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Title:
Analysis of the function of BASP1 in endometrial cancer cells

Analysis of the function of BASP1 in Endometrial cancer cells

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A dissertation submitted to the University of Bristol in accordance with the

requirements for award of the degree of MSc by research in the faculty of

Biomedical Sciences

School of Cellular and molecular medicine

August 2018

Word count: 18,491

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Abstract

BASP1 is a multifunctional protein which has been found to regulate transcription in several different cell lines through acting as a co-factor for WT1 or ERα. BASP1 has been reported to possess either tumour suppressor or tumour promoting activity in multiple cancer types across multiple tissues. In breast cancer cells, BASP1 acts as a tumour suppressor and enhances the effects of tamoxifen treatment. Tamoxifen is an ER modulator commonly used to treat breast cancer but long term treatment is strongly associated with increased risk of endometrial cancer occurrence. Although tamoxifen acts as an ER antagonist in the breast, in the endometrium, tamoxifen acts as an ER agonist.

This study investigated the expression and functions of BASP1 in Ishikawa endometrial cancer cells, as well as its effects on tamoxifen action. After confirming the presence of endogenous BASP1 in Ishikawa cells, cell line derivatives were generated that either knock down or overexpress BASP1. These cells were used to determine how BASP1 regulates cell growth, tumourigenicity and transcription of ER target genes. BASP1 was found to be primarily cytoplasmic in Ishikawa cells but still affected the transcription of a subset of ERα target genes. BASP1 was also found to reduce proliferation and colony formation efficiency in Ishikawa cells, indicating tumour suppressor activity. qPCR analysis revealed that BASP1 enhances the effects of tamoxifen treatment on the expression of a subset of ERα target genes: PGR, Greb1 and TFF1.

The tumour suppressor activity of BASP1 in Ishikawa cells and the modulation of tamoxifen action is similar to that which was reported in breast cancer cells. However, in Ishikawa cells, BASP1 targeted a different set of ER target genes. The work presented here suggests that BASP1 may play a role in the differential effects of Tamoxifen in different cell types.

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ....████████████████████.... DATE:..... 12/08/2018

Acknowledgements

Firstly, I would like to thank my supervisor, Stefan Roberts, for his valuable support during the project and the production of this dissertation. Despite an extremely busy year, he was able to provide a perfect balance of input, project guidance and support, while still allowing me the freedom to work and progress the project independently. This has been optimum for my development as a researcher and I am very grateful for everything he has done.

I also want to thank Amy Dey for teaching me most of the experimental procedures used in this project and providing valuable advice, throughout the majority of the year, particularly when navigating any experimental difficulties that arose. My thanks are also extended to Alice Sherrard who, in the absence of Amy, helped me towards the end of the project, particularly with DNA fragmentation.

In addition, I would like to thank Ann Williams for monitoring my progression and providing advice on prioritising certain experiments and organising my time in order to achieve as much as possible by the end of the year.

Finally, I would like to thank Jazzmin Huber, a talented and hardworking Medicine student choosing to do a 5 week placement in our laboratory, who provided some excellent work in helping to obtain sufficient repeats of the qPCR expression analysis and growth assays for this project.

Abbreviations and acronyms

AML   Acute Myelogenous Leukaemia

BASP1   Brain Acid Soluble Protein 1

BRG1   Brahma Related Gene 1

CBP   CREB Binding Protein

CDK   Cyclin Dependent Kinase

DBD   DNA Binding Domain

DCIS   Ductal Carcinoma in situ

DDS   Denys-Drash Syndrome

DSRCT   Desmoplastic Small Round Cell Tumour

E2   Estradiol

ER   Estrogen Receptor

ERE   Estrogen Response Elements

FLI1   Flightless 1

GAP-43   Growth Associated Protein 43

HDAC   Histone Deacetylase

HEK   Human Embryonic Kidney

LBD	Ligand Binding Domain
MAPK/ERK	Mitogen Activated Protein Kinase/Extracellular Signal Related Kinases
MYO1C	Myosin -1C
NCAM	Neural Cell Adhesion Molecule
NCoR	Nuclear Co-Repressor
NLS	Nuclear Localisation Sequence
PEST	Proline, Glutamic acid, Serine, Threonine
PI3K/Akt	Phosphatidylinositol-4,5-bisphosphate 3 Kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein Kinase C
PR	Progesterone receptor
SERM	Selective Estrogen Receptor Modulator
SMRT	Silencing Mediator for Retinoid or Thyroid
SRC	Steroid receptor coactivators
TGF-β	Transforming Growth Factor β
UPR	Unfolded Protein Response
WAGR	Wilms tumour, Aniridia, Genitourinary abnormalities and mental Retardation
WT1	Wilms Tumour 1

1 Introduction

1.1   BASP1

1.1.1   BASP1

Brain acid soluble protein 1 (BASP1) is a multifunctional protein found in many different tissues and cell types throughout the human body. The RNA and protein expression levels of BASP1 are particularly high in the brain and are found across a vast array of cell types within multiple areas of the brain including neuronal cells and glial cells of the cerebral cortex, hippocampus and caudate, endothelial cells and neuropils of the cerebral cortex and cells of both the granular layer and the molecular layer of the cerebellum (*1*). BASP1 was first characterised in the axonal termini of neuronal cells as a member of the GAP-43 family, a group of proteins which were known to be involved in axon growth and memory formation (*2*) However, BASP1 has since been identified in a vast array of different organs, tissues and cell types throughout the body including but not limited to those of the endocrine tissues, bone marrow, lungs, stomach, kidneys and skin, as well as both male and female reproductive tissues (*1*). Depending on the cell type and location, BASP1 has been proposed to perform a range of different context specific activities and functions (*3*).

1.1.2   BASP1 structure

The primary structure of BASP1 is highly conserved between species and in most cases BASP1 knockdown is lethal in animal models (*4*). Notable structural motifs of BASP1 are its PEST sequences, phosphorylation sites, nuclear localisation sequence and N-terminal myristoylation motif (*5-7*) (fig.1).
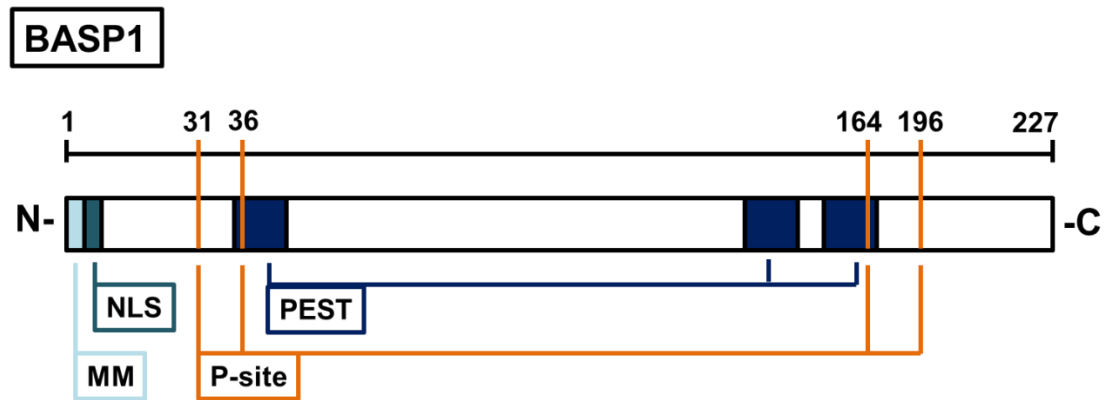
Figure 1 - Structure of human BASP1
Notable regions are denoted by: MM (Myristoylation motif), NLS (Nuclear Localisation Sequence), P-site (phosphorylation sites) and PEST (PEST motifs). Residue number is indicated by a scale above the diagram. Many more phosphorylation sites have now been proposed but only the four most referenced sites were included (*8*).

PEST motifs are regions of protein rich in proline, glutamic acid, serine and threonine that are strongly associated with a fast protein turnover rate (*5*). The multiple phosphorylation sites can be targeted by protein kinases such as protein kinase C (PKC) which can affect BASP1 activity and membrane binding capability (*7*). The N-terminal myristoylation motif allows BASP1 to anchor to lipid membranes with some specificity for certain lipids such as neutral phosphatidylcholine (*6*). Once bound to the membrane, BASP1 is able to modify the lipid makeup of the membrane in close proximity by accumulating cholesterol at the site, in some cases to such an extent that cholesterol crystals can form (*9, 10*). The N-terminal myristoyl motif provides BASP1 with some hydrophobic character, despite it being a mostly hydrophilic protein (*5*). N-terminal myristoylation of this motif is required for many of BASP1s functions (*11, 12*).

1.1.3   BASP1 functions

BASP1 can have many different functions depending on the tissue environment, cell type and presence of other cofactors and transcription factors.

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Within neurones, BASP1 has been proposed to have a range of functions that include involvement with synaptic signal transmission, neuronal signal transduction, neurite growth, regeneration and memory formation. BASP1 can affect neurotransmitter signalling by interacting with calcium bound calmodulin and PKC in the membranes of synaptic vesicles (*13*). In addition, BASP1 has been shown to form cation selective ion channels in lipid bilayers which affects membrane conductance in neurones (*14*). BASP1 has also been shown to be upregulated following multiple forms of nerve injury in several neurone subtypes and this upregulation is associated with neurone regeneration (*15*). Increased expression of BASP1 has also been shown to promote both NCAM (neural cell adhesion molecule) independent neurite outgrowth and nerve sprouting. This is an essential process in the development of proper brain structure, particularly in the later stages of development, and has implications for the potential roles of BASP1 in determining neurone plasticity and memory formation (*11*). This process is an example of BASP1 activity which is dependent on N-terminal myristoylation of BASP1 (see section 1.1.2).

It is now known that BASP1 likely has additional functions in other cell types. BASP1 has been shown, in the presence of WT1(Wilms tumour 1), to be capable of altering K562 myelogenous leukaemia cell's differentiation pathway to produce cells with neurone like morphology (*16*). BASP1 also likely has roles in the development of other tissues, such as the kidneys. BASP1 is expressed during kidney nephrogenesis and continues to be expressed into adulthood, with expression being limited to the podocyte cells (*17*). This expression pattern seen in kidney development is shared by the transcription factor WT1 that is essential for proper kidney development (*18*). Other functions of BASP1 that have been proposed include the modulation of myc in fibroblasts (*19*) and the promotion of apoptosis of renal tubular cells (*20*).

1.2 WT1

1.2.1 WT1

WT1 is a zinc finger protein that is essential for the development of multiple different mammalian tissues including the mesothelium, gonads, heart, blood vessels, blood, spleen, nervous system, eyes, lungs, liver and kidneys (*21-23*). In humans WT1 is highly expressed in the spleen, smooth muscle and kidney as well as both male and female reproductive systems (*24*). Similar to BASP1, WT1 can exhibit a range of different activities and functions, in part due to the alternative splicing of the WT1 gene. The four main splice variants of WT1 arise because of the presence or absence of an additional 17 amino acid sequence encoded by exon 5 (between the N-terminus and the zinc finger domains) and a possible 3 amino acid insertion of lysine, threonine and serine in exon 9 (between the third and fourth zinc fingers domain) called KTS (*25*) (fig.2).

1.2.2 WT1 structure

WT1 can bind to target DNA using its c-terminal zinc finger motifs in order to modulate transcription. WT1 zinc finger motifs are also able to bind to RNA which could potentially result in altered alternative splicing or translation of the targets. These activities are controlled by repression and activation domains in the N-terminal and central regions of the protein (*26*). Transcriptional co-modulators such as BASP1 are able to bind to WT1 within the repression domain to affect WT1 activity.
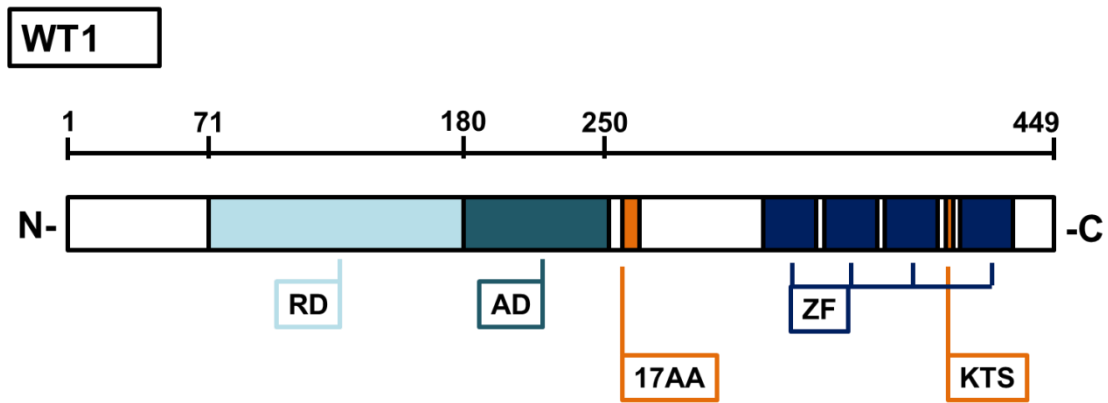
Figure 2 - Structure of human WT1
Notable regions are denoted by: RD (repression domain), AD (activation domain), 17AA (17 amino acid splice variation site), ZF (zinc finger motifs) and KTS (KTS slice variation site). Residue number is indicated by a scale above the diagram.

1.2.3 BASP1-WT1 interaction

In human embryonic kidney (HEK) cells, BASP1 was shown to translocate to the nucleus where it regulates WT1 to exert its effect on WT1 target genes (*17*). The mechanism of transcriptional regulation by BASP1 involves the recruitment of a large protein complex. BASP1's nuclear localisation sequence (NLS) allows it to translocate to the nucleus where it binds to WT1 at the promoters of WT1 target genes (*17*).
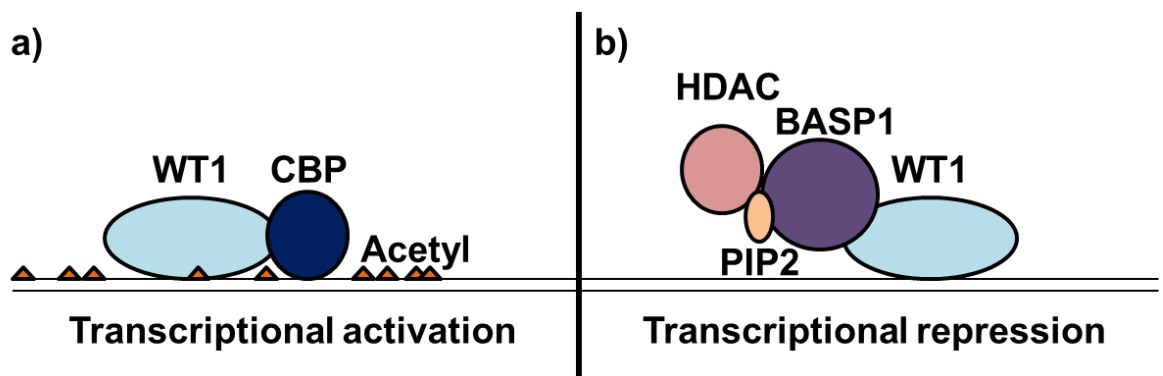
Figure 3 - BASP1 acting as a transcriptional co-suppressor for WT1.
a) WT1 (Wilms tumour 1) binds to target genes and recruits CBP (CREB binding protein), resulting in histone acetylation and activation of transcription. **b)** BASP1 binding to WT1 results in the dissociation of CBP and the recruitment of HDAC1 (Histone Deacetylase) resulting in deacetylation and repression of transcription.

The N-terminal myristoyl group of myristoylated BASP1 allows the recruitment of phosphatidylinositol 4,5 bisphosphate (PIP2) to the BASP1-WT1 complex located at the WT1 target gene promoter (fig.3). BASP1 binding of PIP2 in turn allows the recruitment of histone deacetylase 1 (HDAC1) (*12*). HDAC1 is a histone remodelling factor that removes the acetyl side chain from a histone lysine residue (H3K9) resulting in chromatin condensation. The recruitment of HDAC1 to a WT1 target gene results in local condensation of chromatin and thus transcriptional repression of the target (fig.3b). BASP1 is also able to recruit prohibitin, another transcriptional co-repressor. Prohibitin, in turn, recruits ATP dependent chromatin remodelling factors such as brahma related gene 1 (BRG1) which results in the dissociation of CREB binding protein (CBP) from the promoter (*27*). CBP has histone acetyl transferase activity (results in relaxation of chromatin structure) and is a scaffold protein for the transcriptional complex (*28*). The dissociation of CBP therefore results in further transcriptional repression of WT1 target genes. BASP1 is therefore able to convert WT1 activity from a transcriptional activator to a transcriptional repressor of WT1 target genes.

1.3 BASP1 and WT1 in Cancer

1.3.1 WT1 in embryonal cancers

WT1 is involved in the formation and progression of many different solid tumours and leukaemias, either as an oncogene or a tumour suppressor gene. WT1 dependent tumours can be broadly divided into two groups: embryonal and adult. The most well documented WT1 dependent embryonal tumour is the Wilms Tumour. WT1 loss of function mutations are some of the highest frequency mutations found in Wilms tumour (nephroblastoma) (*29*). This tumour commonly develops in patients with WAGR syndrome (Wilms tumour- Aniridia- Genitourinary abnormalities- mental Retardation) in which the WT1 gene is deleted (*30*). Wilms tumour is also commonly found in patients with Denys Drash Syndrome (DDS) due to a high frequency of mutation in the DNA binding domain of the WT1 gene (*31*). Another tumour shown to be embryonal in origin is desmoplastic small round cell tumour (*32*). WT1 is also frequently mutated in DSCRT of the abdomen. This solid tumour can be caused by a reciprocal translocation that results in the formation of and, unlike in Wilms tumour, overexpression of an EWS-WT1 fusion protein (*33*). In this cancer type, WT1 acts as a tumour suppressor which can be silenced by mutation but this is not the case in all cancer types.

1.3.2 WT1 in adult cancers

Leukaemias, melanomas and breast cancers are all examples of adult cancers which, in some cases, can be WT1 dependent. In adult cancers in which WT1 is implicated, WT1 often exhibits oncogenic activity. WT1 can even be found to be expressed in adult cancers of tissues in which WT1 would not normally be expressed. Adult cancers which have been found to be WT1 dependent in some cases include leukaemias, melanomas, renal cancers and breast cancers (*34-36*).

WT1 is overexpressed in as many as 80% of cases of some subsets of leukaemia which indicates that WT1 can also have oncogenic activity (*35*). In addition, WT1 mutations can also be associated with poor prognosis such as in both adult and childhood cases of acute myelogenous leukaemia (AML) (*37, 38*). WT1 therefore is a highly complex, context specific transcription factor with multiple different functions and mutations in different cancer types. WT1 has even been found to be overexpressed in some leukaemia cases and have loss of function mutations in others (*35, 39*).

1.3.3 BASP1 in cancer

Unsurprisingly, BASP1 too has multiple different functions, activities and mutations in a variety of different cancer types. BASP1 has been shown to exhibit various tumour suppressive activities in acute myelogenous leukaemia cell lines including proliferation inhibition and increased apoptosis. In addition, BASP1 promoter DNA methylation and silencing by the AML1-ETO fusion protein has been observed in AML cells (*40*). As WT1 is overexpressed in many cases of AML it is possible that BASP1 exerts its tumour suppressor activity by co-repression of WT1 target genes. Similarly, BASP1 has been shown to be downregulated by DNA methylation in some hepatocellular carcinoma cases (*41*). BASP1 has also been shown to have reduced expression in thyroid cancer cells compared with normal tissue, and restoration of BASP1 resulted in inhibition of cell growth and migration (*42*). These reports show that BASP1 can act as a tumour suppressor in multiple cancer types and is often a target of repression in cancer cells. However, one exception has been demonstrated in cervical cancer cells. BASP1 expression was shown to promote cancer cell proliferation, colony formation efficiency and tumourigenicity as well as being associated with increased stage progression and poor prognosis (*43*).

