A Transcriptional Analysis of Sirtuins in Breast Cancer

by

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Preface

The work presented in this thesis was performed solely by the author, except as acknowledged. This thesis has not been previously submitted for a degree or diploma at this or any other institution.

Nadeem Ashraf

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Table of Contents

List of Contents

List of Figures

List of Tables

List of Publications

Papers

1. **Ashraf N.**, Zino S., Kingsmore D., Payne A.P., George, W.D. and Shiels, P.G. (2006) Altered sirtuin expression is associated with node positive breast cancer. *British Journal of* Cancer, **95**, 1056-61.

2. Sklavounou E, Hay A, **Ashraf N**, Lamb K, Brown E, MacIntyre A, George WD, Hartley R and Shiels PG. (2006) Superior nitrone based anti-oxidants mitigate the effects of telomere independent stasis in primary human diploid fibroblasts. *Biochemical Biophysical Research Communications*, **347**, 420–427.

3. **Ashraf N.**, Zino S., Kingsmore D., Payne A.P., George, W.D. and Shiels, P.G. (2006) Sirtuin expression as an indicator of damage responses in breast cancer therapy. *Breast Cancer Research and Treatment*, Submitted.

Abstracts

1. **Ashraf, N.**, Zino, S., George, W.D. and Shiels, P.G. (2006) Sirtuin expression correlates with irradiation and paclitaxel treatment in breast cancer. *European Journal of Surgical Oncology*, 32, Supplement 1, S81.

2. Zino, S., **Ashraf, N.**, Kingsmore, D.B., Payne, A.P., George, W.D. and Shiels, P.G. (2006) Altered SIRT2 and SIRT6 expression is associated with breast cancer. *European Journal of Surgical Oncology*, 32, Supplement 1, S90.

3. Zino, S., **Ashraf, N.**, Kingsmore, D.B., Payne, A.P., George, W.D. and Shiels, P.G. (2006) Sirt6 expression is altered in breast cancer. *European Journal of Surgical Oncology*, , 32 (9), p1048.

4. **Ashraf, N.**, George, W.D. and Shiels, P.G. (2006) Altered sirtuin expression is associated with prognostically poor breast cancer. *British Journal of Surgery*, **93**, Supplement 1, p161.

5. **Ashraf, N.**, George, W.D. and Shiels, P.G. (2004) P53 dependence of sirtuin expression in response to ionizing radiation in mammary cancer cell lines. *Abstract book of the 7th Cancer Research UK Beatson International Cancer Conference*, Glasgow, U.K.

Abbreviations

AceCS	Acetyl CoA Synthetase
ATM	Ataxia telangectasia gene
CDK	Cyclin-dependent kinase
CR	Caloric restriction
DSB	Double stranded break (DNA)
ER	Estrogen receptor
ERC	Extrachromosomal ribosomal DNA circles
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HMEC	Primary human mammary epithelial cells
HMLa	Homothallic left
HMRα	Homothallic right
HPRT	Hypoxanthine phosphoribosyltransferase
HR	Homologous recombination
mMPTP	Mitochondrial membrane permeability transition pore
MTR	Mitochondrion, telomere nucleo-protein and ribosome
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide
NHEJ	Non-homologous end joining
OAAR	O-acetyl-ADP-ribose
PCAF	p300/CBP-associated factor
PEV	Positional effect variegation
PI	Propidium iodide

Pol I	RNA polymerase I
rRNA	Ribosomal RNA
rDNA	Ribosomal DNA
RENT	Regulator of nucleolar silencing and telophase exit
ROS	Reactive oxygen species
RS	Replicative senescence
SA-β-Gal	Senescence-associated β-galactosidase
SIR2	Silent information regulator 2
siRNA	Short interfering RNA
SIRT	Sirtuin
SSB	Single stranded break (DNA)
STASIS	Stress or aberrant signalling-induced senescence
TUNEL	Terminal UdTP nick end labelling
UCP-2	Uncoupling protein 2 (mitochondrial)

Summary

Breast cancer is the most common cancer to affect women, and is associated with abnormalities in normal growth and proliferation. An alternative way for understanding this disease, is to view it as an aberration of normal biological ageing. The mitochondrion, telomere nucleo-protein complex and ribosomal DNA, comprise the MTR, and link energy production, protein synthesis and DNA damage responses. Sirtuins act within the various components of the MTR, imbalance of which, may result in accelerated ageing, disease and tumorogenesis, a hypothesis first described by Shiels and Davies (2003).

Consistent with this viewpoint, altered sirtuin expression is associated with a number of ageing-related diseases, progeroid conditions and malignancies. This observation makes their study particularly pertinent for understanding how breast cancer develops and progresses from a platform of aberrant biological ageing. Similarly, the study of sirtuins may be relevant to the study of breast cancer therapy, as these treatments induce cellular senescence and apoptosis, abnormalities of which are also implicated in ageing. Furthermore, the ability of breast cancer cells to withstand the insult of cytotoxic treatments may be determined by the biological age of tumour cells.

The aim of the research presented within this thesis, was to study breast cancer as an aberration of normal biological ageing, with respect to sirtuin expression. It specifically sought to determine whether there was an association between the transcriptional expression of sirtuins and breast cancer pathogenesis and treatment.

The transcriptional expression of sirtuins was initially investigated in primary and immortalised mammary epithelial cells, as a function of *in vitro* growth. In order to determine whether changes in biological ageing, observed in primary mammary epithelial cells, were pertinent to breast cancer *in vivo*, transcriptional expression of sirtuins was studied in non-malignant and malignant breast tissue biopsies.

Transcriptional studies were also performed to determine the relationship between common therapies used for the treatment of breast cancer, namely radiotherapy and chemotherapy, and sirtuin expression. Specifically, sirtuin expression was investigated in breast cancer cells in response to cellular irradiation and paclitaxel treatment.

The expression of *SIRT3* and *SIRT7*, the gene products of which act at the mitochondrion and ribosomal DNA components of the MTR, was found to increase as cells underwent replicative ageing. Similarly, the transcriptional expression of *SIRT3* and *SIRT7* was elevated in lymph node positive breast cancer biopsies compared to non-malignant breast biopsies. In contrast, *SIRT1* and *SIRT2* showed no difference in expression in either replicatively ageing cells or between malignant and non-malignant breast biopsies. The increased *SIRT3* and *SIRT7* expression observed in lymph node positive breast cancer biopsies, suggests that tumours associated with more invasive disease display perturbation within components of the MTR, indicative of increased biological ageing..

In breast cancer cells exposed to irradiation and paclitaxel treatment, *SIRT2* and *SIRT7* transcriptional expression was found to increase. In contrast, *SIRT1* and *SIRT3*

expression remained relatively unchanged. The increased expression of *SIRT2* and *SIRT7* in response to these treatments, suggests that these therapies target components of the MTR that are associated with mitotic growth arrest. In particular, SIRT7, is associated with ageing and indicates that these agents induce ageing in these tumour cells.

In summary, this research shows aberrant sirtuin expression to be a feature of primary mammary epithelial cell senescence, breast cancer pathogenesis and damage responses in breast cancer cells, secondary to ionising radiation and paclitaxel therapy.

Chapter 1

Introduction

1.1 Breast cancer

Breast cancer is the most common cancer to affect women with over 40,000 new cases being detected and around 13,000 women dying from the disease each year (CancerStats, 2005). Breast cancer represents a heterogeneous group of diseases that display a wide spectrum of behaviour ranging from slow growing, non-metastatic disease with an excellent prognosis through to fast growing disease with a propensity for early metastasis and rapid death. Traditionally, tumour stage and grade have been useful in predicting prognosis but fail to predict the clinical course of the disease. The identification of factors that can help understand the natural history of breast cancer as well as identify appropriate treatments is therefore a major area of cancer research.

1.1.1 Tumour Biology

Studies looking at the molecular biology of breast cancer seek to gain a better understanding of this disease to aid diagnosis and management. The incidental discovery by Beatson, that oophorectomy resulted in breast cancer remission, led to the understanding that many breast cancers are oestrogen dependent and resulted in the development of hormone therapies that include tamoxifen and peripheral aromatase inhibitors. More recently, upregulation of telomerase and altered *ERBB2* gene expression have been shown to be features of breast cancer (Russo and Russo, 2003).

An improved understanding of breast cancer biology provides an alternative way of predicting tumour behaviour, distinct from those that have looked at features such as tumour size, histological grade and nodal status. Recent studies using DNA microarrays suggest that breast cancer can be classified into at least four distinct forms based upon the hierarchical clustering of microarray data. These classes are Luminal/ER positive, Basal, ERBB2 positive and Normal-like types (van 't Veer et al., 2002). This classification has been shown to predict prognosis independent of traditional features such as age, lymph node and estrogen receptor (ER) status (Sorlie et al., 2001, van 't Veer et al., 2002). This system also indicates that tumour behaviour may be understood outwith factors such as tumour growth and proliferation. An example of this is the discovery that ERBB2 positive tumours display lower levels of proliferation than Luminal and Normal-like types, despite being associated with a poorer prognosis.

This observation highlights the deficiency of breast cancer studies that have focused on transformation as a result of abnormalities in normal growth and proliferation. It is for this reason that the study of breast cancer as an aberration of normal biological ageing is particularly pertinent. Studying breast cancer from this perspective provides an alternative platform for understanding the disease. Specifically, a better understanding of the molecular processes involved in sensing, assessing and signalling cellular damage might be informative in such a context (Shiels and Davies, 2003).

1.1.2 Cancer therapy

The relationship between ageing and breast cancer may reflect the accumulation of cellular damage over a cell's lifespan, as well as a decline in the cell's ability for damage repair. Similarly, the ability of breast cancer cells to withstand the insult of cytotoxic therapies may also be influenced by genes involved in biological ageing. Therapies commonly used for the treatment of breast cancer, including radiotherapy and chemotherapy, often result in senescence and apoptosis, processes that also contribute to ageing and the pathogenesis of age-related diseases (Shay and Roninson, 2004, Shiels and Davies, 2003). Understanding the biology of breast cancer therapies, may help in tailoring treatment to individuals and their disease biology, as well as helping in the development of new therapies.

Genes central to the control of biological ageing include those that maintain telomere structure, control protein synthesis and regulate mitochondrial energy production. The *SIR2*, silent information regulator, family of genes have been hypothesised to link the functions of the mitochondrion, telomere nucleo-protein complex and ribosome production (the MTR) and contribute to a wide range of ageing related diseases including cancer (Shiels and Davies, 2003, Shiels, 1999).

The functions of Sir2 have been best characterised in the baker's yeast, *Saccharomyces cerevisiae*, and include silencing at telomeres, DNA repair, cell-cycle control and the regulation of ageing (Guarente, 1999). These functions bear close parallels to the function

of Sir2 orthologues, known as sirtuins, in humans. An understanding of sirtuin biology may, therefore, help us in understanding how breast cancer develops and progresses from a platform of biological ageing.

1.2 SIR2

The *SIR2* family of genes comprises a large group of highly conserved genes in organisms ranging from bacteria to man (Brachmann et al., 1995, Frye, 1999). As previously mentioned, *SIR2* in *S. cerevisiae* is the best characterised of these and functions in a variety of essential cell processes, ranging from silencing at telomeres and mating type loci, chromosome segregation, DNA repair, regulation of the cell-cycle, meiosis and ageing (Guarente, 1999). The process of silencing and each of these functions are explored below.

1.2.1 Silencing

Unlike promoter, or sequence-specific transcriptional control of genes, silencing is the reversible, heritable, transcriptional repression of large chromosomal domains regulated in a position-dependent manner (Loo and Rine, 1995). Classic examples of this include mammalian X chromosome inactivation and Drosophila eye colour mosaicism (Lyon, 1961).

The Sir2 proteins are thought to perform this function by enzymatically modifying histone proteins within chromatin (Guarente, 1999). Indeed, silenced heterochromatin, found at telomeres, centromeres and within ribosomal DNA, contains hypoacetylated histones, unlike its transcriptionally active, euchromatin counterpart (Braunstein et al., 1993, Loo and Rine, 1994, Bi and Broach, 1997). This heterochromatin is compact, with

restricted access to transcription, replication and recombination machinery (Tse *et al.*, 1998). Recent studies, however, suggest that Sir2-mediated transcriptional repression does not occur simply by preventing access to transcriptional machinery, but occurs downstream from gene activator protein binding (Chen and Widom, 2005).

1.2.2 Biochemistry

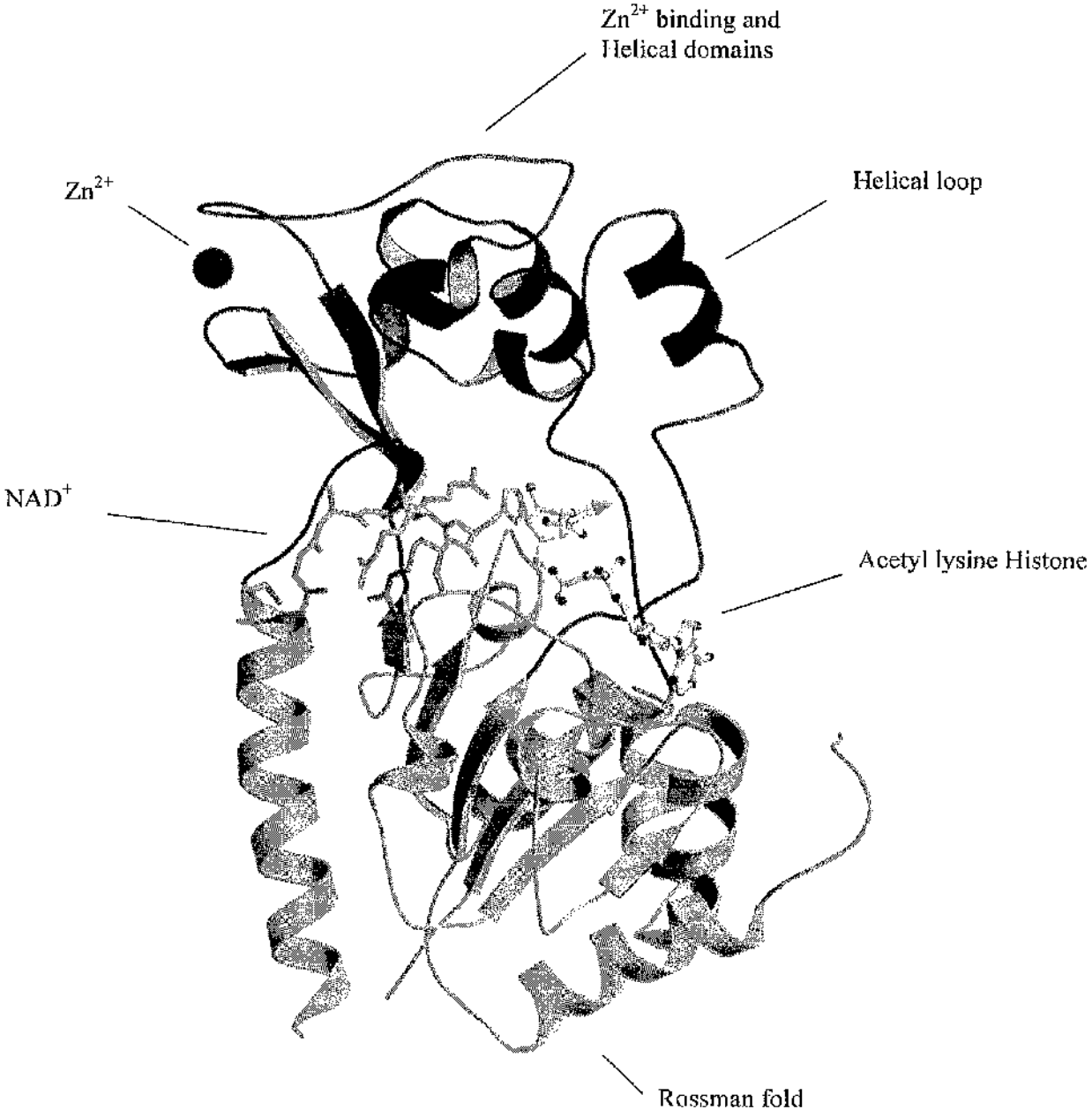
1.2.2.1 Structure

The Sir2 protein family has been sub-classified into five classes, I, II, III, IV and U, based upon sequence homology (Frye, 2000).

In *S. cerevisiae*, four additional Sir2-related proteins exist, encoded by *HST1-4* (homologues of sir two). All of the Sir2 proteins possess a specific C-terminal core region of around two hundred and seventy five amino acid residues that is widely conserved and vital for silencing activity (Brachmann et al., 1995, Cockell and Gasser, 1999).

Four major parts constitute the Sir2 structure, a Rossman fold, a flexible loop and helical and zinc-binding domains (Finnin et al., 2001, Min et al., 2001). The enzymatic co-substrate, NAD, binds in a pocket between the helical domain and Rossman fold, and is flanked by the loop (Figure 1.1).

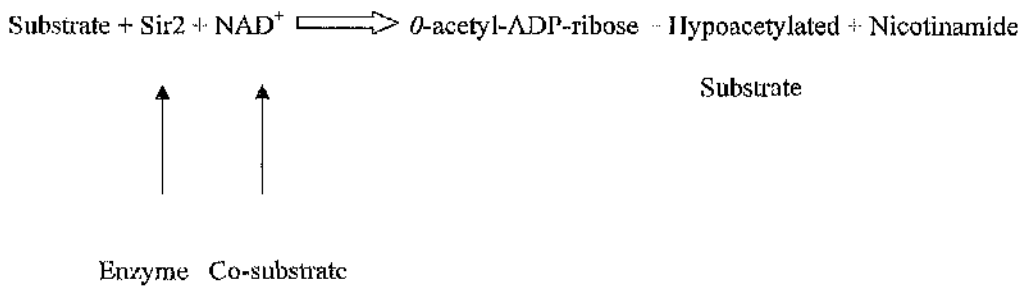
Figure 1.1 Structure of Sir2. The figure shows a schematic representation of the Sir2 protein interacting with its NAD co-substrate and acetyl lysine histone substrate. [Figure adapted from (Marmorstein, 2004)]



1.2.2.2 Enzymatic activity

The Sir2 proteins are class III histone deacetylases (Tanny et al., 1999, Frye, 1999) and have an NAD-dependent function (Imai et al., 2000, Landry et al., 2000, Smith et al., 2000, Tanner et al., 2000). Unlike the other deacetylase classes, they do not have a zinc catalysed function and are not inhibited by trichostatin. The Sir2-dependent deacetylation reaction is tightly coupled with NAD cleavage and the formation of an *O*-acetyl-ADP-ribose (OAAR) product (Figure 1.2).

Figure 1.2 Mechanism of Sir2 enzymatic activity.



The Sir2 proteins also possess ADP-ribosyltransferase activity that may be important in DNA repair (Kreimeyer et al., 1984, Pero et al., 1985). The production of the OAAR metabolite may itself, perform an important function (Tanner *et al.*, 2000). Indeed, OAAR has been shown to play an important role in delaying/blocking oocyte maturation as well as embryo cell division in blastomeres (Borra *et al.*, 2002).

Finally, reversible protein deacetylation is known to be an important post-translational modification in the regulation of many biological processes (Kouzarides, 2000, Sterner

and Berger, 2000). Although Sir2 activity was originally thought to be restricted to the acetyl lysine residues on histones three and four in nucleosomes (Braunstein *et al.*, 1993), the discovery of Sir2 orthologues in organisms that do not have histones, as well as orthologues with extranuclear locations (Zemzoumi et al., 1998, Afshar and Murnane, 1999, Perrod et al., 2001, Vergnes et al., 2002), suggests that targets of Sir2 also include non-histone proteins. Indeed, Sir2 orthologues have been shown to deacetylate a various substrates, including p53 in humans (Luo et al., 2001, Vaziri et al., 2001) and the TAF_I68 subunit of the TATA-box binding protein-containing factor in mice (Muth *et al.*, 2001).

1.2.2.3 Nicotinamide

Nicotinamide, a form of vitamin B_3, is a product of the Sir2 deacetylase reaction and potent non-competitive inhibitor of Sir2, NAD-dependent deacetylase activity (Landry *et al.*, 2000). It is also a substrate used by nicotinamidase, encoded by *PNC1*, for NAD synthesis. Although the 1:1 requirement of NAD for Sir2 deacetylase activity predicts that NAD levels may be a rate limiting component for its activity, cellular NAD levels remain relatively stable. In fact, no change in overall NAD levels are observed in cells lacking *PNC1* despite a resultant fall in Sir2-dependent silencing (Sandmeier *et al.*, 2002). This observation suggests that it is nicotinamide rather than NAD levels that determine the degree of SIR2 silencing. However, it may be that specific compartmental changes in NAD, or changes in the levels of the NAD pre-cursor, nicotinic acid mononucleotide, regulates Sir2 activity (Bitterman *et al.*, 2002).

1.2.3 Functions

SIR2 provides a functional link between the telomere and nucleolar ribosomal DNA cluster integrity, thus impacting on ribosome production and enabling the cell to respond to stress-induced redox state changes within the mitochondrion (Guarente, 1999). Consequently, damage responses in the cell can be modulated to match the degree of insult. The functions of Sir2, as a consequence of endogenous and exogenous stressors, are explored below.

1.2.3.1 Silencing at mating type loci

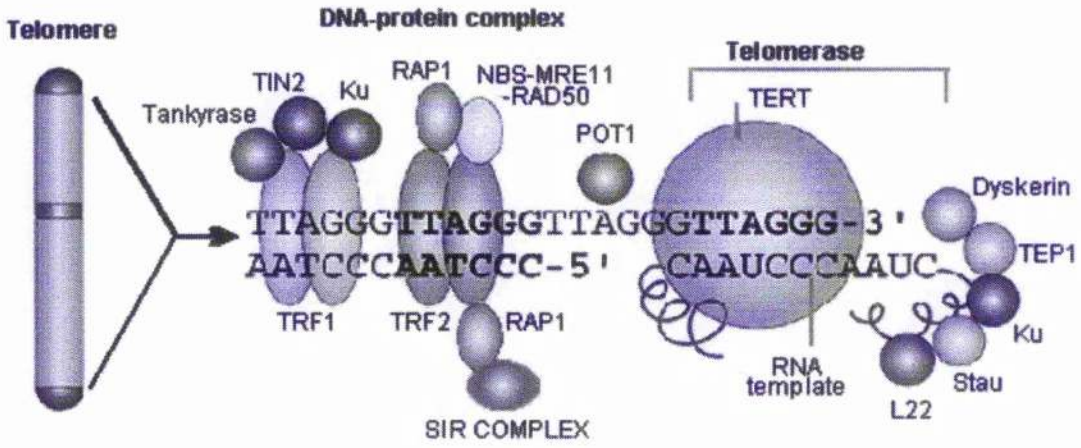
Sir2 silencing activity was originally described for *S. cerevisiae*, and confers it with the capability of expressing two very distinct mating phenotypes, depending on nutritional availability. Silencing at the mating type loci, homothallic left, HML**a**, and homothallic right, HMRα, on chromosome three results either in the expression of the **a** or α mating phenotype (Rine and Herskowitz, 1987). If there are mutations in *SIR2*, silencing at both of these regions is lost, with resultant co-expression of the **a** and α mating type genes and sterility in haploid strains (Kaeberlein *et al.*, 1999)

1.2.3.2 Telomeric silencing

Although the majority of Sir2 is found within the nucleolus (Gotta *et al.*, 1997), Sir2 proteins, along with other telomere binding proteins, are also visualised at the telomeres (Figure 1.3) (Moretti *et al.*, 1994). Sir2 helps preserve and stabilise telomeres and also suppress the transcription of reporter genes placed at telomere-proximal sequences, a

process known as positional effect variegation (PEV) (Palladino et al., 1993, Gottschling et al., 1990). *SIR2* mutations therefore result not only in a loss of PEV and constitutive expression of telomeric reporter genes, but also in telomere shortening (Aparicio et al., 1991, Palladino et al., 1993).

