ being classified as significant. n=3 (except leptin block where n=2) with triplicate results in each n. Treatment of both cell-lines with ACM, lead to significant increases in both viability and proliferation compared to serum-free media control. However when treated with leptin, IL-6 and hepcidin inhibitors/blockers, the increased in proliferation and viability was reversed.

Leptin and IL-6 acting through hepcidin?

To investigate whether any of the adipokines work through stimulating colorectal carcinoma cells to secrete other cytokines, the ELISAs were performed using secretomes of cells treated with leptin, IL-6 or hepcidin. After stimulation with an adipokine for 18 hours, all cells were washed thoroughly so any adipokine present was due to the cells secretion rather than leftover stimulation. As Figure 18A illustrates treatment with all three adipokines induced the cells to produce leptin and IL-6; even treatment with leptin and IL-6, induced a positive feedback situation with increased concentrations of the corresponding adipokine after treatment with it. Treating cells IL-6 and leptin also significantly induced hepcidin production compared to control (Figure 18); however treatment with hepcidin did not produce the positive feedback observed with the other adipokines. This suggests that perhaps the viability and proliferation observed through IL-6 and leptin stimulation, may act through hepcidin. To test this hypothesis, hepcidin expression levels were assessed through qRT-PCR, but unfortunately hepcidin was undetected in all samples; however the laboratory has previously had issues with the hepcidin probe therefore different methods were utilised to test this hypothesis.