1.3.4 BASP1 in breast cancer

The effects of BASP1 expression on breast cancer cells has also been investigated and the results indicated that, in this cell type, BASP1 acts as a tumour suppressor (*44*). In addition to exhibiting tumour suppressor activity, BASP1 expression has been associated with increased breast cancer patient survival. Interestingly, BASP1 was also found to colocalise with several components of the nuclear actin network including β-actin, gelsolin, myosin-1C (MYO1C) and flightless 1 (FLI1). These nuclear actin network proteins are known to interact with ERα, the hyper proliferative hormone receptor protein target of the breast cancer drug tamoxifen (*45*). In the same study, BASP1-ERα colocalisation was then observed and it was shown that BASP1 colocalises with ERα in the nucleus, suggesting BASP1 as an ERα cofactor. Subsequent investigation of the effect of BASP1 expression on tamoxifen action was conducted and it was shown that 40% of the genes regulated by tamoxifen in MCF7 cells were BASP1 dependent. BASP1 expression was also found to enhance the inhibitory effects of tamoxifen on cell proliferation and colony formation (*44*).

The effects of BASP1 expression on specific genes associated with tamoxifen resistance were also investigated. BASP1 was found to affect tamoxifen induced gene expression changes in several genes associated with tamoxifen resistance, including DLC1, BMPER, SOX2, XBP1, Rab31 and Serpina3 (*44*). These results together indicated that BASP1 plays a major role in tamoxifen response in breast cancer cells.

1.4 Tamoxifen and the ER

1.4.1 Tamoxifen

Tamoxifen is a non-steroidal selective estrogen receptor modulator (SERM) used to treat ER+ breast cancers, which make up approximately 70% of all human breast cancer cases (*46, 47*). Tamoxifen can be used to treat either primary or secondary breast cancer cases in both females and males. In some cases tamoxifen can also be used to treat progesterone receptor positive (PR+) ER- breast cancer, which is extremely rare, and ductal carcinoma in situ (DCIS), which makes up approximately 12% of breast cancer cases, but tamoxifen treatment for these conditions is less effective (*48, 49*). Tamoxifen has also been shown to reduce the incidence of invasive and non-invasive breast cancer recurrence and breast cancer occurrence in high risk individuals. It can therefore be administered as a preventative treatment for patients with a high incidence of breast cancer occurrence in their family (*50, 51*). Tamoxifen is a cytostatic drug that exerts its anti-proliferative effect on breast cancer cells by acting as an ER antagonist (*52*). This inhibits estrogen signalling and thus reduces the hyper-proliferative activity of ERα. As a prodrug, Tamoxifen is administered orally and then is metabolised in the liver into its active forms, N-desmethyl-tamoxifen and 4-hydroxytamoxifen, by cytochrome p450 enzymes (*53*). N-desmethyl-tamoxifen is the most readily produced metabolite of tamoxifen but 4-hydroxy-tamoxifen is often studied due to its greater anti-estrogenic activity (*54*).

1.4.2 ER

Estrogen receptors are nuclear receptors that are essential for the proper development of many organs including the uterus, ovary, mammary gland, prostate,

lungs and brain (*55*). Notable structural components of ER are its DNA binding domain, hinge region, ligand binding domain and two activation functions (fig.4).
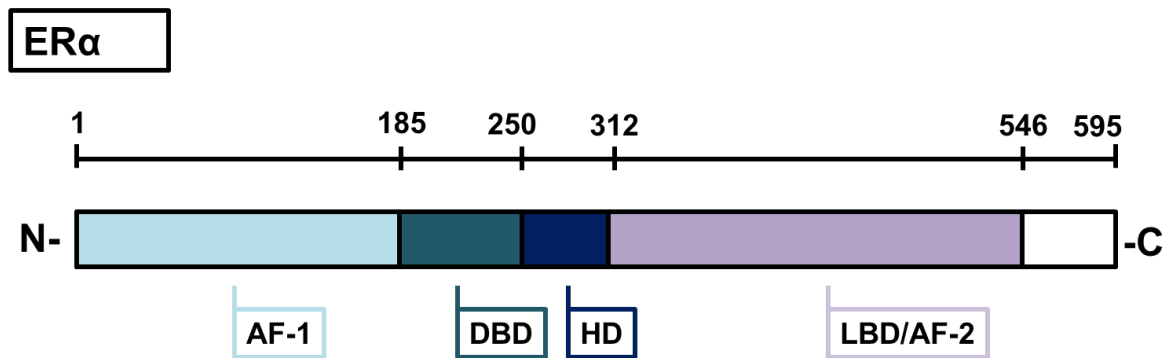
Figure 4 - Structure of human ERα
Notable regions are denoted by: AF-1 (Activation Function 1), DBD (DNA binding domain), HD (Hinge region), and LBD (Ligand Binding Domain). Residue number is indicated by a scale above the diagram.

The DNA binding domain (DBD), located in the centre of the protein, contains two zinc finger motifs and is responsible for recognition and binding of specific DNA sequences (*56*). The hinge region connects the DBD and ligand binding domains and contains a nuclear localisation sequence (NLS) for nuclear translocation (*57*). The two activation domains are found in the N-terminus (AF-1) and the ligand binding domain (AF-2) and are responsible for the recruitment of transcriptional co-regulators to determine activity (*58*). AF-1 is a ligand independent constitutively active domain while AF-2 depends on ligand binding for its activation. AF-2 is found in the C-terminal ligand binding domain (LBD), which is responsible for the binding of ligands such as estrogens and coactivators, as well as ER dimerization (*59*). ERs can be divided into two groups: ERαs and ERβs. The DBDs of the two groups are highly conserved, meaning that their DNA binding affinity and specificity is very similar, but they have variable LBDs, resulting in differing activities (*60*).

1.4.3 ER activation

ERs can be activated by several different mechanisms, some ligand dependent and some ligand independent. The ligand dependent pathways of ER activation require ligands such as estrogens to bind to the AF-2 in the ligand binding domain of the receptors while the ligand independent pathways require kinases, activated by growth factors, to phosphorylate the ER. The most prevalent estrogen in humans is estradiol (E2). In the ligand dependent mechanism of ER activation, a ligand binds to the LBD of the ER, resulting in a conformational change and dissociation of chaperones and other binding proteins from the ER (*61*). This allows the ER to dimerise and translocate in to the nucleus where it exerts its effects on transcription (*62*). The ligand independent pathways of ER activation do not require estrogenic compounds but instead require phosphorylation of the ER. This phosphorylation of ERs can be induced by several different pathways including those of protein kinases (PKA/PKC), growth factor signalling or cyclin dependent kinases (*63-66*). Phosphorylation at various sites within the ER can increase dimerization and cofactor interaction to allow translocation to the nucleus and transcriptional regulation of target genes (*58*).

1.4.4 ER mechanisms of action

In addition to having multiple mechanisms of activation, ERs have multiple mechanisms of function as well. These can be divided into three main groups. The first and most well studied ER mechanism of activity is the classical pathway (fig.6a), in which ER dimers in the nucleus bind to specific DNA sequences called estrogen response elements (EREs) (*58*). Once bound, ER recruits a complex of coactivators, including chromatin remodelling factors such as steroid receptor coactivators (SRCs) and CBP, resulting in the loosening of chromatin and activation of target genes (fig.5) (*67*).
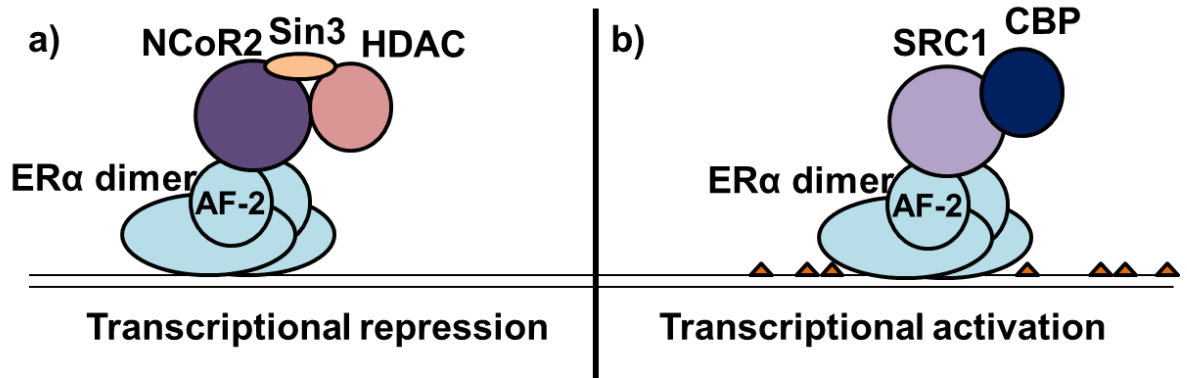
Figure 5 –ERα co-repressor and co-activator complexes
a) ERα (Estrogen Receptor α) binds to target genes and recruits NCoR-2 (Nuclear co-repressor 2), which results in recruitment of corepressors such as HDAC (Histone Deacetylase) to repress transcription. **b)** Ligand bound ERα recruits SRC1 via its LLXXL motif in the AF-2 region. SRC1 recruitment results in the recruitment of coactivators such as CBP (CREB Binding Protein) to acetylate histones and activate transcription.

The second mechanism of ER activity is that of ERE independent genomic action. Similarly to the classical pathways, ER must first dimerise and translocate to the nucleus, but instead of binding directly to DNA via EREs, ER binds indirectly to gene promoters by interacting with other transcription factors (fig.6b) (*68*). This allows the ER to affect transcription of genes that do not contain EREs. The third and final mechanism is that of non-genomic action. In this mechanism, ligand bound ER outside the nucleus produces kinase signalling cascades which can affect the activity of other transcription factors (fig.6c) or even affect already transcribed and translated genes by affecting proteins outside the nucleus (*69*).
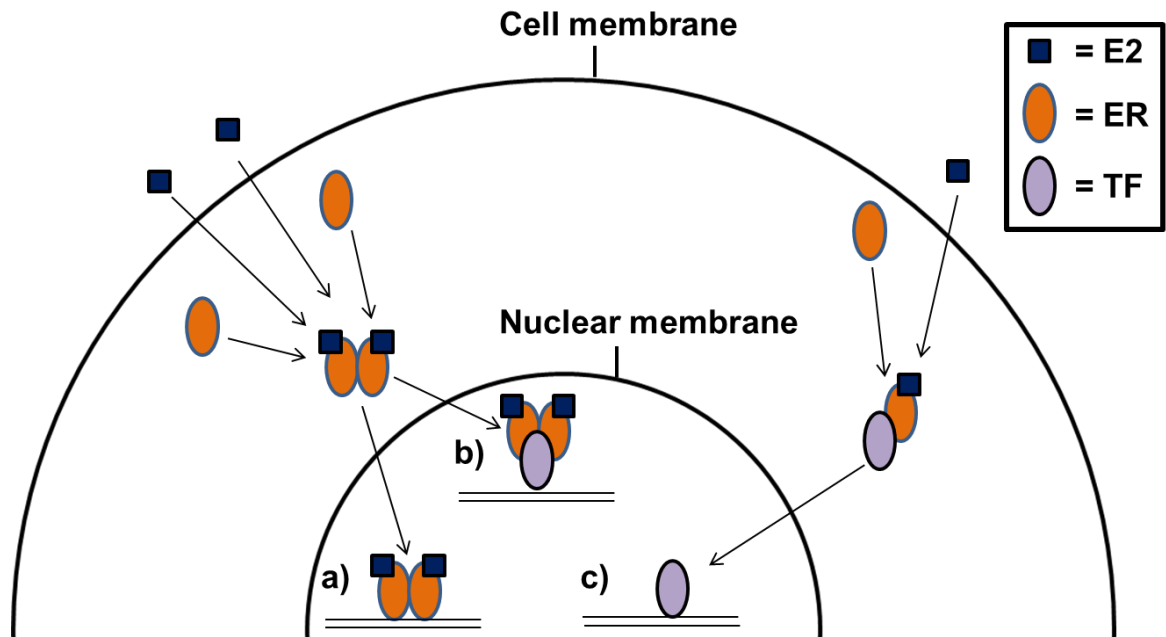
Figure 6 - Estrogen dependent mechanisms of ERα activity
Key to the right of the diagram indicates E2 (estradiol), ER (Estrogen receptor) and TF (other Transcription Factors). **a)** E2-ERα dimer enters nucleus and binds to EREs. **b)** E2-ERα dimer enters nucleus and modulates other transcription factors. **c)** E2-ERα dimer phosphorylates or otherwise modifies other transcription factors in the cytoplasm.

1.4.5 Tamoxifen mechanism in breast cancer

In ER+ breast cancer, in the absence of tamoxifen, estrogen can bind to ERα, forming an activated complex that drives proliferation of the cancer cells (*70*). E2 binding to ERα results in a conformational change, positioning the twelfth helix (H12) of the ERα LBD (fig.3) over the dimerization domain, allowing dimerization and nuclear localization (*71, 72*). This conformation also allows the LBD to bind to transcriptional co-activators via the co-activators LXXLL helix, resulting in transcriptional activation of target genes (*73*). Tamoxifen treatment reduces proliferation of breast cancer cells by competitively inhibiting E2 from binding to ERα. Tamoxifen is similar in structure to E2 and binds to the same region of the ERα LBD, similarly inducing a conformational change that permits dimerization and nuclear localisation. However, Tamoxifen possesses an additional side chain which prevents the proper relocation of H12. H12 is repositioned in such a way that it sterically

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hinders LXXLL helix binding to the LBD, preventing association of co-activators and thus preventing transcriptional activation of target genes (*73*). Instead, TAM-ERα recruits a complex of transcriptional corepressors such as N-CoR (Nuclear Receptor Co-Repressor) and SMRT (Silencing Mediator for Retinoid or Thyroid hormone receptor) (*74*). Since tamoxifen binding doesn't affect DNA binding specificity, this mechanism results in inhibited estrogen signalling and the active repression of ERα target genes.

It should be noted that tamoxifen has been shown to also have some ER independent activity. In ER- cancer cells tamoxifen has been shown in some cases to inhibit protein kinase C (PKC), transforming growth factor β (TGF-β) and cyclin dependent kinases (CDKs) either by direct interaction or by enhancing other inhibitors (*75-77*). However, the effects of tamoxifen on ER- breast cancer cells are variable and tamoxifen is only very rarely used to treat non ER+ breast cancer because of its negative side effects.

1.5 Tamoxifen as an estrogen agonist

1.5.1 Tamoxifen as an estrogen agonist

One side effect of tamoxifen is an increased risk of endometrial cancer later in life. While tamoxifen acts as an ER antagonist in certain tissues such as breast and brain tissue, in other tissues such as the uterus, bone and liver tissue tamoxifen acts as an ER agonist (*78*). This estrogenic activity presents a problem for tamoxifen treatment of breast cancer because the induction of ER activity in the uterus is a risk factor for endometrial cancer formation (*79*). Estrogen replacement therapies have been shown to increase the risk of endometrial cancer and now include progesterone treatment to reduce this symptom (*80*). Long term tamoxifen treatment has similarly been strongly associated with increased incidence of endometrial cancer. The relative risk of endometrial cancer formation in long term tamoxifen treated patients compared with untreated subjects has been shown to increase 1.2-19.4 fold, depending on duration of treatment (*81*). The level of increased risk of endometrial cancer formation has also been shown to increase with duration of tamoxifen treatment (*81*). The frequency of endometrial cancer formation, however, is still relatively low. The most recent statistics from cancer research UK place frequency of endometrial cancer incidence at 0.04-0.1% of the European female population depending on age group (*82*). In tamoxifen treated patients, therefore, this can increase to as much as 2%. For this reason, despite the risks, tamoxifen is still used to treat the majority of ER+ breast cancer patients because the benefits to survival of patients with breast cancer and the reduction in recurrence outweigh the risk of endometrial cancer later in life (*81*).

1.5.2 Endometrial cancer

Endometrial cancer is a cancer of the inner epithelial lining of the uterus. This lining separates the inner uterine cavity from the myometrium (*83*). The endometrium itself is made up of two layers: the functionalis (adjacent to the uterine cavity) and the basalis (adjacent to the myometrium) (*83*). The functionalis is the hormone responsive layer of cells that thickens in response to estrogen and sheds in response to progesterone during the menstrual cycle. The basalis effectively forms the base of the endometrium and it is from this base that the functionalis regenerates (*83*). Tamoxifen is associated with endometrial cancer formation, tumour stage progression and poor prognosis but is still commonly used to treat breast cancer because the benefits outweigh the risks (*81*). Analysis of the mortality rates among tamoxifen induced endometrial cancer cases has produced varying results but it is now thought that mortality is higher than in non-tamoxifen induced endometrial cancer (*84*).

1.5.3 ER independent endometrium tamoxifen mechanism

There are many proposed mechanisms for tamoxifen's effect on endometrial carcinogenesis and stage progression but the exact processes are not known for certain. This could be, in part, due to the majority of gene expression studies investigating the short term effects of tamoxifen treatment, while endometrial cancer risk is associated with very long term tamoxifen use. In addition, Tamoxifen appears to modulate gene expression in endometrial cells by a multitude of different mechanisms, some ER dependent and some ER independent. Substantial crossover between tamoxifen and estradiol induced gene expression changes has been observed in endometrial cells but tamoxifen is also known to regulate other genes, independent of the ER (*85*).

One proposed mechanism of ER-independent tamoxifen associated endometrial carcinogenesis is the increased rate of DNA damage induced by tamoxifen treatment. Some tamoxifen metabolites have been shown to form DNA adducts but their role in endometrial carcinogenesis has not yet been determined (*86, 87*).

Another proposed mechanism is alteration of the UPR (Unfolded Protein Response) and mTOR (Mammalian Target of Rapamycin) signalling pathways. In healthy cells, the UPR pathway activates in order to control apoptosis in response to endoplasmic reticulum related stress but this process can often be inappropriately activated in breast cancer cells in response to long term endocrine cancer therapy (*88*). In endometrial cells, tamoxifen has been shown to increase expression of BiP (Binding Immunoglobulin Protein), an endoplasmic reticulum stress response chaperone, which could suggest that the UPR pathway can be over-activated in endometrial cells as well (*87*). In addition, in breast cancer cells, BiP is known to regulate expression of mTOR (*89*) which has also been associated with tamoxifen induced endometrial cancer (*90*).

Tamoxifen can also modulate the expression of the ER gene but results have been highly variable, with some studies indicating an upregulation of ERα in the endometrium and others indicating downregulation (*81, 91*). One isoform of ERα, ERα-36, has been shown to be overexpressed in endometrial cancer cells and can activate the MAPK/ERK and PI3K/Akt pathways in the presence of tamoxifen. Activation of these pathways affects differentiation and apoptosis, as well as reducing the control of expression of c-myc, a potent driver of cell proliferation (*92*).

1.5.4 ER dependent endometrium tamoxifen mechanism

In addition to these non-estrogenic mechanisms, tamoxifen is known to modulate ER activity in endometrial cells. In breast cancer cells, tamoxifen functions

by acting as an ER antagonist but in endometrial cells tamoxifen acts more as an ER agonist. It is not fully understood why tamoxifen acts as an agonist in endometrial cells but it has been shown that TAM-ER recruits a very different set of co-modulators in endometrial cells compared with breast cells. While in breast cells TAM-ER recruits co-repressors such as N-CoR and SMRT, in endometrial cells, TAM-ER recruits coactivators such as SRC-1 and CBP (*93*). This co-activator complex bears more similarity to that which is recruited by the E2-ER complex in breast cells. It is therefore possible that differing expression levels and activities of TAM-ER coactivators and corepressors between the two cell types could be responsible for some of the opposing effects on the ER.