Figure 1.3 Telomeric nucleo-protein complex. The figure shows the interaction of Sir2 with the DNA-protein complex and the telomerase enzyme at the telomere. Collectively these structures form part of the telomeric nucleo-protein complex [Figure adapted from Hodes *et al.*, (2002)]



The Sir2 protein complex interacts with telomeres via a set of DNA binding factors, including Rap1 and the Ku heterodimer. Rap1, has itself been shown to bind directly to telomere-repeat sequences (Moretti *et al.*, 1994). As with *Sir* mutants, *Ku* mutants also display a loss of telomeric silencing and have a more splayed telomeric arrangement (Laroche *et al.*, 1998).

1.2.3.3 DNA repair

Sir2 proteins are also required for the repair of double stranded breaks (DSB) in DNA. Following the initial recruitment of Ku and Rap1, the Sir protein complex redistributes from telomeres to sites of DSB (Martin et al., 1999, Mills et al., 1999). Ku may bind the ends of the DSBs, protecting them from nucleolytic degradation, thereby allowing Sir proteins to generate heterochromatin to constrain the ends and facilitate end joining by ligase activity (Tsukamoto et al., 1997, Jackson, 1997).

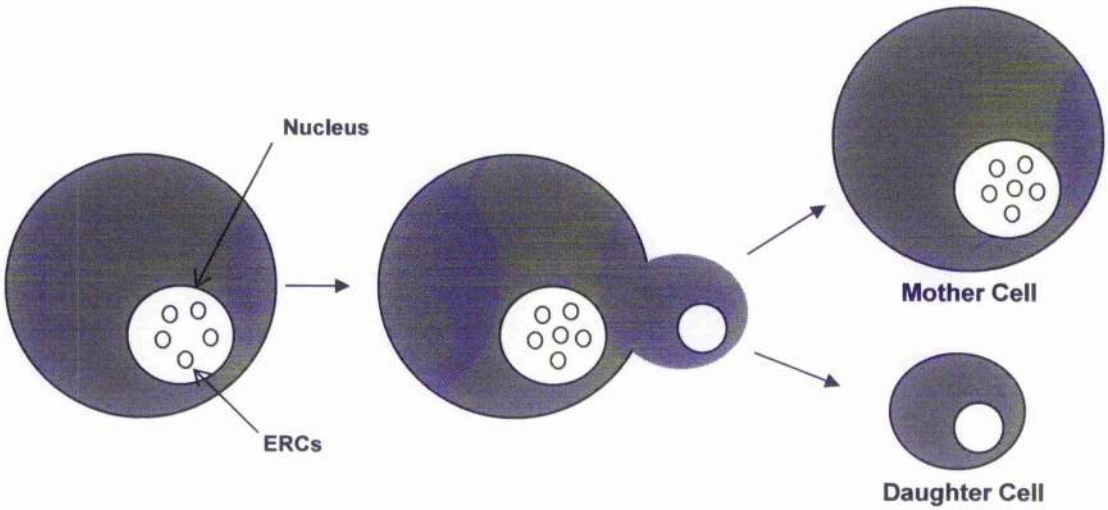
Despite DSBs in yeast being repaired mainly by an homologous recombination (HR) pathway, Sir2-mediated repair occurs by non-homologous end joining (NHEJ) (Critchlow and Jackson, 1998, Tsukamoto et al., 1997). NHEJ, however, is the main method of DSB repair in mammals. Yeast cells harbouring mutant *SIR2* display a significant decrease in NHEJ, less efficient DSB ligation and increased radiosensitivity (Astrom et al., 1999, Martin et al., 1999, Tsukamoto et al., 1997).

1.2.3.4 Ageing

Sir2 is also involved in the silencing of the ribosomal DNA (rDNA) cluster on chromosome twelve. This DNA contains one to two hundred repeats of a nine kilobase unit, which encodes ribosomal RNA (rRNA) that is transcribed and assembled into ribosomes within the nucleolus (Shaw and Jordan, 1995). Sir2 has been shown to relocate to the nucleolus with increasing age, and Sir2 concentration is directly related to lifespan (Kaeberlein *et al.*, 1999).

HR-mediated repair of DSB occurring within the rDNA results in the formation of extrachromosomal ribosomal DNA circles (ERC) (Park *et al.*, 1999). These structures are segregated in a biased manner towards the yeast mother cell. Once accumulation of these ERCs reaches a critical level the mother cell can no longer divide (Figure 1.4) (Sinclair and Guarente, 1997).

Figure 1.4 Nucleolar ERC formation and preferential segregation to mother cells. The figure shows the preferential segregation of extrachromosomal ribosomal DNA circles (ERC) to the mother cells with cell division.



Sir2 is involved in transcriptional silencing at the rDNA as well as in recombinational suppression, thus preventing the accumulation of ERCs (Gottlieb and Esposito, 1989, Bryk et al., 1997, Smith and Boeke, 1997). Loss of function mutations in *SIR2* results in an increase in ERC generation and cell ageing.

1.2.3.5 Cell cycle

The ability of *S. cerevisiae* to initiate DNA replication requires the assembly of pre-replicative complexes (pre-RCs). The origin replication complex involved in formation of silenced chromatin, is essential for DNA replication and binds to and recruits factors to pre-RCs. This results in DNA unwinding and recruitment of polymerases, and thereby allows DNA replication. In a study by Pappas *et al.*, (2004), Sir2 was shown to inhibit pre-RC assembly and therefore DNA replication. Sir2 function prevents chromosomal DNA replication, thereby preventing an increase in ploidy and resultant genomic instability.

Sir2 along with the net1, cdc14 and nan1 proteins forms the RENT (regulator of nucleolar silencing and telophase exit) complex, that controls cell-cycle progression (Shou et al., 1999, Straight et al., 1999). Cdc14 functions to inactivate cyclin-dependent kinase (cdk) activity at the end of mitosis. Cdc14 is kept anchored in an inactive state within the nucleolus by the RENT complex but can redistribute into the cytoplasm, thereby allowing re-entry into the G_1 phase. Cells lacking Cdc14 arrest have spindle abnormalities, chromosomal defects and genome instability (Pringle and Hartwell, 1981).

1.2.3.6 Meiosis

Sir2 also functions in a meiotic checkpoint to monitor chromosome segregation. The pch2 protein has been shown to be required for the pachytene checkpoint of meiosis as yeast mutants, deficient in pch2, are able to bypass this pachytene phase arrest. These mutants can sporulate and undergo meiotic recombination (Sym et al., 1993, San-

Segundo and Roeder, 1999). Nucleolar co-localisation with Sir2 is thought to be necessary for the action of pch2. In *Sir2* mutants, nucleolar pch2 co-localisation is lost and the meiotic checkpoint disrupted resulting in an increased rate of meiotic recombination.

1.2.4 Caloric restriction

The diverse functions of Sir2 in yeast are closely linked and reflect the central role that Sir2 plays in growth and proliferation, DNA damage repair and ageing in response to exogenous stresses and nutritional availability. One of the most exciting discoveries to be made in this regard is that caloric restriction (CR) results in a Sir2-dependent prolongation of lifespan (Howitz *et al.*, 2003).

This lifespan extension can be explained by the attenuation of oxidative damage and changes in fuel utilisation. The reliance of Sir2 on its NAD co-substrate, is central to this lifespan extension. Increased mitochondrial NAD, as a result of CR, may result in a concomitant increase in Sir2 activity at telomeres and within the nucleolar rDNA (Lin *et al.*, 2002). The decrease in oxidative stresses may also result in a fall in DNA damage, and increased concentration of Sir2 at telomeres and the nucleolus.

Interestingly, the plant polyphenol, resveratrol, found in large quantities in red wine, has been found to be a potent Sir2 activator and can, like CR, increase DNA stability and extend lifespan in yeast (Howitz *et al.*, 2003).

1.3 Sirtuins

In mammals, seven Sir2-like proteins, termed sirtuins, have been discovered (Table 1.1) (Brachmann et al., 1995, Frye, 1999, Frye, 2000). Like Sir2, all contain a core domain comprising approximately two hundred and seventy five amino acid residues. Based on molecular phylogenetic analyses of both prokaryotic and eukaryotic sirtuins, sirtuins have been designated into five distinct classes, classes U and I-IV (Frye, 2000). The seven human sirtuins belonging to classes I-IV.

The human sirtuins possess variable NAD-dependent deacetylase and ADP-ribosyltransferase enzymatic activities (Schwer *et al.*, 2002). Sirtuins also vary in their intracellular location and targeting, with some members being predominantly nuclear, cytoplasmic or mitochondrial (Figure 1.9). Although human sirtuins are expressed in a wide variety of human tissue (Michishita *et al.*, 2005), they show a preponderance for metabolically active tissue, such as the heart and skeletal muscle.

Table 1.1 Classification of human sirtuins. This figure provides an overview of the molecular and biochemical features of the human sirtuins. [a]NCBI Gene ID is available from the National Center for Biotechnology Information, (NCBI), at http://www.ncbi.nlm.nih.gov.

Sirtuin	Class	[a]NCBI Gene ID	Location	Transcript(s) length (base pairs)	Amino acid residues	Molecular weight (kilodaltons)
SIRT1	Ia	23411	10q21.3	4086	747	82
SIRT2	Ib	22933	19q13.2	1963, 1931	399	43
SIRT3	Ib	23410	11p15.5	2900, 2574	399	44
SIRT4	II	23409	12q24.23	1163	314	35
SIRT5	III	23408	6p23	2350, 1670	310	34
SIRT6	IV	51548	19p13.3	1638	355	39
SIRT7	IV	51547	17q25	1718	400	45

1.3.1 Sirtuin 1

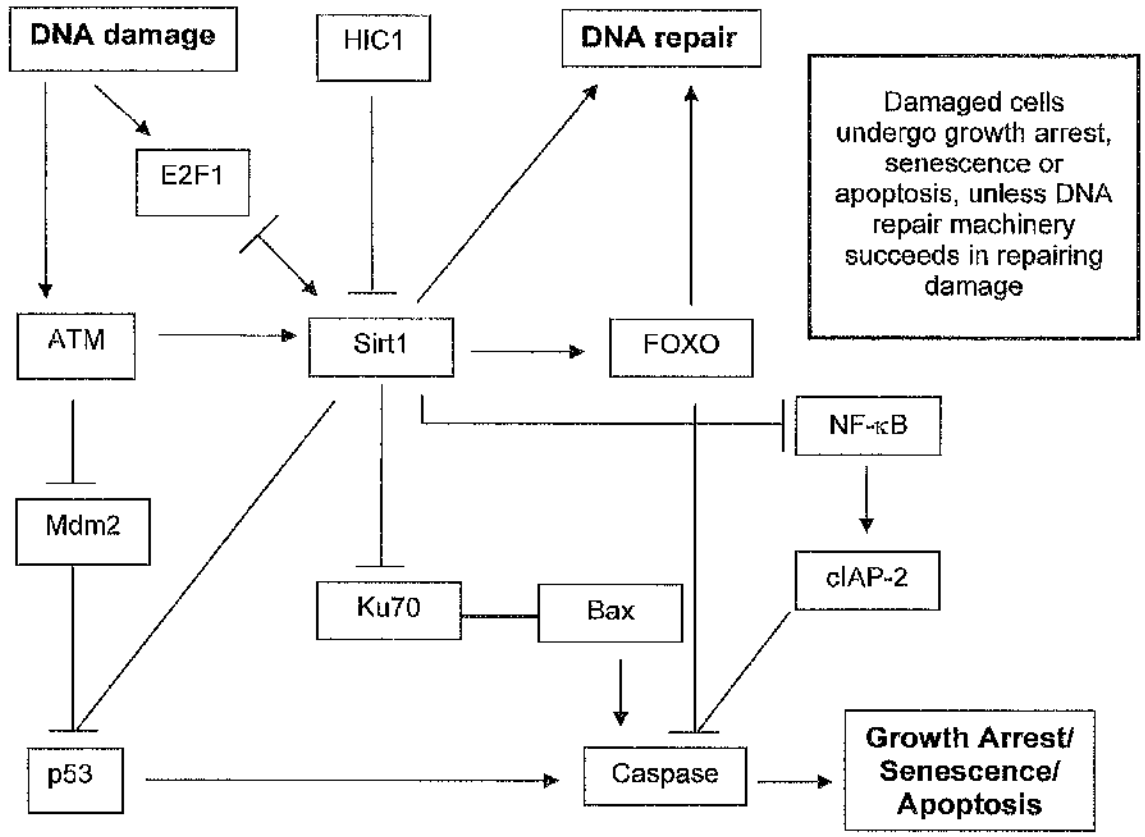
Based on protein sequence homology analysis, Sirtuin 1 (Sirt1) is the closest orthologue of yeast Sir2 (Imai et al., 2000, Frye, 1999). It is also the best characterised of the human sirtuins, unlike the remainder of which little is still known.

Like yeast Sir2, Sirt1 exhibits predominantly nuclear localisation and also interacts with and deacetylates histones, to promote the formation of repressive facultative heterochromatin. (Imai et al., 2000, Senawong et al., 2003, Vaquero et al., 2004). Sirt1 also has a non-histone substrates including p53, PCAF, p300, MyoD, the Foxo transcription factors, NF-κB and E2F1 (Yeung et al., 2004, Vaziri et al., 2001, Luo et al., 2001, Cohen et al., 2004a, Fulco et al., 2003, Motta et al., 2004, Brunet et al., 2004, van der Horst et al., 2004, Kobayashi et al., 2005, Wang et al., 2006). In addition, Sirt1 interacts with the Hes1/Hey2 factors, CTIP2 and PPAR-γ (Takata and Ishikawa, 2003, Senawong et al., 2003, Picard et al., 2004). Interaction of Sirt1 with these substrates, allows Sirt1 to be recruited to chromatin regions and specific promoters, to influence the transcription of other genes. Through these interactions, Sirt1 is thought to function in a wide variety of important cell processes, including apoptosis (Luo et al., 2001, Vaziri et al., 2001, Cohen et al., 2004a), development and embryogenesis (Sakamoto et al., 2004, Fulco et al., 2003, Cheng et al., 2003, Takata and Ishikawa, 2003, McBurney et al., 2003), glucose and fat metabolism (Moynihan et al., 2005, Nemoto et al., 2005, Rodgers et al., 2005), muscle differentiation (Fulco et al., 2003, Machida and Booth, 2004) and axonal degeneration (Araki *et al.*, 2004).

1.3.1.1 Apoptosis

Sirt1 is thought to play an important regulatory role in mediating cell survival by preventing p53-mediated growth arrest, senescence and apoptosis. Sirt1 mediates apoptosis in response to stimuli including oxidative damage, ionising radiation and exposure to TNFα (Luo et al., 2001, Vaziri et al., 2001, Yeung et al., 2004). It is thought to do this through p53-dependent and independent pathways (Figure 1.5).

Figure 1.5 Putative role of Sirt1 in the DNA damage response. This figure is an overview of the interactions of Sirt1 with key players in the DNA damage response.



1.3.1.1.1. p53

Human Sirt1 has been shown to be involved in the negative regulation of p53 in the presence of both oxidative stress (Luo *et al.*, 2001) and γ-radiation induced damage (Vaziri *et al.*, 2001). Control of p53 activity primarily involves post-translational modifications, such as phosphorylation and acetylation (Appella and Anderson, 2000). In the absence of DNA damage, Mdm2 is the major negative regulator for p53 (Prives and Hall, 1999, Vogelstein et al., 2000). In response to DNA damage, ATM phosphorylates p53, resulting in its stabilisation and activation, thereby preventing it from binding Mdm2 (Lambert *et al.*, 1998). ATM also phosphorylates Mdm2, decreasing its inhibitory potential on p53. ATM may also, like its yeast MEC1 kinase counterpart, participate in the recruitment of Sirt1 to sites of DNA damage. In addition, the tumour suppressor HIC1 represses *SIRT1* transcription permitting p53 to mediate growth arrest and apoptosis (Chen *et al.*, 2005). In response to DNA damage, Sirt1 binds, deacetylates and thereby inhibits p53 activity (Figure 1.5) (Vaziri et al., 2001, Luo et al., 2001). This is a simplistic model, as Sirt1 has also been shown to have binding sites for p53, that can itself stimulate Sirt1 transcription, in a Foxo-dependent manner (Nemoto *et al.*, 2004).

1.3.1.1.2. FOXO transcription factors

In response to oxidative stress, Sirt1-mediated deacetylation of the Foxo forkhead family of transcription factors also occurs (Motta et al., 2004, Greer and Brunet, 2005, Brunet et al., 2004, van der Horst et al., 2004, Yang et al., 2005). The Foxo factors induce the transcription of wide variety of genes including MnSOD, GADD45, $p27^{KIP1}$, Bim and Fas (Daitoku et al., 2004, Brunet et al., 2004). These genes are involved in the oxidative

stress response, DNA repair, cell-cycle control and apoptosis respectively (Accili and Arden, 2004). The effects of Sirt1 on Foxo-controlled genes vary between tissues and range from their activation to repression. Overall, it appears that Sirt1 activity results in a decrease in Foxo3-mediated apoptosis, an increase in Foxo3-mediated DNA repair and cell-cycle arrest and an increase in both Foxo1 and Foxo4-mediated stress resistance (Motta et al., 2004, Brunet et al., 2004, van der Horst et al., 2004, Kobayashi et al., 2005, Daitoku et al., 2004). The overall effect is to shift Foxo-induced responses away from apoptosis towards cell-cycle arrest, perhaps allowing cells time to repair the DNA damage and detoxify free radicals. Through its interaction with the Foxo factors, Sirt1 mediates stress resistance in a p53-independent manner (Figure 1.5). Evidence supporting this comes from the observation that apoptosis in cells lacking *SIRT1* is only partially rescued by transfection with a dominant negative *p53* mutant (Vaziri *et al.*, 2001) and, that in cells lacking functional p53, the Sirt1 activator, resveratrol, can still increase resistance to γ–radiation (Howitz *et al.*, 2003).

1.3.1.1.3. Ku70 (G22P1)

The p53 apoptotic response is multifaceted, as it activates numerous pro-apoptotic genes including Bax and PUMA. Bax is normally rendered inactive by its tight association with the Ku70 protein, a component of the Ku DNA helicase encoded by *G22P1*. In response to acute cell damage or stress, Ku70 is acetylated by the CREB-binding protein (CBP) and the acetyltransferase, p300/CBP-associated factor (PCAF), disrupting the Ku70-Bax association. The Bax protein then initiates apoptosis by relocalizing from the cytoplasm to the outer mitochondrial membrane. The result is the release of cytochrome and

downstream events including caspase activation (Cohen et al., 2004a, Bossy-Wetzel and Green, 1999). Sirt1 deacetylates residues on Ku70, keeping Bax sequestered from the mitochondria and thereby prevents apoptosis (Figure 1.5).

1.3.1.1.4. E2F1

The E2F family of transcription factors have roles in regulating cell proliferation and apoptosis (Nahle *et al.*, 2002). Similar to ATM and p53, DNA damage also results in the activation of the E2F1 apoptotic factor (Lin *et al.*, 2001). In a negative feedback loop, E2F1, induces *SIRT1* expression, which binds to and inhibits E2F1 activity (Figure 1.5) (Wang *et al.*, 2006). Inhibition of Sirt1 results in an increase in E2F1-mediated apoptosis. Sirt1 may prevent E2F1-mediated apoptosis, thereby allowing DNA damage repair.

1.3.1.1.5. NF-κB

Although Sirt1 provides a survival advantage by binding, deacetylating and thereby decreasing p53, Foxo and Ku70-mediated apoptosis, it is also known to deacetylate and inactivate NF-κB, thereby sensitising cells to TNFα-induced apoptosis (Yeung *et al.*, 2004).

NF-κB, is responsible for the regulation of genes that control cell survival. It exists as a heterodimer, composed of p50 and RelA/p65 polypeptides. Within the cytoplasm it is normally bound to members of the IκB family of inhibitory proteins. Once activated NF-κB is released into the nucleus where it enhances transcription by tethering histone acetyltransferases (HAT) that result in an open chromatin structure. Histone deacetylases

(HDAC) including Sirt1 inhibit NF-κB transcription (Yeung *et al.*, 2004). Sirt1 does this by associating with and deacetylating the RelA/p65 subunit of NF-κB. Treatment with, resveratrol, a sirtuin activator and known inhibitor of NF-κB transcription, results in a fall in the transcription of genes, including a fall in the anti-apoptotic cIAP-2 gene following TNFα stimulation. The cIAP-2 gene product inhibits TNFα-induced caspase activation and therefore resveratrol results in sensitisation to TNFα-induced apoptosis. This effect is Sirt1-dependent, as loss of Sirt1 expression completely inhibits this TNFα-induced apoptosis (Figure 1.5).

1.3.1.2 Growth and differentiation

Sirt1 expression influences cell survival and division in the face of stress and signals for terminal differentiation. Specifically, Sirt1 is able to inhibit skeletal muscle gene expression and differentiation. Sirt1 does this by interacting with and enhancing the transcriptional repression of Hes1 and Hey2, members of Hairy-related protein subfamilies (Takata and Ishikawa, 2003). These are transcriptional repressors that prevent cellular differentiation (Fisher and Caudy, 1998). Sirt1 may also act by being recruited to chromatin by the Hes1 and Hey2 DNA-binding factors, thereby allowing direct enzymatic modification of histones by Sirt1. Finally Sirt1 may prevent muscle differentiation by associating with and deacetylating PCAF, thereby causing an increase in the interaction between Hey1 and the muscle differentiation transcription factor, MyoD, and also by repressing MyoD directly by interacting with the two MyoD regulated promoters, MHC and myogenin (Fulco *et al.*, 2003).

Increased levels of Sirt1 are also seen in satellite muscle cells from older animals. These muscle cells play a role in the regeneration and repair of damaged muscle (Hawke and Garry, 2001). In aged rats, there is lower muscle satellite cell proliferation and in a study looking at the protein expression in muscle cells from older rats, p21, p53, Sirt1 and Foxo1 were all found to be higher than in cells from younger animals (Machida and Booth, 2004). Rather than controlling muscle cell growth, increased Sirt1 in older animals may reflect the cells attempt at limiting the p53 and Foxo-mediated apoptosis in muscle cells, as a result of ageing.

1.3.1.3 Glucose and fat metabolism

Skeletal muscle is also the primary site of insulin-mediated glucose uptake and Sirt1 in muscle tissue may influence glucose uptake in response to food intake, fasting and exercise. In pancreatic β cells of transgenic mice that overexpress *SIRT1*, there is an improvement in glucose tolerance and enhanced insulin secretion in response to glucose stimulation (Moynihan *et al.*, 2005). Sirt1 does this by repressing expression of the mitochondrial uncoupling protein, UCP-2 (Bordone *et al.*, 2006). SIRT1 deacetylation of Foxo1 also promotes resistance to oxidative stress in pancreatic β cells (Kitamura *et al.*, 2005).

Sirt1 modulates hepatic gluconeogenesis and glycolysis in response to fasting. In this situation, Sirt1 expression increases in a Foxo3a and p53-dependent manner (Nemoto *et al.*, 2004). Sirt1 then deacetylates and decreases the expression of the mitochondrial biogenesis coactivator, PGC-1α, a regulator of cellular metabolism in response to fasting

(Nemoto *et al.*, 2005), Sirt1 thereby induces gluconeogenic genes and glucose release and modulates PGC-1α mediated repression of glycolytic genes (Rodgers *et al.*, 2005).

Genes regulating fat development and mobilisation are also under the control of Sirt1. In response to food deprivation, Sirt1 promotes fat mobilisation in white adipocytes by binding to and repressing genes controlled by the fat regulator, peroxisome proliferator-activated receptor-γ, PPAR-γ. It does this by binding with its cofactors, nuclear receptor co-factor, NcoR, and the silencing mediator of retinoid and thyroid hormone receptors, SMRT. The result is the mobilisation of fatty acids from white adipocytes and decreased lipogenesis and fat storage. Overexpression of Sirt1, as well as treatment with resveratrol results in an increased Sirt1-mediated reduction in fat (Picard *et al.*, 2004).

Sirt1 also deacetylates the metabolic enzyme acetyl co-synthetase 1, AceCS1, resulting in an increase in acetyl CoA synthesis from free acetate (Hallows *et al.*, 2006). Although the significance of this acetyl CoA synthesis is unclear, one of its uses may be in fat metabolism (Figure 1.7).