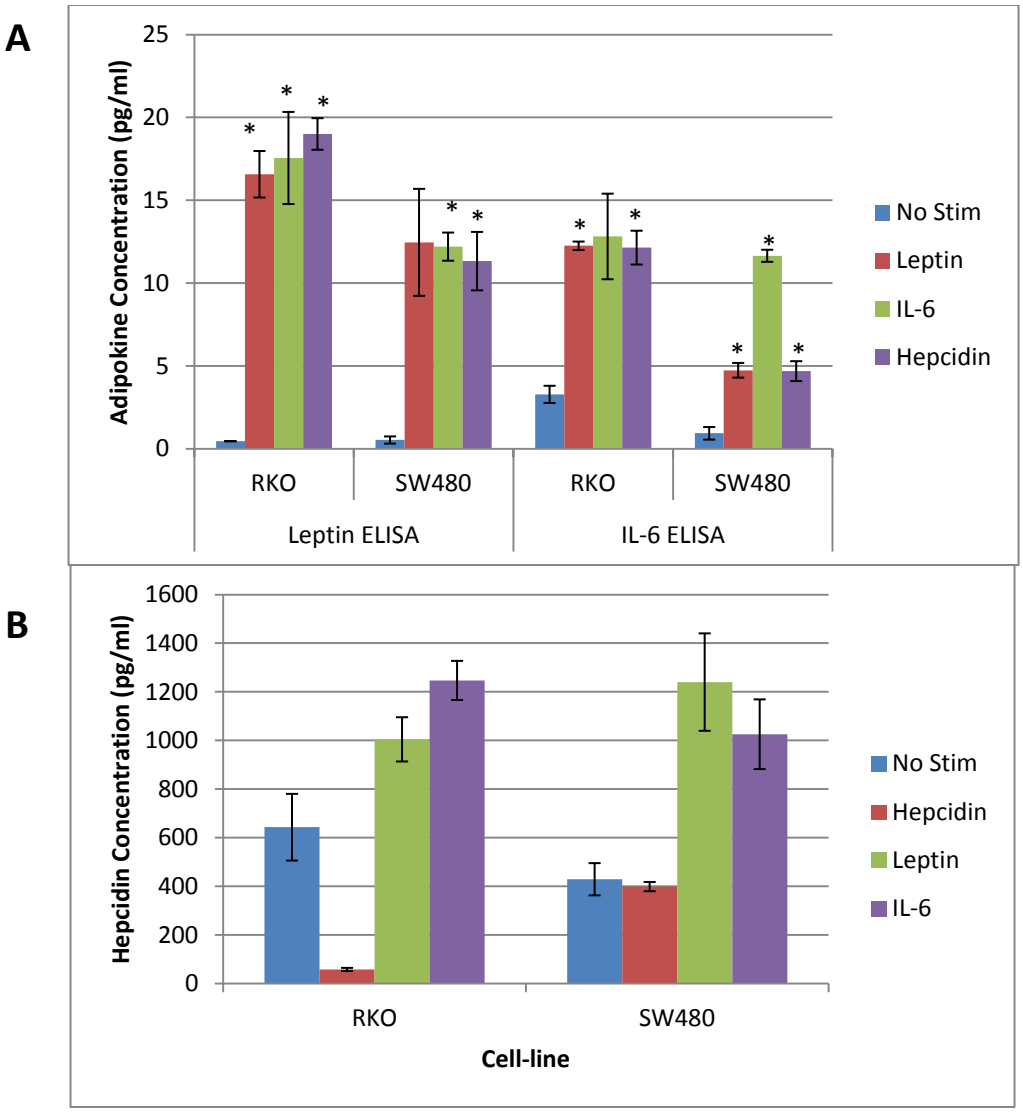


Figure 18 – Leptin and IL-6 appear to work through Hepcidin – RKO and SW480 cells were stimulated with leptin (60ng/ml), IL-6 (10ng/ml), hepcidin (1µM) or a no-stim control for 24 hours. Cells were thoroughly washed before serum-free media added and the secretomes being collected 18 hours later. Secretomes from all treatments were then subjected leptin, IL-6 and hepcidin ELISAs. (A) shows the different stimulation effects on secreted concentrations of leptin and IL-6 as determined by ELISAs. * represents statistical significance ($p<0.05$), as determined by a paired, two-tailed student T-test, compared to control of no stimulation. n=3 with triplicate results in each n. (B) shows how different stimulations affect secreted hepcidin concentrations as determined by ELISA. n=3 with triplicate results in each n.

To test the hypothesis that leptin and IL-6 may be acting through hepcidin, viability and proliferation assays were performed using Leptin and IL-6 stimulation but also the hepcidin antagonist peptide. In RKO cells, blocking hepcidin when treating with leptin, significantly decreased both viability and proliferation, compared to leptin stimulation alone, this was also observed with the proliferation of SW480s (Figure 19). When cells were treated with IL-6 and hepcidin was blocked there was evidence that viability and proliferation decreased in RKO cells, compared to IL-6 stimulated alone, this can also be observed with proliferation of SW480s. The results across cell lines, treatments and assays are not fully consistent, however, there is emerging evidence that leptin and IL-6 may be functioning through hepcidin.