1.5.5 BASP1 novel ER modulator

As previously stated, BASP1 and ERα have been shown to co-localise in the nucleus of breast cancer cells. It was also shown by RNA sequencing that 40% of the genes regulated by tamoxifen were BASP1 dependent. This was then confirmed by Chromatin Immuno-precipitation (ChIP) of four of the regulated genes: XBP1, Rab31, Serpina3 and PGR. Both BASP1 and ERα were detected at the promoters of these ER target genes (*44*). These results indicated that BASP1 could be a novel ER cofactor in breast cancer cells which affects TAM-ER activity. As BASP1 is also expressed in the endometrium (*1*), it is possible that BASP1 functions differently in the two cell types which could potentially contribute to the differences in TAM-ER function between the cell types.

1.6 Research aims

BASP1 has recently been shown to act as an ERα cofactor and tumour suppressor in breast cancer cells (*44*). In breast cancer treatment, tamoxifen is often used to inhibit the hyper-proliferative effects of ERα (*47*). While tamoxifen exhibits ER antagonist activity in breast cells, it exhibits mostly ER agonist activity in endometrial cells (*78*). BASP1 is normally expressed in the adult endometrium but ceases to be expressed in the adult breast after puberty (*1, 94*). This suggests that BASP1 has different functions in the two tissue types. It is also possible that BASP1 has roles in tamoxifen response in endometrial cells as well as breast cancer cells.

The aim of this project was to investigate the effects of BASP1 expression in endometrial cancer cells. Ishikawa cells, an ER+ endometrial adenocarcinoma cell line, were used to produce stable cell lines in which BASP1 expression was manipulated. These cell lines were then used to investigate the effects of BASP1 expression on proliferation, gene expression and response to tamoxifen treatment in endometrial cancer cells.

The first specific objective of the project was to examine the effects of changing BASP1 expression on growth and survival of Ishikawa cells. Growth assays and colony formation assays using Ishikawa cells were performed to examine whether BASP1 possesses tumour suppressor activity in Ishikawa cells. BASP1 has been shown previously to possess tumour suppressor activity in breast cancer cells (*44*). Since BASP1 is not normally expressed in adult breast tissue, it is likely that BASP1 becomes reactivated upon transformation of the breast cells. The purpose of this phenotype investigation is to determine whether BASP1 possesses this same tumour suppressor activity in endometrial cancer cells, or whether its functions are different, (as the difference in expression between the two adult tissues indicates).

The second specific objective of the project was to examine the effects of changing BASP1 expression on Ishikawa cell gene expression. BASP1 has been shown to act as a cofactor for transcription factors WT1 and ERα in other tissues (*17, 44*). For this reason, the investigation of BASP1 dependent gene expression changes in Ishikawa cells was focused on ERα and WT1 target genes.

The third and final specific objective of the project was to examine the effects of changing BASP1 expression on tamoxifen response in Ishikawa cells. Since BASP1 has been shown to modulate approximately 40% of tamoxifen induced gene expression changes in breast cancer cells, it was hypothesised that BASP1 could possibly be able to modulate tamoxifen induced gene expression changes in endometrial cancer cells as well. This was investigated using growth assays, colony formation assays and qPCR of tamoxifen treated and non-treated cells.

2 Materials and methods

2.1 Materials

2.1.1 Materials

The following chemicals and reagents were bought from Sigma Aldrich

Ishikawa cells, Foetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), 4OH-Tamoxifen (TAM), Ethanol, Methanol, Crystal violet, Bovine Serum Albumin (BSA), Triton and Paraformaldehyde (PFA).

The following chemicals were bought from Thermo Fisher Scientific/Gibco

Phosphate Buffered Saline (PBS), Pierce ECL Western Blotting Substrate, CL-Xposure film, SYBR green assay reagents, agarose, Tris-Acetate-EDTA (TAE) buffer, Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, Penicillin Streptomycin (PS), L-Glutamine (LG), spectra BR, filter paper, 6X DNA loading dye, LR DNA ladder gene ruler, TWEEN, Tris and Ammonium Persulphate (APS).

The following chemicals were bought from Invitrogen/Ambion

One-shot top10 chemically competent e-coli, pcDNA3 plasmids, pSilencer plasmids, MagG dynabeads, proteinase K and RNase A

Other products were bought from the following manufacturers

MCF7 cells and K562 cells were bought from the European Collection of Authenticated Cell Cultures (ECACC). Effectene transfection reagent, QIA-prep Spin Mini-prep Kit 250, RNeasy kit and QIA-Quick PCR purification kit were from QIAGEN. Immobilon-P membranes, NP40 and protease inhibitor cocktail set III were from Merck Millipore. Bradford 1X dye reagent, iScript reagents and acryl-bis mix were from Bio-Rad. NaCl and ethidium bromide were from VWR Prolabo Chemicals. PCR primers were from Integrated DNA technologies (IDT). Other reagents include

TEMED (Flowgen Bioscience), MgCl$_2$ (Acros Organics), molecular biology water (Pannreac AppliChem), glycine (Promega), and G418 (Melford).

2.1.2 Antibodies

ERα HC-20 (sc-543) and Lamin A/C (E-1) antibodies were from Santa Cruz (Santa Cruz, CA, USA). β-Actin (A5316) antibodies were from Sigma Aldrich (St Louis, MO, USA). β-tubulin (10068-I-AP) antibodies were from Protein-tech (Manchester, UK). Secondary antibodies, anti-rabbit (32260) and anti-mouse (32230), were from Thermo Fisher Scientific (Paisley, UK). WT1 and BASP1 antibodies were from SAPU (Edinburgh, UK). Normal rabbit IgG antibody (12-370) was from Merck Millipore (Burlington, MA, USA).

2.2 Solutions and buffers

Blocking buffer: 50mM Tris, 150mM NaCl, 0.05% (v/v) TWEEN, 1% (w/v) BSA.

Cell signalling lysis buffer: 20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% (v/v) Triton.

Cytoplasmic lysis buffer: 10mM Tris-HCl pH8.1, 10mM NaCl, 1.5mM $MgCl_2$, 0.5% (v/v) NP40.

High salt ChIP buffer: 500mM NaCl, 50mM Tris-HCl pH 8.0, 5mM EDTA, 0.5% (v/v) NP40, 1% (v/v) Triton X-100.

LiCl buffer: 10mM Tris-HCl pH 8.0, 250mM LiCl, 1mM EDTA, 1% (v/v) NP40, 1% (w/v) sodium deoxycholate.

Low salt ChIP buffer: 150mM NaCl, 50mM Tris-HCl pH8.0, 5mM EDTA, 0.5% (v/v) NP40, 1% (v/v) Triton X-100.

PK buffer: 125mM Tris-HCl pH 8.0, 10mM EDTA, 150mM NaCl, 1% (w/v) SDS.

Resolving gel: 375mM Tris pH 8.8, 10% (w/v) acryl/Bis gel mix, 0.1% (w/v) SDS.

Stacking gel: 125mM Tris pH6.8, 3.75% (w/v) acryl/Bis gel mix, 0.1% (w/v) SDS

TE buffer: 10mM Tris-HCl pH 8.0, 1mM EDTA.

Transfer buffer: 20% (v/v) methanol, 1X Tris-Glycine (25mM Tris, 250mM Glycine)

2.3 Tissue culture

2.3.1 Ishikawa cells

Ishikawa cells were bought from Sigma. Ishikawa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) from Gibco (41966-029) with 5% (v/v) Foetal Bovine Serum (FBS) (Sigma) and 1% (v/v) Penicillin Streptomycin (PS) (Gibco) incubated at $37^{\circ c}$ and 5% CO_2.

2.3.2 MCF7 cells

MCF7 cells were bought from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Stable MCF7 cells used were set up in our laboratory by transfection with pSilencer plasmids as detailed previously (*44*). MCF7 stable cell lines were grown in DMEM (Gibco) with 10% (v/v) FBS (Sigma), 1% (v/v) PS (Gibco) and 700µg.mL^{-1} G418 from Melford (Ipswich, UK), incubated at $37^{\circ c}$ and 5% CO_2.

2.3.3 K-562 cells

K562 cells were bought from ECACC. Stable K562 cells used were established by transfection with pcDNA3 plasmids as detailed previously (*17*). K562 stable cell lines were grown in Roswell Park Memorial Institute (RPMI) medium (Gibco, 31870-025) with 10% (v/v) FBS (Sigma), 1% (v/v) PS (Gibco) and 1% (v/v) L-Glutamine (LG) (Gibco).

2.4 Stable transfection of Ishikawa cells

2.4.1 E-coli culture

Liquid LB medium used was prepared in house by the University of Bristol media preparation laboratory. Transformation competent E-coli cells were One-shot top10 chemically competent e-coli, bought ready to use from Invitrogen (C404006) (Carlsbad, CA, USA).

2.4.2 Preparation of DNA

Stable Ishikawa cell lines were set up using pcDNA3 plasmids (Invitrogen) and pSilencer plasmids (5765G) from Ambion (Invitrogen). The B-Ishikawa cell line was set up using pcDNA3 plasmids containing a BASP1 expressing region as detailed previously (*17*). The sh-BASP1 cell line was set up using pSilencer plasmids containing an sh-RNA expression region which targets BASP1 as detailed previously (*44*). Before transfection, pSilencer plasmids were expanded by transformation of competent E. coli cells. Ligation DNA was added at a volume of 3µL to 200µL of competent cell E. coli and kept on ice for 30 minutes. Samples were heat shocked at 42˚C for 2 minutes and after cooling were spread onto 100 µg/mL ampicillin plates and incubated at 37˚C overnight. Plasmids were isolated from samples using a QIA-prep Spin Mini-prep Kit 250 from QIAGEN (Hilden, Germany).

2.4.3 Ishikawa cell Transfection

Ishikawa stable cell lines were set up using pcDNA3 (Invitrogen) or pSilencer 2.1 neo (Ambion) vectors detailed in section 2.4.2. Transfection was conducted using QIAGEN effectene transfection reagent. 10^6 cells were seeded in 10cm plates and incubated for 24 hours before treatment with transfection complexes for 48 hours. Media was then changed to DMEM (as described in section 2.3.1) with 1mg/mL G418 disulphate (Melford).

2.5 Immunoblots

2.5.1 Western blotting

Western blots were performed with either whole cell samples or nuclear and cytoplasmic fractions mixed with SDS loading dye. For whole cell samples, cells were extracted by scraping on ice and harvested by centrifugation at 1400 RCF for 3 minutes before being re-suspended in PBS (Fisher). The appropriate volume of loading dye was added and the samples were then heated to $95^{\circ c}$ for 10 minutes. Nuclear and cytoplasmic fractionation is detailed in section 2.5.2.

Gels used consisted of a resolving gel and a stacking gel (see section 2.2). To 12mL of resolving gel, 30μL APS (10% (w/v) Ammonium Persulphate) and 30μL TEMED from Flow-gen Bioscience (Nottingham, UK) were added before being poured between glass plates. Water was poured above the gel to prevent surface dehydration and ensure that it remained level. The resolving gel was allowed to polymerise for approximately 25 minutes and water was poured away before 8mL of stacking gel, with 30μL APS and 30μL TEMED, was added. This was allowed to polymerise, with a gel comb inserted, for approximately 18 minutes. The gels were then placed in a BIO-RAD Mini-Protean Tetra system gel tank (BIORAD) which was filled with 10X SDS PAGE running buffer. To each well, 10-15μL of cell samples were added and resolved against 5μL Spectra BR marker (Thermo). Gels were electrophoresed at 100V for 15 minutes and then at 220V for approximately a further 30 minutes. Proteins were transferred from the gel to an Immobilon-P membrane (Mercks) using a BIO-RAD TRANS-BLOT Semi Dry transfer cell. Gels were placed on a membrane soaked in methanol, in between two pieces of filter paper (Thermo) soaked in transfer buffer (see section 2.2). Transfer was performed at 25V for 45 minutes.

The membrane was washed in 10mL blocking buffer (see section 2.2), on a rocking platform, for 1 hour at room temperature. This buffer was then removed and appropriate volumes of primary antibodies (see section 2.1) were added in 10mL fresh blocking buffer. The membranes were then incubated with antibodies on a rocking platform at 4°C overnight. Antibody buffer was removed and the membrane was washed in 10mL PBST on a rocking platform for 5 minutes. This process was repeated 3 times. Membranes were then incubated with 10mL blocking buffer containing 1/5000 secondary antibody on a rocking platform for 1 hour. Membranes were then washed a further 3 times in PBST. Membranes were then soaked in Pierce ECL Western Blotting Substrate (Fisher) and exposed to CL-Xposure film (Fisher).

2.5.2 Nuclear and cytoplasmic fractionation

Ishikawa cell samples were extracted by scraping on ice and harvested at 1400 RCF for 3 minutes before being re-suspended in 500μL cytoplasmic lysis buffer (see section 2.2) and held on ice for 15 minutes. Samples were centrifuged at 13000 RCF at 4^{oc} for 5 minutes, forming a pellet* and a supernatant. The supernatant was transferred to a new tube, centrifuged for a further 10 minutes and the resulting supernatant was again transferred to a new tube, producing the cytoplasmic fraction. The pellet* was re-suspended in 500μL cytoplasmic lysis buffer, held on ice for a further 15 minutes and then centrifuged again at 13000 RCF. The resulting pellet was then re-suspended in cell signalling lysis buffer (see section 2.2), with 0.5% (w/v) SDS added before use, and held on ice for 10 minutes. This was centrifuged at 13000 RCF at 4^{oc} for 10 minutes and the resulting supernatant was transferred to a new tube, producing the nuclear fraction. Protein concentrations of the fractions were equalized based on their absorbance at 595nm which was measured in an Ultrospec 3000pro spectrophotometer using quick start Bradford 1X dye reagent (BIORAD).

Fractions were then diluted in the appropriate volume of PBS (Fisher) and SDS

loading dye.

2.6 RNA analysis

2.6.1 RNA and cDNA preparation

Ishikawa cells were extracted by scraping on ice and harvested at 1400 RCF for 3 minutes before being re-suspended in 1mL PBS (Sigma). RNA was isolated from cells using an RNeasy kit from QIAGEN and cDNA was prepared from this using the iScript cDNA synthesis kit from BIORAD. The volume required for 1μg cDNA was measured and mixed with 4μL 5X iScript reaction mix, 1μL of reverse transcriptase (or molecular biology water for NRT controls) and enough water to make the mixtures up to 20μL. This was placed in a TC-3000 thermocycler from TECHNE, which incubated the mixtures at 25°C for 5 minutes, followed by 42°C for 30 minutes and then 85°C for a further 5 minutes.

2.6.2 Real Time PCR

Real time PCR was performed using a BIORAD Mini Opticon system and SYBR Green assay reagents (Fisher). Each well was made up to 20μL with 10μL SYBR green (Fisher), 7μL molecular biology water (Panreac AppliChem), 1μL 10μM oligonucleotide primers and 2μL cDNA. PCR primers used were from Integrated DNA Technologies (Coralville, IO, USA). Primer sequences were as follows: GAPDH forward 5′-GAAATCCCATCACCATCTTCCAGG-3′ and reverse 5′-GAGCCCCAGCCTTCTCCATG-3′; XBP1 forward 5′-GCGCCTCACGCACCTG-3′ and reverse 5′-GCTGCTACTCTGTTTTTCAGTTTCC-3′; Rab31 forward 5′-CGAGCACATGATGGCGATACG-3′ and reverse 5′-GTCCTTCAGCAGTGCACAGGA-3′; PGR forward 5′-CGCGCTCTACCCTGCACT-3′ and reverse 5′-TGAATCCGGCCTCAGGTAGTT-3′; TFF1 forward 5′-

CATCGACGTCCCTCCAGAAGAG-3' and reverse 5'-

CTCTGGGACTAATCACCGTGCTG-3'; Greb1 forward 5'-

CAAAGAATAACCTGTTGGCCCTGC-3' and reverse 5'-

GACATGCCTGCGCTCTCATACTTA-3'; VDR forward 5'-

CTGACCCTGGAGACTTTGAC-3' and reverse 5'- TTCCTCTGCGAGACTTTGAC-3'.

2.7 Colony formation assays

2.7.1 Cell seeding and growth

Stable Ishikawa cell lines (V-Ishikawa, B-Ishikawa, shNeg Ishikawa, shBASP1 Ishikawa), detailed in section 2.4, were seeded in 2mL DMEM growth media (with G418 as in section 2.3.1) at 500 cells per well. Cells were counted before seeding using an Invitrogen Countess automated cell counter. After incubating (as in section 2.3.1) for 24 hours, the cells were treated with 100nM tamoxifen (Sigma) or vehicle control ethanol (Sigma). Cells were incubated with 100nM tamoxifen media for 72 hours before media was changed to fresh DMEM growth media (without tamoxifen). Cells were grown for a further 9 days before fixation.

2.7.2 Cell fixing

The media was removed and plates were washed with 2mL PBS (Sigma) before being fixed with 1mL methanol for 10 minutes. Methanol (Sigma) was then aspirated and the plates were stained in 0.5% (w/v) crystal violet (Sigma) with 25% (v/v) methanol (Sigma) for a further 10 minutes. Crystal violet was then removed and plates were washed with distilled water before being photographed. Colonies visible with the naked eye were counted and used to calculate colony forming efficiencies.

2.8 Growth assays

Ishikawa stable cell lines (V-Ishikawa, B-Ishikawa, shNeg Ishikawa, shBASP1 Ishikawa), detailed in section 2.4, were seeded onto 6cm plates at 2×10^{5} cells per plate. Cells were seeded in 4mL DMEM media (with G418 as in section 2.3.1) per plate. Cells were incubated for 24 hours before being treated with 100nM tamoxifen (sigma) or vehicle control ethanol (sigma) for the remainder of the assay. Total plate cell number was counted, using an Invitrogen countess automated cell counter, every 24 hours for 96 hours and results were used to plot growth curves.

2.9 ChIP analysis

2.9.1 Cell sample fixing

ChIP assays were performed on Ishikawa cells. 5µL protein magG dynabeads (Invitrogen) were mixed with 0.6mL low salt IP buffer (see section 2.2) and 1mL 10mg/mL Bovine Serum Albumin (BSA) Sigma in three tubes. Anti IgG, BASP1 and ERα antibodies were added to the separate tubes. Antibody-bead mixtures were rotated at $4^{\circ c}$ for a minimum of 4 hours *. $1X10^7$ Ishikawa cells were extracted per ChIP experiment and centrifuged. Cells were re-suspended in PBS (Fisher) to make a concentration of $1X10^6$cells.mL^{-1}. 40µL 37% (v/v) PFA (Sigma) was added per mL of sample and the resulting mixture was then incubated on a rocker at room temperature for 15 minutes. 141µL.mL^{-1} of 1M ice cold glycine (Promega) was added and incubated as before for a further 5 minutes. Cells were harvested, supernatant was removed and pellet was re-suspended in PBS.

2.9.2 Sonication

The resulting sample (see 2.10.1) was harvested again, the supernatant was removed and the pellet was re-suspended in 1mL low salt ChIP buffer (see section 2.2) with 1X protease inhibitor (Calbiochem). This was held on ice for 15 minutes before being centrifuged at 4000RCF at $4^{\circ c}$ for 5 minutes. The resulting pellets were then re-suspended in 1mL low salt ChIP buffer (see section 2.2) with 1X protease inhibitor (Calbiochem) and subjected to sonication. Sonication was performed using a QSONICA sonicator. Sonication settings were varied (see section 4.11) but in all cases the probe was inserted just over halfway into the sample and was not allowed to touch the edges of the tube **.

2.9.3 Agarose gel electrophoresis

For sonication optimisation, DNA fragment size was measured using agarose gel electrophoresis. After sonication ** (see 2.10.2) 20µL samples of the sonicated mixtures were added to 30µL of PK buffer (see section 2.2) and 1µL RNase A (Invitrogen). Samples were incubated at 37^{oc} for 30 minutes. To each of the samples, 1µL proteinase K (Ambion) was added, and these were then incubated at 62^{oc} for 2 hours. Following de-crosslinking 5µL of each sample was added to 1µL 6X DNA loading dye (Thermo) and resolved on an agarose gel alongside gene ruler low range DNA ladder (Thermo). The agarose gel was made up with 1% agarose (Fisher). Gels were run in 1X Tris-Acetate-EDTA (TAE) buffer (Fisher) at 120V for approximately 40 minutes in a BIO-RAD Mini-sub Cell GT. After electrophoresis, gels were placed in 1X TAE buffer (Fisher) with 1/10000 ethidium bromide (VWR Prolabo). Gels were then visualised using a GelDoc-It TS Imaging System.