1.3.1.4 Embryonic development

Multi-cellular organisms employ apoptosis widely during embryogenesis and have adapted this mechanism to rid the organism of redundant tissue during organogenesis. The expression of Sirt1 is particularly high during embryogenesis. In animal studies, Sirt1 levels were highest in early embryogenesis and, in particular, in embryonic heart and neural tissue. Furthermore, transgenic mice deficient in *SIRT1*, display cardiac and

neurological defects (McBurney et al., 2003, Cheng et al., 2003). These observations suggest that Sirt1 may play a role in both cardio- and neurogenesis.

Sirt1 enhances the transcriptional repression of the Hes1 and Hey2 transcription factors. Hes1 and Sirt1 are both expressed in neural precursor cells and, both their expression decreases during neurogenesis (Sasai et al., 1992, Sakamoto et al., 2004). Hes1 is though to prevent neural differentiation, therefore, it may be speculated that downregulation of both Hes1 and Sirt1 may be necessary for normal neural development. Hey2 is also detectable in the primitive ventricle during embryogenesis and is thought to regulate its morphogenesis (Leimeister *et al.*, 1999). Here too, Sirt1 may interact with Hey2 to perform this function. Sirt1 may affect organogenesis by either fulfilling an antiapoptotic function or by regulating the transcription of other genes that control growth and differentiation.

1.3.1.5 Axonal degeneration

Axonal degeneration is an active process and is involved in physiological and pathological conditions including Alzheimer's disease, Parkinson's disease and nerve injury (Raff *et al.*, 2002). NAD and Sirt1 have been shown to prevent axonotomy-associated axonal degeneration (Araki *et al.*, 2004). Treatment with sirtuin inhibitors prior to injury decreased this protection and, pre-treatment with the Sirt1 activator resveratrol, replicated the axonal protection seen with NAD.

1.3.1.6 Haematopoeisis

Sirt1 is also implicated in transcriptional repression mediated by chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2, CTIP-2 (Senawong *et al.*, 2003). CTIP-2 itself enhances the transcriptional repression of COUP-TF. The CTIP proteins were originally identified in cells of lymphoid origin and are implicated in normal haematopoietic cell development. Moreover, the CTIP proteins are implicated in the aetiology of haematopoietic malignancies.

1.3.2 Sirtuin 2

Sirtuin 2 (Sirt2) is closely related to yeast Hst2 and, is also a predominantly cytoplasmic protein (Afshar and Murnane, 1999, Perrod et al., 2001). As with Sirt1, it possesses both NAD-dependent deacetylase and ADP ribosyltransferase activities (Frye, 1999). It has been shown to interact with and deacetylate the α-tubulin subunit of microtubules (North et al., 2003, Dryden et al., 2003).

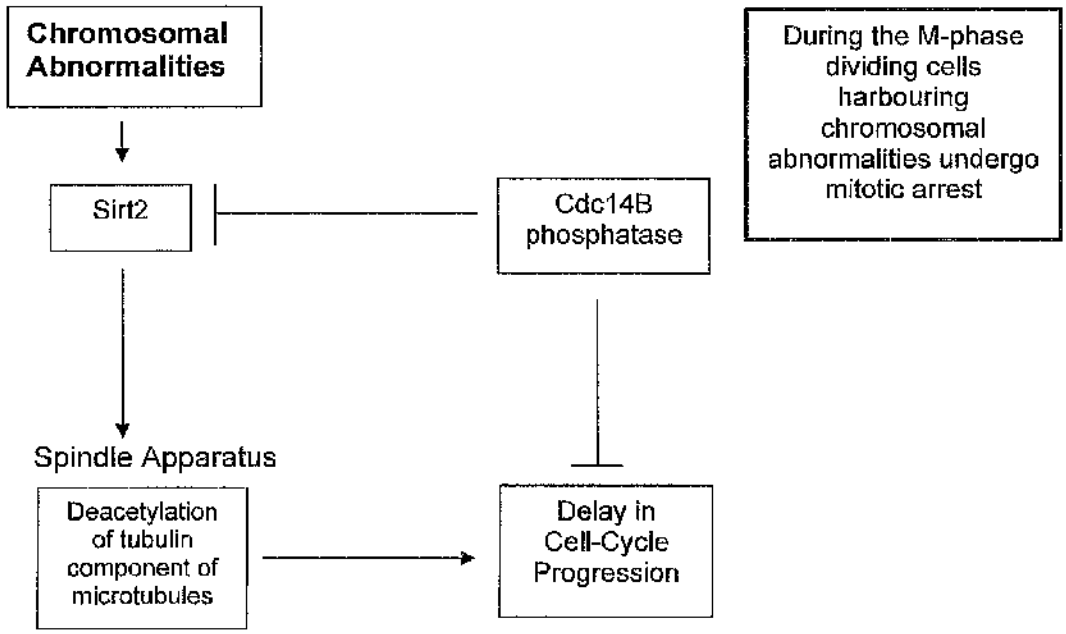
Microtubules are formed by the polymerization of α- and β-tubulin units and play an important role in cell shape, motility, intracellular transport as well as division (Nogales, 2000). Many post-translational modifications of microtubules are known to occur including both acetylation and deacetylation (Piperno et al., 1987, Nogales et al., 1999).

1.3.2.1 Cell cycle

Sirt2 has been shown to dramatically increase in abundance within the cytoplasm during mitosis. Indeed, hyperphosphorylated, activated forms of Sirt2 are seen to be confined to the M phase of the cell-cycle at the G_2/M phase transition. Overexpression of the enzymatically active Sirt2 results in a delay in cell-cycle progression (Dryden *et al.*, 2003). Deacetylation of microtubules in the spindle apparatus result in arrest during cytokinesis (North *et al.*, 2003). Furthermore, the cdc14B phosphatase released in late M-phase, may act upstream of Sirt2 by indirectly targeting it for turnover by the 26S proteosome (Dryden *et al.*, 2003). This phosphatase would therefore inhibit the Sirt2-mediated delay in cell-cycle progression, thereby allowing cell-cycle progression. The

cdc14B protein and its interaction with Sirt2, mirror that of its cdc14 yeast orthologue and its interaction with the Sir2-containing RENT complex (Shou et al., 1999, Straight et al., 1999). As with yeast Sir2, Sirt2 may participate in a late mitotic check-point to ensure correct chromosome segregation during cytokinesis (Figure 1.6).

Figure 1.6 Sirtuin 2 mediated cell-cycle regulation. This figure shows the putative role of the Sirt2 protein in cell-cycle regulation.



As well as controlling mitotic exit, by modulating microtubule dynamics, Sirt2 may also control intracellular transport, motility and cell storage. Sirt2 has also been shown to interact with the homeobox transcription factor, suggesting a role for it in mammalian development (Bae *et al.*, 2004). Finally, nucleo-cytoplasmic shuttling of Sirt2 also occurs

in response to ionising radiation suggesting a possible role for it in the DNA damage response (Inoue *et al.*, 2006)

1.3.3 Sirtuin 3

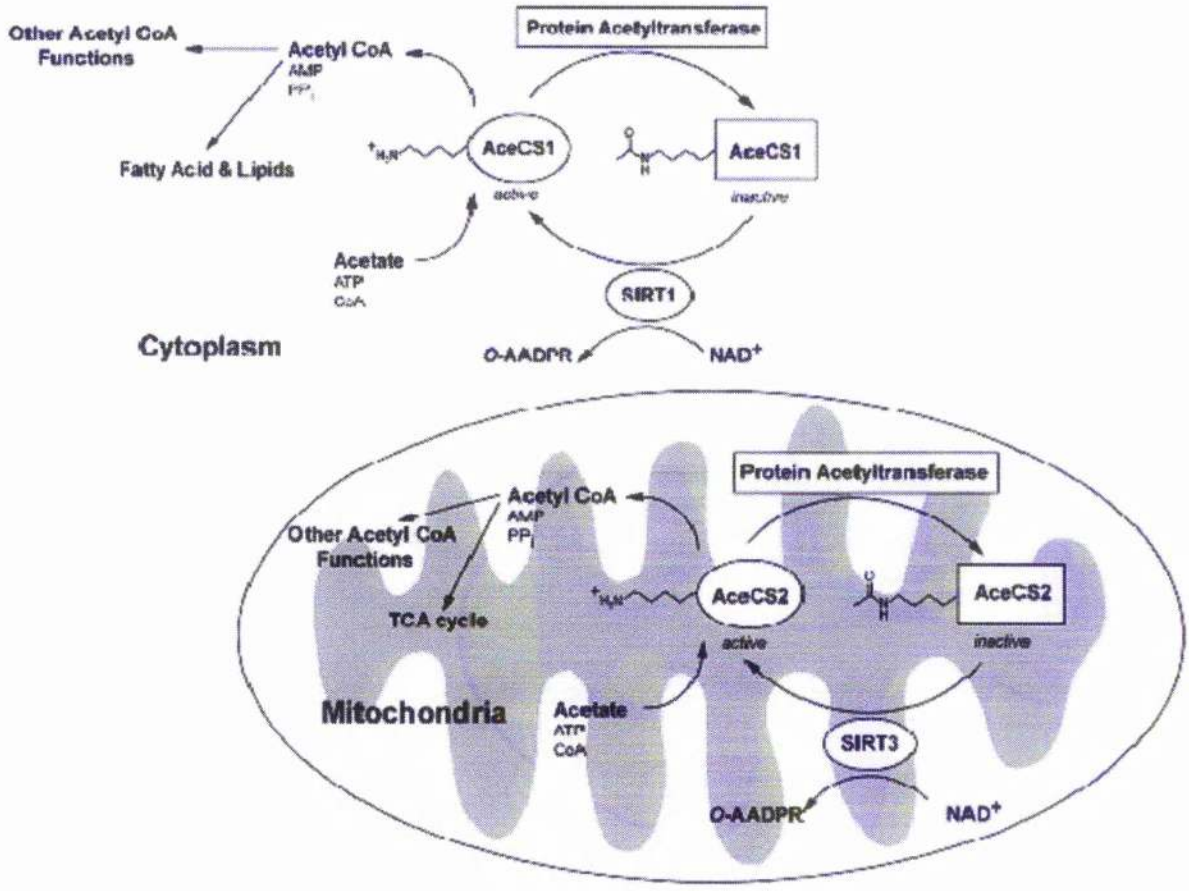
Sirtuin 3 (Sirt3) is a cytoplasmic protein that is imported into and activated within the mitochondrion (Schwer et al., 2002, Onyango et al., 2002). Sirt3 is then able to deacetylate and thereby activate the mitochondrial matrix protein, acetyl CoA synthetase 2 (AceCS2) (Figure 1.7). The cell requires acetyl CoA as an intermediate for the Krebs' cycle, as well as other metabolic processes, including lipogenesis. Although acetyl CoA is ordinarily produced via the enzymatic conversion of pyruvate, the production of acetyl CoA by AceCS2 is particularly high during ketogenic conditions such as prolonged fasting or diabetes.

Indeed, the abundance of AceCS2 within heart and skeletal muscle under ketogenic conditions, suggests that Sirt3 may play an important role in AceCS2 activation and acetyl CoA production (Fujino *et al.*, 2001). Substantiating this, high levels of *SIRT3* expression are found in metabolically active tissue such as muscle, liver, kidney and heart (Onyango *et al.*, 2002).

In response to oxidative stress, there may be a decrease in Sirt3 activity. In these cases, oxidative damage would cause an opening of the mitochondrial membrane permeability transition pore (mMPTP) and result in NAD moving into the inter-membrane space

where it would be rapidly hydrolysed by NADase. This fall in NAD levels would result in a concomitant fall in Sirt3 activity. In this manner, analogous to Sirt1, Sirt3 may also act as a sensor of the redox state of the cell, permitting the cell to regulate metabolism, DNA repair, cell-cycle control and ageing in response to oxidative stimuli (Shiels, 1999, Smith et al., 2000).

Figure 1.7 Interaction of sirtuins with acetyl CoA transferases. This figure represents a model for the interaction of Sirt1 and Sirt3 with the acetyl CoA transferases, AceCS1 and AceCS2. These sirtuins appear to activate these transferases and may result in changes in metabolism and energy utilisation [Figure taken from Hallows *et al.*, (2006)].



1.3.4 Sirtuins 4 – 6

Although little is known about Sirtuins 4 – 6 (Sirt4-6), recent discoveries suggest that these sirtuins may also play a part in stress responses (Haigis et al., 2006, Mostoslavsky et al., 2006). As with Sirt3, the gene products of Sirt4 and Sirt5 are localised to the mitochondrion. Sirt4 has NAD-dependent ADP-ribosyltransferase activity and has been reported to downregulate mitochondrial glutamate dehydrogenase within pancreatic β cells (Haigis *et al.*, 2006). This effect of Sirt4 may be inhibited by CR, perhaps as a result of alterations in the NAD/NADH ratio. The resultant reduction in glutamate dehydrogenase represses glucose and amino acid-induced insulin secretion. This activity is in contrast to that of Sirt1, which increases insulin secretion (Moynihan *et al.*, 2005). Sirt1-mediated repression of UCP-2 is alleviated by acute starvation, and that of Sirt4 on glutamate dehydrogenase, alleviated by chronic CR (Haigis *et al.*, 2006). These may reflect sirtuin regulation of insulin in response to varying types of nutritional stress.

Sirt6 also has ADP-ribosyltransferase activity (Liszt *et al.*, 2005) and has been shown to display nuclear localization, associating with heterochromatic regions that include centromeres and telomeres (Michishita *et al.*, 2005). Sirt6 has recently been shown to play an integral role in DNA repair and the maintenance of genomic stability in cells (Mostoslavsky *et al.*, 2006). The loss of *SIRT6* expression, results in a fall in Base Excision Repair (BER). In transgenic mice deficient in *SIRT6*, this manifests in the development of a progeroid phenotype, characterised by loss of subcutaneous fat, lordokyphosis and severe metabolic defects (Mostoslavsky *et al.*, 2006). This observation

is congruent with the MTR hypothesis linking sirtuins, telomere maintenance, DNA repair, metabolism and ageing (Shiels and Davies, 2003).

1.3.5 Sirtuin 7

Sirtuin 7 (Sirt7) maps to chromosome 17q25.3, a region frequently affected by chromosomal alterations in acute leukaemias and lymphomas. As with Sirt6, Sirt7 also displays nuclear localisation, but specifically associates with nucleolar rDNA (Ford et al., 2006, Michishita et al., 2005). Here, Sirt7 interacts with and increases the transcription of RNA polymerase I (Pol I). Decreased Sirt7 activity reduces cell proliferation and triggers apoptosis and increased Sirt7 expression encourages cell growth and prevent apoptosis (Ford *et al.*, 2006).

1.3.5.1 Growth and proliferation

As with their yeast counterparts, mammalian cells also contain around one hundred copies of the rDNA gene that encodes rRNA. The rate at which these genes are transcribed by Pol I, matches the cell's metabolic activity and demand for ribosomes. In response to changes in the cellular environment, rRNA transcription determines the potential for cell growth and proliferation (Shiels and Davies, 2003, Shiels, 1999). Interestingly, unlike Sirt7, Sirt1 has been shown to repress Pol I transcription by deacetylating the TAF_I68 protein in mice (Muth *et al.*, 2001).

1.4 Sirtuins, ageing and cancer

The discovery that, in *S. cerevisiae*, lifespan extension in response to CR is Sir2-dependent, raises the possibility that sirtuins may have an analogous role in humans. Indeed, the consumption of red wine, that contains the potent Sirt1 activator, resveratrol, is associated with longevity in humans (Howitz *et al.*, 2003) and may explain the 'Mediterranean paradox' of why ageing-related diseases such as atherosclerotic arterial disease are less common in this population..

In yeast, Sir2 mediates lifespan extension by linking mitochondrial energy production, protein synthesis and telomere maintenance. It is hypothesised that, like yeast Sir2, human sirtuins may similarly link the functions of the mitochondrion, telomere nucleo-protein complex and ribosomal machinery (the MTR) (Figure 1.8).

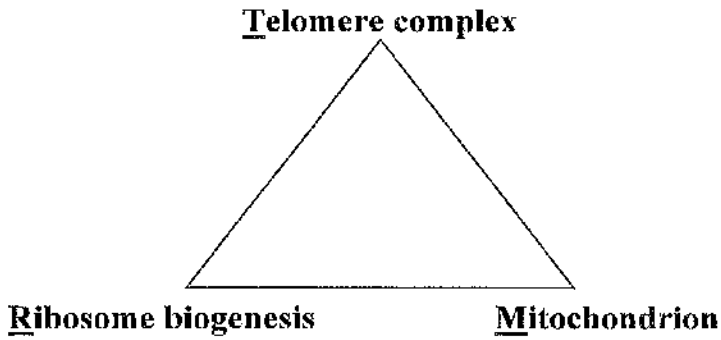


Figure 1.8 The MTR. The interacting components of the MTR.

Sirt1 may do this by interacting with apoptotic factors to prevent DNA and telomeric damage, Sirt3 by altering energy production in response to the cell's redox state and Sirt7 by controlling ribosomal transcription and consequently protein synthesis. Furthermore, Sirt2 is involved in cell-cycle regulation and Sirt6 in DNA damage repair (Figure 1.9). This is a simplistic model, as Sirt1 also acts in the cytoplasm and mitochondria and Sirt2 in the nucleus.

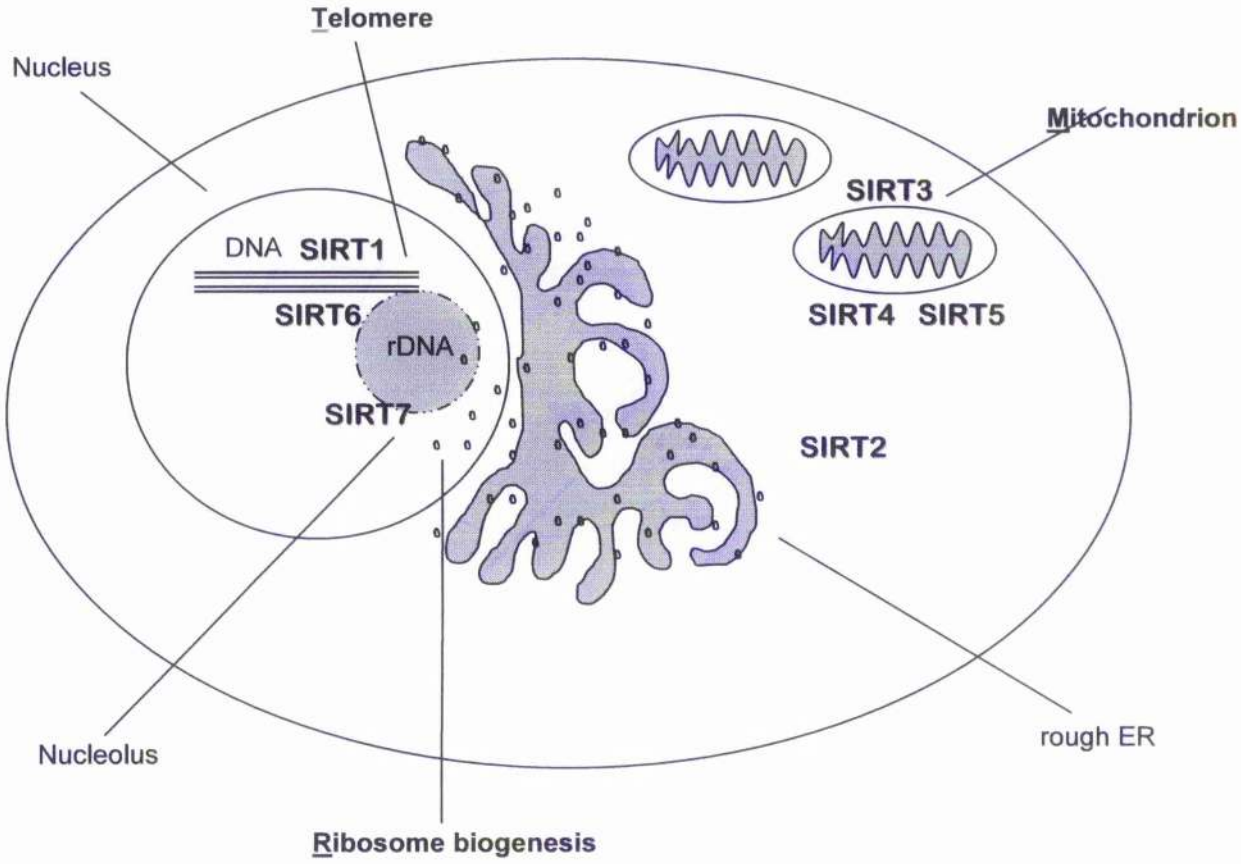


Figure 1.9 Sirtuins and the MTR. This figure shows the areas where sirtuins are thought to function within the cell, in particular, at the telomere, mitochondria and ribosomal DNA, to protect against cell stress and damage.

Sirtuin activity at the MTR, allows the cell to regulate telomeric damage, oxidant load and protein biosynthesis. An imbalance in any one of these components would have a knock on effect on the others. For example, the accumulation of damage over a cell's lifespan may be associated with accumulating DNA damage and telomere shortening, as well as inefficiency in mitochondrial fuel utilisation, energy production and decreased protein biosynthesis.

Sirtuin activity at each of these sites may counteract the accumulation of changes associated with the development of biological ageing. Indeed, accelerated ageing is linked with the development of a number of pathologies, and may be associated with aberrant sirtuin expression at any of these sites (Shiels and Davies, 2003). It is also established that factors that promote longevity result in a predisposition to cancer (Campisi, 2000). Aberrant sirtuin activity may, therefore, contribute to the pathogenesis of cancer.

The roles of sirtuins in preventing apoptosis in response to DNA damage (Luo et al., 2001, Vaziri et al., 2001, Yeung et al., 2004), regulating the cell-cycle in response to chromosomal abnormalities (Dryden et al., 2003, North et al., 2003), controlling rRNA transcription (Ford *et al.*, 2006) and participating in DNA damage repair (Mostoslavsky *et al.*, 2006), provide areas where aberrant sirtuin activity may contribute to cancer pathogenesis. Indeed, increased *SIRT1* expression is a feature of a number of malignancies (Lim, 2006b, Kuzmichev et al., 2005, Chen et al., 2005, Yeung et al., 2004,

Bradbury et al., 2005), decreased *SIRT2* expression a feature of gliomas (Hiratsuka *et al.*, 2003) and gastric cancer (Inoue et al., 2006), and increased *SIRT7* expression a feature of thyroid cancer (Frye, 2002, de Nigris et al., 2002).

The transcriptional analysis of sirtuin expression is, therefore, useful for the study of both biological ageing and cancer. In the proceeding chapters we will explore how sirtuin biology contributes to senescence, tumorogenesis as well as the cell damage response in breast cancer.

1.5 Aims

The aim of this thesis was to study breast cancer as an aberration of normal biological ageing, with respect to sirtuin expression. Specifically, it sought to determine the role of sirtuins, that link components of the MTR, in mammary epithelial cell senescence as well as in the pathogenesis and progression of breast cancer. It also sought to determine the relationship between common therapies used for the treatment of breast cancer, namely radiotherapy and chemotherapy, and sirtuin expression.

In order to do this, transcriptional studies of sirtuin expression were performed to:

1. Determine the relationship between sirtuin expression in primary mammary epithelial cells as they age.

2. Investigate sirtuin expression in malignant and non-malignant breast tissue biopsies.

3. Determine the relationship between both ionising radiation and paclitaxel chemotherapy and sirtuin expression.

Chapter 2

Materials and Methods

2.1 Materials

Ambion, UK

DNA-free Kit

Biosource UK Ltd, UK

Taqman primer/probe sets

Bristol-Myers Squibb, USA

Paclitaxel (6mg/ml)

Clontech, UK

Apoalert DNA Fragmentation Assay Kit

Eurogentech, UK

Taqman mastermix

Invitrogen, UK

Superscript first-strand synthesis system for RT-PCR

GE Healthcare, UK

X-Ray films

Invitrogen, UK

TRIzol reagent

SuperScript II reverse transcription system

Nalge Nunc International, Fisher Scientific, UK

Labtek II chamber slide system

Applied Biosystems, UK

MicroAmp optical 96-well PCR plate

MicroAmp optical cap strips

Other chemicals used were supplied from Sigma Chemical Company Ltd, UK unless otherwise stated.