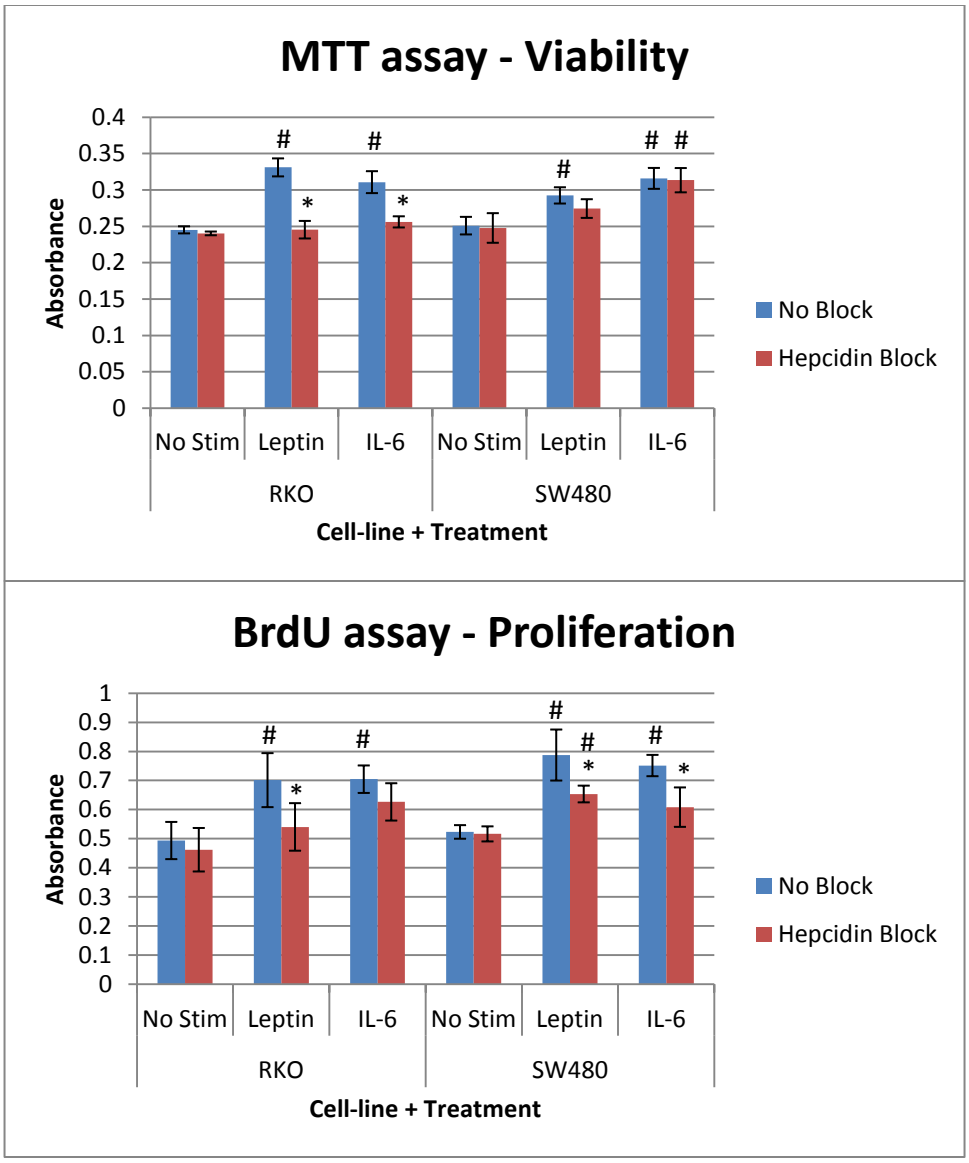


Figure 19 - Blocking Hepcidin decreases proliferation with Leptin and IL-6 stimulation – RKO and SW480s were stimulated with IL-6 (10ng/ml), leptin (60ng/ml) or a no-stim control for 24 hours. Along with stimulation, half the samples were also treated with a hepcidin antagonist (1µM) for the 24 hours before MTT and BrdU assays were performed (see materials and methods). Assays can be quantified by changes in absorbance (at 450nm and 405nm for MTT and BrdU respectively). Mean data ±SEM is plotted with # representing statistical significance compared to the relevant no stim/no block control in that cell-line and * representing statistical significance with hepcidin block compared to the respective stimulation with no block. Statistical significance was determined by paired, two-tailed student T-tests with $p<0.05$ being determined significant. n=3 with triplicate results in each n.

A hepcidin reporter assay was performed to further assess leptin and IL-6 effect on its expression. The assay showed that IL-6 significantly increased hepcidin promoter activity in both cell-lines (Figure 20). Leptin significantly increased hepcidin expression in SW480 cells but had no effect in RKO cells. This supports the previous data suggesting both leptin and IL-6 may be functioning through hepcidin.

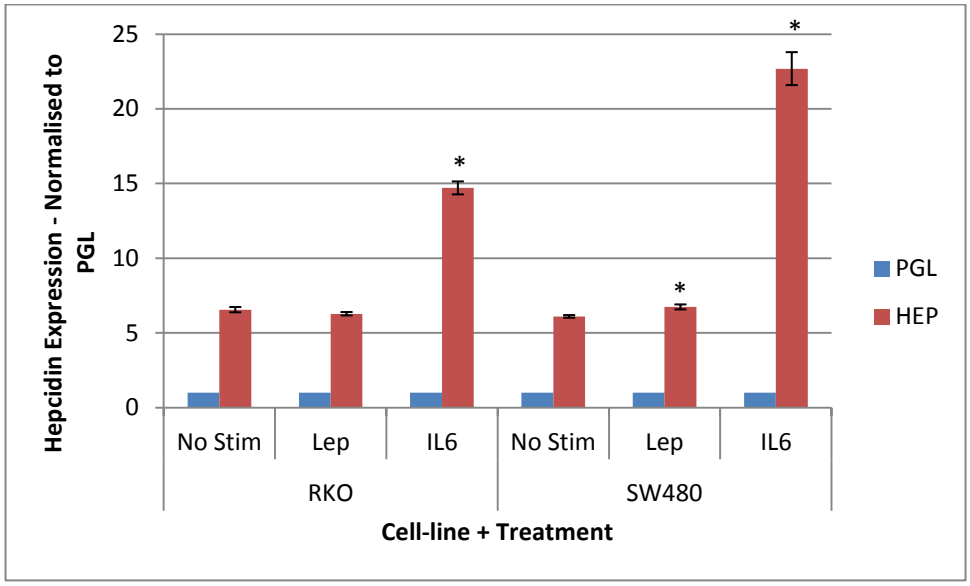


Figure 20 – IL-6 induces Hepcidin expression – RKO and SW480 cells were transfected with PGL plasmids with (HEP) or without (PGL) hepcidin regulatory elements upstream from the firefly luciferase gene, for 24 hours. After transfection, cells were stimulated with leptin (60ng/ml), IL-6 (10ng/ml) or no stim control for 24 hours, before the dual luciferase reporter assay was carried out (see materials and methods). PGL provided a control of background luminescence as it did not contain hepcidin regulatory elements, data from HEP transfected cells was normalised to this to allow comparison between different stimulations. IL-6 significantly increased hepcidin expression in both cell-lines whereas leptin only significantly increased promoter activity in SW480s. The mean data ± SEM was plotted with * representing statistical significance ($p<0.05$), determined using a paired, two-tailed student T-test, compared with no stim control HEP in each cell-line. n=3 with duplicate results in each n.

Mechanism of increased viability and proliferation

To establish exactly how cells become more viable and proliferative after incubation with differentiated ACM and stimulation with leptin, IL-6 and hepcidin, several different experiments were undertaken. Wnt signalling was initially assessed using a TOPFLASH assay, as increased signalling by this pathway may be responsible for the increased viability and proliferation observed. The TOPFLASH assay showed that both Leptin and IL-6 increased Wnt signalling in both cell-lines significantly (Figure 21). SW480s have more Wnt activity under normal conditions as they are APC null. The data suggested the adipokines may be inducing Wnt signalling in colon cancer cells, however even though the data is statistically significant, a larger fold change, particularly in the RKO cells, that contain WT APC, would be expected.