2.9.4 ChIP protocol

After sonication ** (see 2.10.2) samples were centrifuged at 12000RCF for 10 minutes at 4^{oc} and the resulting supernatant was transferred to new tubes. These were each mixed with 10µL protein magG dynabeads (Invitrogen), and then rotated at 4°C for 1 hour. Sample-bead mixtures were magnetised for 1 minute to harvest the beads and 200µL of the remaining sample was added to each of the bead-antibody mixtures * (see 2.10.1). To an empty tube, 4µL of the remaining sample was added, to be used as a pre-clear, and stored at -20°C. The sample-bead-antibody mixtures were rotated overnight at 4^{oc}.

Sample-bead-antibody mixtures were magnetised for at least 1 minute, the remaining liquid was removed, and the beads were re-suspended in 1mL ice cold low Salt IP buffer (see section 2.2) without inhibitors. The resulting mixture was held on

ice for 3 minutes. This was repeated with high salt ChIP buffer (see section 2.2), followed by LiCl buffer (see section 2.2), and then by TE buffer (see section 2.2). TE buffer samples were magnetized for at least one minute and the remaining liquid was removed. Each of the resulting bead samples and the pre-clear from -20$^{\circ c}$ storage were separately re-suspended in 100µL PK buffer (see section 2.2) and incubated at 65°C overnight.

2.9.5 DNA purification and PCR

Beads were re-suspended by vortexing and 1µL 20mg/mL Proteinase K (Ambion) was added before incubating at 55$^{\circ c}$ for 3-4 hours. Following incubation, the samples were centrifuged at 13000 RCF for 5 minutes and supernatants were transferred to new tubes. DNA was extracted using a QIA-Quick PCR purification kit (QIAGEN) and DNA samples were incubated at 95˚C for 10 minutes. The resulting DNA samples were then used to perform qPCR analysis (as described in section 2.7.2) to quantify the extent of protein binding to target genes relative to BAX21nt. The following ChIP primers were used: PGR forward 5′-AATGAGGCTGACATTCTGGGA-3′ and reverse 5′-GTTGACCTCATTCCAAGGCAG-3′; Greb1 forward 5'-TCTGAAGGGCAGAGCTGATAA-3' and reverse 5'-GAATGACCCAGTTGCCACAC-3'; BAX21nt forward 5'-TTCTGACGGCAACTTCAACTG-3' and reverse 5'-CTTGGTGCACAGGGCCTTGAG-3'.

3 Characterisation of Ishikawa cells

3.1 Introduction

As described earlier (see section 1.3.4) a previous publication from our laboratory established that, in MCF7 breast cancer cells, BASP1 interacts with and colocalises with ERα in the nucleus. In addition, it was revealed that BASP1 was required for approximately 40% of gene expression changes induced by Tamoxifen treatment in these cells (*44*). This indicated that BASP1 could be a novel ER modulator capable of affecting cancer cell response to tamoxifen. It is therefore possible that BASP1 activity or function is different in endometrial cancer cells from breast cancer cells and that this could potentially contribute to the difference in tamoxifen response between the cell types. In order to test this possibility, the response of ER+ endometrial cells to differing levels of BASP1 expression and tamoxifen treatment was investigated. This study used Ishikawa cells (see section 2.3.1), an ER+ endometrial adenocarcinoma cell line, to produce stable cell lines that either over or under express BASP1. In order to investigate BASP1 function in these cells, it was first necessary to confirm the expression of BASP1, ERα and WT1. WT1 was included because BASP1 is known to act as a transcriptional co-suppressor for WT1 in other cell types (see section 1.2.3) (*17*).

In addition to the interaction between BASP1 and ERα that has recently been observed in MCF7 cells (Marsh), BASP1 has been found to exhibit tumour suppressor activity in multiple other cancer cell types (*40-42*) and also to exhibit tumour promoting activity in cervical cancer cells (*43*). It is currently not known by which mechanisms BASP1 exerts this activity. It is possible that the BASP1-ERα interaction also occurs in other cancer cell types than breast cancer cells, but it is also possible that BASP1 exerts these effects by interaction with WT1 or by novel, presently unknown mechanisms of action. For this reason, it was of interest to observe the changes in phenotype induced by changes in BASP1 expression as well as the changes in gene expression.

The experiments presented in this chapter were conducted to confirm the expression of BASP1, ERα and WT1 in Ishikawa cells and investigate the subcellular localisation of BASP1 and ERα. In addition, qPCR analysis of the Ishikawa cell response to E2 and tamoxifen treatment was conducted to confirm that tamoxifen acts as an ER agonist in these cells, in accordance with the literature on the subject (*95*). Finally, growth assays and colony formation assays of Ishikawa stable cell lines were conducted in order to determine the effects of BASP1 expression on Ishikawa cell growth and survival.

3.2 Ishikawa cell expression of BASP1, WT1 and ERα

Ishikawa whole cell extracts were subjected to immunoblotting to determine the expression levels of BASP1, ERα and WT1 in the cells. Whole cell extracts were prepared from several cell lines as follows. MCF7 cells that contain a negative control shRNA (sh-Neg MCF7), MCF7 cells that contain an shRNA that targets BASP1 (sh-BASP1 MCF7), Ishikawa cells, K562 cells that contain a control pcDNA3 vector (V-K562) and K562 cells that contain a pcDNA3 vector driving expression of BASP1 (B-K562) were subjected to immunoblotting. Samples were probed for β-actin, BASP1, ERα and WT1. β-actin served as a control to demonstrate the similar overall protein concentrations between samples. V-K562 cells served as a negative control as they do not express BASP1. B-K562 cells served as a positive control as they highly express C-terminal HA tagged BASP1. The results confirmed that Ishikawa cells express BASP1, ERα and WT1.
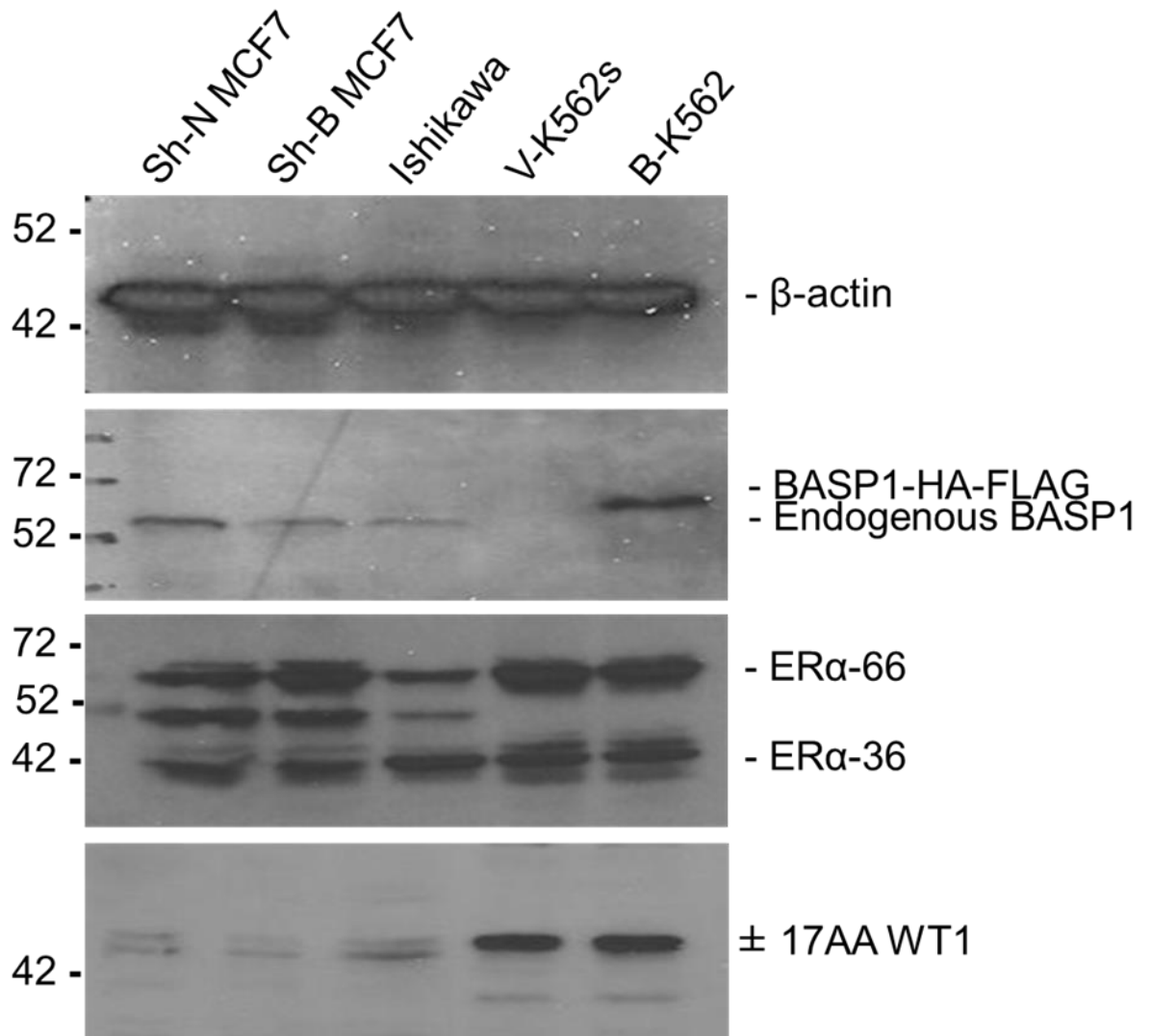
Figure 7 - Immunoblotting of Ishikawa cells for BASP1, ERα and WT1.
BASP1, ERα and WT1 are all expressed by Ishikawa cells. Whole cell extracts of shNeg MCF7, shBASP1 MCF7, Ishikawa, V-K562 and B-K562 cell lines were used to perform western blots. Cell samples were immunoblotted with antibodies for β-actin, BASP1, ERα and WT1. β-actin served as a control to ensure similar overall protein concentration between samples. Molecular weight markers are shown to the left of the blots (kDa). The blots shown are representative of 3 independent experiments. B-K562 cells contain a pcDNA3 vector that expresses BASP1 with HA and FLAG tags which cause the protein to run slower on the gel. BASP1 expressed in MCF7 and Ishikawa cells is endogenous BASP1. Three isoforms of ERα are expressed in MCF7 and Ishikawa cells, including ERα-36 and ERα-66. Both +17AA and -17AA WT1 isoform bands are clearly visible in MCF7s and Ishikawa cells.

Figure 7 shows that Ishikawa cells express BASP1, ERα and WT1. While

BASP1 expression appears lower in the Ishikawa cells than in MCF7 cells, the β-

actin concentration is clearly higher in the MCF7 cell line samples so this comparison

cannot be drawn. In addition to the similar expression of BASP1 between the cell

types, BASP1 also has similar mobility in both Ishikawa cells and MCF7 cells. As expected, BASP1 in the B-K562 cell lane has reduced mobility compared with the endogenous BASP1 expressed in the other cell types. This reduced mobility is due to the presence of C-terminal HA and FLAG tags in BASP1 that is expressed from pcDNA3 vectors. WT1 expression is also similar in both MCF7 cells and Ishikawa cells. Both the 17AA+ and 17AA- isoforms of WT1 are clearly visible in Ishikawa cells and MCF7s. This can be compared to the K562 cell lines which predominantly express the 17AA+ isoform, as indicated by the strong lower motility WT1 bands in these wells. ERα expression and motility was also similar between MCF7 cells and Ishikawa cells. The presence of slower migrating ERα-66 and faster migrating ERα-36 bands are clearly visible in both cell types, in addition to a third ERα isoform with intermediate mobility. This could be one of two more known isoforms, ERα-47 or ERα-53. ERα expression was also detected in K562 cells, and this result was reproducible. This was not anticipated and has not been reported before. However, the presence of the additional third ERα isoform found in Ishikawa cells and MCF7 cells was not detected in K562 cells. Taken together, these results confirm that Ishikawa cells express BASP1, ERα and WT1 and indicate that the expression and mobility of these proteins in Ishikawa cells is similar to that of MCF7 cells.

3.3 Subcellular localisation of BASP1 and ERα

It was previously shown in our laboratory that BASP1 was located in both the nucleus and the cytoplasm of MCF7 cells and K562 cells (*16, 44*). Nuclear and cytoplasmic fractionation was performed to determine the intracellular localisation of BASP1 and ERα in Ishikawa cells. Overall protein concentrations of subcellular fractions were equalized based on absorbance at 595nm, measured by Bradford assays. The fractions were then immunoblotted using antibodies for LAMIN A/C, β-tubulin, BASP1 and ERα. Lamin A and Lamin C are proteins found exclusively in the nucleus and served as a control to monitor any nuclear contamination of the cytoplasmic fraction. β-tubulin is found exclusively in the cytoplasm and served as a control to evaluate cytoplasmic contamination of the nuclear fraction.
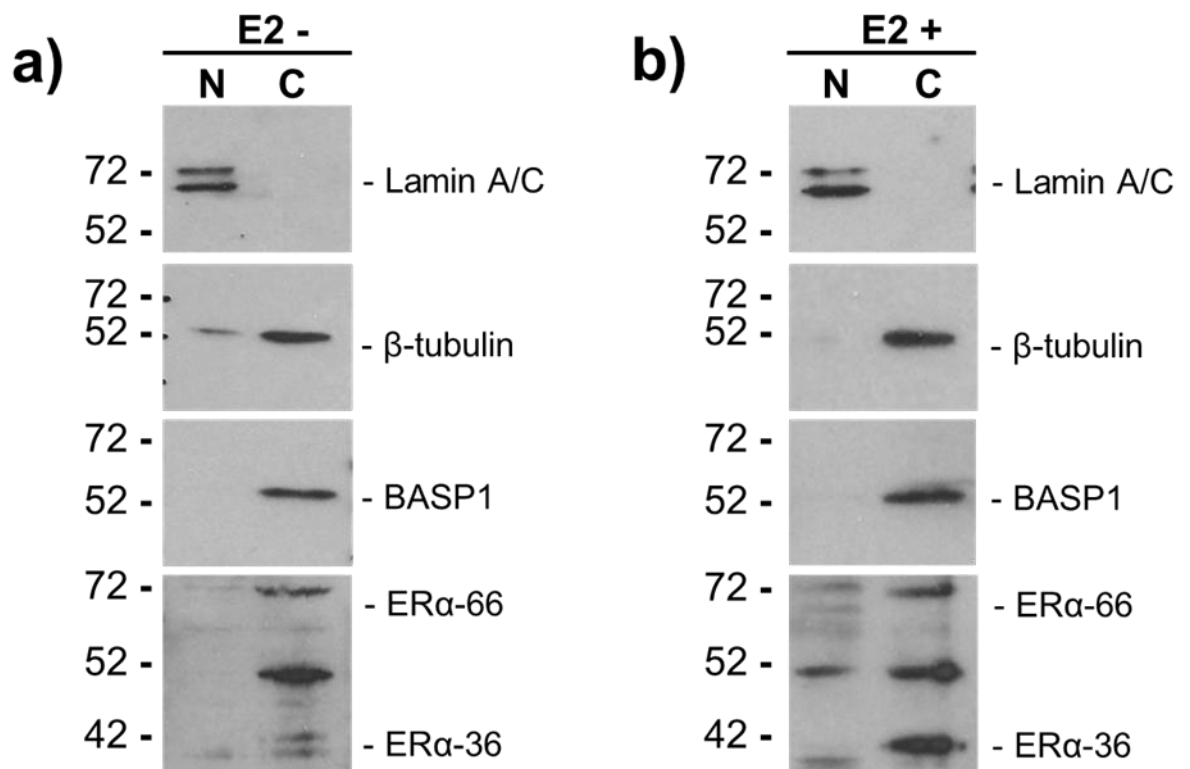
Figure 8 - Subcellular fractionation and immunoblotting, for BASP1 and ERα, of Ishikawa cells +/- E2.
Ishikawa cells were incubated for 24 hours with either 100nM E2 or the equivalent volume of the vehicle control, H$_2$O. Treatment is indicated above the figure by either E2- (H$_2$O treated) or E2+ (E2 treated). Cells were collected, nuclear and cytoplasmic fractionation was conducted and overall protein concentrations of the fractions were equalized based on

absorbance at 595nm, measured by Bradford assay. Fractions were subjected to immunoblotting with antibodies for Lamin A/C, β-tubulin, BASP1 and ERα. Lamin A/C served as a nuclear protein control and β-tubulin served as a cytoplasmic protein control. Molecular weight markers are shown to the left of the blots (kDa). Blots shown are representative of 3 independent experiments.

In non-E2 treated Ishikawa cells (fig.8a), no nuclear contamination of the cytoplasmic fraction by Lamin A/C was detected (panel 1), while very slight cytoplasmic contamination of the nuclear fraction by β-tubulin was evident (panel 2). This could be caused by a small amount of cytoplasmic protein remaining adhered to the nuclei or by experimental error during fractionation. BASP1 was only detected in the cytoplasmic fraction (panel 3) indicating either that it is not located in the nucleus or that it is present in levels too low to be detected by immunoblotting. ERα was primarily detected in the cytoplasmic fraction (panel 4). There was some very slight detection of ERα in the nucleus; however this may have been due to cytoplasmic contamination of the nuclear fraction.

In E2 treated Ishikawa cells (fig.8b), no nuclear contamination of the cytoplasmic fraction by Lamin A/C was detected (panel 1) and very low cytoplasmic contamination of the nuclear fraction by β-tubulin was detected (panel 2). ERα was detected in both the cytoplasmic and nuclear fractions (panel 4), indicating that E2 treatment resulted in the nuclear localisation of the ER. This is supported by numerous publications that have demonstrated this effect in a host of different cell types (*96*). BASP1 was detected primarily in the cytoplasmic fraction (panel 3). An extremely faint band is just visible in the nuclear fraction but this could be due to very slight cytoplasmic contamination of the nuclear fraction (as evidenced in the β-tubulin blot).

These results confirm that, in Ishikawa cells, E2 treatment results in nuclear localisation of ERα. The results also indicate that, in Ishikawa cells, BASP1 is primarily a cytoplasmic protein. However, it is possible that BASP1 is present in the

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nucleus at levels too low to be observed by immunoblotting. This is in contrast to what was previously observed in MCF7 and several other cell lines in our laboratory, in which BASP1 was detected in the nuclear extract (*16, 44*). The nuclear/cytoplasmic distribution of BASP1 was not altered by treatment of Ishikawa cells with E2.