2.2 Cell lines

The following cell lines were used in this study (Table 2.1). The MCF-12A cells were gifts from Dr Susan Jamieson (Department of Pathology, Western Infirmary, University of Glasgow) and MCF-7 cells from Dr Robert Mairs (Department of Radiation Oncology, University of Glasgow).

Table 2.1 Cell lines used in the *in vitro* studies.

Cell line	Organism	Tissue	Characteristics	Source
HMEC	Human	Mammary epithelial cells	Primary	Clonetics, Cambrex Bioscience, UK
MCF-12A	Human	Mammary epithelial cells	Immortalized non-tumorogenic	ECACC, UK
MDA-MB-231	Human	Mammary epithelial cells	Immortalized tumorogenic mutant *p53*	ECACC, UK
MCF-7	Human	Mammary epithelial cells	Immortalized tumorogenic wild type *p53*	ECACC, UK

2.2.1 Growth conditions

The primary human mammary epithelial cells (HMEC) were grown in mammary epithelial cell growth medium supplemented with bovine pituitary extract (BPE), 20ng/ml epidermal growth factor, insulin and 500ng/ml hydrocortisone.

The MCF-12A cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented with 5% foetal calf serum, 20ng/ml epidermal growth factor, 100ng/ml cholera toxin, 0.01 mg/ml bovine insulin and 500ng/ml hydrocortisone.

The MCF-7 cells were grown in RPMI medium supplemented with 10% foetal calf serum.

The MDA-MB-231 cells were grown in Leibovitz's L-15 medium with 2mM L-glutamine supplemented with 10% foetal calf serum.

2.3 Breast biopsies

Archival breast biopsies stored in liquid nitrogen and held by the Department of Surgery, Western Infirmary, Glasgow, were used for the biopsy study. These biopsies came from patients with potentially curable, invasive ductal adenocarcinomas who had given permission for the storage and future analysis of samples of tissue. All patients had surgery with a curative intent (mastectomy or breast conservation surgery, and level II axillary clearance) performed by a single operator between 1996 and 2000. The pathological characteristics of each group were selected to include equal distribution by nodal status (12 lymph node positive and 12 lymph node negative), and tumour grade. A total of 24 breast cancer samples were chosen and matched with 21 'normal', histologically non-malignant breast tissue biopsies from the breasts of cancer patients.

2.4 Extraction of total RNA

The TRIzol method of total RNA extraction was used for total RNA extraction from cell lines and breast biopsy tissue. Cells grown in tissue culture flasks were mixed with 1ml of TRIzol reagent. 0.2mls of chloroform was added for each 1ml of TRIzol reagent used. The tubes were left at room temperature for 3 minutes. Samples were then centrifuged at 12,000G for 15 minutes at 4°C. The resultant aqueous phase was carefully removed, and transferred to an epindorf containing 0.5ml isopropanol, for each 1ml of TRIzol reagent used. The tubes were mixed and incubated at room temperature for 10 minutes, to allow RNA to precipitate before being centrifuged at 12,000G for 10 minutes at 4°C to pellet the RNA. The supernatant was removed and the RNA pellet washed by adding 1ml of 75% ethanol, vortexing and centrifuging at 7,500G for 5mins at 4°C. The RNA pellet was air-dried and resuspended in DEPC-treated water. Samples were heated for 10 minutes at 60°C before being put on ice. For the extraction of total RNA form biopsy tissue, breast glandular tissue was carefully dissected free from surrounding fatty tissue. 1ml of TRIzol was added per 50mg of tissue. Tissue was then homogenised using a mechanical tissue homogenizer at 0°C. The resultant homogenate was then processed as above.

The quality of the RNA was tested by running 500ng of the sample on a 0.5% agarose gel containing 0.5µg/ml Ethidium bromide and visualising under UV light. The RNA concentration and purity was determined by measuring absorbance of the sample at 260nm using a GeneQuant capillary spectrophotometer (Pharmacia Biotech). Samples were stored at –80°C.

2.5 Taqman Real-time PCR

Taqman real-time PCR is a cDNA-specific 5' nuclease assay for quantitatively detecting PCR products using a non-extendable oligonucleotide hybridisation probe. The probe is labelled with a reporter fluorescein dye, 6-carboxy-fluorescein (FAM), at the 5' end and a quencher fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA), at the 3' end. When the probe is intact, the reporter dye emission is quenched due to the physical proximity of the reporter and quencher fluorescent dyes. During the extension phase of the PCR cycle, the nucleolytic activity of the Taq DNA polymerase cleaves the hybridisation probe and releases the reporter dye from the probe (Figure 2.1). With each cycle of PCR amplification there is an increase in fluorescence emission which is monitored in real-time using the ABI Prism 7700 sequence detector (Heid *et al.*, 1996).

The sequence detector is a combination thermal cycler, laser and detection software system that automates 5' nuclease-based detection and quantitation of nucleic acid sequences. A computer algorithm compares the amount of reporter dye emission (R_{n+}) with the quenching dye emission (R_{n-}) during the PCR amplification, generating a ΔR_n value. The ΔR_n value reflects the amount of hybridised probe that has been degraded. The algorithm fits an exponential function to the mean ΔR_n values of the last three data points for every PCR extension cycle, generating an amplification plot.

Taqman PCR was performed for specific genes and simultaneously performed using primer/probe sets for either hypoxanthine phosphoribosyltransferase (*HPRT*) or the *18S* ribosomal subunit, housekeeping genes used as internal references. The amplification of the endogenous control allowed us to standardise the amount of RNA in each reaction.

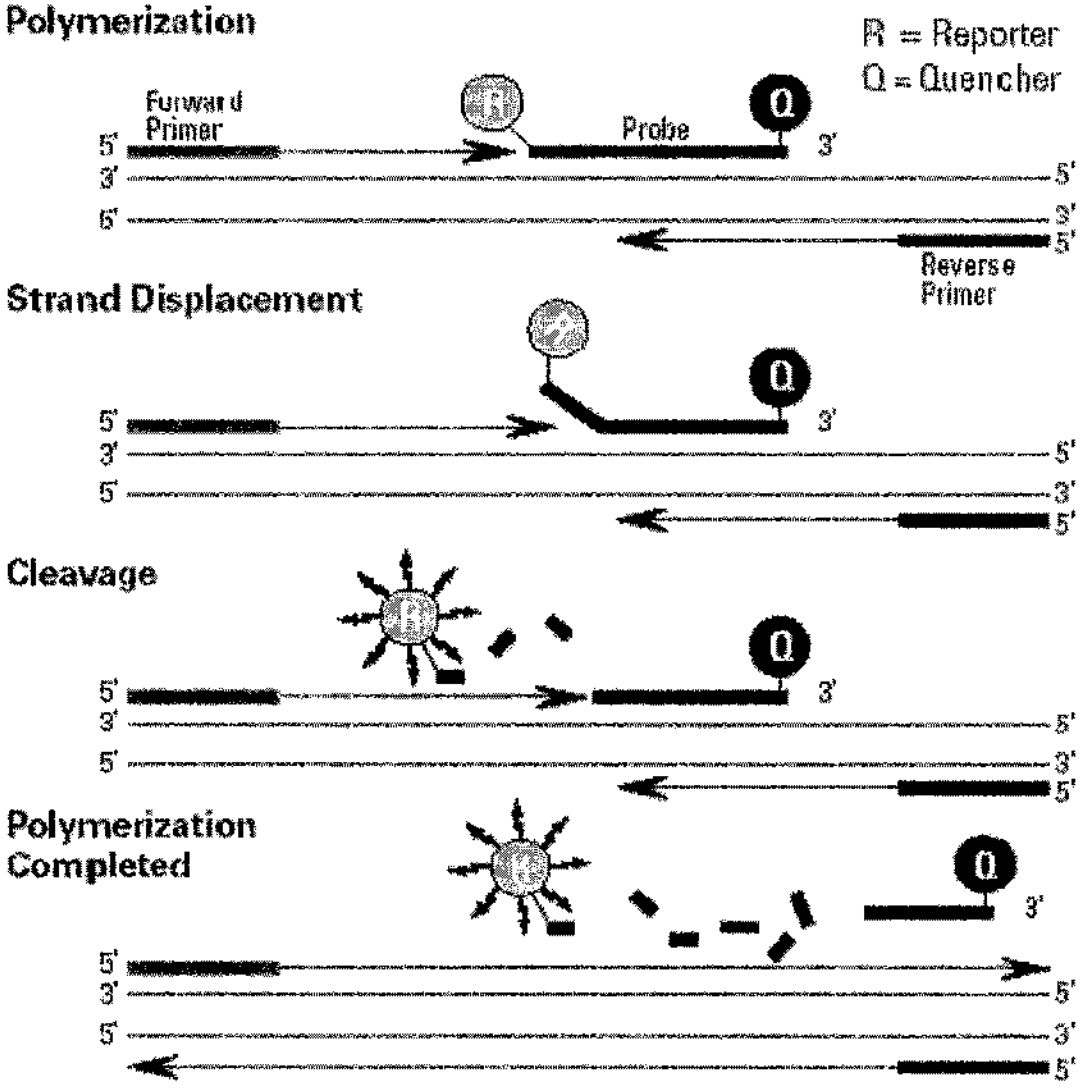
A threshold was set at a point where this amplification appeared linear. The resultant threshold cycle number (Ct) for both of these genes was recorded for each sample. This allowed the expression of the gene of interest to be normalized to the endogenous housekeeping gene. This was performed by calculating the ΔCt_{Sample} by subtracting the Ct value of the endogenous housekeeping gene from the Ct vale of the gene of interest ($\Delta Ct_{Sample} = Ct_{Gene\ of\ interest} - Ct_{Housekeeping\ gene}$)

The comparative Ct method, also known as the $2^{-\Delta\Delta Ct}$ method, was used for calculating the relative gene expression. This involved comparing the ΔCt values of the samples with a control or calibrator, such as RNA from a non-treated sample or normal tissue. This involves calculating the ΔΔCt, by subtracting $\Delta Ct_{Calibrator}$ from the ΔCt_{Sample}.

For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference housekeeping gene must be approximately equal. This can be established by looking at how ΔCt_{Sample} varies with template dilution. If the gradient of cDNA dilution versus ΔCt_{Sample} is close to zero, it implies that the amplification efficiencies of the target and housekeeping genes are similar (Figure 2.2 and 2.3).

Figure 2.1 Taqman PCR. This is a diagrammatic representation of the steps involved in the real-time Taqman PCR reaction [Illustration adapted from Applied Biosystems Taqman Manual]

1. The primers and probe anneal to the cDNA transcript. There is no fluorescence because the reporter dye emission is quenched.
2. The primers are extended during the extension phase of the PCR cycle.
3. The 5'-3' exonuclease activity of the DNA polymerase cleaves the hybridised probe and releases the reporter dye emission resulting in an increase in reporter fluorescent dye emission.
4. The primers continue to be extended until polymerisation of the amplicon is complete.


Polymerization
R = Reporter
Q = Quencher
Forward Primer
Probe
Reverse Primer
Strand Displacement
Cleavage
Polymerization Completed
5'
3'

2.5.1 DNase treatment

DNAse treatment was performed using the commercially available DNA-Free Kit (Ambion). RNA was added to a tube containing 2-3 units of DNase I and 0.1 volume of 10× DNase I Reaction Buffer. The contents of the tube were mixed gently and incubated for 30 minutes at 37°C. The DNase I was then inactivated by adding 0.1 volume of DNase Inactivation Reagent. The mixture was incubated for 2 minutes at room temperature. The solution was then centrifuged for 1 minute to pellet the DNase Inactivation Reagent. The supernatant contained the DNase-treated RNA.

2.5.2 cDNA Synthesis

Two micrograms of RNA was then reverse transcribed into cDNA using the Superscript First-strand Synthesis System for reverse transcriptase PCR (Invitrogen) recommendations. This part of the study was performed in duplicate with each sample being treated with or without the reverse transcriptase (RT) enzyme. Random hexamer based transcription was performed for the breast biopsy studies and oligonucleotide (oligodTs) based transcription for experiments using mammary cell lines. Random hexamer-based transcription has been shown to generate a more normalised cDNA pool from sources that may contain partially degraded RNA (Schwabe *et al.*, 2000).

For the hexamer-based RT reaction 1µl of 10mM dNTP mix and 2µl hexamers were added for every 2µg of RNA to be reverse transcribed. The total volume was made up to

10µl using DEPC-treated H_2O_2. The mixture was then incubated at 65°C for 5 minutes before being placed on ice. 2µl of 10× RT Buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT and finally 1µl of the RNase inhibitor, RnaseOUT, were then added, bringing the total volume up to 19µl. This mixture was gently mixed by brief centrifugation before being incubated at 20°C for 2 minutes. 1µl of the reverse transcriptase enzyme, SuperScript II, was then added, but only to those samples to be reverse transcribed and not to the no-RT, negative controls. The total mixture was then incubated at 25°C for 10 minutes, at 42°C for 50 minutes, terminated at 75°C for 15 minutes and finally chilled on ice for 5 minutes. Finally, the reactants were treated with 1µl of RNAse at 37°C for 20 minutes. The specimens were then stored at -20°C.

For the oligo dT-based RT reaction 1µl of 10mM dNTP mix and 1µl oligo d'l's was added for every 2µg of RNA to be reverse transcribed. The total volume was made up to 10µl using DEPC-treated H_2O_2. The mixture was then incubated at 60°C for 5 minutes before being placed on ice. 2µl of 10× RT Buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT and finally 1µl of the RNase inhibitor, RnaseOUT, were then added, bringing the total volume up to 19µl. This mixture was gently mixed by brief centrifugation before being incubated at 42°C. 1µl of the reverse transcriptase enzyme, SuperScript II, was then added, but only to those samples to be reverse transcribed and not to the –RT, negative controls. The total mixture was then incubated at 42°C for 50 minutes, terminated at 70°C for 15 minutes and finally chilled on ice for 5 minutes. Finally, the reactants were treated with 1µl of RNAse at 37°C for 20 minutes. The specimens were then stored at -20°C.

2.5.3 Taqman PCR

Taqman PCR was carried out on the cDNA samples using the ABI Prism 7700 sequence detector (Applied Biosystems) in a 25µl reaction. The mixture consisted of 10–100ng of cDNA, 300nM of each primer, 200nM Taqman probe, 12.5µl of Taqman Universal PCR Master Mix (Eurogentech, UK) and H_2O_2. Cycle conditions were 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

2.5.4 Design of Taqman primers and probes

Taqman primer and probe sequences for all genes (Table 2.2) were designed de novo from sequences in the Genebank database using Primer Express software. All primer/probe sets were designed such that at least one of the oligonucleotides crossed an intro/exon boundary.

All primer/probe sets were subsequently tested by blasting and amplifying the cDNA. This was to ensure that the primers and probe did not bind other sequences and that primer dimers were not formed.

Table 2.2 A list of the primer and probe sequences used for Taqman analysis.

Transcript	Forward Primer (5'-3')	Reverse Primer (5'-3')	Taqman™ Probe (5' FAM – TAMRA 3')
SIRT1	TAGAGCCTCACATGCAAGCTCTA	GCCAATCATAAGATGTTGCTGAAC	ACTCCAAGGCCACGGATAGGTCCATATACTT
SIRT2	GGCATCCCCGACTTTCG	CCTCTGGGTAGGGAAGATGGT	CCATCCACCGGCCTCTATGACAACCTA
SIRT3	CATTCGGGCTGACGTGATG	AACCACATGCAGCAAGAACCT	TGCACCGGCGTTGTGAAGCC
SIRT7	CGTCCGGAACGCCAAATAC	GACGCTGCCGTGCTGATT	TGGTCGTCTACACAGGC
p21	GCAGACCAGCATGACAGATTTCTA	GCGGATTAGGGCTTCCTCTT	CACTCCAAACGCCGGCTGATCTTC
p16	CATAGATGCCGCGGAAGT	CCCGAGGTTTCTCAGAGCCT	CCTCAGACATCCCCGATTGAAAGAACC
HPRT	CTTGCTCGAGATGTGATGAAGG	CAGCAGGTCAGCAAAGAATTTATAG	ATCACATTGTAGCCCTCTGTGTGCTCAAGG
18S	ACCTGGTTGATCCTGCCAGTAG	AGCCATTCGCAGTTTCACTGTAC	TCAAAGATTAAGCCATGCATGTCTAAGTACGCAC

2.5.5 Validation studies

For comparative PCR to be valid, the efficiencies of the target amplification and the amplification of the reference gene must be approximately equal. To check this, an initial experiment was set up using dilutions of cDNA as follows.

Table 2.3 Concentrations of cDNA template amplified for validation studies on each primer/probe set.

Target gene					Reference gene				
500ng	100ng	20ng	4ng	800pg	500ng	100ng	20ng	4ng	800pg

Each reaction was run in duplicate. The PCR reaction mix for Taqman validation (25µl) consisted of 1× Taqman Universal PCR Master Mix, 300nM of both forward and reverse primers, 200nM Taqman probe and 800pg to 500ng of mammary epithelial cell line cDNA and breast biopsy cDNA (Table 2.3). The cycling conditions were as described in 2.5.3.

The ΔCt for each gene primer/probe set, i.e. values of $Ct_{\text{Gene of interest}} - Ct_{\text{Housekeeping gene}}$ was relatively constant for *SIRT2,3,7* and *p21*, using *HPRT* as the internal reference gene (Figure 2.3). Likewise the ΔCt was relatively constant for *SIRT2*, *SIRT3* and *SIRT7*, using *18S* as the internal reference gene (Figure 2.4). The efficiencies of the target amplification of *SIRT1* and that of both *HPRT* and *18S* was only equal between a template concentration of 0.8 and 20ng. 10ng of template were, therefore, used for the

Taqman experiments. No amplification was seen in the wells containing the 'no RT' controls, or in the wells lacking any template.

Figure 2.2 Relative efficiencies of SIRT1,2,3,7 and p21 with reference to HPRT. These figures show how the ΔCt for *SIRT1,2,3,7* and *p21* varies with $\log_{10}$ [template concentration] with reference to the *HPRT* housekeeping gene.

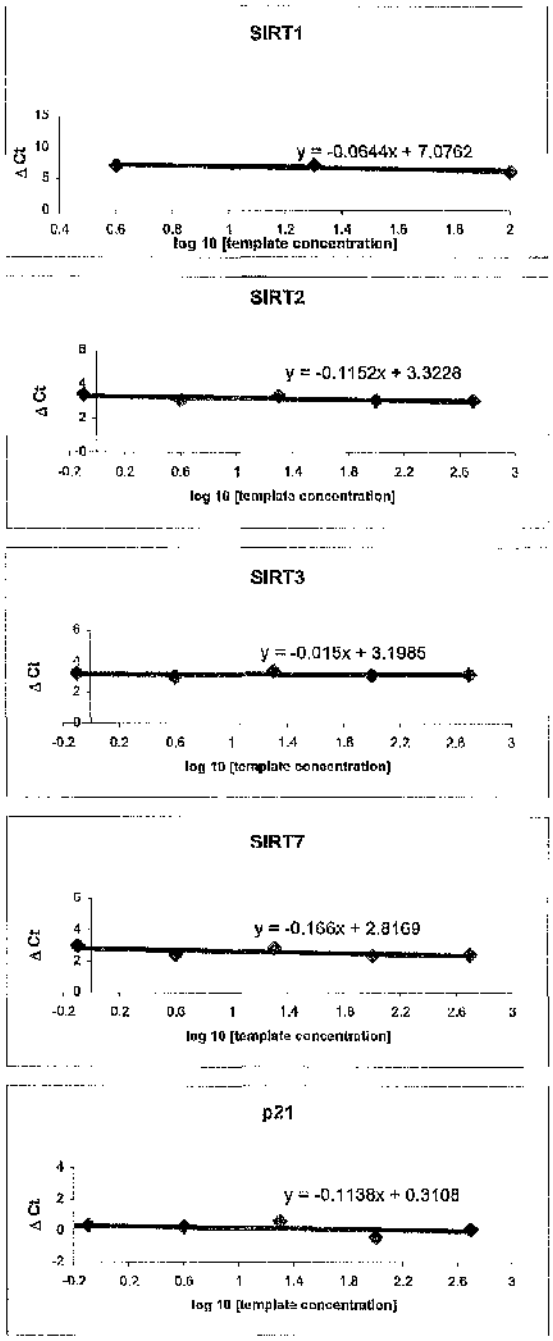
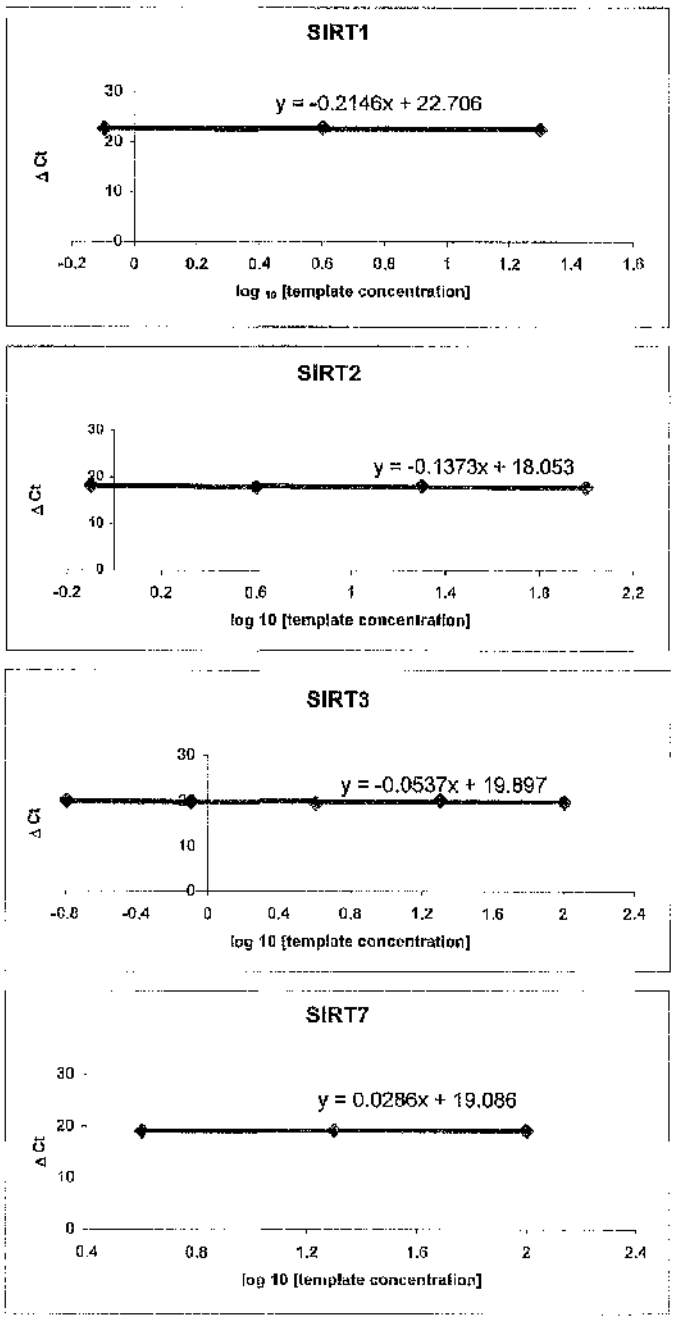


Figure 2.3 Relative efficiencies of SIRT1,2,3 and 7 with reference to 18S. These figures show how the ΔCt for *SIRT1,2,3* and *7* varies with $\log_{10}$ [template concentration] with reference to the *18S* housekeeping gene.



2.5.6 Primer and probe optimisation

The purpose of this procedure was to ensure that all primer/probe sets gave amplification that was both detectable, and shown to increase exponentially with each PCR cycle. Each reaction was, again, run in duplicate. The PCR reaction mix for primer/probe optimization (25µl) consisted of 1× Taqman Universal PCR Master Mix, 300nM of both forward and reverse primers, 200nM Taqman probe and 100ng of mammary epithelial cell line cDNA. The cycling conditions were as described in 2.5.3.

These primer/probe concentrations provided satisfactory results for all but the original SIRT7 primer/probe set designed. Despite running an optimisation plate (Table 2.4) with varying concentrations of both forward and reverse primers, no amplification was seen. SIRT7 was therefore re-designed and this time the new primer/probe set worked.