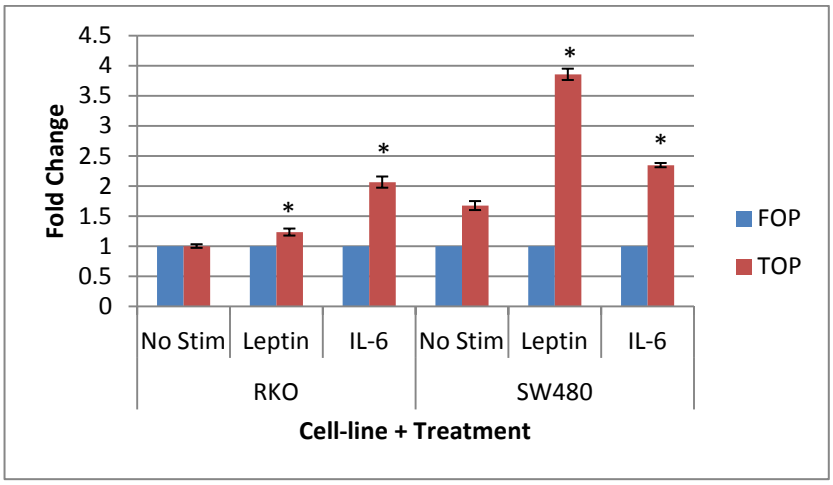


Figure 21 - Adipokines increase Wnt signalling levels – RKO and SW480 cells were transfected with plasmids with WT (TOP) or mutated (FOP) Wnt signalling regulatory elements upstream from the firefly luciferase gene, for 24 hours. After transfection, cells were stimulated with leptin (60ng/ml), IL-6 (10ng/ml) or no stim control for 24 hours, before the dual luciferase reporter assay was carried out (see materials and methods). FOP provided a control of background luminescence as it did not contain Wnt signalling regulatory elements, data from TOP transfected cells was normalised to this to allow comparison between different stimulations. TOPFLASH assay shows treating cells with leptin and IL-6 increased Wnt signalling in both cell cell-lines but particularly in the APC null SW480 cells. The mean data ± SEM was plotted with * representing statistical significance ($p<0.05$), determined using a paired, two-tailed student T-test, compared with no stim control TOP in each cell-line. n=3 with duplicate results in each n.

To further assess this proposed mechanism, qRT-PCR for Lgr5 was performed, unfortunately no Lgr5 was detected in any of the samples; however the lack of Lgr5 presence in any of the samples is unusual, as constitutive expression would be expected in SW480 cells that have constitutive Wnt signalling - this suggested the Lgr5 primers/probe did not function properly.

Myc and cyclin D1 expression were observed in the context of qRT-PCR. C-myc expression was upregulated compared to the control when both cell-lines were treated with ACM, particularly pre-differentiated ACM (Figure 22). The same increase was not observed when cells were stimulated with leptin, hepcidin and IL-6, suggesting that these adipokines are not responsible for the increase in c-myc levels. The large increase in c-myc levels, when treated with pre-differentiated is to be expected, as pre-differentiated ACM has been shown to contain increased insulin levels, which has in turn been shown to stimulate c-myc (Sun and Jin, 2008). Cyclin D1 levels decreased in RKOs when treated with differentiated ACM and leptin and IL-6 stimulations yet increased with the same treatments in SW480 cells. As the results are so varied and there is no apparent correlation, it would suggest cyclin D1 is not key contributor to the increased viability and proliferation.