3.4 Effect of E2 and Tamoxifen treatment on ER target gene expression in Ishikawa cells

As stated previously, endometrial cells have been shown to respond differently to Tamoxifen treatment than breast cancer cells (*78, 79*). Tamoxifen generally stimulates ER target genes in endometrial cancer cells (*97*). The experiments in the following figure were conducted to confirm this ER agonist activity in the Ishikawa cells before going on to analyse the effects of BASP1 on tamoxifen action. Ishikawa cells were incubated with either 100nM E2, E2 vehicle control (H_2O), 100nM tamoxifen or tamoxifen vehicle control (ethanol) for 24 hours before the cells were collected and RNA was prepared. The RNA served to prepare cDNA which was then subjected to qPCR using primers for Greb1, PGR and TFF1. Greb1 and TFF1 genes are both regulated by ERα and both contain EREs, indicating the classical pathway of ER activity (*98, 99*) (see section 1.4.4). PGR is regulated by ERα but does not contain any EREs near transcriptional start sites, indicating other mechanisms of ERα activity (*100*). Greb1, PGR and TFF1 were selected because they are all well-established ERα target genes shown to be upregulated by E2 treatment in MCF7 cells (*44*). Expression of the ERα target genes relative to GAPDH was quantitated.
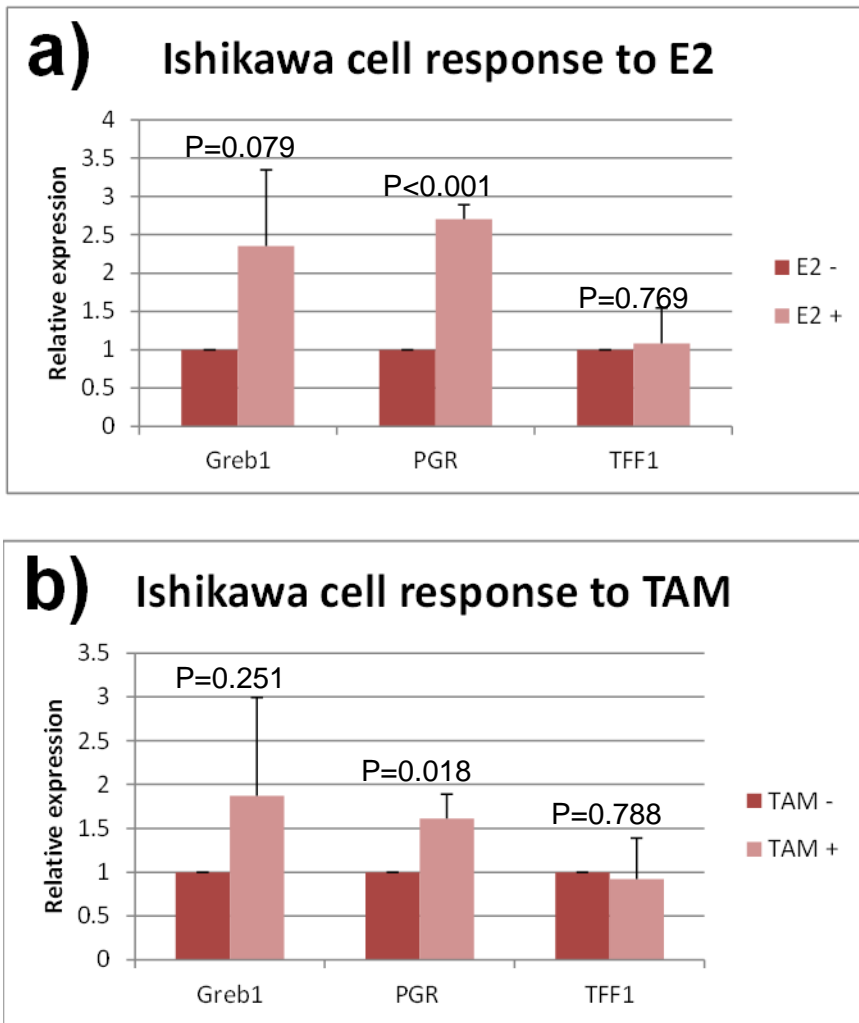
Figure 9 - qPCR to analyse the expression of ERα target genes Greb1, PGR and TFF1 in Ishikawa cells +/-E2 and +/-TAM.
a) Ishikawa cells were incubated for 24 hours with either 100nM E2 or the equivalent volume of the vehicle control H_2O. Treatment is indicated to the right of the figure by either E2- (H_2O treated) or E2+ (E2 treated). RNA and cDNA were prepared. GREB1, PGR and TFF1 expression levels, relative to the control GAPDH, were quantified by qPCR. Error bars are standard deviation from the means for each gene (n=3). Unpaired two tailed t-tests were performed on the data to determine the significance of difference in expression of each gene between treated and untreated cells. P values are indicated above each bar in the graph. **b)** Same protocol as part a, but Ishikawa cells were incubated with 100nM tamoxifen or the vehicle control (ethanol) rather than E2 and H_2O. Treatment is indicated to the right of the figure by either TAM- (ethanol treated) or TAM+ (tamoxifen treated).

The results indicated that E2 treatment of Ishikawa cells resulted in increased expression of Greb1 and PGR (fig.9a). Unpaired two tailed t-tests were performed on the data. The increase in expression of PGR was extremely statistically significant (P<0.001) but the increase in Greb1 was considered not quite statistically significant

(P=0.079). However, only three repeats were conducted and one repeat showed very little Greb1 increase. If further repeats are conducted, it is possible that the increase in Greb1 expression will then be considered significant. Interestingly, TFF1 was unaffected by E2 treatment (fig.9a) (P=0.769). This is contrary to what was seen in MCF7 cells which suggests that there is some difference in estrogen receptor activity between the two cell types. The results also indicated that tamoxifen had a similar effect to that observed with E2 on the expression of these genes in Ishikawa cells. The increase in expression of PGR was significant (P=0.0178) while the increase in Greb1 expression was not (P=0.2510). Again, there was no significant change in TFF1 expression. The similar effects of E2 and tamoxifen on the expression of these genes confirm that, unlike in MCF7s, tamoxifen exhibits ER agonist activity in Ishikawa cells.

3.5 Effect of BASP1 expression on Ishikawa cell proliferation

With the estrogenic activity of Tamoxifen in Ishikawa cells confirmed, experiments were conducted to examine the effects of changing BASP1 expression on tamoxifen action in the cell type. Ishikawa cell phenotype was investigated by conducting growth assays and colony formation assays using stable Ishikawa cell line derivatives that express different levels of BASP1 (see section 2.4).

To produce pcDNA3 stable cell lines, Ishikawa cells were transfected with either an empty pcDNA3 vector (V-Ishikawa) or a pcDNA3 vector that contains a BASP1 expressing region (B-Ishikawa). This produces a stable cell line that expresses HA-FLAG tagged BASP1 in addition to endogenous BASP1. To produce pSilencer stable cell lines, Ishikawa cells were transfected with either a control pSilencer vector (sh-NEG Ishikawa), resulting in expression of a negative control shRNA, or a pSilencer BASP1 vector (sh-BASP1 Ishikawa), resulting in expression of an shRNA that targets BASP1. Both pcDNA3 and pSilencer vectors contain a neomycin resistance region. Successfully transfected cells can then be selected using G418, a neomycin sulfate analogue. First, to identify the optimum concentration of G418, a G418 titration was conducted. Ishikawa cells were seeded onto a 6 well plate and treated with a range of G418 concentrations from 80-240µg.mL^{-1}. Optimum G418 concentration was found to be 200µg.mL^{-1} which resulted in total Ishikawa cell death after 8 days. All four Ishikawa stable cell lines produced were pools of cells.

V-Ishikawa and B-Ishikawa stable cell line whole cell extracts were subjected to immunoblotting using antibodies for β-actin and BASP1. β-actin served as a control to demonstrate the similar overall protein concentrations between samples. V-K562 cells served as a negative control as they do not express BASP1. B-K562 cells served as a positive control as they highly express C-terminal HA tagged BASP1. Results confirmed that the B-Ishikawa stable cell line overexpresses BASP1

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compared to the V-Ishikawa cell line. Similar to the B-K562 cells, the ectopically expressed epitope-tagged BASP1 exhibits slower migration in the gel compared to the endogenous BASP1 in the Ishikawa cells. For growth assays, V-Ishikawa cell proliferation rate was compared to that of B-Ishikawa cells. Cells were seeded at 150,000 cells per plate and grown for 96 hours, with cell counts every 24 hours. After the first 24 hours, cells were treated with either 100nM tamoxifen or the equivalent volume of the vehicle control ethanol.
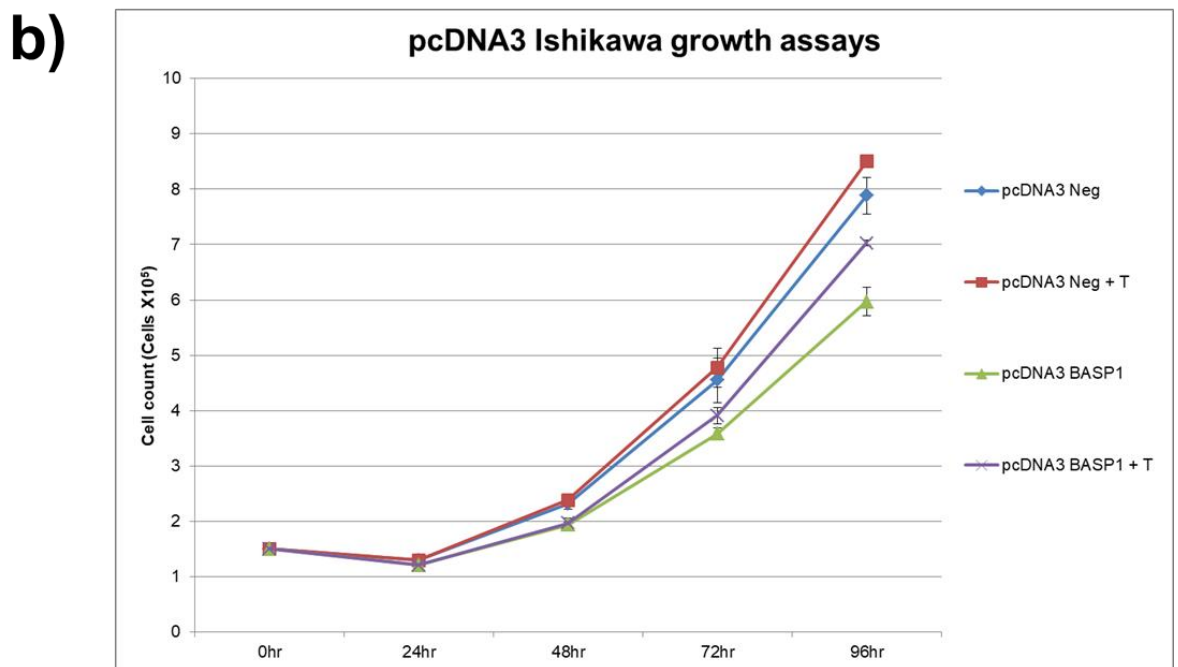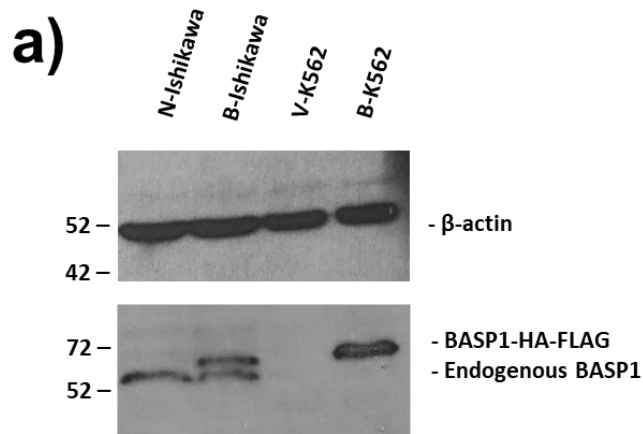
Figure 10 - Growth assays of V-Ishikawa and B-Ishikawa cell lines +/- TAM
a) Whole cell extracts of V-Ishikawa and B-Ishikawa stable cell lines, with V-K562 and B-K562 cells, were used to perform western blots. Cell samples were immunoblotted with antibodies for β-actin and BASP1. β-actin served as a control to ensure similar overall protein concentration between samples. Molecular weight markers are shown to the left of the blots (kDa). Blots shown are representative of 3 independent experiments. **b)** V-Ishikawa and B-Ishikawa cell lines were seeded at 1.5×10^5 cells per plate. After 24 hours, cells were treated with 100nM TAM or the equivalent volume of the vehicle control ethanol. Cells were grown for a total of 96 hours with cell counts being taken every 24 hours. Error bars are standard deviation from the mean at each time point (n=3). Repeated measures two-way ANOVA with a Bonferroni's post hoc analysis and a student's t-test were performed for each time point.

The results indicated that increased BASP1 expression reduces proliferation rate in Ishikawa cells. Two-way ANOVA testing revealed that B-Ishikawa cell count was significantly less than that of the V-Ishikawa cell line after 24 hours (0.000652) and at every subsequent time point. Whilst TAM treated cells appear to grow marginally faster than untreated cells, this difference was shown to be not significant at the majority of time points. At the 48 hour and 72 hour time points, TAM treatment resulted in no significant change in cell number. However, Tamoxifen treated B-Ishikawa cells were found to have proliferated significantly more than untreated V-Ishikawa cells, at the 96 hour time point only (P=0.004659). Tamoxifen treated B-Ishikawa cell number was not quite significantly different from that of untreated V-Ishikawa cells (P=0.061057).

To determine whether decreasing BASP1 expression had the opposite effect, growth assays were conducted using the sh-Neg and sh-BASP1 Ishikawa cell lines. Immunoblotting and growth assay protocols were the same as in figure 10, but sh-Neg and sh-BASP1 Ishikawa cells were used instead of V-Ishikawa and B-Ishikawa cells. Also, K562 cells were not included in the immunoblotting as there was no need to show the different migratory speeds of endogenous and HA-FLAG tagged BASP1. Immunoblotting results confirmed that the sh-BASP1 Ishikawa stable cell line under-expresses BASP1 compared to the sh-Neg Ishikawa cell line.
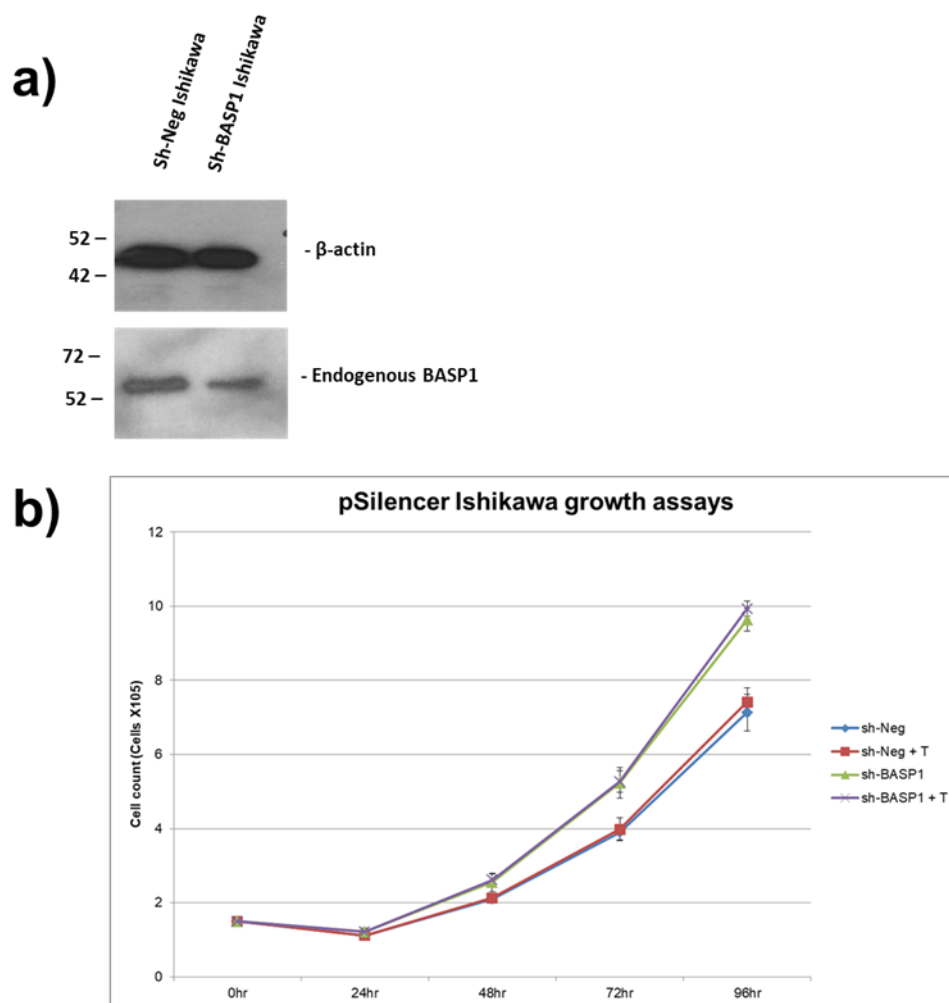
Figure 11 - Growth assays of sh-Neg and sh-BASP1 Ishikawa cell lines +/- TAM
a) Whole cell extracts of sh-Neg and sh-BASP1 Ishikawa stable cell lines were used to perform western blots. Cell samples were immunoblotted with antibodies for β-actin and BASP1. β-actin served as a control to ensure similar overall protein concentration between samples. Molecular weight markers are shown to the left of the blots (kDa). Blots shown are representative of 3 independent experiments. **b)** sh-Neg and sh-BASP1 Ishikawa cell lines were seeded at $1.5X10^5$ cells per plate. After 24 hours, cells were treated with 100nM TAM or the equivalent volume of the vehicle control ethanol. Cells were grown for a total of 96 hours with cell counts being taken every 24 hours. Error bars are standard deviation from the mean at each time point (n=4). Repeated measures two-way ANOVA with a Bonferroni's post hoc analysis and a student's t-test were performed for each time point.

The results of the growth assays indicated that reducing BASP1 expression increases the proliferation rate in Ishikawa cells. Two-way ANOVA testing revealed that sh-BASP1 Ishikawa cell count was significantly greater than that of the sh-Neg Ishikawa cell line after 48 hours (P=0.005291) and at every subsequent time point. Tamoxifen treatment produced no significant change in cell count at any time point.

3.6 Effect of BASP1 expression on Ishikawa cell colony formation efficiency

Growth assays of Ishikawa cells indicated that increased BASP1 expression results in reduced proliferation and that decreased BASP1 expression results in increased proliferation. Colony formation assays were performed to further investigate the effects of BASP1 expression on Ishikawa cell proliferation and survival. Colony formation efficiency of B-Ishikawa cells was compared to that of V-Ishikawa cells.