Table 2.4 Combinations of forward and reverse primers for primer/probe set optimisation.

50fwd/50rev	50fwd/300rev	50fwd/900rev
300fwd/50rev	300fwd/300rev	300fwd/900rev
900fwd/50rev	900fwd/300rev	900fwd/900rev

2.6 TUNEL assay

Apoptosis was detected using the Apoalert DNA Fragmentation Assay Kit (Clontech). This assay detects DNA strand breaks by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labelling (TUNEL). TdT catalyses the incorporation of fluorescein-dUTP at the free 3'-hydroxyl ends of fragmented DNA. The fluorescein-labelled DNA can then be detected using fluorescence microscopy.

2.6.1 Sample preparation

Apoptosis was detected in cells grown on Labtek II Chamber Slides (Nalge Nunc). Following treatment slides were washed twice in PBS. Cells were then fixed by incubating slides for 25 minutes in 4% formaldehyde/PBS at 4°C. Slides were then immersed in PBS for 5 minutes and again washed twice in PBS. Cells were then permeabilised by incubating cells for 5 minutes in pre-chilled 0.2% Triton X-100/PBS.

A DNase-treated positive control was also prepared at this stage. This slide was incubated in 100µl of DNase I buffer at room temperature. Following this, the slide was incubated in 100µl of DNase I buffer containing 0.5-1µg/ml DNase for 10 minutes. The slide was then washed 3-4 times in H_2O before being treated with the other samples as follows.

Slides were then incubated for a further 5 minutes in PBS at room temperature. This step was repeated. Slides were now ready for the tailing reaction.

2.6.2 Tailing reaction

The tailing reaction describes the incorporation of fluorescein-dUTP to the free 3'-hydroxyl tails of fragmented DNA.

100µl of equilibration buffer was then added to the slides for 10 minutes at room temperature. TdT incubation buffer was then prepared for all of the samples. This consisted of:

Equilibration buffer	45µl
Nucleotide mix	5µl
TdT enzyme	1µl

A TdT-minus, negative control was also prepared. Deionised H_2O was substituted in place of the TdT. 50µl of TdT incubation buffer was then placed on the cells. Coverslips were added and the Samples were then placed for 60 minutes in a dark, humidified 37°C incubator. The tailing reaction was terminated by immersing the slides in 2×SSC at room temperature for 15 minutes. Cells were then washed by immersing in PBS at room temperature for 5 minutes. This was repeated twice. Cells were then stained with propidium iodide(PI)/PBS for 5-10 minutes at room temperature. Cells were then washed by incubating with deionised H_2O at room temperature for 5 minutes. This was repeated twice. Anti-fade solution was then added and slides were covered with glass coverslips.

2.6.3 Fluorescence microscopy

The stained specimen was then observed in a triple band-pass filter using the Zeiss Axioplan 2, epifluorescence microscope. Apoptotic cells exhibited a strong, nuclear green fluorescence using a standard fluorescein filter set (520±20nm). All cells stained with PI exhibited strong red cytoplasmic fluorescence when viewed at >620nm (Figure 2.6). To determine the degree of apoptosis, two experiments in total were performed, and the percentage of cells exhibiting apoptosis in a field of approximately 100 cells was recorded.

2.7 Senescence studies

2.7.1 Mammary cell line studies

Three different types of mammary epithelial cell lines were used in the investigations described in this thesis. These cell lines were a primary human mammary epithelial cell line (HMEC), an immortalized non-tumorogenic human mammary cell line (MCF-12A) and immortalized tumorogenic human mammary cell lines (MCF-7 and MDA-MB-231). Cells were seeded and collected at successive passages with three population doublings between each passage. The number of population doublings were calculated by growing cells to sub-confluence before splitting them in a 1 in 8 manner. RNA was extracted from these cells before being subject to Taqman analyses for established senescent associated genes (SAGs) including Sirtuins 1,2,3 and 7.

2.7.2 Senescence-associated β galactosidase assay

Cells to be assayed were washed twice in PBS then treated with 1ml of 1× fixation solution (2% formaldehyde/0.2% gluteraldehyde) at room temperature for 10 minutes. Cells were then washed twice with PBS.

Cells were then stained using the staining solution which was constituted as follows:

Volume per well	**Final concentration (per 1ml)**
50 µl X-Gal (20mg/ml)	1mg
12.5µl Potassium Ferrocyanide (400mM)	5mM
12.5µl Potassium Ferrocyanide (400mM)	5mM
10µl $MgCl_2$ (200mM)	2mM
100µl NaCl in 1× PBS (1.5M)	150mM
Citric acid/ sodium phosphate pH 6.0 in 1× PBS	40mM

1ml of staining solution was then added to each well and cells were incubated at 37°C (without CO_2) for 8-12 hours. 100 cells were then examined under a light microscope for SA-β-gal staining. A positive control at pH 4.0 was also analysed.

2.8 Cancer therapy experiments

2.8.1 Cellular irradiation

The MDA-MB-231 and MCF-7 cells respectively were grown in 25cm^2 tissue culture flasks and glass chamber slides (Labtek) to 70% confluence and then subjected to varying doses of radiation. Cellular irradiation was performed using a ^{60}Co source with a 0.8-cm Perspex 'build up' to ensure maximum energy deposition. The dose rate was approximately 0.5Grays/min, and the treatment dose ranged from 2 to 10 Grays.

All experiments were performed in duplicate. Cells were harvested at two distinct time points after irradiation, 24 hours and 48 hours prior to further investigations.

2.8.2 Taxol treatment

The MCF-7 cells were grown in 25 cm^2 tissue culture flasks to 70% confluence and then subjected to different doses of paclitaxel treatment (10nM to 100μM). Cells were incubated for 24 hours in the presence of the drug before being harvested.

2.9 Statistical analyses

Analysis of individual genes expression between each of biopsy groups (i.e. non-malignant, lymph node negative and lymph node positive breast biopsies) was performed using an analysis of variance (ANOVA). Post-hoc comparisons were then performed using the independent student's t-test.

Linear regression analyses were performed to determine if any relationship existed between passage in replicatively ageing mammary epithelial cells and sirtuin expression as well as between irradiation and paclitaxel treatment and sirtuin expression. Linear regression analyses were also performed to determine whether there was an association between SA-β-Gal staining and replicative age. Both R^2 and p-values were calculated to show the 'goodness of fit' of the models as well as their statistical significance.

Chapter 3

Investigations into Sirtuin Expression in Mammary Epithelial Cells In Vitro

3.1 Introduction

Cells explanted from living tissue into culture undergo a finite number of divisions before entering growth arrest (Hayflick and Moorhead, 1961). This phenomenon is known as replicative senescence (RS) and has been likened to a tumour suppressor mechanism, as it must be overcome for cells to achieve immortalisation (Campisi, 2000). Sirtuins are implicated in preventing replicative senescence in a number of cells (Langley et al., 2002, Lim, 2006a, Kim et al., 2004) and may perform this function by functioning within the MTR. Specifically, they may help stabilise telomeres and repair DNA damage, improve the efficiency of energy production and fuel utilisation and regulate the rate of protein biosynthesis (Shiels and Davies, 2003). Understanding the changes that occur in sirtuin expression within ageing mammary epithelial cells may help in understanding breast tumorogenesis. Here follows a review of senescence, as well as the role sirtuins may play in this process.

3.1.1 Replicative senescence

Replicative senescence is characterised by an irreversible arrest of cell proliferation, as well as altered cell function. Various models of senescence exist although these are not mutually exclusive. The most celebrated model of senescence is the telomere-based model.

3.1.2 Telomere model of senescence

The telomere model of RS predicts senescence to occur once telomeres reach a critically shortened length. Telomeres are specialised nucleoprotein structures found at the end of eukaryotic chromosomes and consist of tandemly repeated TTAGGG motif bound by a variety of proteins. Telomeres have numerous roles, including the maintenance of chromosome structure and integrity. As cells divide, DNA polymerases are incapable of completing replication at telomeric ends, with the result that telomeres progressively shorten, culminating in growth arrest once they reach a critically shortened length (Olovnikov, 1973). This model is overly simplistic, as it has been since shown that it is altered telomere state and not telomere length that results in senescence (Karlseder *et al.*, 2002). Telomeric alterations may also result in the expression of sub-telomeric genes that contribute to the senescent phenotype (Baur *et al.*, 2001). Expression of the telomerase enzyme maintains telomere lengths and can result in immortalisation in certain cell types. Telomerase has been shown to be up-regulated in over 80% of tumours, with the remainder possibly employing a mechanism known as alternate lengthening of telomeres (ALT) to maintain their telomere length (Stewart and Weinberg, 2000).

3.1.3 Stress or Aberrant Signalling Induced Senescence

Stress or Aberrant Signalling Induced Senescence (STASIS) is the name given to the senescence state induced by cellular damage (Wright and Shay, 2002). As cells age they accumulate damage at the nuclear, mitochondrial and cytoplasmic level (Shiels and

Davies, 2003). Once this damage reaches a critical limit, cells undergo senescence. Independent of primary telomeric changes, this senescent state can be induced *in vitro* by the activation of oncogenes (Serrano *et al.*, 1997), exposure to sub-cytotoxic stressing agents (Chen *et al.*, 1995), agents that perturb chromatin structure (resulting in a loss of silencing) (Ogryzko *et al.*, 1996) as well as by primary changes in either the p53 or the pRb pathways (McConnell *et al.*, 1998). The accumulation of damaged, dysfunctional mitochondria resulting in inefficiencies in fuel utilisation, reduced energy availability, impaired cellular function and eventually cell death are also associated with senescence, disease and ageing (Linnane et al., 1989, Brunk and Terman, 2002).

3.1.4 Cell-cycle checkpoints and senescence

The cell-cycle is normally governed by interactions between cyclins and cyclin-dependent kinase (cdk) proteins which form complexes that regulate the cell-cycle at various checkpoints. Phosphorylation and thereby, inactivation, of the retinoblastoma protein, pRb, by cyclin-cdk protein complexes, mediates the release of E2F transcription factors that interact with histone acetylases. These open chromatin structure and promote the expression of genes required for cell-cycle progression.

Telomere shortening, secondary to replicative ageing, results in the induction of p53 and may lead to elevation of the cdk inhibitor, $p21^{CIP1}$. The $p21^{CIP1}$ protein prevents phosphorylation of pRb, thereby repressing gene expression and resulting in cell-cycle arrest. Although $p21^{CIP1}$ transcription is primarily activated by p53, p53-independent

pathways also exist. Following changes in $p21^{CIP1}$ expression, levels of the cdk-inhibitor $p16^{INK4a}$ also increase (Sklavounou *et al.*, 2006). These cdk inhibitors bind cdk proteins, thereby, preventing their interaction with cyclins (Sherr and Roberts, 1999). Elevated levels of $p16^{INK4a}$ are thought to be required for maintenance of the senescent state (Stein *et al.*, 1999).

3.1.5 Senescence and cancer

The discovery that many of the stressors that precipitate senescence are oncogenic, has led to the understanding that senescence can be thought of as a mechanism by which higher organisms suppress the proliferation of cells at risk of malignant transformation (Campisi, 2000, Shay and Roninson, 2004). Indeed, many of the inducers and mediators of senescence are aberrant in cancer cells. The study of senescence therefore not only helps in understanding ageing and degenerative disease, but also in understanding tumorogenesis.

3.1.6 Senescence-associated changes

Senescent cells are not only growth arrested and unresponsive to mitogenic stimuli, but also display an altered function (Joosten et al., 2003, Campisi, 2000). These senescence-associated changes are both morphological, biochemical and molecular. Morphological changes include the acquisition of flattened shape and altered size (Bayreuther *et al.*, 1988) and biochemical changes, the increased expression of senescent-associated

biomarkers such as senescence-associated-β-galactosidase (Dimri *et al.*, 1995). Established molecular changes of senescence, include the overexpression of cdk inhibitors such as $p16^{INK4a}$ and $p21^{CIP1}$ (Stein and Dulic, 1998, Sklavounou et al., 2006). These proteins are implicated in inhibiting cyclin and cdk-mediated cell-cycle progression. The $p21^{CIP1}$ protein progressively accumulates in ageing cells, reaching a peak at the early stages of senescence, before declining. In contrast, levels of $p16^{INK4a}$ increase in the terminal stages of senescence and are, thereafter, maintained (McConnell et al., 1998, Stein and Dulic, 1998).

3.1.7 Sirtuins and senescence

In *S. cerevisiae*, Sir2 is involved in the recombinational suppression of ERCs (Kaeberlein *et al.*, 1999), the repair of DNA damage (Tsukamoto *et al.*, 1997) as well as the maintenance of telomeres (Grunstein, 1997, Aparicio et al., 1991, Moretti et al., 1994). Disruption of these Sir2-mediated functions can result in senescent-like growth arrest. Furthermore, the NAD-dependence of Sir2 activity links senescence with the cell's ability to utilise fuel (Shiels, 1999). Indeed, in *S. cerevisiae* and the nematode, *C. elegans*, lifespan extension, secondary to caloric restriction, is linked to Sir2 activity, with absolute levels of Sir2 determining when senescence occurs (Lin et al., 2000, Guarente and Kenyon, 2000).

Analogous to yeast Sir2, human sirtuins may link the mitochondrion, telomere nucleo-protein complex and ribosome production (the MTR) with senescence (Shiels and

Davies, 2003, Shiels, 1999). Sirtuins function within various components of the MTR and, perturbation of sirtuin activity at any one of these sites, may impact on global sirtuin activity and result in growth arrest and senescence.

Sirt1 has been shown to be a negative regulator of p53 and has been suggested to play a part in the ability of stressed cells to repair damage and resist both apoptosis and senescence (Luo et al., 2001, Vaziri et al., 2001, Langley et al., 2002, Chua et al., 2005). Deacetylation of p53 by Sirt1, within pro-myelocytic leukaemia nuclear bodies, has been shown to antagonise p53 and cellular senescence (Langley *et al.*, 2002). Decreasing levels of *SIRT1* transcription and protein levels are also seen in ageing primary human fibroblasts and epithelial cells in culture (Michishita et al., 2005, Sklavounou et al., 2006). Selective knockdown of the *SIRT1* gene using short interfering RNA (siRNA) causes senescence, in both non-malignant and malignant cells (Ota *et al.*, 2005). Sirtuin activators have also been implicated in the prolongation of lifespan and delaying senescence (Howitz et al., 2003, Wood et al., 2004). This is further substantiated by *in vivo* studies of Sirt1 and senescence. With increasing donor age, decreased levels of Sirt1 and increased levels of hyperacetylated p53 and $p21^{CIP1}$ proteins are seen in human gingival fibroblasts (Kim et al., 2004, Araki et al., 2004). These observations collectively suggest that Sirt1 may protect against senescence and absolute levels of Sirt1 may determine when cells enter into this state.

Sirt2 participates in the regulation of the cell-cycle and functions to deacetylate α-tubulin thereby arresting growth (Dryden et al., 2003, North et al., 2003). Acetylated α-tubulin is

a key component of stable microtubules and is generated at the time of the cell-cycle by mammalian cells. These stabilised microtubules help coordinate cytokinesis and cell-cycle transition (Piel *et al.*, 2001). Microtubule dysfunction is associated with polyploidy and replicative senescence (Wagner *et al.*, 2001) and agents that cause microtubule stabilisation result in accelerated senescence (Klein *et al.*, 2005). Sirt2 may respond to chromosomal abnormalities that result from senescence, increasing mitotic arrest.

Sirt3 is localised and activated within the mitochondria. Sirt3 activity is linked with the availability of fuel as well as with the oxidative state of the cell via its NAD-dependent enzymatic activity (Hallows *et al.*, 2006). In response to stresses such as chronic starvation, increased levels of Sirt3 and NAD promote acetyl CoA production from acetate to fuel the Krebs' cycle. This increases the efficiency of fuel utilisation and energy production thereby decreasing the cell's oxidant load and promoting longevity (Shiels and Davies, 2003). In contrast, acute oxidative stresses result in a fall in NAD levels and, therefore, Sirt3 activity and result in a decreased efficiency of fuel utilisation and energy production that may contribute to senescence and ageing. The role of the other mitochondrial sirtuins, Sirt4 and Sirt5, may be similar to Sirt3 in improving the utilisation of fuel in times of stress and promoting survival (Haigis *et al.*, 2006). In addition, as with Sirt1, the mitochondrial sirtuins may play a part in protecting against the accumulation of mitochondrial damage, a process that is also implicated in biological ageing and senescence (Brunk and Terman, 2002).

Sirt6 has been shown to be involved in DNA base-excision repair, with absence of Sirt6 resulting in a progeroid phenotype in transgenic mice (Mostoslavsky et al., 2006). This suggests that Sirt6 helps prevent the accumulation of damage that results in cellular and subsequently physiological senescence.

As previously mentioned, Sir2 determines lifespan in *S. cerevisiae* by repressing the recombination formation of ERCs within rDNA at the nucleolus (Kaeberlein et al., 1999, Guarente, 1997). Sirt7 is the only human sirtuin to localise to the nucleolus but, unlike yeast Sir2, has been shown to activate Pol I transcription at the rDNA, encouraging growth and proliferation (Ford *et al.*, 2006). However, analogous to the role of yeast Sir2, intrinsic Sirt7 levels may determine when a cell becomes senescent. This theory is supported by the observation that Sirt7 protein levels fall in both ageing human epithelial cells and fibroblast *in vitro* (Michishita *et al.*, 2005).

Furthermore, the redistribution of sirtuins from areas of silencing to areas of DNA damage over a cells lifespan may result in a gradual decline in sirtuin activity and fall in the integrity of silenced chromatin. In this way, decreased silencing may, with resultant genomic instability, may contribute to the senescent phenotype (Neumeister *et al.*, 2002). It is perhaps this loss of silencing that results in the gradual reactivation of the silenced mammalian X-chromosome with age (Surralles *et al.*, 1999).

Finally, rather than changes within the chromatin or altered telomeric state stimulating senescence, senescence-inducing signals may come from the loss of silencing and

expression of sub-telomeric genes (Baur *et al.*, 2001). Over a cells life, the cumulative sirtuin reduction from telomeric regions may not only destabilise the telomeres and result in telomeric damage, but also result in the expression of sub-telomeric genes, that induce senescence (Shiels and Davies, 2003). Evidence to suggest this comes from studies that show altered *SIRT3* expression to be associated with survival in the elderly (Rose *et al.*, 2003).

In summary, senescence may be regarded as a tumour suppressor mechanism that must be overcome for immortalisation. Furthermore, sirtuins within individual components of the MTR may be related to damage responses that contribute to biological ageing and senescence. Subsequently, perturbation of sirtuin activity may be associated with tumorogenesis. The study of sirtuin expression in normal replicative senescence is, therefore, of value in understanding cancer.

This thesis has sought to establish the transcriptional expression profile for individual sirtuins as a function of normal biological ageing, and to compare it with expression profiles in immortalised and cancer cells.

3.1.8 Aims

In this part of the study, sirtuin gene expression was investigated in primary and immortalised mammary epithelial cells as a function of *in vitro* growth.

Individual questions addressed were:

1. How does sirtuin transcriptional expression alter in replicatively ageing mammary epithelial cells?

2. Are these changes associated with established molecular and biochemical markers of senescence?

3. How does this compare to sirtuin transcriptional expression profiles in immortalised mammary cell lines?

3.2 Results

Sirtuin expression was measured in mammary epithelial cell lines as they underwent replicative ageing. These cell lines included a primary mammary epithelial cell line (HMEC), an established immortalised non-tumorogenic breast cell line (MCF-12A) and a tumorogenic breast cancer cell line (MCF-7). Gene expression was measured using Taqman real-time relative quantitative PCR and Senescence-associated β-galactosidase (Scn-β-gal) assays were performed.

3.2.1 Replicative senescence

Primary human mammary epithelial cells (HMEC) from a culture grown for seven population doublings (PD) were seeded and serially passaged. Cells grown to sub-confluence were split in a 1 in 8 manner ensuring that there were approximately three PD between each successive passage. After three passages, that is after nine PD, the HMEC stopped proliferating and displayed a senescent morphology. The acquisition of the senescent phenotype by the HMECs, with serial passage, was confirmed by a transcriptional analysis of the senescence-associated genes, $p16^{INK4a}$ and $p21^{CIP1}$. The expression of both, $p16^{INK4a}$ and $p21^{CIP1}$, showed an increase with successive passage in the HMEC, unlike that of the immortalised, non-tumorogenic MCF-12A cell line and immortalised, tumorogenic MCF-7 cell line (R^2 = 0.933, p=0.002 and R^2 = 0.944, p=0.001 respectively) (Table 3.1 and Figure 3.1). *SIRT1* and *SIRT2* showed no obvious pattern of change in transcriptional expression in successively passaged cells, unlike *SIRT3* and *SIRT7*, which showed a progressive increase in transcriptional expression with

passage in the HMEC compared to the immortalised mammary cell lines (R^2 = 0.825, p=0.012 and R^2 = 0.772, p=0.021 respectively) (Table 3.1 and Figure 3.2). Similarly, SA-β-gal assays showed increased staining with successive passage in the HMEC, but not in the immortalised MCF-12A cell line (R^2 = 0.969, p=0.000) (Figure 3.3).