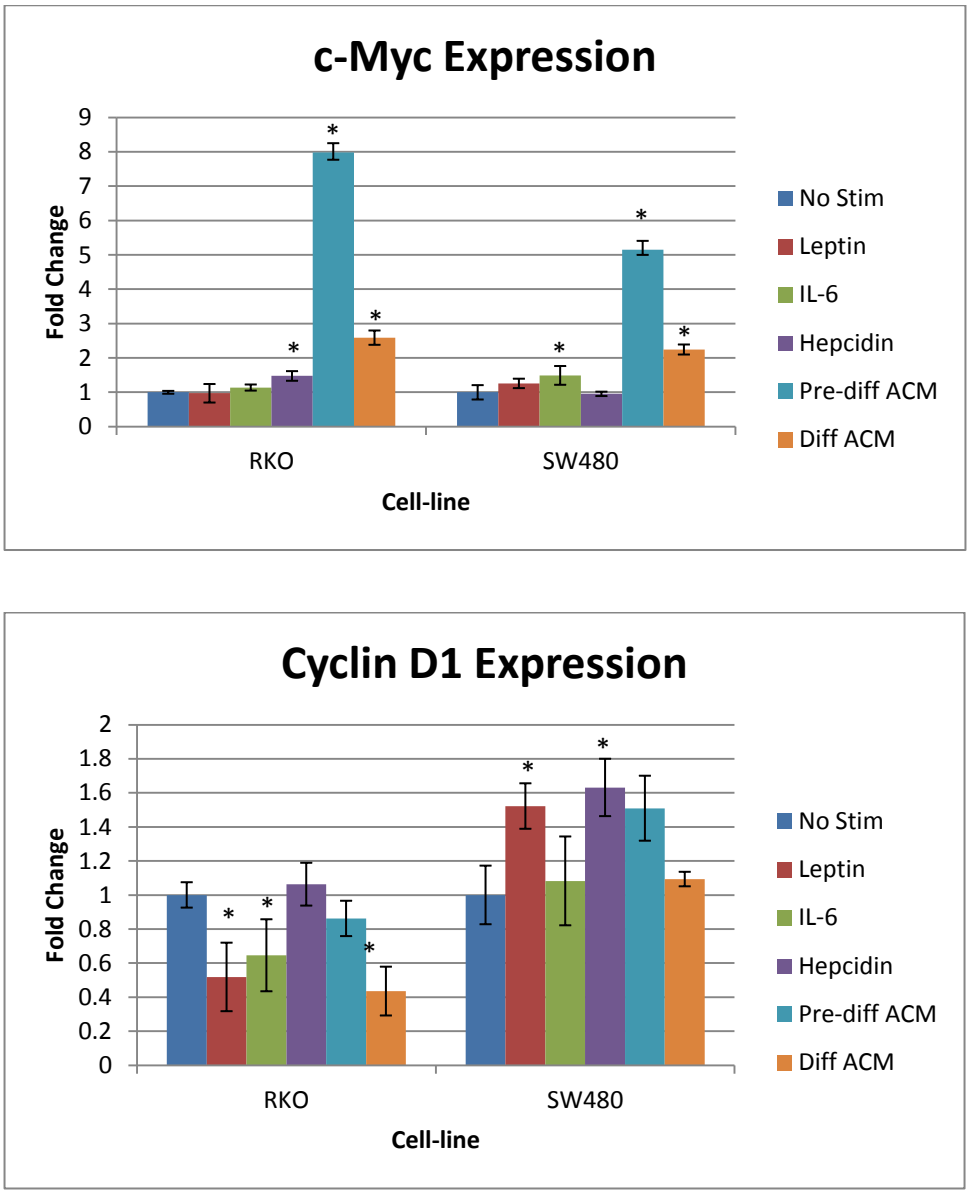


Figure 22 - Adipokine and ACM Stimulation increase c-myc levels but has varying effects on Cyclin D1 levels – RKO and SW480 cells were treated with leptin (60ng/ml), IL-6 (10ng/ml), hepcidin (1µM), pre-differentiated ACM, differentiated ACM or no stim control for 24 hours. cDNA was generated from RNA extracted from cells and qRT-PCR carried out probing for myc and cyclin D1 (see materials and methods). The mean data ± SEM was plotted with * representing statistical significance ($p<0.05$), determined using a paired, two-tailed student T-test, compared with no stim control TOP in each cell-line. n=3 with duplicate results in each n.

Discussion

Adipocyte Conditioned Media

Differentiated and pre-differentiated ACM were observed to be vastly different in this study, differing in both adipokines present and their effect on colorectal cancer cell-lines; these observed differences coincide with the literature and previous studies on the changes of adipokine secretome prior to and after adipogenesis (Zhong et al., 2010). Differentiated ACM had the most profound effect on colorectal cell lines, increasing both viability and proliferation significantly in both RKO and SW480 cells; compared to pre-differentiated ACM that increased viability but decreased proliferation. The most abundant adipokine in pre-differentiated ACM was insulin, however insulin has been shown to increase proliferation and viability of colorectal cells (Fenton and Birmingham, 2010), so the observed effects are likely to be induced by a factor, not detected in the adipokine array.

As hypothesised, differentiated ACM was observed to accelerate colorectal cancer cell proliferation and viability. The differentiated ACM appeared to be, comparatively, more complex containing elevated levels of a diverse range of adipokines. IL-6 was the most abundant factor, and with obesity being more often described as a state of chronic inflammation, it is no surprise that IL-6, an inflammatory mediator, is present in such quantities. ENA-78, the second most abundant adipokine, according to the antibody array, has previously been shown to increase proliferation and migration in colorectal cancer cell-lines (Kawamura et al., 2011). Other adipokines found in the differentiated ACM have also been associated with increasing the proliferation of colon cells such as PAI-1 (plasminogen activator inhibitor-1) (Ho et al., 2012) and IL-8 (Ning and Lenz, 2012).

The antibody array was useful as a quick assessment of whether a diverse range of molecules were present; however, they are not always specific enough to detect all molecules. In this study, the

antibody array detected very little leptin in differentiated ACM compared to pre-differentiated ACM, however when a leptin ELISA was utilised, a fold increase of 11 was actually observed. This illustrates the limitations of the array compared to the ELISA technique. Another limitation is that they may not contain antibodies for all the molecules of interest, for example in this study, hepcidin was an adipokine of primary interest, yet unfortunately was not array; however, utilisation of an ELISA specific for hepcidin confirmed its presence in the ACM.