For colony formation assays, cells were seeded onto 6-well plates at 500 cells per well and grown for 13 days before fixing with methanol. After the first 24 hours cells were treated with either 100nM tamoxifen / vehicle control (ethanol) or 100nM estradiol / vehicle control (water) for 72 hours. Colonies with a cell number greater than 50 were counted and used to calculate colony formation efficiency.
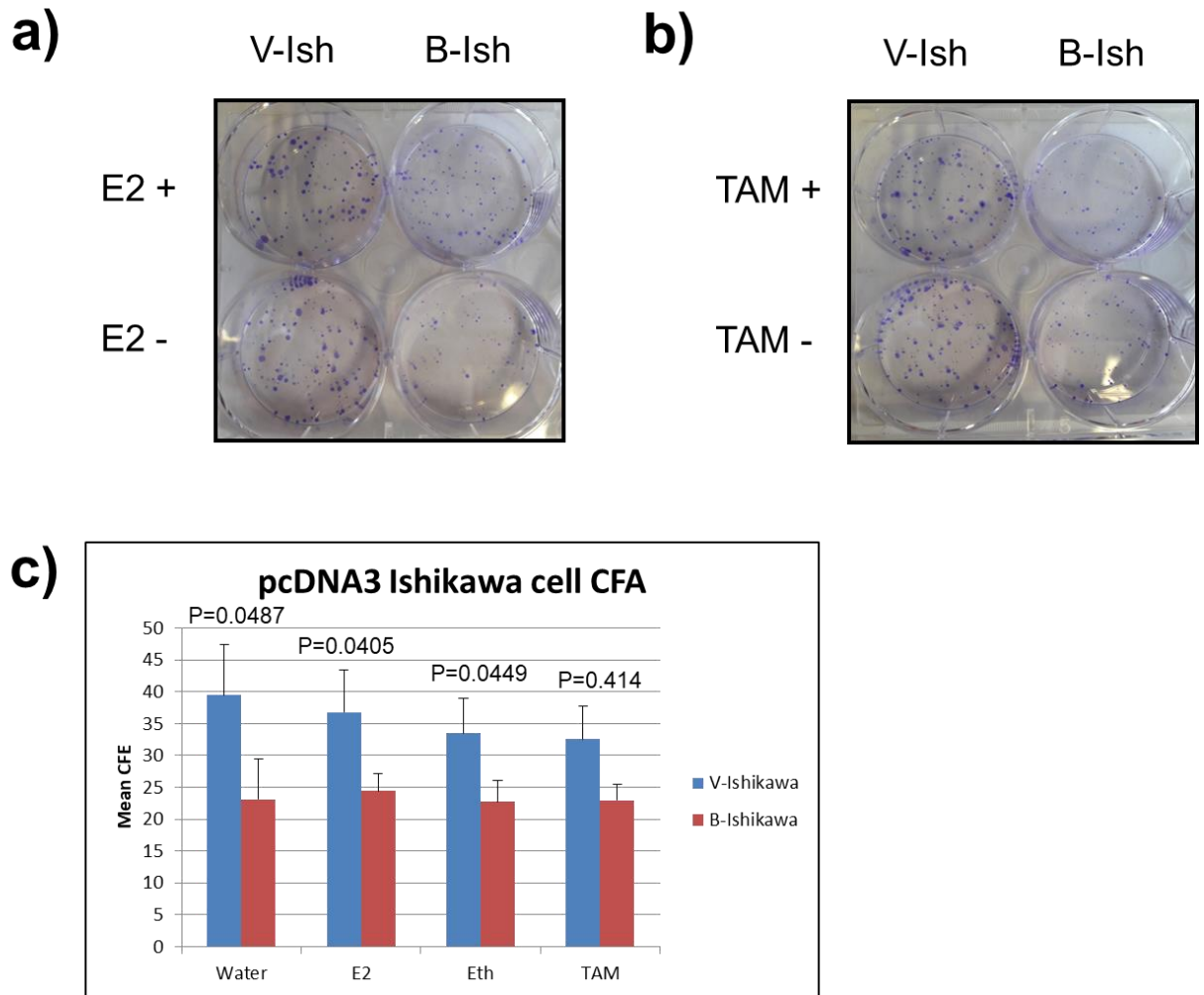
Figure 12 - Colony formation assays of V-Ishikawa and B-Ishikawa cells +/- TAM and E2
V-Ishikawa and B-Ishikawa stable cell lines were seeded onto 6 well plates at 500 cells per well. After 24 hours, cells were incubated with either 100nM E2, E2 vehicle control H_2O, 100nM TAM or TAM vehicle control ethanol for 72 hours. Cells were grown for a further 11 days before colonies were fixed in methanol and stained using crystal violet. Total numbers of colonies visible with the naked eye were counted and used to calculate colony forming efficiencies (n=3). Repeated measures two-way ANOVA with a Bonferroni's post hoc analysis and a student's t-test were performed for each time point. T-test P values are indicated above each bar in the graph.

Repeated measures two-way ANOVA testing indicated that there was a significant difference in colony formation efficiency between V-Ishikawa and B-Ishikawa cell lines, but that there was no significant difference between drug and vehicle control treatments. Unpaired two tailed t-test confirmed that colony formation efficiency was significantly lower in the B-Ishikawa cells compared to the V-Ishikawa

cells in all cases. In addition, colonies formed by the B-Ishikawa cells appear to be smaller on average than those of the V-Ishikawa cells.

To further investigate the effect of altered BASP1 expression on colony formation efficiency, colony formation assays were conducted using the sh-Neg and sh-BASP1 Ishikawa cell lines. Assay protocols were the same as in figure 12, but sh-Neg and sh-BASP1 Ishikawa cells were used instead of V-Ishikawa and B-Ishikawa cells.
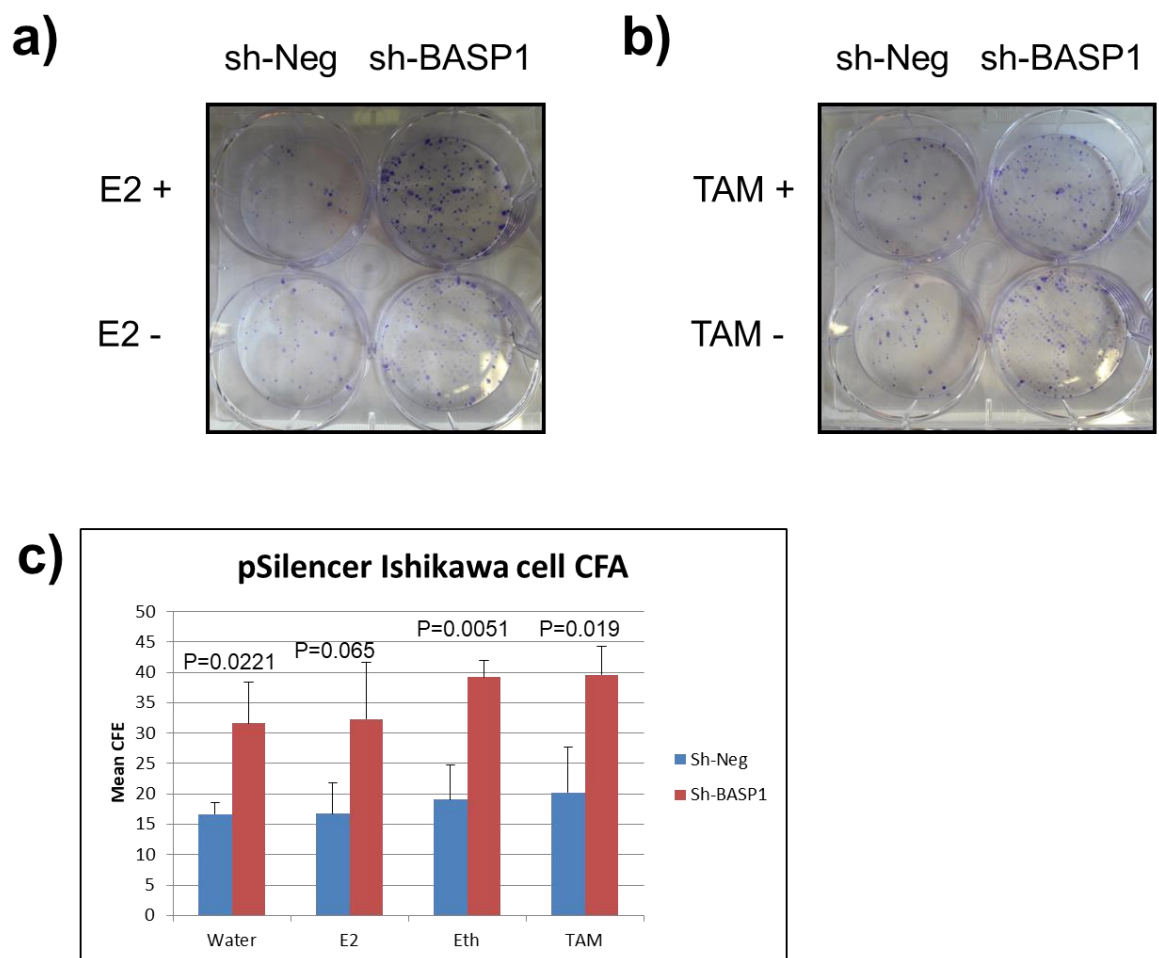
Figure 13 - Colony formation assays of sh-Neg and sh-BASP1 Ishikawa cells +/- TAM and E2

sh-Neg and sh-BASP1 Ishikawa stable cell lines were seeded onto 6 well plates at 500 cells per well. After 24 hours, cells were incubated with either 100nM E2, E2 vehicle control H_2O, 100nM TAM or TAM vehicle control ethanol for 72 hours. Cells were grown for a further 9 days before colonies were fixed in methanol and stained using crystal violet. Total numbers of colonies visible with the naked eye were counted and used to calculate colony forming efficiencies (n=3). Repeated measures two-way ANOVA with a Bonferroni's post hoc analysis

and a student's t-test were performed for each time point. T-test P values are indicated above each bar in the graph.

As was seen in the pcDNA3 Ishikawa cell lines, repeated measures two-way ANOVA testing indicated that there was a significant difference in colony formation efficiency between sh-Neg and sh-BASP1 Ishikawa cell lines. This was confirmed by unpaired two tailed t-tests which indicated that the colony formation efficiency of sh-BASP Ishikawa cells was significantly greater than that of sh-Neg Ishikawa cells in all conditions apart from the E2 treated cells. The E2 treated sh-BASP1 Ishikawa cells colony formation efficiency was slightly more variable than the untreated and TAM-treated cells. Since the colony formation efficiency of E2 treated sh-BASP1 Ishikawa cells was higher than the colony formation efficiency of E2 treated sh-Neg Ishikawa cells in all repeats, and the increase was taken to be only slightly non-significant, it is probable that another repeat at these conditions would produce a significant result. Additionally, colonies formed by the sh-BASP1 Ishikawa cells appear to be larger on average than those of the sh-Neg Ishikawa cells.

3.7 Conclusion

The results in this section have confirmed the expression of BASP1, ERα and WT1 in Ishikawa cells (fig.7), and have confirmed the ERα agonist activity of tamoxifen (fig.9). However, subcellular fractionation and immunoblotting failed to detect the presence of BASP1 in the nucleus of Ishikawa cells (fig.8). It has previously been shown that, in MCF7 cells, BASP1 is present in the nucleus at levels detectable by immunoblotting and that BASP1 can be found at the promoters of ER target genes. Since it has been shown that the tamoxifen response in MCF7s is affected by BASP1 expression, it is possible that this difference in nuclear localisation of BASP1 between the MCF7 cells and Ishikawa cells could result in differences in tamoxifen response.

It should be noted that it is possible that BASP1 is present in the nucleus at levels too low to be detected by immunoblotting but it is also possible that it is an entirely cytoplasmic protein, under these conditions, in this cell type. BASP1 has previously been shown to regulate gene expression in other cell types, despite being primarily cytoplasmic in these cells. In iPSCs, inhibition of BASP1 has been shown to result in increased WT1 activation (*101*) despite not being detected in the nucleus by immunofluorescence. It is possible that low levels of BASP1 in the nucleus are sufficient to mediate transcriptional regulation. Alternatively, BASP1 could influence transcription from the cytoplasm by interacting with other transcription factors. In Ishikawa cells ERα can be found in both the nucleus and the cytoplasm (fig.8). WT1 has also been shown to be present in both the nucleus and cytoplasm in other cell types (*102*). It is therefore possible that BASP1 might retain either WT1 or ERα in the cytoplasm, or otherwise influence their nuclear import, resulting in changes in gene expression. In future, immunofluorescence could be conducted to analyse the nuclear and cytoplasmic distribution of BASP1 in order to confirm the subcellular fractionation results.

Quantitative PCR to measure the mRNA of ERα target genes in Ishikawa cells following E2 or tamoxifen treatment revealed multiple differences in tamoxifen treatment response between the two cell types. Tamoxifen exhibited estrogenic activity in Ishikawa cells which is in contrast to its effects in MCF7 cells which are anti-estrogenic. These results are supported by numerous other studies on the subject (*78, 79, 97*). It was expected that tamoxifen would exhibit estrogenic activity on key ER target genes and this was confirmed in the case of PGR expression. However, TFF1 expression was unchanged in response to either E2 or tamoxifen treatment (fig.9). This data suggests that there may be differences in ERα target genes and mechanisms of activity between the cell types, in addition to the difference in ER agonist/antagonist response to tamoxifen. These results are consistent with previous RNA sequencing data of Ishikawa cells which showed that PGR and Greb1 were significantly upregulated by E2 and Tamoxifen treatment but TFF1 was not (*103*).

Colony formation assays showed that increased BASP1 expression resulted in a decrease in CFE (%) and decreased BASP1 expression resulted in an increase in CFE (%) in all repeats. ANOVA testing indicated significant difference in colony formation efficiency between the B-Ishikawa and V-Ishikawa and between the sh-BASP1 Ishikawa and sh-Neg Ishikawa cell lines. Student's t-tests indicated that these changes could be accepted as significant in all conditions apart from the E2 treated pSilencer Ishikawa cells. In this case, the slightly non-significant result was partially due to the higher level of variation in colony formation efficiency in the E2 treated sh-BASP1 cells. A possible reason for the high level of variation in CFE (%) is that the Ishikawa cells used grow well at high cell densities but exhibit highly variable growth and survival when at very low cell numbers. This hypothesis is supported by the results of growth assays showing that BASP1 expression reduces proliferation in Ishikawa cells. In the growth assays, B-Ishikawa cells showed reduced proliferation

compared with V-Ishikawa cells in all cases and sh-BASP1 Ishikawa cells showed increased proliferation compared with sh-Neg Ishikawa cells in all cases. For the growth assays, cells were seeded in much higher numbers which could have reduced the variation caused by Ishikawa cells inconsistent growth and survival rates when at very low cell numbers. The results of the growth assays and colony formation assays, taken together, indicate that BASP1 possesses tumour suppressor activity in Ishikawa cells.

Another observation from the colony formation assay was that BASP1 expression appeared to reduce the average colony size. These colony formation assays and growth assays, taken together, indicate that BASP1 exhibits tumour suppressor activity in Ishikawa cells. This activity would suggest that BASP1 is capable of regulating transcription. It is not known by what mechanism BASP1 elicits these effects, but it is possible that this could be being carried out by very low, undetected levels of BASP1 in the nucleus, or by interactions with other transcription factors in the cytoplasm such as ERα, WT1 or c-myc.

The effects of BASP1 on Ishikawa cell growth do not appear to have been changed by E2 or TAM treatment in either the growth assays or the colony formation assays. However, in the pcDNA3 Ishikawa cell line growth assays, Tamoxifen treated B-Ishikawa cells were found to have proliferated significantly more than untreated B-Ishikawa cells, at the 96 hour time point, while tamoxifen treated V-Ishikawa cell number was not quite significantly different from that of untreated V-Ishikawa cells (fig.10). This could suggest that BASP1 overexpression may enhance the proliferative effect of Tamoxifen on Ishikawa cells, but more repeats with later time points would be required to test this.

BASP1 has previously been shown to enhance the effects of TAM treatment in both MCF7 and T47D breast cancer cells (*44*). While the effects of TAM treatment

in breast cancer cells is actually anti-proliferative, it is possible that BASP1 may enhance the effects of TAM by the same mechanism across the different cell types. Subsequent experiments aimed to investigate whether BASP1 exhibits transcriptional modulation activity in Ishikawa cells, despite being primarily cytoplasmic, and whether changes in BASP1 expression are able to affect the tamoxifen response.

4 Analysis of the role of BASP1 in gene regulation in Ishikawa cells

4.1 Introduction

In the previous chapter, immunoblotting failed to detect the presence of BASP1 in the nucleus of Ishikawa cells (fig.8) but growth assays and colony formation assays indicated that BASP1 may possess tumour suppressive activity in the cell type (fig.11). This suggests that BASP1 could modulate gene expression through low level BASP1 in the nucleus, or by interacting with other transcription factors in the cytoplasm. In other cell types BASP1 has been shown to interact with WT1 and ERα and localise at gene promoters (*16, 44*).

Growth assays and colony formation assays indicated that BASP1 possesses tumour suppressor activity in Ishikawa cells, similarly to that which has been seen in MCF7 breast cancer cells (*44*). However, BASP1 does not appear to alter the effects of TAM treatment in Ishikawa cells. The only indication that BASP1 may promote the effects of TAM treatment was the 96 hour time point of the pcDNA3 Ishikawa cell growth assays. It is possible that this effect would consistently be seen in growth assays at later time points, but these experiments will have to be repeated with a longer time scale in order to confirm this. These results indicate that BASP1 may be capable of modulating gene expression to affect proliferation, but it is not clear whether it is capable of modulating Tamoxifen response. This raises the question of whether BASP1 mediates any effects through the gene regulatory functions of ER.

The following experiments were conducted to investigate: the effects of BASP1 on gene expression of ERα and WT1 targets, the effects of BASP1 on tamoxifen induced gene expression changes, and possible BASP1 binding to ERα target gene promoters. Specific transcript levels were analysed using qPCR in an attempt to demonstrate possible gene expression modulation by BASP1, as well as possible effects of BASP1 on TAM induced gene expression changes. ChIP was

performed in an attempt to detect possible low level BASP1 localisation at gene promoters in the nucleus of Ishikawa cells.

4.2 Effects of BASP1 on gene expression

In order to investigate possible gene expression modulation by BASP1, qPCR for ERα and WT1 target genes was performed on Ishikawa stable cell lines. Expression of the chosen genes in B-Ishikawa cells were compared to that of V-Ishikawa cells. Expression of the chosen genes in sh-BASP1 Ishikawa cells were compared to that of sh-Neg Ishikawa cells. RNA was prepared from each of the cell lines and cDNA prepared from this before being subjected to qPCR using primers for Greb1, PGR, TFF1, Rab31 and VDR. Expression of the ERα target genes (Greb1, PGR, TFF1 and Rab31) and the WT1 target gene (VDR) relative to GAPDH was quantitated.
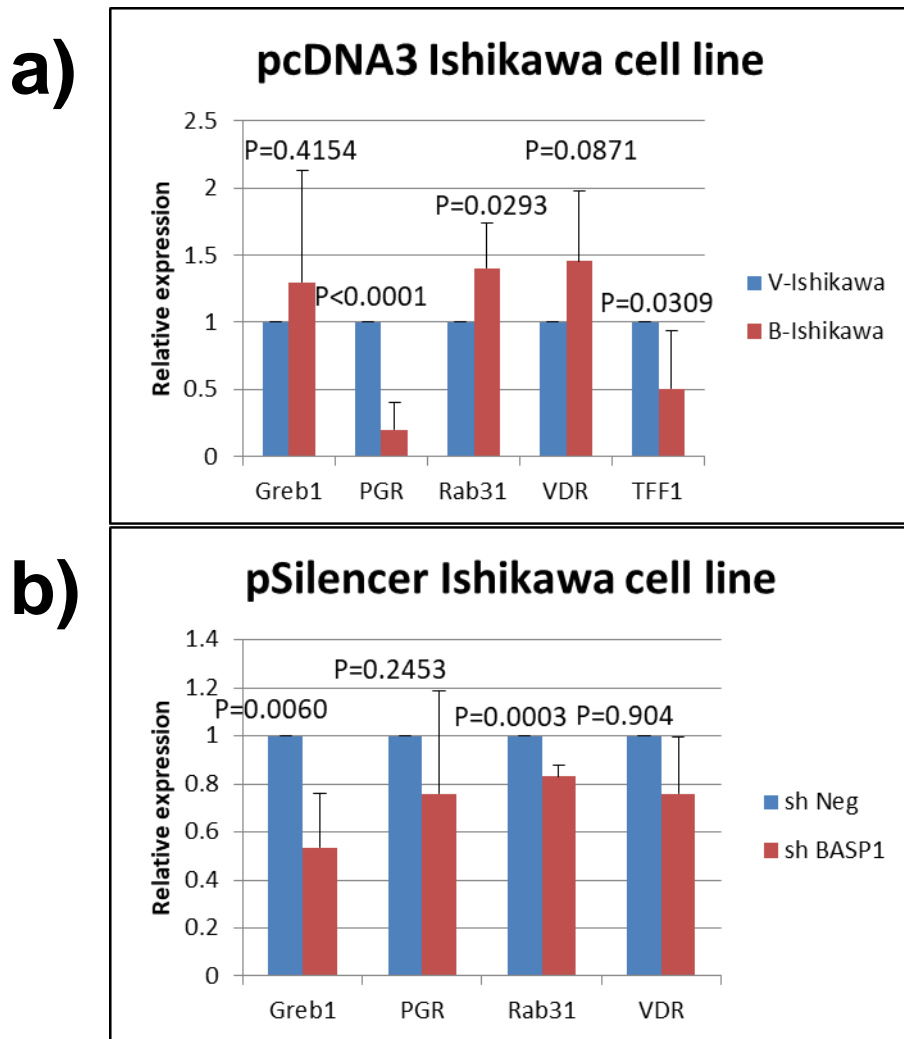
Figure 14 - qPCR for ERα and WT1 target genes in Ishikawa stable cell lines with altered BASP1 expression.
RNA and cDNA were prepared from V-Ishikawa, B-Ishikawa, sh-Neg Ishikawa and sh-BASP1 Ishikawa stable cell lines. GREB1, PGR, TFF1, Rab31 and VDR expression levels, relative to the control GAPDH, were quantified by qPCR. Error bars are standard deviation from the means for each gene (n=6). Unpaired two tailed t-tests were performed on the data to determine the significance of difference in expression of each gene, between cell lines with differing BASP1 expression. P values are indicated above each bar in the graph.