Table 3.1 Relationship between replicatively ageing mammary epithelial cells and sirtuin, p16 and p21 expression. The table shows linear regression analyses for the relationship between replicative ageing and sirtuin, *p16* and *p21* transcriptional expression in primary human mammary epithelial cells. R^2 values and p-values are displayed. *p<0.01, **p<0.05

HMEC	R^2	p-value
P16	0.933	0.002*
P21	0.944	0.001*
SIRT1	0.222	0.346
SIRT2	0.212	0.358
SIRT3	0.825	0.012*
SIRT7	0.772	0.021**

Figure 3.1 Senescence-associated gene expression in proliferating human mammary epithelial cells. Histograms shows mean p16 and p21 gene expression in primary cell cultures (HME) and immortalised breast cell lines (MCF-12A and MCF-7). Data is plotted as means ± s.d. (n=2)

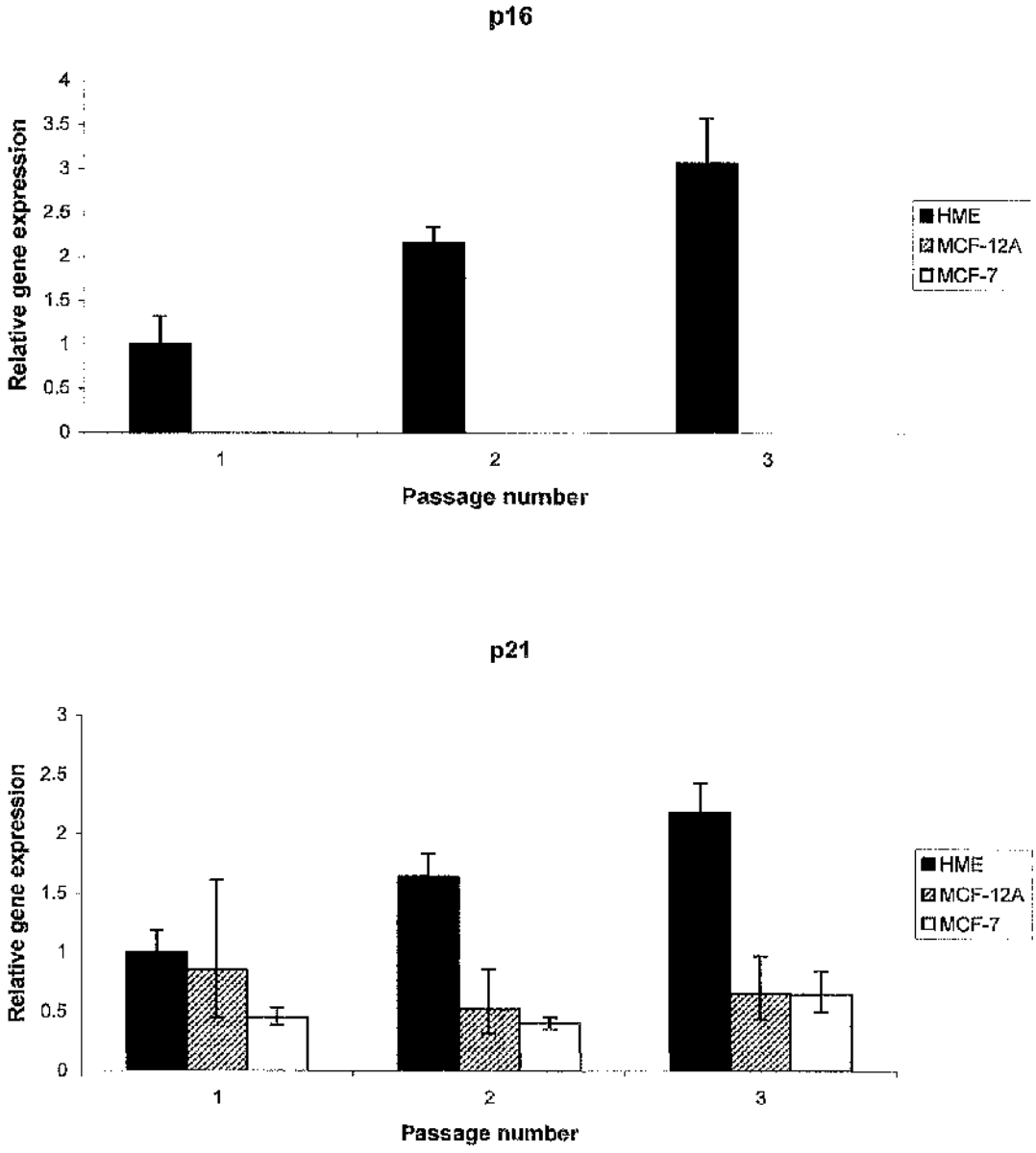
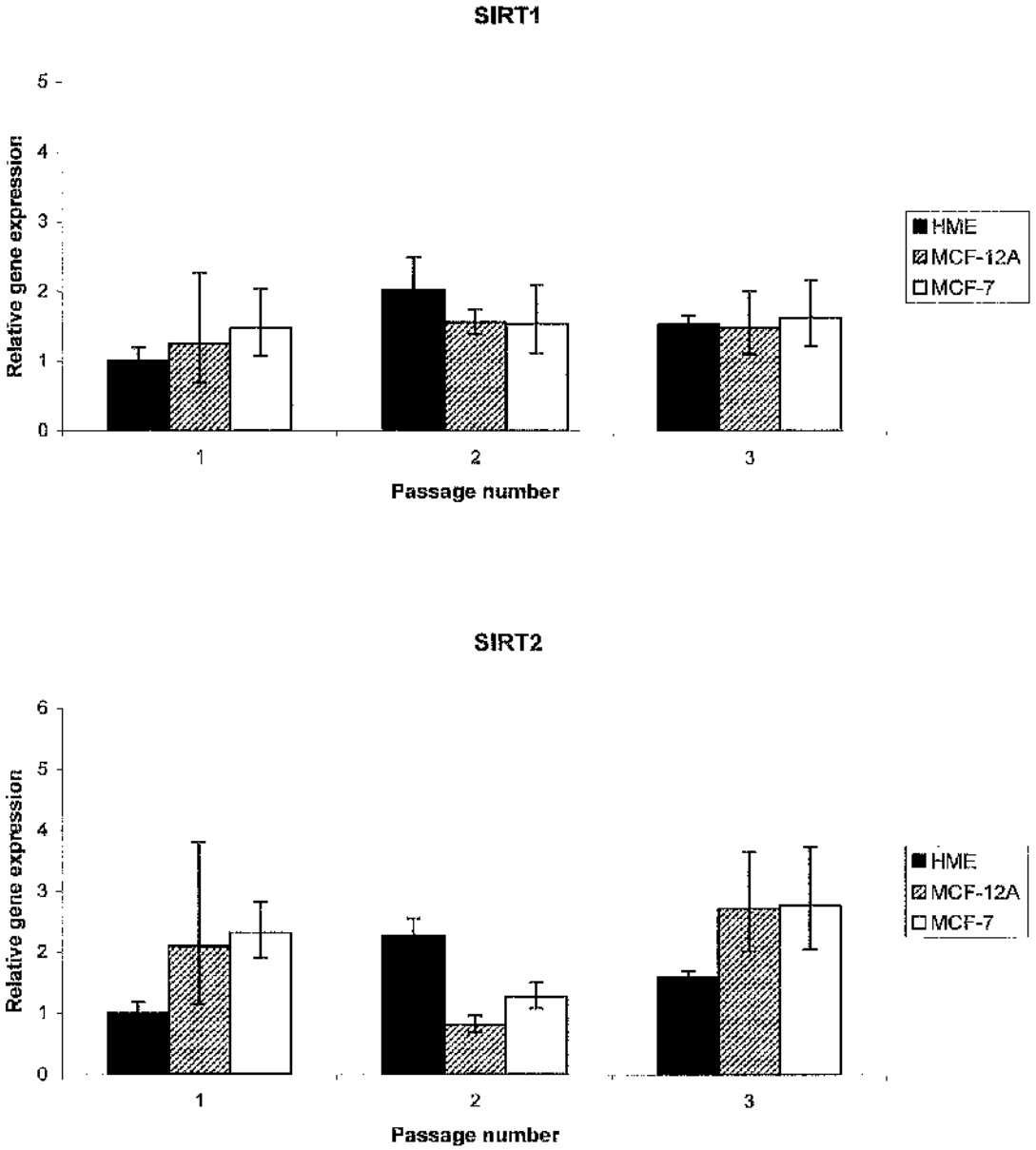
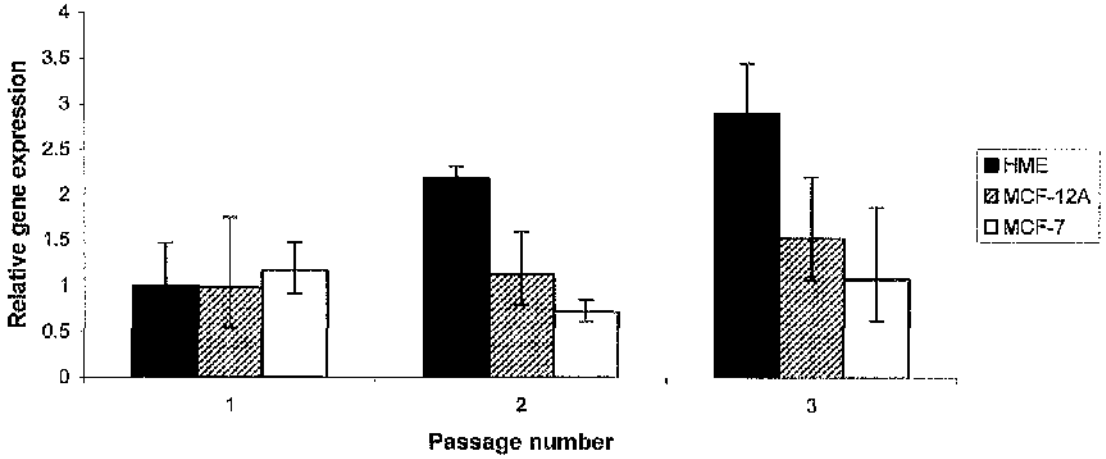


Figure 3.2 Sirtuin expression in proliferating human mammary epithelial cells. Histograms shows mean *SIRT1,2,3* and *7* gene expression in primary cell cultures (HME) and immortalised breast cell lines (MCF-12A and MCF-7). Data is plotted as means ± s.d. (n=2)



SIRT3


Relative gene expression
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
Passage number
HME
MCF-12A
MCF-7

SIRT7

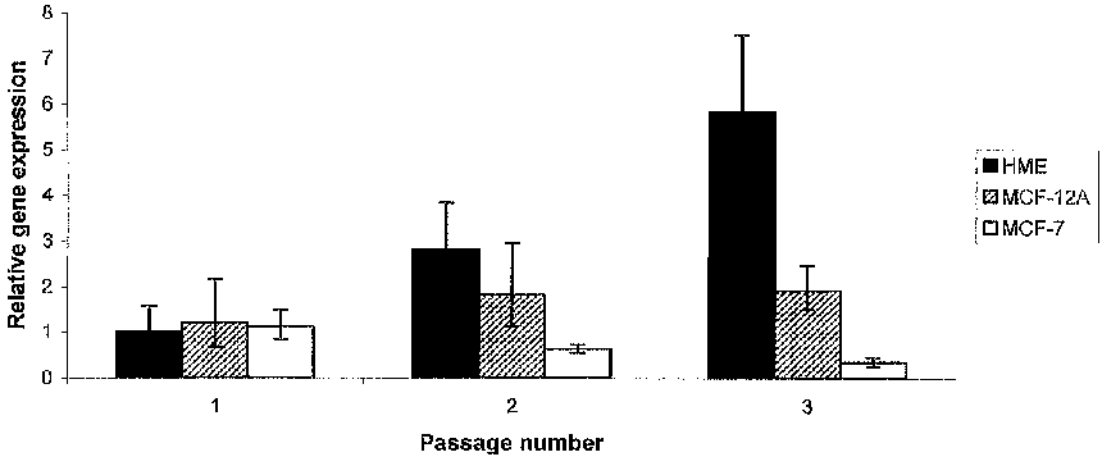
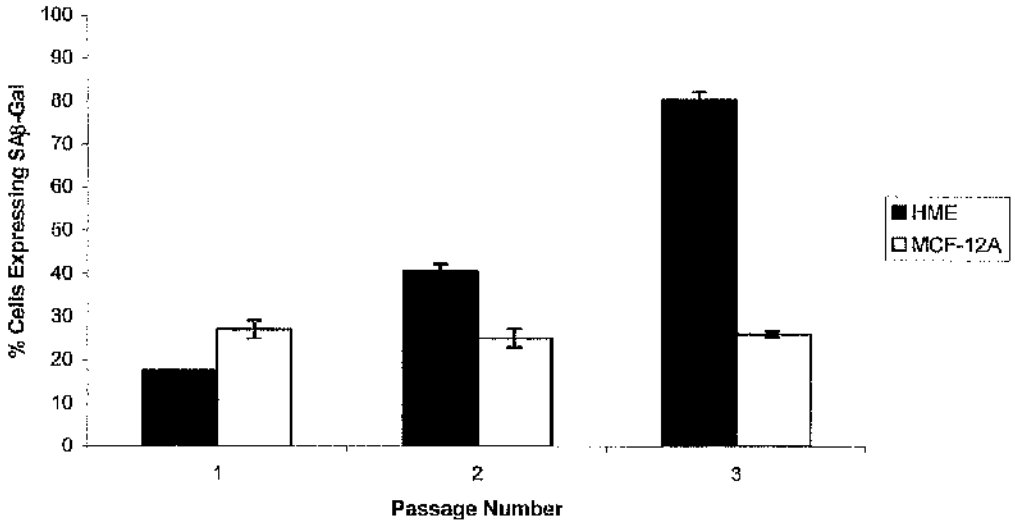

Relative gene expression
8
7
6
5
4
3
2
1
0
1
2
3
Passage number
HME
MCF-12A
MCF-7

Figure 3.3 Senescence-associated β-galactosidase staining in successively passaged human mammary epithelial cells. Histograms shows mean shows mean Senescent-β-galactosidase expression in successively passaged primary cell cultures (HME) and immortalised breast cell lines (MCF-12A). Data is plotted as mean ± s.e.m. (n=2)



3.3 Discussion

Cellular senescence represents a mechanism by which an organism suppresses proliferation of cells at risk of malignant transformation (Campisi, 2000). Studying the transcriptional of human sirtuins in primary mammary epithelial cell ageing may, therefore, help us understand breast cancer pathogenesis from the perspective of biological ageing as a platform for disease.

3.3.1 Senescence-associated gene expression and SA-β-Galactosidase staining

The transcriptional expression of $p16^{INK4a}$ and $p21^{CIP1}$, as well as, SA-β-Gal staining were studied in replicatively ageing cells to determine whether these cells were displaying a replicative senescence state. Transcriptional expression of $p16^{INK4a}$ and $p21^{CIP1}$ and SA-β-Gal staining were noted to increase in serially passaged primary HMEC. The changes in $p16^{INK4a}$ expression observed confirm previous observations (Kim *et al.*, 2002). The lack of expression of $p16^{INK4a}$ in either of the immortalised cell lines is predictable, as deletion of the cdk inhibitor, $p16^{INK4a}$, is a recognised step in the immortalisation of mammary cancer cell lines (Musgrove *et al.*, 1995). Elevated levels of $p21^{CIP1}$ in the HMEC with serial passage, may reflect a function of either pre-senescent cells still in the culture (Schwarze *et al.*, 2001), or of continuing damage to senescent cells that are attempting to repair damage, independent of their growth arrested state. Overall, the changes within the HMEC are consistent with an *in vitro* increase in biological age as a

function of time. These results suggest that this experiment is a useful model of biological ageing and for determining the role of sirtuins in senescence and cancer.

3.3.2 Changes in sirtuin expression

Sirtuins 1,2,3 and 7 (Sirt1,2,3, and 7) were specifically studied as they have been shown to participate within the MTR and may provide information on how the MTR senses, assesses and signals changes associated with senescence (Ford et al., 2006, Onyango et al., 2002, Schwer et al., 2002). In this study, *SIRT1* and *SIRT2* transcriptional expression showed no obvious pattern of change in successively passaged HMEC, unlike *SIRT3* and *SIRT7* expression, which showed a progressive increase in transcriptional expression with increasing passage.

The transcriptional expression of *SIRT3* increased in replicatively ageing HMEC. Sirt3 activity is linked with the availability of fuel, as well as with the oxidative state of the cell, via its NAD-dependent enzymatic activity on AceCS2 (Hallows *et al.*, 2006). In HMEC undergoing RS, stresses experienced by the cell may alter NAD levels and result in a concomitant fall in Sirt3-mediated enzymatic activity (Schwer *et al.*, 2002). This reduction in Sirt3 activity may result in a fall in the efficiency of fuel utilisation and contribute to senescent growth arrest. Increased *SIRT3* transcription may result in order to increase the efficiency of fuel utilisation and energy production, thereby preventing this growth arrest. Sirt3, along with Sirt2, mediates susceptibility to oxidative death via activation of the mitochondrial transient receptor potential melastatin-related channel 2

(TRPM2) (Grubisha *et al.*, 2006). Increasing levels of Sirt3, with replicative age, may reflect the cellular response to mitochondrial damage that accrues with time, leading to senescent growth arrest. High levels of Sirt3 are protective in this context. *In vivo* evidence to support this include the finding that reduction in Sirt3 expression is detrimental for longevity in the elderly (Rose et al., 2003, Bellizzi et al., 2005). Our data, that show increasing transcriptional expression of *SIRT3* in replicatively ageing mammary epithelial cells, are supportive of this.

SIRT7 expression was also seen to increase with RS. As with yeast Sir2, Sirt7 is localised to the nucleolus, the site of rDNA where it is also known to associate with condensed chromosomes during mitosis (Michishita *et al.*, 2005). In *S. cerevisiae*, Sir2 functions at the rDNA to prevent the recombinational formation of ERCs (Park *et al.*, 1999). Although the accumulation of ERCs does not occur in humans, Sirt7 has been shown to activate Pol I-mediated transcription of rRNA, encouraging growth and proliferation (Ford *et al.*, 2005). Paradoxically, senescent fibroblasts exhibit a higher rRNA synthesis rate when compared to young cells at similar growth rates, resulting in the generally observed higher rRNA content of senescent cells (Halle *et al.*, 1997). Increasing *SIRT7* expression may reflect this increased rRNA synthesis that occurs as cells become senescent. Finally, rRNA transcription may also act as a direct sensor for accumulation of DNA damage over a cell's lifespan (Shiels and Davies, 2003). The repetitive nature of the rRNA genes, in combination with the dense loading of the Pol I complexes at the active templates, provides a high probability of a polymerase encountering, and stalling at, sites of DNA damage. The accumulation of signals associated with such stalled DNA

polymerases may activate p53 and DNA-damage response pathways that contribute to senescence. Again, our data that show increased SIRT7 expression in replicatively ageing mammary epithelial cells are supportive of this.

SIRT1 is thought to modulate cellular senescence by negatively regulating p53 (Langley et al., 2002, Chua et al., 2005). *SIRT1* is known to act as a negative regulator of p53 and may be important in allowing a cell to resist the gradual accumulation of sub-lethal damage that may contribute to the senescent phenotype. *SIRT1* expression may, therefore, be expected to fall as cells undergo RS. This study did not show a fall in *SIRT1* expression in ageing primary HMEC. Although this observation is counterintuitive, the evidence for the role of Sirt1 in senescence is also conflicting. Although over-expression of *SIRT1* has been shown to rescue mouse epithelial fibroblasts from primary senescence (Langley *et al.*, 2002), other studies have shown that transfection of human fibroblasts or human epithelial cells with *SIRT1* does not affect when senescence occurs (Michishita *et al.*, 2005). In addition, *SIRT1* transcription and protein expression appears to decrease in some fibroblast cell lines but not in others (Michishita et al., 2005, Sklavounou et al., 2006). Although this supports the observation that over-expression of *SIRT1* in human cancer cells has no effect on their growth (Afshar and Murnane, 1999), the Sirt1 inhibitor, Sirtinol, has been shown to induce senescence-like growth arrest in MCF-7 cells (Ota *et al.*, 2005). The role of Sirt1 on senescence may therefore depend on numerous factors including cell type, as well as whether senescence is replicative or stress and aberrant signalling-induced (Wright and Shay, 2002). This is analogous to the contrasting role that

Sirt1 has in mediating apoptosis; that is of inducing TNFα-mediated apoptosis but protecting against p53-mediated apoptosis (Yeung *et al.*, 2004).

Finally, levels of *SIRT2* also showed no change within the HMEC undergoing RS. This is in keeping with the previous observation that *SIRT2* expression remains unchanged in ageing human fibroblasts (Michishita *et al.*, 2005). Sirt2 appears to participate in a cell-cycle checkpoint during the M phase of mitosis. Although senescence in mammary epithelial cells is associated with the accumulation of chromosomal abnormalities their presence does not appear to result in an increase in *SIRT2* expression at the transcriptional level. RS does not, therefore, appear to be associated with Sirt2-mediated cell-cycle arrest.

This study has shown that the transcriptional expression of *SIRT3* and *SIRT7* is associated with replicatively ageing primary mammary epithelial cells. These changes are associated with established molecular and biochemical markers of senescence, namely increased transcriptional expression *p21* and increased SA-β-Gal staining.

Chapter 4

Sirtuin Expression in Non-malignant and Malignant Breast Biopsies

4.1 Introduction

Sirtuins link the functions of the mitochondrion, telomere nucleo-protein complex and ribosome production (the MTR), and may be important contributors to a wide range of ageing-related diseases including cancer (Shiels and Davies, 2003). Over a cell's lifespan, perturbation in sirtuin activity at each of these loci may result in biological ageing. Similarly, changes in sirtuins acting within the MTR may be expected to occur in cancer. Indeed, human sirtuins have been implicated in senescence growth arrest (Langley *et al.*, 2002), apoptosis (Luo et al., 2001, Vergnes et al., 2002) and cell-cycle regulation (Dryden *et al.*, 2003), processes that are all involved in suppressing the growth of abnormal, rapidly proliferating cells, a hallmark of cancer.

Supporting this, are the changes in sirtuin transcriptional expression observed within ageing primary mammary epithelial cells. Specifically, changes in *SIRT3* and *SIRT7* expression, involving the mitochondrial and ribosomal units of the MTR, may be predictive of similar changes in sirtuin expression in breast cancer.

4.1.1 Sirtuin expression in cancer

Increased histone deacetylase activity has been shown to provide a growth and survival advantage in certain cancers (Marks *et al.*, 2001). In keeping with this, *SIRT1* expression has been shown to be higher in a number of cancers (Lim, 2006b, Kuzmichev et al., 2005, Chen et al., 2005, Yeung et al., 2004, Bradbury et al., 2005). Sirtuin activators are able to

cause cellular lifespan extension (Wood *et al.*, 2004) and *SIRT1* siRNA can cause cancer cells to become senescent (Ota et al., 2005, Ford et al., 2005). Sirt1 may contribute to tumorogenesis by inhibiting tumour cell apoptosis. It may do this via its interactions with proteins that include $p33^{ING1b}$, p53, NF-κB and the Foxo transcription factors (Luo et al., 2001, Vaziri et al., 2001, Tran et al., 2002, Yeung et al., 2004, Kataoka et al., 2003). Normally, Sirt1 interacts with these substrates to encourage growth, prevent apoptosis as well as to increase a cell's resistance to stress. Increased expression of Sirt1 may provide a growth and survival advantage thereby contributing to tumorogenesis. Finally, Sirt1 might prolong survival of cells at risk for transformation by participating in the silencing of tumour suppressor genes (Chen et al., 2005, Pruitt et al., 2006).

The *SIRT2* gene is located in a position that frequently undergoes loss of homozygosity in human gliomas. Sirt2 is thought to regulate the cell-cycle by preventing its progression in cells harbouring chromosomal abnormalities (Dryden *et al.*, 2003). Proteomic studies show a downregulation of Sirt2 to be a feature of gliomas (Hiratsuka *et al.*, 2003) as well as gastric cancers (Inoue *et al.*, 2006). Marked aneuploidy in gliomas suggest that a defective mitotic spindle checkpoint resulting in chromosomal instability occurs (Cleveland *et al.*, 2003). *SIRT2* may function as a tumour suppressor gene as its downregulation may be responsible for the chromosomal instability that leads to tumorogenesis.

The activity of the Sirt3 deacetylase is linked to the oxidative state of the cell. Analogous to the role of Sirt1 and Sirt2, Sirt3 may also have a role in survival and function to

prevent cells accumulating mitochondrial damage that may contribute to senescent growth arrest (Shiels and Davies, 2003). The *in vitro* studies have shown that *SIRT3* expression increases in ageing mammary epithelial cells, perhaps in response to the stresses associated with replicative ageing. Furthermore, variability of the *SIRT3* gene has been linked to survival in the elderly (Rose *et al.*, 2003).

Sirt3 may also have a direct role in tumorogenesis. The gene encoding it has been shown to map to a sub-telomeric region that is subject to genomic imprinting. This region also contains a major but as yet unidentified tumour suppressor gene as well as a locus associated with Beckwith-Wiedemann Syndrome, a syndrome associated with both prenatal overgrowth and predisposition to cancer (Shiels, 1999, Shiels and Jardine, 2003, Feinberg, 2000, Weksberg et al., 1993, Lee et al., 1999a).

The expression of *SIRT7* is invariably elevated in malignant thyroid cancer cell lines and biopsies indicating that this represents an important step in thyroid cancer pathogenesis (de Nigris et al., 2002, Frye, 2002). In addition, the *SIRT7* gene maps to chromosome 17q25.3; a region that is frequently affected by chromosomal alterations in acute leukaemias and lymphomas (Voelter-Mahlknecht *et al.*, 2006). Increased nucleolar yeast Sir2 activity results in a decrease in ERCs and prolongation of lifespan. Similarly, Sirt7 also displays nucleolar localisation and has been shown to activate Pol I and rDNA transcription, thereby promoting cell growth and proliferation (Ford *et al.*, 2006). The *in vitro* studies suggest that, like yeast Sir2, Sirt7 levels may determine the stage at which

cells become senescent. An increased expression of *SIRT7* may provide tumour cells with a survival advantage contributing to tumorogenesis.

In summary, as with senescence, tumorogenesis may be associated with changes within the components of the MTR. Specifically, in cancer cells, altered sirtuin expression may be associated with a perturbation in damage responses that normally contribute to biological ageing and senescence. The study of sirtuin expression, relating to components of the MTR, in cancer may help in understanding how cancer develops and progresses from a platform of biological ageing. In the previous chapter, sirtuin transcriptional expression was demonstrated to change as a functional of biological ageing. This analysis can be extended to *in vivo* situation by looking at how sirtuin expression in breast tissue biopsies is tied to disease status.

4.1.2 Aims

The purpose of this part of the study was to determine if changes in biological ageing observed in the HMEC are pertinent to the situation *in vivo*. This was achieved by studying sirtuin expression in non-malignant and malignant breast tissue biopsies.