Leptin, IL-6 and Hepcidin

Leptin, IL-6 and hepcidin were all confirmed by ELISA to be significantly elevated in differentiated ACM, compared to pre-differentiated ACM. When assessed as individual molecules, they all increased proliferation and viability of colorectal cancer cell-lines; yet when individually blocked in differentiated ACM, the increased proliferation and viability was reversed. The decrease in the viability and proliferation observed when the three adipokines were inhibited, compared to ACM treatment alone, shows how vital they are for the observed phenotype. The ELISA revealed that stimulation with leptin increased leptin, IL-6 and hepcidin levels in both cell-lines and likewise with IL-6 stimulation, suggesting the molecules are all individually important and capable of inducing cells, to secrete factors to further increase viability and proliferation. Leptin has previously been shown to induce IL-6 production in B cells (Agrawal et al., 2011) and IL-6 to induce leptin production in macrophages (Loffredal et al., 1998), while both have been shown to stimulate hepcidin (Chung et al., 2007)(Wrighting and Andrews, 2006).

Hepcidin: a mediator?

Leptin and IL-6 stimulation increased the levels of hepcidin, as seen by ELISA, in colorectal cancer cell-lines, although the results were not quite significant (p values= 0.056-0.180). These results suggested that the adipokines may be acting through hepcidin; to confirm this, hepcidin was blocked, as cells were stimulated with IL-6 and leptin with proliferation and viability being assessed. In RKO cells, proliferation and viability significantly decreased when cells were stimulated with leptin and hepcidin was blocked, as did the viability when stimulated with IL-6. Blocking hepcidin had no effect on the viability when SW480 cells were stimulated with either leptin or IL-6, but proliferation was significantly decreased with both stimulations. The hepcidin reporter assay also showed that IL-6 increased hepcidin expression, with leptin only increasing hepcidin promoter activity in SW480s. Although the results are not fully consistent, there is a trend to suggest that hepcidin is important for the increased the viability and proliferation observed when cells are treated with IL-6 and leptin. This data coincides with current literature; Hintze et al. have previously described that IL-6 and leptin secreted from 3T3-L1, an adipocyte cell-line differentiated from murine fibroblasts, both induced hepcidin production in hepatocytes.

Wnt Signalling

The TOPFLASH reporter assay suggests that Wnt signalling is at least one of the mechanisms that adipokines leptin and IL-6 function through in colorectal cells; however, this mechanism cannot be confirmed without further study. Although the TOPFLASH assay data is significant, the laboratory have previously seen much larger increases in β-catenin promoter activity, when RKO and SW480s have been stimulated with leptin and IL-6; the smaller changes seen in this study, may be due to inefficient transfection resulting from technical issues with the transfection reagent. Repetition of this assay with a different transfection reagent would be appropriate to confirm the data.

Detection of Lgr5 by qRT-PCR would have provided an ideal method of assessing whether Wnt signalling is the pathway involved, but unfortunately technical difficulties rendered Lgr5 undetectable. Cyclin D1 and c-myc levels, both usually induced by Wnt signalling, were not significantly increased when cells were treated with differentiated ACM, suggesting this pathway might not be significantly involved. Literature, however, has previously described a role for Wnt signalling when colorectal cancer cells are stimulated with leptin, as cells with active Wnt signalling have been observed to have increased expression of the leptin receptor (Endo et al., 2011).

Alternative Signalling Pathways

Wnt signalling has been observed to have an important role in colorectal cells, with permutations in the pathway leading to deregulated proliferation and ultimately colorectal cancer. This study has suggested the Wnt signalling pathway may be involved in the effect ACM has on colorectal cancer cell-lines, however it is not the sole pathway, and other pathways must be involved.

Both IL-6 (Fenton and Birmingham, 2010) and leptin (Endo et al., 2011) have been associated with increasing STAT-3 phosphorylation and activation. Furthermore, in hepatocytes, IL-6 has been shown to induce hepcidin expression through STAT-3 (Wrighting and Andrews, 2006); this data coincides with some of the findings of this study and suggests that the JAK/STAT pathway may be a major contributor. Leptin has also been observed to activate other pathways including the MAPK pathway (Hardwick et al., 2001) and PI3K/Akt (Uddin et al., 2009) so these pathways may also have a role. Further work would be needed to elucidate whether any of the mentioned pathways are involved in increasing proliferation and viability of colorectal cancer cells, after treatment with adipokines.