The results indicated that BASP1 regulates transcription of a subset of ERα target genes. Expression of both PGR and TFF1 was significantly reduced in B-Ishikawa cells compared with V-Ishikawa cells. These suggest that BASP1 may repress expression of PGR and TFF1, which are both known ERα targets. However, there was no significant difference in PGR expression between the two pSilencer cell lines and TFF1 data was not obtained for the pSilencer cell lines. Expression of Greb1, Rab31 and VDR was increased in the pcDNA3 BASP1 Ishikawa cell line but

only the expression change of Rab31 was found to be statistically significant. Greb1 is another ERα target which has been implicated in the proliferative effect of estradiol in breast cancer (*104*). Rab31 is also an ERα target and is a known target of MUC1-C induced tamoxifen resistance in some breast cancer cases (Kharbanda). VDR is a WT1 target (*105*) and is known to act as a transcription factor which has been implicated in several cancer types (*106*). Greb1, Rab31 and VDR expression was reduced in sh-BASP1 Ishikawa cells compared with sh-Neg Ishikawa cells but only the expression changes in Greb1 and Rab31 were statistically significant. Taken together, these results indicate that BASP1 is able to affect transcription of a subset of ERα target genes in Ishikawa cells.

4.3 Effects of BASP1 on tamoxifen induced gene expression changes in pcDNA3 Ishikawa cells

Growth assays indicated that BASP1 expression reduces the proliferation rate of Ishikawa cells and qPCR indicated that BASP1 modulates the expression of a subset of ERα target genes. However, it is not known whether BASP1 is able to modulate tamoxifen action on the ER in Ishikawa cells, as has been shown previously in MCF7 cells (*44*). The following experiments were conducted in order to investigate the effect of BASP1 expression on tamoxifen induced gene expression changes. Expression of key ER target genes in the absence or presence of tamoxifen was quantified by qPCR. The differences in gene expression between the tamoxifen treated and untreated cells in B-Ishikawa cells were compared to that of V-Ishikawa cells.

Cells were incubated with either 100nM TAM or equivalent volume of vehicle control (ethanol) for 24 hours before the cells were collected and RNA was prepared. cDNA was prepared and was then subjected to qPCR using primers for Greb1, PGR, TFF1, XBP1, Rab31 and VDR. Expression of the ERα target genes (Greb1, PGR, TFF1, XBP1 and Rab31) and the WT1 target gene (VDR) relative to GAPDH was quantitated.

a)

PGR expression

Greb1 expression

TFF1 expression

b)

Rab31 expression

XBP1 expression

VDR expression

Figure 15 - qPCR for ERα and WT1 target genes in V-Ishikawa and B-Ishikawa +/- TAM
RNA and cDNA were prepared from B-Ishikawa and V-Ishikawa stable cell lines. Cells were incubated for 24 hours with either 100nM TAM or the equivalent volume of the vehicle control ethanol. Treatment is indicated to the right of the figure by either TAM- (ethanol treated) or TAM+ (TAM treated). GREB1, PGR, TFF1, Rab31 and VDR expression levels, relative to the control GAPDH, were quantified by qPCR. Error bars are standard deviation from the means for each gene (n=6). Unpaired two tailed t-tests were performed on the data to determine the significance of difference in expression of each gene. P values are indicated above each bar in the graph.

The results indicated that BASP1 overexpression results in the upregulation of a subset of ERα target genes in response to tamoxifen treatment. In B- Ishikawa cells, PGR, Greb1 and TFF1 were all upregulated significantly more in response to tamoxifen treatment, than in V- Ishikawa cells. There were no significant expression changes induced by tamoxifen treatment of XBP1 or Rab31 in either the V-Ishikawa cells or B-Ishikawa cells. There was also no significant difference between the two cell lines in expression change of these genes. Significant VDR downregulation in

response to tamoxifen treatment was observed in V-Ishikawa cells but not in B-Ishikawa cells.

These results indicate that BASP1 is capable of modifying tamoxifen action in Ishikawa cells. Tamoxifen induced expression change was significantly different between the two cell types for PGR, Greb1 and TFF1, which are all known ERα targets. In addition, neither TFF1 nor Greb1 showed any significant change in expression in response to tamoxifen treatment in V-Ishikawa cells but both showed a significant increase in expression in B-Ishikawa cells. This suggests that BASP1 could be required for tamoxifen to exert its effects on gene expression at a subset of ERα target genes.

4.4 Effects of BASP1 on tamoxifen induced gene expression changes in pSilencer Ishikawa cells

qPCR of the pcDNA3 Ishikawa stable cell lines indicated that some of the expression changes induced by tamoxifen treatment are BASP1 dependent. In order to confirm this, the same experiments were conducted on sh-Neg and sh-BASP1 Ishikawa stable cell lines. Cells were incubated with either 100nM TAM or equivalent volume of vehicle control (ethanol) for 24 hours before the cells were collected and RNA was prepared. cDNA was prepared from this RNA and was then subjected to qPCR using primers for Greb1, PGR, XBP1, Rab31 and VDR. Expression of the ERα target genes (Greb1, PGR, XBP1 and Rab31) and the WT1 target gene (VDR) relative to GAPDH was quantitated.

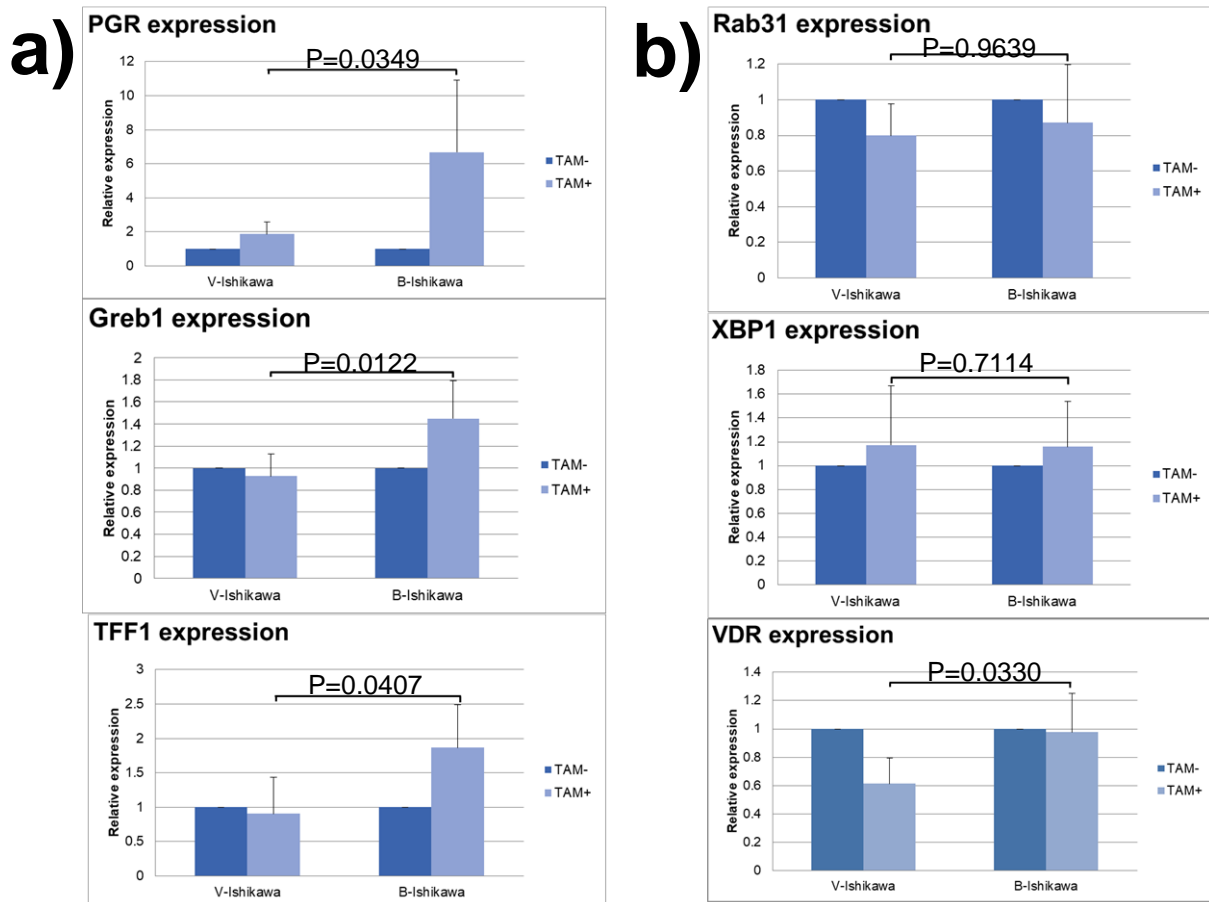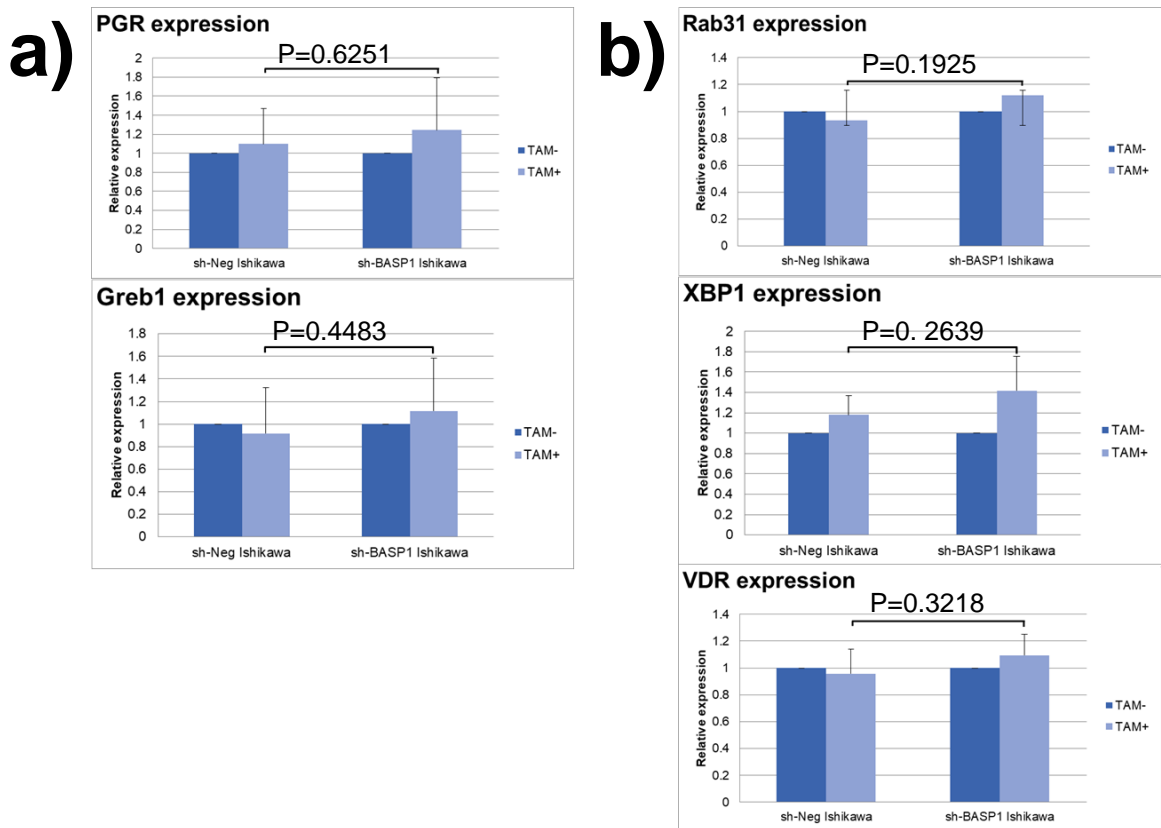**Figure 16 - qPCR for ERα and WT1 target genes in sh-Neg and sh-BASP1 Ishikawa cells +/- TAM**

RNA and cDNA were prepared from sh-Neg and sh-BASP1 Ishikawa stable cell lines. Cells were incubated for 24 hours with either 100nM TAM or the equivalent volume of the vehicle control ethanol. Treatment is indicated to the right of the figure by either TAM- (ethanol treated) or TAM+ (TAM treated). GREB1, PGR, Rab31 and VDR expression levels, relative to the control GAPDH, were quantified by qPCR. Error bars are standard deviation from the means for each gene (n=6). Unpaired two tailed t-tests were performed on the data to determine the significance of difference in expression of each gene. P values are indicated above each bar in the graph.

The results indicate that reduced BASP1 expression does not affect tamoxifen induced expression changes of these genes. Unlike, in B-Ishikawa cells, neither the sh-Neg nor sh-BASP1 stable cell lines showed any significant changes in PGR, Greb1 or TFF1 expression induced by tamoxifen treatment. Similar to the observations in pcDNA3 Ishikawa cells, in pSilencer Ishikawa cells there were no significant expression changes, induced by tamoxifen treatment, of XBP1 or Rab31.

Also, the downregulation of VDR seen in V-Ishikawa cells was not seen in sh-Neg

Ishikawa cells.

4.5 Sonication optimisation for ChIP with Ishikawa cells

The qPCR results indicate that manipulation of the level of BASP1 in Ishikawa cells alters the regulation of a subset of ER target genes by tamoxifen (fig.17). It has previously been shown in our laboratory that, in MCF7 cells, BASP1 expression is required for 40% of Tamoxifen induced expression changes. The proposed mechanism for this effect was direct ERα complex binding by BASP1 in the nucleus because BASP1 and ERα were shown to co-localise in the nucleus and BASP1 was found at the promoters of ERα target genes by ChIP (*44*). Co-IP experiments in Ishikawa cells were not conducted because, if there is any nuclear localisation of BASP1, it was likely too low to be detected by immunoblotting (fig.8). However, it is possible that very low levels of BASP1 could be detected at the promoters of ERα target genes in Ishikawa cells. ChIP experiments were therefore initiated to determine if BASP1 can localise to the promoters of ER target genes in Ishikawa cells.

In order to conduct ChIP experiments, it was first necessary to optimise the sonication protocol for Ishikawa cells. Ishikawa cells were subjected to varying levels of sonication time and power and the resulting fragmented chromatin was resolved by agarose gel electrophoresis to determine the size of the resulting DNA fragments.
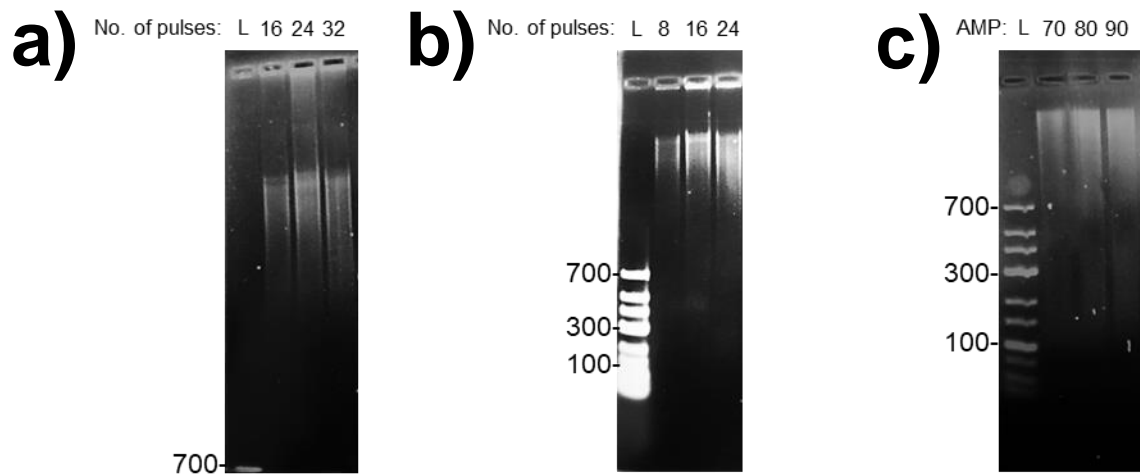
Figure 17 - Sonication optimisation for ChIP of Ishikawa cells
Chromatin fragments resolved by agarose gel following sonication with varying sonication times, repetitions and amplitude. **a)** Pulses of sonication for 3s, followed by 2s on ice, for differing number of rounds (16-32), at 60% amplitude. **b)** Pulses of sonication for 10s, followed by 30s on ice, for differing numbers of rounds (8-24) at 50% amplitude. **c)** Pulses of sonication for 30s, followed by 30s on ice, for 16 rounds, with varying amplitudes (70-90%). DNA size markers are shown to the left of the gels, indicated by 'L' above the marker well (bp).

The Results indicated that increased sonication intensity was required to break down the Ishikawa cell chromatin. The results also showed that increasing sonication time per pulse had a greater effect on chromatin fragmentation than increasing the number of pulses or the amplitude of the sonicator. DNA fragmentation improved but a wide spectrum of DNA fragment sizes was produced even by the highest intensity sonication. Further optimisation of both the sonication and cross-linking protocols is required in order to produce a smaller band of more consistent DNA fragment size.

4.6 Investigating localisation of ERα and BASP1 at ERα target gene promoters in Ishikawa cells

Sonication optimisation produced samples of fragmented chromatin that included small enough fragments to perform ChIP experiments on. However, the DNA fragment sizes produced were highly variable so the cross linking and sonication experiments should ideally be further optimised in order to produce reliable ChIP data. In an effort to produce preliminary, indicative results, ChIP was conducted using the sonication settings set out above.

Ishikawa cells were incubated with E2 for 24 hours to increase nuclear localisation of ERα (fig.8). Cells were collected and DNA was crosslinked with PFA followed by fragmentation by sonication. Chromatin was fragmented by pulses of sonication for 30s, followed by 30s on ice, for 16 rounds, at 70% amplitude. ChIP was performed with control IgG, BASP1 and ERα magG bead-bound antibodies. The immuno-precipitated DNA was subjected to qPCR using ChIP promoter primers for Greb1 and PGR, and as a negative control the Bax gene.
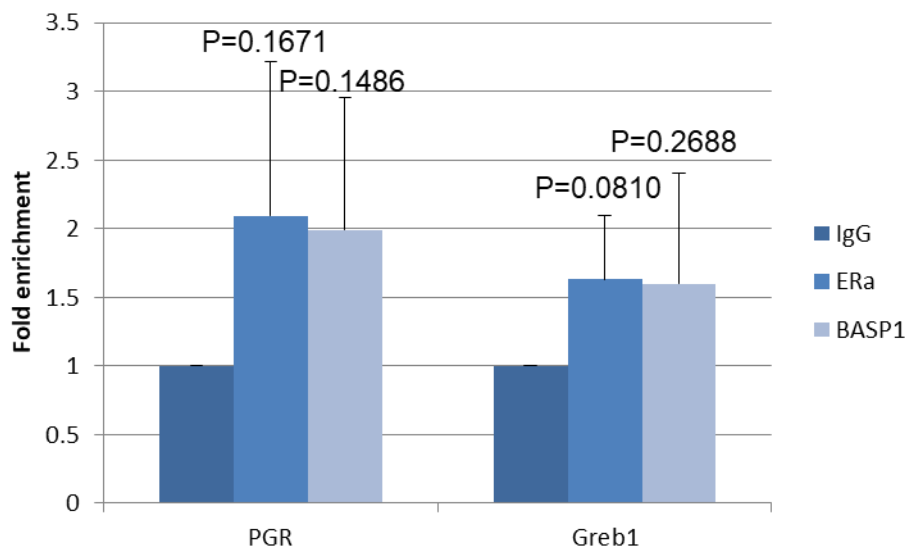
Figure 18 - ChIP of Ishikawa cells for ERα and BASP1 binding to ERα target genes
Ishikawa cells were extracted following 24 hour incubation with 100nM E2 and cross linked with PFA. Chromatin was extracted and fragmented by sonication. Chromatin fragments were probed with antibodies for IgG, BASP1 and ERα antibodies. Binding of BASP1 and ERα to

target genes (PGR and Greb1) was quantified relative to the control, BAX1. Fold enrichment was calculated relative to IgG. Unpaired two tailed t-tests were performed on the data to test significance of difference between ERα/BASP1 and IgG (n=3). P values for each gene are indicated above the bars.