Individual questions addressed were:

1. Does sirtuin transcriptional expression differ between non-malignant and malignant breast tissue biopsies?

2. Is the transcriptional expression of sirtuins indicative of breast cancer histopathology or tumour stage?

4.2 Results

Sirtuin expression was investigated in breast cancer to determine if changes in the transcriptional expression of individual sirtuins correlated with malignancy and disease stage. Sirtuin expression was measured, in archival non-malignant and malignant breast biopsies collected and stored within the Department of Surgery at the University of Glasgow, using Taqman real-time relative quantitative PCR.

4.2.1 Summary of breast biopsies analysed

The pathological characteristics of the breast biopsies were selected to include equal distribution by nodal status (12 lymph node positive and 12 lymph node negative) as nodal status has been shown to be one of the most important indicators of breast cancer prognosis (Russo and Russo, 2003). A total of 24 breast cancer samples were chosen and matched with 21 non-malignant breast tissue biopsies from the breasts of cancer patients. Both groups were well matched with the mean age of the patients in the cancer biopsy group being 62.3 years (SEM 3.1) and that of the non-malignant biopsy group, 61.4 (SEM 3.1). All tumours were ductal adenocarcinomas and both groups had a similar histopathology, oestrogen receptor status and size (Table 4.1).

Table 4.1 Summary of pathological prognostic factors of breast biopsies.

	No	Age years (SEM)	Nodal Status	Size mm (SEM)	ER Status		LVi	Grade		NPI (SEM)
					+ve	– ve		II	III	
Normal	21	61.4 (3.1)	N/A	N/A	N/A		N/A	N/A		N/A
Cancer	24	62.3 (3.1)	+ ve	30.7 (3.1)	9	3	6	4	8	4.1 (0.2)
			– ve	35.7 (3.1)	8	4	5	5	6	6.0 (0.3)

SEM = standard error of the mean, N/A = not applicable, ER=estrogen receptor, LVi = Lymphovascular invasion. NPI = Nottingham Prognostic Index

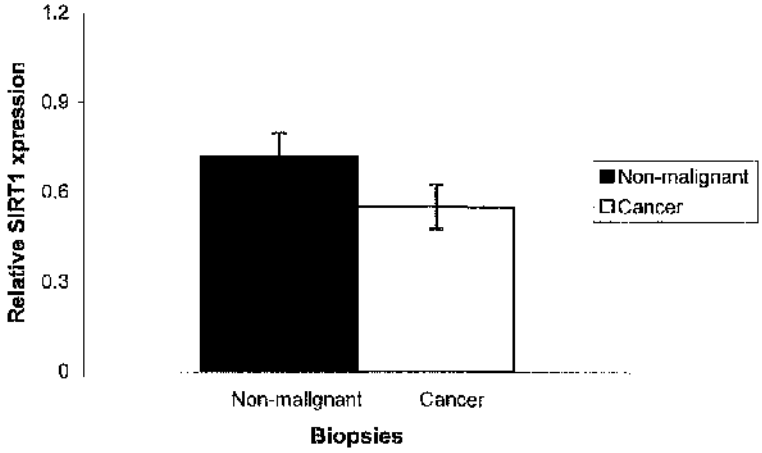
4.2.2 Sirtuin expression in breast biopsies

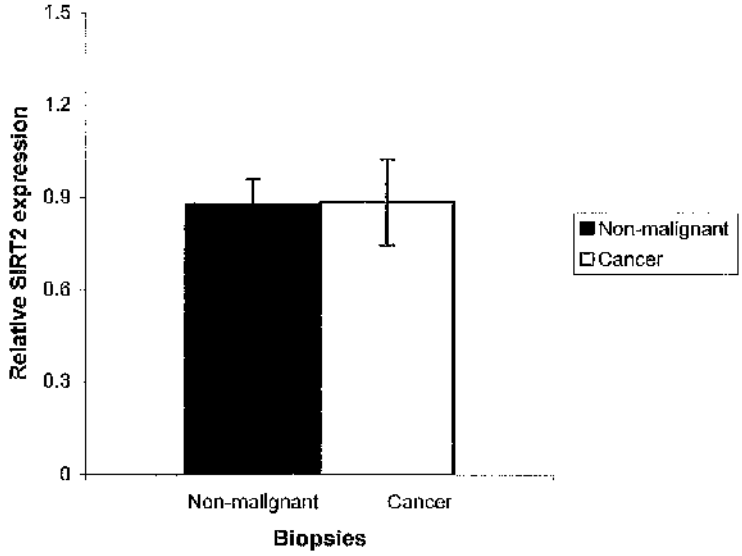
Relative *SIRT7* gene expression was significantly greater in biopsies from breast cancers compared to non-malignant breast tissue 1.45 (95% C.I. 1.19 – 1.71) vs. 0.83 (95% C.I. 0.68 – 0.99), $p<0.001$ (Figure 4.1). The relative expression of *SIRT7* was also significantly higher in node positive compared to non-malignant breast biopsies 1.63 (95% C.I. 1.27 – 1.99) vs. 0.83 (95% C.I. 0.68 – 0.99), $p<0.001$ (Figure 4.2).

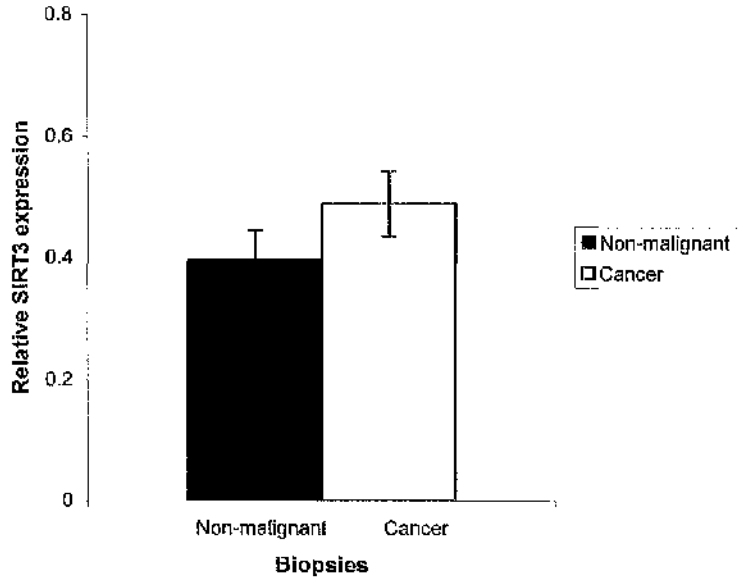
No significant differences in *SIRT1*, *SIRT2* and *SIRT3* gene expression were observed in a simple comparison of breast cancer biopsies with non-malignant breast tissue biopsies (Figure 4.1). However, analogous to *SIRT7* expression, *SIRT3* expression was also significantly higher in lymph node positive breast cancer biopsies when compared to non-malignant breast biopsies, 0.62 (95% C.I. 0.46 – 0.78) vs. 0.40 (95% C.I. 0.30 – 0.49), $p<0.05$ (Figure 4.2).

Sirtuin expression was similarly compared by grade, oestrogen receptor status and the presence of lymphovascular invasion in both node positive and node negative cancers. No significant association between sirtuin expression and these factors was observed.

Figure 4.1 Sirtuin expression in non-malignant and breast cancer biopsies. Histograms show relative sirtuin gene expression in non-malignant and malignant breast biopsies, relative to an 18S rRNA control. Data show mean sirtuin expression ± s.e.m.






0.8
0.6
0.4
0.2
0
Relative SIRT3 expression
Non-malignant
Cancer
Non-malignant
Cancer
Biopsies

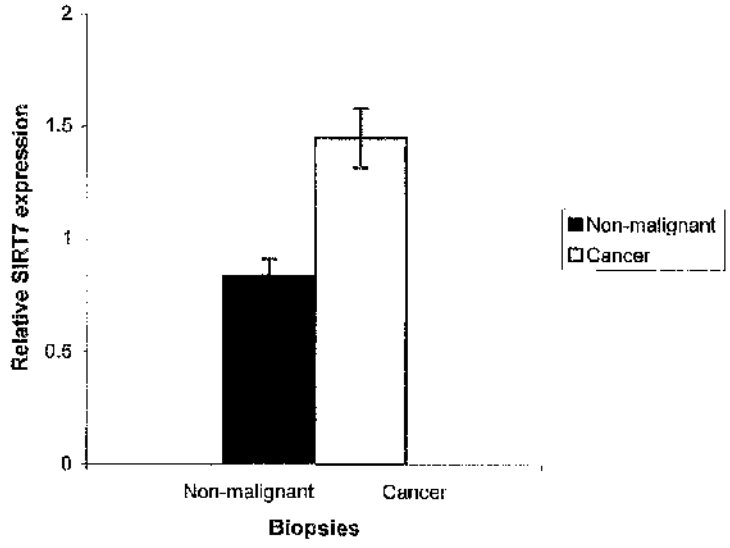
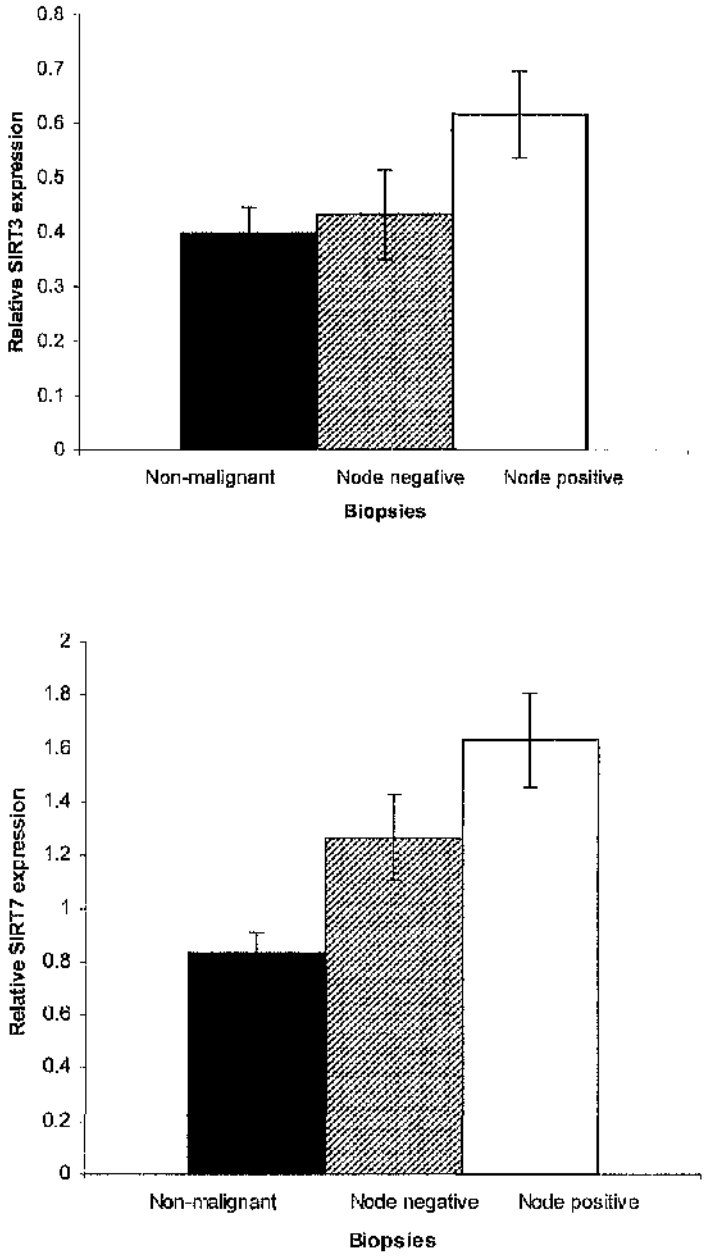

2
1.5
1
0.5
0
Relative SIRT7 expression
Non-malignant
Cancer
Non-malignant
Cancer
Biopsies

Figure 4.2 SIRT3 and SIRT7 expression in breast biopsies by nodal status. The histogram shows relative *SIRT3* and *7* gene expression in non-malignant and lymph node positive and negative breast cancer biopsies. Data are plotted as mean sirtuin expression ± s.e.m.



4.3 Discussion

4.3.1 Sirtuin expression in breast cancer

A number of lines of evidence suggest sirtuins are implicated in cancer (de Nigris et al., 2002, Frye, 2002, Pruitt et al., 2006, Ota et al., 2006, Neumeister et al., 2002, Hiratsuka et al., 2003, Chen et al., 2005). Our data are consistent with these, showing increased *SIRT7* expression to be a feature of breast cancer. This increased expression may reflect a molecular change necessary for breast cancer tumorogenesis. Furthermore, *SIRT7* expression was also associated with nodal invasion and, therefore, with more invasive tumours and more aggressive disease. Proliferating cells have a higher demand for ribosomes than slowly proliferating cells. Since the rapid growth of tumours requires elevated rates of ribosome biosynthesis, Pol I transcription may need to increase during tumorogenesis. Indeed, levels of rDNA transcription are increased in transformed cells and the corresponding increase in nucleolar size is often used as a prognostic marker for the rapidity of cancer-cell proliferation (Russo and Russo, 2003).

The question remains as to whether the deregulation of rRNA synthesis contributes to the increased proliferative potential of mammary cancer cells, plays a secondary, but necessary, part in supporting the increased cell growth or, alternatively, is a compensatory mechanism as a consequence of the increased proliferative state. The latter hypothesis is supported by observations on the activity of the yeast sirtuin orthologue, *SIR2*. Nucleolar Sir2 activity is a limiting factor for determining when the cell undergoes senescence (Guarente, 1999). The high proliferative rate in many tumours, often results in

them having shortened telomeres as a consequence of rapid cell turnover outstripping telomerase-mediated telomere repair. This may, analogous to the situation in yeast (Guarente, 1999), lead to an up-regulation of sirtuin activity in an attempt to stabilise the nucleolus. The increased *SIRT3* and *SIRT7* transcription observed in primary mammary epithelial cells, as a consequence of increased oxidant load with *in vitro* growth, is also supportive of such a scenario.

At first glance, no difference in *SIRT3* transcriptional expression was observed in breast cancer biopsies. However, on subdividing tumours by nodal status, levels of *SIRT3* are observed to be higher in the prognostically poorer lymph node positive tumours compared to normal breast biopsies. Similarly, increased *SIRT3* expression was observed in ageing primary mammary epithelial cells. Sirt3 has been shown to be specifically targeted and converted into its active form within the mitochondria (North et al., 2003, Dryden et al., 2003). Cumulative mitochondrial damage contributes to a fall in relative NAD levels, a concomitant fall in Sirt3 activity, and is associated with growth arrest, senescence and apoptosis (Shiels and Davies, 2003). Correspondingly, increased growth and proliferation is associated with an increase in mitochondrial activity and Sirt3-mediated energy production (Hallows *et al.*, 2006). Increased *SIRT3* expression, observed in the node positive breast cancer biopsies, may reflect this. Analogous to Sirt1 and Sirt2, increased *SIRT3* expression may provide ageing mammary epithelial cells with a growth and survival advantage and may also contribute to tumorogenesis in breast cancer. Although differences in *SIRT3* expression were only observed on subdividing tumours by nodal status, it may be that, by increasing the study size, we find a difference in *SIRT3* expression between non-malignant and breast cancer biopsies.

Surprisingly, transcriptional changes in *SIRT1* expression did not appear to be implicated in breast cancer pathogenesis in this study. This is substantiated by the results of studies on ageing primary mammary epithelial cells which showed no association between *SIRT1* transcriptional expression and replicative senescence. Collectively, these findings suggest that aberrant Sirt1 transcriptional activity is not implicated in breast cancer pathogenesis. This data appear to be at odds with those from groups that have shown increased levels of *SIRT1* expression in a number of cancers (Luo et al., 2001, Lim, 2006b, Yeung et al., 2004, Kuzmichev et al., 2005, Chen et al., 2005). Indeed, Sirt1 interacts with a number of proteins involved in cancer pathogenesis including $p33^{ING1b}$, p53, HIC1, the Foxo transcription factors and NF-κB (Kataoka et al., 2003, Chen et al., 2005, Yeung et al., 2004, Langley et al., 2002, Yang et al., 2005). Therefore, although these data suggest that Sirt1 is not implicated in breast cancer pathogenesis, it cannot be excluded, as these experiments looked at a small number of tumour biopsies, did not look at whether *SIRT1* expressed was mutated and, crucially, did not look at any putative post-transcriptional regulation.

Finally, no difference in *SIRT2* transcriptional expression was observed in breast cancer biopsies compared with non-malignant breast biopsies. Again, this substantiates the findings from the *in vitro* studies and, like Sirt1, suggests that changes in Sirt2 are not implicated in breast cancer pathogenesis. Sirt2 has been shown to interact with and deacetylate the α-tubulin subunit of microtubules and is thought to participate in a late mitotic check-point to ensure correct chromosome segregation during cytokinesis (North et al., 2003, Dryden et al., 2003). Sirt2 may perform a regulatory function that prevents

cells with chromosomal abnormalities from dividing. Chromosomal fusions are commonly seen in cancer cells and decreased *SIRT2* expression is a feature of gliomas and gastric cancer (Hiratsuka et al., 2003, Inoue et al., 2006). Chromosomal instability appears early during breast carcinogenesis and is considered a major driving force in malignant transformation (Meeker and Argani, 2004). Unlike defects in *BRCA* gene expression, a common feature of breast cancer and known cause of chromosomal instability and aneuploidy (Daniels et al., 2004, Grigorova et al., 2004), decreased *SIRT2* transcriptional expression does not appear to account for the increased chromosome instability observed in breast cancer. Despite these results, *SIRT2* expression and activity is cell-cycle dependent (Dryden et al., 2003, North et al., 2003) and it may be that more dynamic evaluation of changes in *SIRT2* expression at specific stages of the cell-cycle may reveal abnormal patterns of *SIRT2* expression in breast cancer. Again, it must be noted that these experiment looked at a small number of tumour biopsies and did not look at any putative post-transcriptional regulation.

This study has validated the *in vitro* work, and shown increased transcriptional expression of *SIRT7* to be associated with breast cancer. Furthermore, it has shown that the transcriptional expression of both *SIRT3* and *SIRT7* is associated with lymph node positive disease. These sirtuins act at the mitochondrial and ribosomal components of the MTR and support the hypothesis that perturbation in these components is associated with tumorogenesis.

Chapter 5

Sirtuin Expression as an Evaluator of Breast Cancer Therapy

5.1 Introduction

Breast cancer treatment invariably involves surgery, as well as adjuvant therapy that include radiotherapy and chemotherapy. These treatments result in tumour cell death by inducing apoptosis and replicative cell death. Sirtuins have been implicated in apoptosis and in cell survival in response to a variety of stresses (Mostoslavsky et al., 2006, Tran et al., 2002, Chen et al., 2005, Matsushita et al., 2005, Luo et al., 2001). Sirtuin activity may, therefore, mediate damage responses in response to irradiation and chemotherapy. In the following sections, follows a review of how irradiation and taxane chemotherapy cause cell damage, and of how sirtuins may mediate damage responses.

5.2 Cellular irradiation

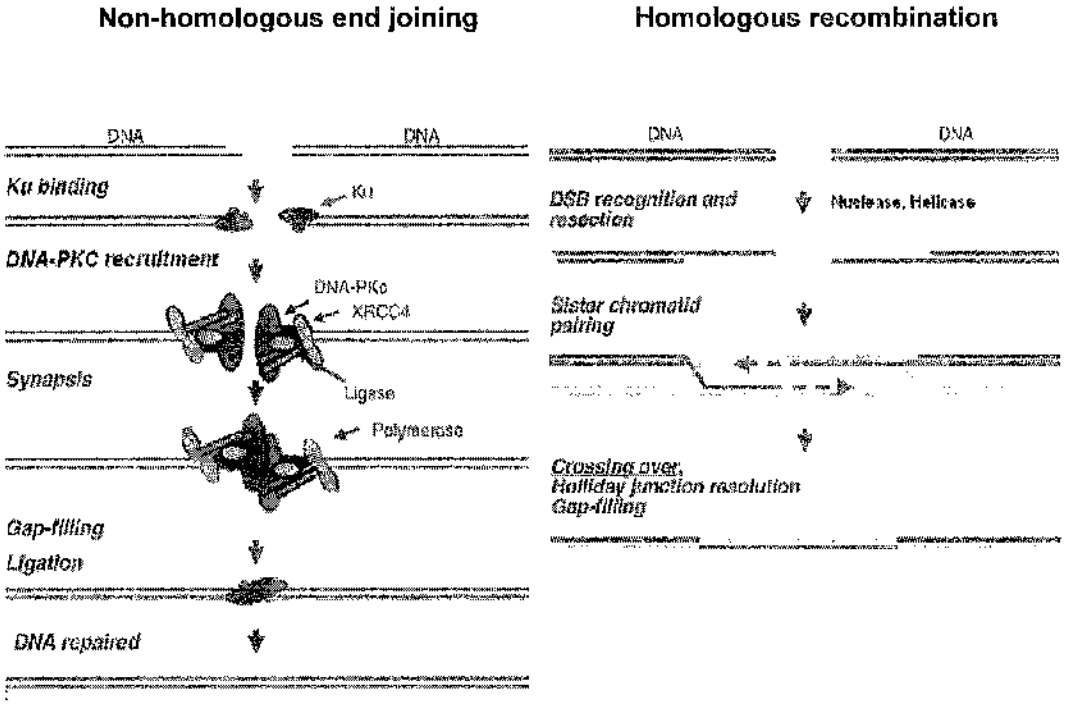
Cellular irradiation is commonly used for the treatment of breast cancer and involves the delivery of ionising radiation with high-energy photons such as γ-rays and x-rays. The ionisation of tissue water results in the formation of reactive oxygen species (ROS) that cause oxidative damage to the cell (Ward, 1988). Radiation produces cell death by either apoptosis or replicative cell death. Apoptosis occurs as a consequence of genotoxic insult, leading to mitochondrial dysfunction and destabilisation of telomere nucleo-protein complexes (Figure 1.5) (Shiels and Davies, 2003). Replicative cell death occurs when cells with unrepaired DNA strand breaks pass through mitosis resulting in the formation of lethal chromosomal aberrations (Revell, 1983). It is replicative cell death that predominates after exposure to ionising radiation (Steel, 2001).

5.2.1 Ionising radiation and DNA repair

Common types of damage induced by ionising radiation include oxidative DNA base damage, as well as single-strand breaks (SSB) and double-strand breaks (DSB) within the DNA duplex. Non-repaired radiation induced DSB are frequently lethal and radiation killing is inversely proportional to their rate of repair (Pawlik and Keyomarsi, 2004). Two major pathways of DSB repair have been identified: homologous and non-homologous recombination (Figure 5.1) (Valerie and Povirk, 2003, Meselson and Radding, 1975).

In homologous recombination (HR), the DSB is processed to a single-stranded region by a nuclease and helicase. A nucleoprotein filament forms on the single-stranded DNA and identifies homologous DNA to be used as a template to direct accurate repair. The missing sequences are restored by the activities of a DNA polymerase and ligase. Finally, resolution of the crossed DNA strands, known as "holiday junctions", yields two intact duplexes (Figure 5.1) (Valerie and Povirk, 2003). Non-homologous recombination or end-joining (NHEJ) predominates in mammalian cells and involves many proteins including the binding to DNA of the Ku heterodimer and the recruitment of DNA-PKC (Hartley *et al.*, 1995). The DNA ends are brought together, processed and ultimately joined by a ligase (Figure 5.1).

Figure 5.1 Homologous and non-homologous DNA repair. This figure shows the major steps in both homologous and non-homologous recombination of DSB repair. [Figure adapted from Valerie and Povirk, (2003)].

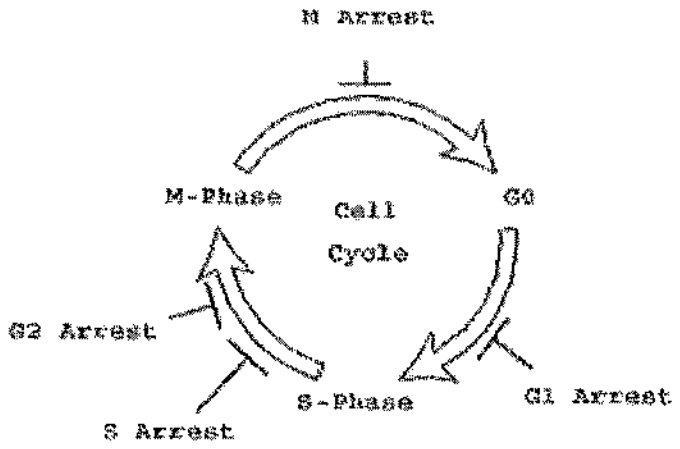


The link between the cell-cycle and DNA damage occurs through cell-cycle sensing of DNA breaks and transduction of signals through key proteins such as ATM and p53 (Samuel *et al.*, 2002). The exact sequence of interactions of these proteins remains to be elucidated.