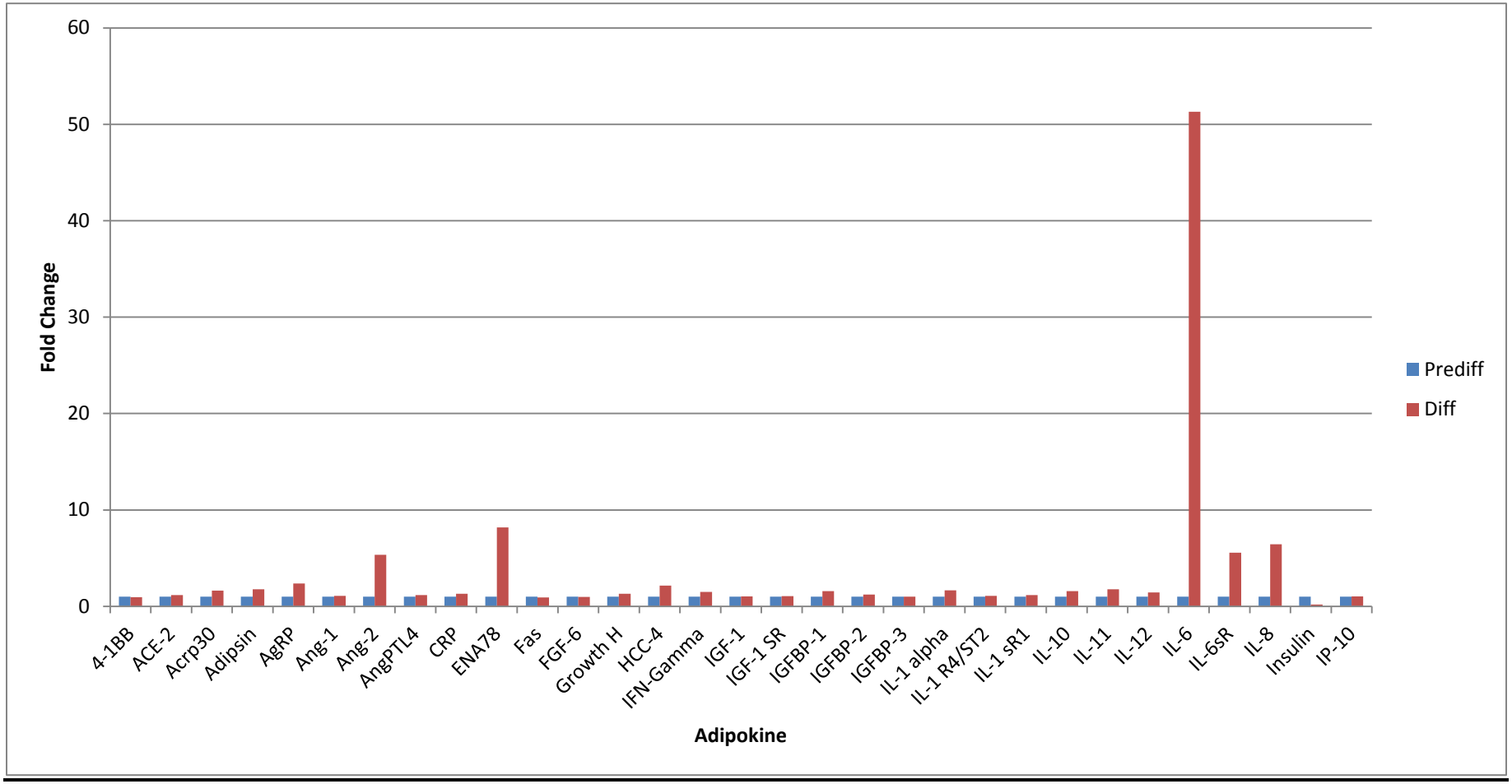
Limitations and Further Work

Although this investigation was a comprehensive study, there are obvious limitations. The largest flaw is the pooling of the ACM; although pooling decreases patient variability and gives an overall insight, it would not have been the preferred method. However, due to the limited availability of patient samples, it was the most applicable method of providing data. If samples were unlimited, it would have been preferable to compare fat samples by patient or pool, according to whether the patient was suffering from colorectal cancer or not. Comparing samples by patient BMI would also be an appropriate study. Another potential flaw is the stimulation of colorectal cells with leptin, IL-6 and hepcidin; although the cells were stimulated with concentrations of the adipokines widely used in the literature, these concentrations have proved to be much higher than the concentrations found in the ACM. Since levels of leptin, IL-6 and hepcidin in ACM have been quantified using ELISA, it would be appropriate to stimulate the cells with the concentrations secreted and further assess cell viability and proliferation. Unlimited samples would also have provided larger volumes of ACM; thus allowing ACM to be used directly in assays such as TOPFLASH and hepcidin reporters.