ChIP revealed that at both the Greb1 and PGR promoters, BASP1 and ERα were both found to have fold enrichments of between 1.59 and 2.10 on average. This fold enrichment over the IgG was taken to be not significant due to the high level of variation in the magnitude of enrichment. However, BASP1 and ERα samples had a fold enrichment of greater than 1 in almost all cases. In addition, it was expected that ERα binding would be significant at these promoters but it showed similar fold enrichment to BASP1.

4.7 Conclusion

Gene expression analysis of Ishikawa stable cell lines with manipulated levels of BASP1 has indicated that BASP1 is able to modulate gene expression of a subset of ERα and WT1 target genes. Since immunoblotting of Ishikawa cell subcellular fractions could not detect any BASP1 in the nucleus (fig.9), it is possible that this effect is produced by very low levels of BASP1 in the nucleus or by cytoplasmic BASP1 activity, such as sequestration of transcription factors. In other cell types, BASP1 has been shown to interact with several transcription factors: WT1, ERα and c-myc (*17, 19, 44*).

Gene expression analysis of tamoxifen treated Ishikawa stable cell lines indicated that BASP1 is required for the estrogenic effect of tamoxifen on ERα target genes: PGR, TFF1 and Greb1 (Fig.13). In MCF7s, approximately 40% of tamoxifen induced gene expression changes were previously shown to be BASP1 dependent. It is therefore possible that, despite the opposing effects of tamoxifen treatment on gene expression in these cell types, a portion of the activity of tamoxifen is BASP1 dependent in the same way. Tamoxifen induced expression change of VDR was also found to be significantly different in B-Ishikawa cells from that of V-Ishikawa cells. VDR is not an ERα target gene but it is possible that this is a secondary effect. However, the significant tamoxifen induced reduction in VDR expression seen in N-Ishikawa cells was not seen in sh-Neg Ishikawa cells. Both V-Ishikawa and sh-Neg Ishikawa cell lines are Ishikawa cells that should have normal BASP1 expression levels. This would suggest either that this difference between B-Ishikawa cells and V-Ishikawa cells was an error or that it was not due to differing BASP1 expression.

Tamoxifen response in Ishikawa cells was altered by increased BASP1 expression but not by decreased BASP1 expression (fig.15 and fig.16). It is possible that the increase in expression of BASP1 in B-Ishikawa cells compared with V-

Ishikawa cells is greater than the decrease in expression of BASP1 in sh-BASP1 Ishikawa cells compared with sh-Neg Ishikawa cells. It is also possible that endogenous BASP1 expression levels are too low in Ishikawa cells for tamoxifen to exert its effects on the expression of these investigated genes. If this is the case, then lowering BASP1 levels even further (as in the sh-BASP1 Ishikawa cell line) would have no effect on tamoxifen induced expression changes. Since this is what was observed (fig.16), it is possible that a certain level of BASP1 expression is required for tamoxifen to exert its effects on gene expression. Another possibility is that nuclear BASP1 is required for these tamoxifen induced changes. It is possible that overexpressed HA-FLAG tagged BASP1 may be localised more in the nucleus than endogenous BASP1. If this is the case, then B-Ishikawa cells could have much higher levels of BASP1 in the nucleus than the other Ishikawa cell lines, since endogenous BASP1 localisation was found to be primarily cytoplasmic (fig.8). This could be tested by conducting immunoblotting and immunofluorescence experiments on the B-Ishikawa stable cell line.

ChIP analysis of BASP1 and ERα binding to ERα target genes in Ishikawa cells produced non-significant results (fig.18). However, only three repeats were conducted, the majority of repeats indicated binding and the cross linking/sonication protocols were not fully optimised (fig.17). ERα localisation was measured to provide a positive control since ERα is known to localise at the promoters chosen, Greb1 and PGR. Since ERα binding was found to be not significant, this would suggest that further optimisation of the protocol is needed. This low level, non-significant ChIP for ERα is comparable to that of BASP1. This raises the possibility that BASP1 might be present at the promoters but further optimisation of the protocol is required in order to test this. Protocol optimisation in this project focused on optimisation of the sonication procedure. It was found that high power and long pulse times were required to improve DNA fragmentation (fig.17). Increasing these further would risk

shearing of the DNA fragments due to increased sample temperature caused by the sonication. To further optimise the protocol, cross-linking can be optimised by varying cross-linker concentration or the incubation time of the cross-linking step. In addition, sonication can be further optimised by varying sample volume and sample cell concentration.

Taken together, the results in this chapter indicate that: BASP1 is able to modulate gene expression of a subset of ERα target genes, BASP1 is required for some tamoxifen induced gene expression changes of ERα target genes, and BASP1 presence at the promoters of ERα target genes is possible but was not demonstrated to be significant. The mechanism of these effects is still unknown but the ChIP data raises the possibility that BASP1 might be acting directly at the target genes that it regulates, as was seen in MCF7 cells (*44*).

5 Discussion

BASP1 as a tumour suppressor 5.1

Increasing evidence implicates BASP1 in transcriptional control and tumourigenicity in a variety of cell and tissue types throughout the human body. BASP1 has been shown to exert tumour suppressor activity and to be a target of repression in a diverse range of cancer cell types including breast cancer cells, acute myelogenous leukaemia cells, hepatocellular carcinoma cells and thyroid cancer cells (*40-42, 44*). Within these cancer cell types, BASP1 has been shown to exert a number of different effects including reducing proliferation, migration and colony formation, as well as increasing apoptosis. New evidence demonstrates the complexity of the functions of BASP1 as it has, by contrast, been found to possess tumour promoting activity in cervical cancer cells (*43*). Again, it was found that BASP1 has multiple effects in the cancer type including increasing proliferation, colony formation and tumourigenicity, as well as being associated with stage progression and poor prognosis.

The results of this study indicate endometrial cancer as another cancer cell type in which BASP1 possesses tumour suppressor activity. Growth assays and colony formation assays showed BASP1 expression reduced cell proliferation and colony formation in all repeats (fig10-13). These results are similar to those seen in acute myelogenous leukaemia cells, hepatocellular carcinoma cells and thyroid cancer cells (*40-42*). This was also a similar effect to that which was shown previously in MCF7 and T47D breast cancer cells (*44*). However, in MCF7 cells the magnitude of colony formation efficiency change induced by altering BASP1 expression was far greater than in Ishikawa cells. It is possible that this was due to a greater over-expression of BASP1 in B-MCF7s and greater under-expression of BASP1 in shB-MCF7s compared with the B-Ishikawa and sh-BASP1 Ishikawa cell lines used in this study. This is supported by immunoblotting of the stable cell lines for BASP1, in which it appears that the changes in BASP1 expression were greater

in the MCF7 cell lines (fig.10 and 11) (*44*). However, it is also possible that this reduced magnitude of difference in Ishikawa cells compared with MCF7 cells is due to the lower nuclear localisation of BASP1.

In MCF7 cells BASP1 was detectable in the nucleus by immunoblotting, while in Ishikawa cells BASP1 was only detected in the cytoplasm (fig.8). The primarily cytoplasmic localisation of BASP1 in Ishikawa cells, coupled with BASP1s exhibition of tumour suppressor activity, is comparable to that previously observed in some other cell types. In iPSCs, BASP1 has been shown to affect transcription despite not being detected in the nucleus by immunofluorescence (*101*). The mechanisms for these effects are not known. It is possible that BASP1 is exclusively cytoplasmic, and exerts its effects on gene expression by modulating activity or nuclear import of other transcription factors. Another possibility is that very low, undetectable levels of BASP1 are localised in the nucleus, and that this low level nuclear BASP1 affects transcription of certain genes directly.

More experimentation could be conducted in order to further investigate the subcellular localisation of BASP1 in Ishikawa cells. One option is to subject the cells to immunofluorescence microscopy, probing for BASP1. This could detect possible low level BASP1 in the nucleus, if it is localised there. However, as previously shown, BASP1 wasn't detectable by immunofluorescence in iPSCs but was shown to affect transcription via regulation of WT1 in iPSCs (*101*). It is possible that, even if immunoblotting and immuno-fluorescence fail to detect nuclear BASP1, BASP1 could be present at extremely low levels in the nucleus. Clear demonstration by ChIP that BASP1 localises to gene promoters would provide evidence for the nuclear localisation of BASP1.

Previous MCF7 data showed BASP1 to reduce colony formation efficiency and to enhance the tamoxifen response (*44*). While similar tumour suppressor

activity was observed in Ishikawa cells, one difference between the cell types was that BASP1 seemed to have very little effect on the phenotype changes induced by tamoxifen in Ishikawa cells (fig.10-13). In both colony formation assays and growth assays, tamoxifen treatment had almost no significant effects on cell proliferation or colony formation. This is in contrast to previous observations in endometrial cells, in which tamoxifen has been shown to enhance cell growth and proliferation (*107*). It is possible that the concentration of tamoxifen used was too low or the incubation time with tamoxifen too short for the full effects to be observed. It is known that tamoxifen has many long term effects on endometrial cancer cells so it is also possible that the experiments used too short a time scale. Since qPCR of Ishikawa stable cell lines indicated that BASP1 did enhance the effects of Tamoxifen treatment on a subset of ERα target genes (fig.15), it is possible that with higher drug concentrations or drug incubation periods, the effects of BASP1 on phenotypic tamoxifen response in Ishikawa cells could be detected.

BASP1 as a transcriptional regulator 5.2

The exact mechanisms by which BASP1 exerts tumour suppressor activity in breast cancer cells, acute myelogenous leukaemia cells, hepatocellular carcinoma cells and thyroid cancer cells are not known, but multiple studies have found that BASP1 possesses transcriptional coregulatory activities. BASP1 has been shown to interact with several major transcription factors including WT1, c-myc and ERα in a variety of cell types (*17, 19, 44*). In the BASP1-WT1 interaction, BASP1 binds to WT1 and converts it from a transcriptional activator to a transcriptional repressor of WT1 target genes. BASP1 does this by inducing the dissociation of CBP and the recruitment of HDAC1 to the WT1 transcriptional complex (*27*). In the case of BASP1-myc interaction, BASP1 has been shown to block myc induced changes in fibroblasts, but it is not known by what mechanism this occurs (*19*). More recently, the BASP1-ERα interaction has been investigated, and BASP1 has been shown, in breast cancer cells, to interact with ERα and localise directly at the promoters of ERα target genes. This interaction was found to have significant effects on the transcriptional activity of ERα in response to treatment with the ERα ligand Tamoxifen (*44*).

In addition to the effects of BASP1 expression on Ishikawa cell proliferation, differing BASP1 expression was shown to result in changes in expression levels of a number of investigated genes (fig.14). PGR, Greb1, TFF1 and Rab31 expression levels were all significantly changed in response to differing levels of BASP1 expression. These four genes are all known ERα target genes, implying that BASP1 could be affecting ERα mediated transcriptional regulation. This activity of BASP1 as an ERα cofactor has already been observed in breast cancer cells (*44*) so it is possible that Ishikawa cells and breast cancer cells share similarities in BASP1 function. However, the BASP1 dependent ERα target genes in breast cancer cells were different from those seen in Ishikawa cells. In MCF7 breast cancer cells,

BASP1 was not shown to regulate PGR, Greb1 or TFF1 and BASP1 did not affect tamoxifen induced expression changes of these genes (*44*). This indicates that, while BASP1 may similarly interact with ERα in the two cell types, the target genes and thus the effects of this interaction may be very different. It should also be noted that the transcriptional effects of BASP1 in other cell types are highly complex and it is possible that these gene expression changes seen in ERα target genes in Ishikawa cells are secondary effects of other transcription factors.

VDR was mostly unaffected by changes in BASP1 expression and tamoxifen treatment (fig.14-16). In one control Ishikawa cell line, VDR was significantly downregulated, but this effect was not reproduced in the other control cell line, nor was it seen in either the B-Ishikawa or sh-BASP1 Ishikawa cell lines. This suggested that the downregulation of VDR in V-Ishikawa cells could have been a false positive result. VDR is a known WT1 target gene but several studies of WT1 have uncovered cell type-specific effects on the genes that it regulates (*108*). This is in contrast to the regulation of VDR by WT1/BASP1 in K562 cells, podocyte cells and iPSCs (*16, 17, 101*) but it further supports the hypothesis that the transcriptional effects produced by BASP1 could be occurring through ERα, as was previously seen in breast cancer cells (*44*). However, gene expression of more WT1 target genes in Ishikawa cells with varying BASP1 expression levels would be required before any conclusions about WT1 in Ishikawa cells could be drawn.

In the BASP1-ERα interaction previously observed in MCF7s, BASP1 localised directly at the promoters of ERα target genes. BASP1 expression was also shown to enhance the tamoxifen induced downregulation of a subset of ERα target genes, including XBP1 and Rab31 (*44*). In Ishikawa cells, tamoxifen treatment had no significant effects on XBP1 or Rab31 and altering BASP1 expression produced no significant change in this result. It is possible that, as mentioned above, tamoxifen concentration or incubation time was too low to produce a detectable effect on these

genes. However, since effects on other genes were detected, it is also possible that this was a result of the differences in BASP1 and ERα mechanisms of action between MCF7 breast cancer cells and Ishikawa cells. Differences between the cell types in BASP1 and ERα mechanisms of action are highly probable since BASP1 has been shown to possess a complex range of transcriptional modulation activities in different cell types (*17, 44, 101*) and tamoxifen induced ER activity is known to be very different in Ishikawa cells from MCF7 cells (*81*).

However, analysis of gene expression in Ishikawa cells by qPCR indicated that BASP1 enhances tamoxifen induced upregulation of a different subset of ERα target genes, PGR, Greb1 and TFF1, none of which were shown to be significantly affected by BASP1 in MCF7 cells. This suggests that BASP1 may modulate the tamoxifen response in Ishikawa cells by a similar mechanism to that which was seen in MCF7 cells, despite the opposing activities of ERα between the two cell types, but that this mechanism targets a different set of genes. In Ishikawa cells, BASP1 overexpression was found not only to enhance the stimulation of PGR by TAM, but also to repress PGR expression in the absence of TAM. This indicates that the effect of BASP1 overexpression on PGR expression was reversed by tamoxifen treatment. These results together suggest a complex relationship between BASP1 and tamoxifen action in Ishikawa cells. In MCF7 cells BASP1 was not found to regulate PGR expression or to affect tamoxifen induced downregulation of PGR. Therefore, these results also highlight the differences in BASP1 functions between MCF7 breast cancer cells and Ishikawa endometrial cancer cells. It is possible that these differences in the functions of BASP1 between breast cancer cells and endometrial cancer cells could contribute to the differences in tamoxifen response between the two tissue types.

The similarities in tumour suppressor activity and enhancement of tamoxifen induced gene expression changes by BASP1 between MCF7 cells and Ishikawa cells

raised the question, does BASP1 localise to ERα target genes in the nucleus of Ishikawa cells? ChIP analysis of BASP1 binding to the promoters of ERα target genes in Ishikawa cells produced insignificant results (fig.18). This was probably due to the requirement for further optimisation of the cross linking and sonication steps of the ChIP protocol (fig.17). While BASP1 binding was not significant, neither was ERα binding which was used as a positive control. The comparable detection of ERα and BASP1 at the promoters raises the possibility that BASP1 could be present at these promoters, but further optimisation and repeats of this ChIP are required before this conclusion can be made.

In all, the results of this study indicate that BASP1 possesses tumour suppressor activity in Ishikawa cells and that this could be in part mediated by co-regulation of ERα. In addition, BASP1 overexpression was shown to enhance tamoxifen induced gene expression changes of a subset of ERα target genes, suggesting similarities in the mechanism of action to that of MCF7 cells, but that these effects of BASP1 target a different set of genes in Ishikawa cells.

5.3 Future directions

5.3.1 Global RNA Sequencing

One of the goals of future work investigating BASP1 in endometrial cancer cells is to perform RNA sequencing on the Ishikawa cells. Gene expression analysis of Ishikawa cells by qPCR indicated that BASP1 enhances the effects of tamoxifen on gene expression of a subset of ERα target genes (fig.15). This effect has been shown in both Ishikawa cells (fig.15) and previously in MCF7s (44). In Ishikawa cells, this effect was shown for PGR, Greb1 and TFF1 but not XBP1 or Rab31 in Ishikawa cells (fig.15) while the opposite was seen in MCF7 cells (44). The effects of BASP1 expression in MCF7s was previously investigated by full RNA sequencing, in addition to qPCR analysis (44). Sequencing showed that 40% of tamoxifen induced gene expression changes in MCF7 cells were BASP1 dependent. RNA sequencing of Ishikawa cells would allow the effects of BASP1 expression on tamoxifen induced gene expression to be compared to those of MCF7 cells. The crossover and differences between the cell types could then be analysed to investigate the differences in BASP1 mechanisms in the two cell types. This data could reveal currently unknown roles of BASP1 in ERα activity in endometrial cancer cells and possibly, currently unknown roles of BASP1 in the oncogenic effect of tamoxifen in endometrial cancer cells.

5.3.2 Other endometrial cell lines

Another goal of future work will be to investigate the effects observed in this study in other endometrial cancer cell lines. The ERα-BASP1 interaction has been demonstrated in two breast cancer cell types: MCF7 cells and T47D cells. In order to

make any conclusions about the effects of BASP1 on ERα activity and the possible roles of BASP1 in the oncogenic effects of tamoxifen treatment on endometrial cells, the results will need to be reproduced in multiple endometrial cancer cell lines. Some endometrial cancer cell options for this include ECC1 and MFE-280 stable cell lines. Both lines, like Ishikawa cells, highly express ERα so BASP1 expression, localisation and effects on ER target genes could be investigated in these cell types. These cell lines would also provide a range of genotypes. Ishikawa cells have an epithelial-like morphology, while ECC1 cells maintain luminal cell morphology and MFE-280 cells have epithelial morphology. Investigation of these phenotypically diverse ER+ endometrial cancer cell lines could indicate whether the effects of BASP1 expression seen in this study are consistent across multiple endometrial cancer cell types.

5.3.3 BASP1 G2A mutant

One additional goal of future work will be to produce BASP1 G2A mutant Ishikawa cells in order to determine whether or not the effects seen in this study are dependent on myristoylation of BASP1. Conversion of glycine 2 in BASP1 to an alanine produces G2A mutant BASP1 which cannot be myristoylated. G2A BASP1 is therefore unable to interact with PIP2 and thus unable to bind to lipid membranes. It's been shown that this myristoylation of BASP1 is required for transcriptional co-suppression of WT1 target genes in embryonic kidney cells (*12*). Investigation of the effects of G2A BASP1 on tamoxifen induced gene expression changes could indicate whether myristoylation of BASP1 is required for the effects on ERα target genes seen in this study and provide a better idea of the mechanisms or localisation of BASP1 in Ishikawa cells.

5.3.4 Endometrial cancer tissue analysis

Finally, another goal for future work will be to analyse endometrial cancer tissue by immunohistochemistry. This would allow comparison of BASP1 expression and localisation between endometrial cancer tissue and normal endometrial tissue. This was previously done in MCF7 cells and it was found that both ERα and BASP1 were upregulated in breast cancer tissue compared with the normal breast tissue (*44*). In addition, it would allow this comparison to be made between benign endometrial tumour tissue and endometrial cancer tissue. This would serve to investigate whether any changes in BASP1 expression and localisation are associated with tumour progression as well as with tumour initiation in endometrial tissue.

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