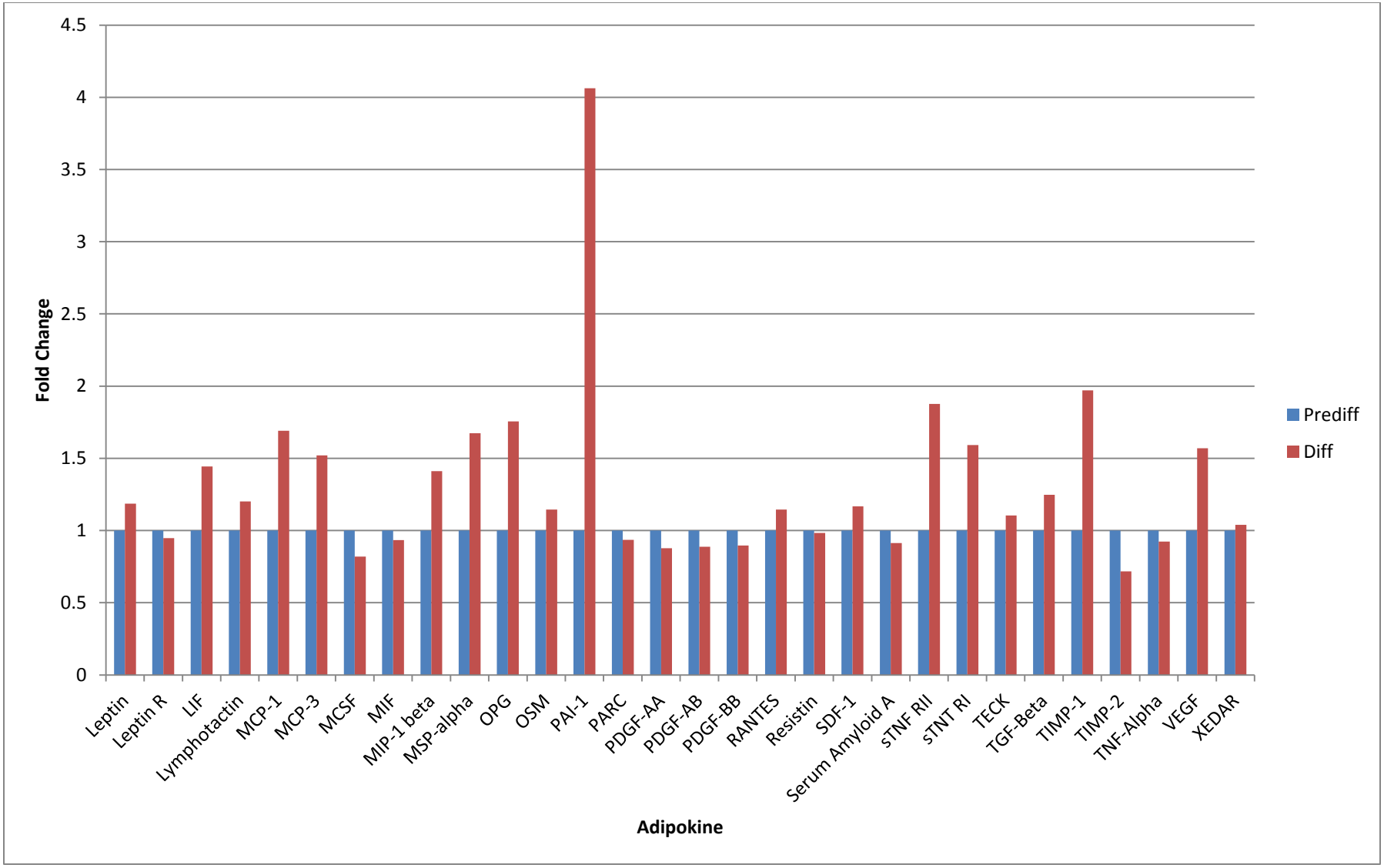
Mass spectrometry (MS) analysis of pre-differentiated and differentiated ACM to identify differences in the secreted factors, was originally one of the main aims of this study. It was planned also to use mass spectrometry to analyse secretomes from SW480 and RKOs treated with leptin, hepcidin and IL-6, to identify factors secreted that may be further promoting viability and proliferation. Methods were optimised and samples were prepared for the analysis; however due to technical issues, the analysis was unable to be completed. To further this work, it would be useful to utilise LC-MS/MS, as planned, to identify any differences between the two ACM and changes to the secretome of cell-lines after adipokine stimulation.

To elucidate a pathway for the increased proliferation and viability there are several experiments that would be essential. To confirm, IL-6 and leptin are signalling through hepcidin, use of functioning hepcidin primers and probe are vital, as utilisation of qRT-PCR would quantify increased hepcidin expression in cells stimulated with IL-6, leptin and differentiated ACM. Wnt signalling could be re-assessed using a TOPFLASH assay with a different transfection reagent, to ensure efficient transfection, along with repeating the Lgr5 qRT-PCR with different primers/probe. Inhibition of Wnt signalling, using a GSK3 inhibitor, such as SB216763, or β-catenin knockdown using specific siRNA, with IL-6, leptin, hepcidin and ACM stimulation, would allow assessment of Wnt signalling's involvement in the increased viability and proliferation observed. If Wnt signalling is determined to not be truly involved, other pathways would then need to be assessed to elucidate the mechanism of this increased proliferation and viability.

Appendices



Appendix 4 – The first 32 adipokines on the antibody array - The values are normalised to the density of pre-differentiated ACM, to allow easy comparison of the differences in the levels of adipokines between differentiated and pre-differentiated ACM.



Appendix 5 - The last 32 adipokines on the antibody array - The values are normalised to the density of pre-differentiated ACM, to allow easy comparison of the differences in the levels of adipokines between differentiated and pre-differentiated ACM.

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