5.2.2 Cell-cycle and ionising radiation

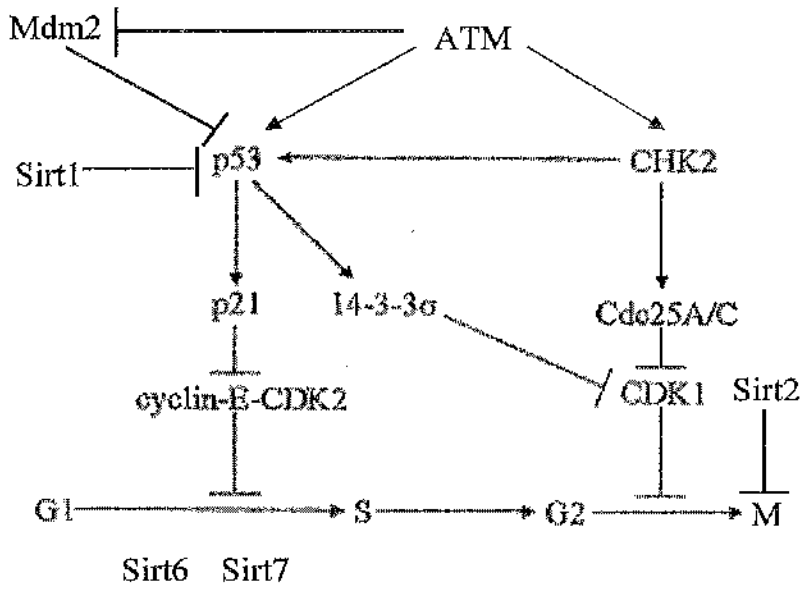
Cells commonly respond to ionising radiation-induced DNA damage by activating cell-cycle checkpoints. Major cell-cycle checkpoints occur at the G_1 and G_2, controlling entry of cells into the S and M phases respectively. Checkpoints also exist during the S and M phases (Figure 5.2).

Figure 5.2 Cell-cycle checkpoints. This figure shows the G_0, M and S phases of the cell-cycle as well as the G_1, S, G_2 and M cell-cycle checkpoints.



The G_1 checkpoint arrest is promoted by p53, with post-translational modifications increasing the protein's half-life and resulting in a transient increase in levels of activated p53 protein (Kastan *et al.*, 1992). DNA strand breaks are the most potent inducer of p53 following ionising radiation. Cell-cycle arrest is induced by the trans-activation of the $p21^{CIP1}$ protein, that interacts with the cyclinE/CDK2 complex, inhibiting the progression of cells into the S phase (Figure 5.3) (Samuel *et al.*, 2002).

Figure 5.3 The cell-cycle and radiation damage. This figure shows where sirtuins may interact with components of the damage response machinery, thereby contributing to cell-cycle arrest, following ionising radiation. [Figure adapted from Samuel *et al.*, (2002)].



Following the G_1 checkpoint, there is the S phase checkpoint. This regulates the initiation and elongation stages of DNA replication and is also inhibited by ionising radiation (Maity *et al.*, 1994). Radiation signalling pathways may interact with DNA replication processes in mammals via ATM, a protein that also plays a role in replicon initiation and chain elongation (Valerie and Povirk, 2003). It has been well established that mutations in the *ATM* gene result in oversensitivity to radiation-induced DNA damage (Taylor *et al.*, 1975).

A prolonged cell-cycle arrest in G_2 occurs in almost all eukaryotic cells following exposure to ionising radiation (Maity *et al.*, 1994). This G_2 arrest may perform a

surveillance function in cells to prevent those with DNA damage from undergoing mitosis. ATM activates the CHK2 protein that subsequently inhibits CDK1 and causes G_2 phase arrest (Figure 5.3) (Samuel *et al.*, 2002). The p53 protein also triggers arrest at this checkpoint by the transcription of 14-3-3σ that can also inhibit CDK1. The M phase checkpoint phase follows the G_2 phase and ensures correct chromosome alignment. It is at this checkpoint that damage to microtubules that would lead to aberrant mitosis is detected. This is pertinent to drug-induced cellular damage from agents that include vinca alkaloids and taxanes.

5.3 Taxanes

Taxanes are employed as adjuvant therapy for the management of breast cancer. The role of this chemotherapeutic is explored below with respect to cell damage responses. Taxanes belong to a class of chemotherapeutic, derived from the bark and leaves of the Pacific yew tree. Their mechanism of action, is via the inhibition of microtubule function, although how this exactly occurs is poorly understood (Hudis, 1999)

5.3.1 Cell-cycle and taxanes

To understand how taxanes function, we must refer to the cell-cycle (Figure 5.1). As previously mentioned, DNA damage prevents G_1 cells from entering the S phase and G_2 cells from entering the M phase of mitosis.

The M phase checkpoint monitors the alignment of chromosomes on the mitotic spindle, ensuring a complete set of chromosomes is distributed accurately to the daughter cells. The failure of one or more chromosomes to align properly on the spindle causes mitosis to arrest at the metaphase, prior to the segregation of the newly replicated chromosomes to daughter nuclei. As a result of this checkpoint, the chromosomes do not separate until a complete complement of chromosomes has been organised for distribution to each daughter cell (Cleveland *et al.*, 2003).

The mechanism of action of the taxane chemotherapeutic, paclitaxel, is thought to be via the disruption of normal microtubule dynamics (Hudis, 1999). This results in mitotic cell-

cycle arrest at the M phase checkpoint and cell death. Paclitaxel exposure is also associated with increased microtubule acetylation and therefore stability (Piperno et al., 1987, Marcus et al., 2005). Deacetylases, such as the sirtuins, can affect microtubule structure and stability, and may determine the efficacy of paclitaxel therapy. Indeed sirtuins may also influence the efficacy of other cancer therapies (Marks *et al.*, 2001).

5.4 Sirtuins and cancer therapy

In the preceding experiments, sirtuin expression was linked with biological ageing in primary epithelial cells, and was also shown to be associated with lymph node positive breast cancer. These results support a role for sirtuins in mammary epithelial cell survival. Similarly, sirtuins may also mediate the response of breast cancer cells to both radiotherapy and paclitaxel chemotherapy (Mostoslavsky et al., 2006, Tran et al., 2002, Chen et al., 2005, Matsushita et al., 2005). In the following sections, the mechanisms by which sirtuins may function in damage responses to both of these treatments are explored.

5.4.1 Radiotherapy

In response to radiation-induced DNA damage, Sirt1 allows the repair of DNA damage by inhibiting the p53 protein (Vaziri et al., 2001, Luo et al., 2001). Substantiating the role of Sirt1 in protecting against radiation-induced apoptosis, cells transfected with *SIRT1* display an increase in radioresistance, while conversely, deletion of *SIRT1* results in radiosensitivity (Luo et al., 2001, Cheng et al., 2003). *In vivo* studies confirming this have

been equivocal, with reports detailing thymocytes from *SIRT1* null mice displaying p53 hyperacetylation after DNA damage and increased radiation-induced apoptosis, and others showing no difference (McBurney et al., 2003, Cheng et al., 2003, Kamel et al., 2006). Although various explanations for these differences have been proposed, the exact reason remains to be determined (Kamel *et al.*, 2006).

Cell irradiation causes chromosomal instability that results in replicative cell death (Revell, 1983). Sirt2 participates in a cell-cycle checkpoint role whereby it detects the presence of chromosomal abnormalities and may arrest the division of irradiated cells that harbour them (Figure 1.6) (Dryden et al., 2003, Inoue et al., 2006). Indeed, increased levels of the Sirt2 protein are seen at the G_2/M phase transition and are persistently high during the M phase of the cell-cycle (Dryden et al., 2003, Vaquero et al., 2006, Inoue et al., 2006).

Irradiation can induce oxidative damage within the cell by the formation of ROS (Maity *et al.*, 1994). The resultant fall in the NAD/NADH ratio may result in decreased Sirt3 and concomitantly, AceS2 activity (Hallows *et al.*, 2006). This would result in a fall in the efficiency of fuel utilisation and energy production and in an increase in the generation of toxic metabolites that contribute to cell death. The mitochondrion itself is also a key regulatory centre of apoptosis (Pollack *et al.*, 2002) and can induce apoptosis in a caspase-dependent or independent manner (Danial and Korsmeyer, 2004). Sirt3 may be implicated in resistance to ionising radiation-induced damage, not only by improving fuel utilisation and energy production but also by directly sensing and responding to the redox state of the cell (Smith et al., 2000, Onyango et al., 2002, Schwer et al., 2002).

Sirt6 and Sirt7 closely associate with chromosomes during mitosis and, like Sirt2, may participate in a cell-cycle checkpoint. Sirt6 has been shown to promote resistance to DNA damage and suppress genomic instability by participating in DNA repair (Mostoslavsky *et al.*, 2006). Sirt7 has been shown to play a role in Pol I-mediated transcription, and its depletion prevents cell proliferation and triggers apoptosis (Ford *et al.*, 2006).

5.4.2 Chemotherapy

Chemotherapeutics act in a number of different ways. Anthracyclines mediate much of their toxicity through the production of free radicals, etoposide by inhibiting topoisomerase II activity and taxanes by the disruption of microtubules. Sirtuins have been implicated in preventing cell death resulting from anthracyclines (Chu *et al.*, 2005) and topoisomerase II inhibitors (Cheng *et al.*, 2003) but have not been studied with respect to taxanes.

Taxane therapy is thought to induce cell damage by microtubule disruption (Horwitz, 1992) and is associated with increased microtubule acetylation (Marcus *et al.*, 2005). Sirt2 deacetylates microtubules at the M phase of the cell-cycle resulting in mitotic arrest. It is hypothesised, that in this way Sirt2 prevents cell division in cells harbouring chromosomal abnormalities (Dryden *et al.*, 2003). Taxanes cause chromosomal abnormalities resulting in M phase arrest and may trigger the expression of *SIRT2*. Recently, Sirt2 has been shown to prevent microtubule poison-induced hyperploid cell formation, via a block of entry to chromosome condensation (Inoue *et al.*, 2006). Interestingly, both Sirt6 and Sirt7 also closely associate with condensed chromosomes during mitosis and may, like Sirt2, be involved in cell-cycle control (Michishita *et al.*, 2005).

A role for Sirt1 has also been postulated for drug resistance in breast cancer treated with paclitaxel (Chu *et al.*, 2005). Pertinent to this, is the observation that *SIRT1* regulates the *mdr1* gene, implicated in paclitaxel resistance in breast cancer (Mechetner *et al.*, 1998).

Treatment with paclitaxel may, therefore, be expected to promote *SIRT1* transcription. In the MCF-7 breast cancer cell line, levels of the Foxo3a protein increase dramatically after paclitaxel treatment (Sunters *et al.*, 2003). Sirt1 increases the ability of Foxo3a to induce cell-cycle arrest and promote resistance to oxidative stress but inhibits its ability to induce cell death. Increased Sirt1 transcription may, therefore, tip Foxo-dependent responses away from apoptosis toward stress resistance facilitating repair (Brunet et al., 2004, Motta et al., 2004, Nemoto et al., 2005).

Paclitaxel has also been reported to affect mitochondrial apoptotic mechanisms by interfering with the mMPTP (Evtodienko *et al.*, 1996). In addition, paclitaxel, may induce apoptosis by directly inducing ROS formation within the mitochondria (Varbiro *et al.*, 2001), These may result in a changes in NAD/NADH dynamics thereby impacting on Sirt3 activity. Specifically, a fall in the cellular NAD/NADH ratio, as a result of ROS formation, would result in inhibition of mitochondrial sirtuin activity. Mitochondrial Sirt3 levels may predict resistance to paclitaxel-induced cell death.

Sirtuin activity, within different components of the MTR, link DNA damage responses. Consequently, an imbalance in sirtuin activity within any individual part of the MTR may result in the dysregulation of normal growth, proliferation and apoptosis (Shiels and Davies, 2003). Abnormalities in sirtuin expression within tumour cells, may affect the way in which these cells response to damage compared to non-malignant tissue (Chu et al., 2005, Ota et al., 2006).

In the preceding studies, sirtuins have been shown to be associated with replicative senescence and cancer. This part of the study, sought to determine the relationship between sirtuin transcriptional expression in tumour cells in response to damage from standard cancer therapies, namely radiotherapy and chemotherapy. Specifically, sirtuin expression was investigated in breast cancer cell lines in response to ionising radiation and paclitaxel therapy. Studies were performed on breast cancer cell lines expressing wild-type and mutant, inactive, *p53*. These breast cancer cell line were selected, as sirtuins are implicated in the regulation of p53 (Vaziri et al., 2001, Langley et al., 2002) and may themselves be regulated by this protein (Nemoto *et al.*, 2004).

Transcriptional expression of $p21^{CIP1}$ was studied as it is a good indicator of radiation damage responses (Figure 5.3) (Samuel *et al.*, 2002). Transcriptional expression of *SIRT1,2,3* and *7* was studied as these sirtuins act within components of the MTR that are implicated in apoptosis and cell-cycle arrest (Figure 1.5 and 1.6) (Dryden et al., 2003, Hiratsuka et al., 2003, Inoue et al., 2006, Vaziri et al., 2001, Luo et al., 2001).

Finally, TUNEL assays were performed to determine whether breast cancer cells studied displayed resistance to radiation-induced apoptosis and, if so, whether this occurred in a sirtuin-mediated manner.

5.5 Aims

In this part of the study, sirtuin expression was used to evaluate breast cancer therapies including ionising radiation and paclitaxel, chemotherapy.

Individual questions addressed were:

1. Does sirtuin transcriptional expression alter following ionising radiation in breast cancer cells?

2. Does sirtuin transcriptional expression alter following paclitaxel chemotherapy in breast cancer cells?

3. Is the transcriptional expression of any individual sirtuin reportive for ionising radiation or paclitaxel chemotherapy?

5.6 Results

Transcriptional expression of sirtuins was evaluated in breast cancer cells following ionising radiation and paclitaxel treatment. The results are described below in separate sections delineating each of these treatments.

5.6.1 Cellular irradiation

Two breast cancer cell lines were chosen for this study, namely the MCF-7 cell line, expressing wild-type *p53*, and the MDA-MB-231 cell line, expressing mutant *p53*. Breast cancer cells were subjected to 2 to 10 Grays of radiation, corresponding to the maximal radiation dose range for the treatment of breast cancer (Arnold *et al.*, 1999). The cultures were assessed for the effects of acute stress via real-time Taqman PCR analysis of $p21^{CIP1}$ transcription, as well as transcription of *SIRT1,2,3* and *7* at 24 and 48 hours after irradiation. Sirtuin transcriptional expression was studied at the 24 and 48 hour time-points as ionising radiation influences cell-cycle progression and cell-cycle checkpoints that result in replicative cell death (Pawlik and Keyomarsi, 2004).

A dose related increase in $p21^{CIP1}$ expression was observed in the MCF-7 cells at 24 hours ($R^2 = 0.856$, $p<0.001$) and at 48 hours ($R^2 = 0.925$, $p<0.001$) after irradiation (Table 5.1, Figure 5.4). A dose related increase in *SIRT2* ($R^2 = 0.687$, $p=0.001$) and *SIRT7* ($R^2 = 0.875$, $p<0.001$) expression was also observed in the MCF-7 cells 48 hours after irradiation (Table 5.1, Figures 5.6 and 5.8). A dose related increase in $p21^{CIP1}$ was only observed in the MDA-MB-231 cell line at 48 hours after irradiation $R^2 = 0.492$, $p=0.011$).

Similar to the MCF-7 cells, an increase in *SIRT2* ($R^2 = 0.560$, p=0.005) and *SIRT7* ($R^2 = 0.710$, p=0.001) expression was also observed at 48 hours after irradiation (Figures 5.6 and 5.8). Neither of the cell lines showed any relationship between cellular irradiation and *SIRT1* or *SIRT3* transcription at either of the timepoints (Figures 5.5 and 5.7).

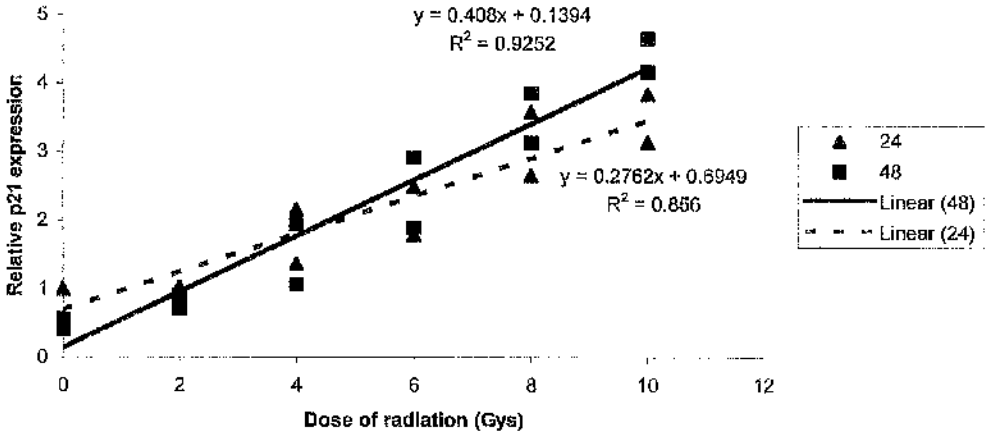
A TUNEL assay was used to evaluate the degree of apoptosis in the irradiated cell lines. Both breast cancer cell lines showed substantial resistance to radiation-induced apoptosis at the radiation doses employed (Figure 5.9 and 5.10).

Table 5.1 Relationship between irradiation dose and sirtuin and p21 expression. The table shows linear regression analyses for the relationship between irradiation dose and sirtuin and $p21^{CIP1}$ transcriptional expression at 24 and 48 hours after treatment in MCF-7 and MDA-MB-231 mammary cancer cell lines. R^2 values and p-values are displayed. *p<0.05, **p<0.01, , ***p<0.001

	24 hrs		48 hrs	
MCF-7	**R^2**	**p-value**	**R^2**	**p-value**
P21	0.856	0.000**	0.925	0.000**
SIRT1	0.157	0.202	0.055	0.465
SIRT2	0.277	0.079	0.687	0.001**
SIRT3	0.156	0.203	0.147	0.219
SIRT7	0.241	0.105	0.875	0.000**
MDA-MB-231				
P21	0.086	0.355	0.492	0.011*
SIRT1	0.002	0.888	0.062	0.436
SIRT2	0.287	0.073	0.560	0.005**
SIRT3	0.008	0.789	0.212	0.132
SIRT7	0.065	0.425	0.710	0.001***

Figure 5.4 p21 expression in irradiated mammary cancer cell lines. The scatterplots show irradiation dose plotted against *p21*^{CIP1} transcriptional expression at 24 and 48 hours after treatment in MCF-7 and MDA-MB-231 mammary cancer cell lines. Lines of 'best fit' are displayed where tests of linear regression showed a statistically significant relationship. (n=2)

MCF-7



MDA-MB-231

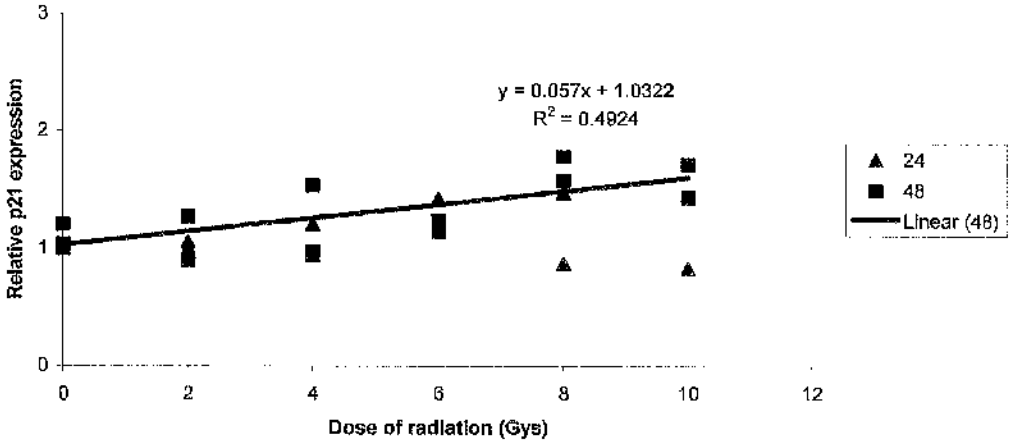
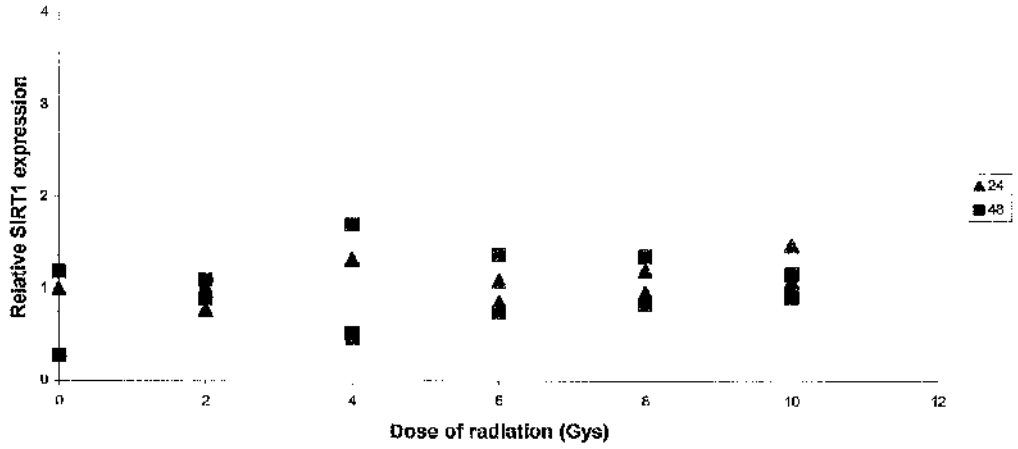


Figure 5.5 SIRT1 expression in irradiated mammary cancer cell lines. The scatterplots show irradiation dose plotted against *SIRT1* transcriptional expression at 24 and 48 hours after treatment in MCF-7 and MDA-MB-231 mammary cancer cell lines. Lines of 'best fit' are displayed where tests of linear regression show a statistically significant relationship. (n=2)

MCF-7



MDA-MB-231

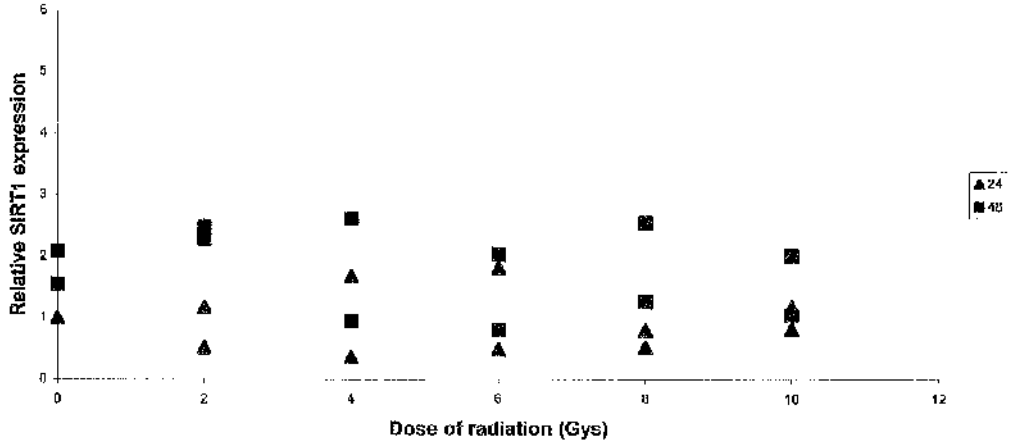
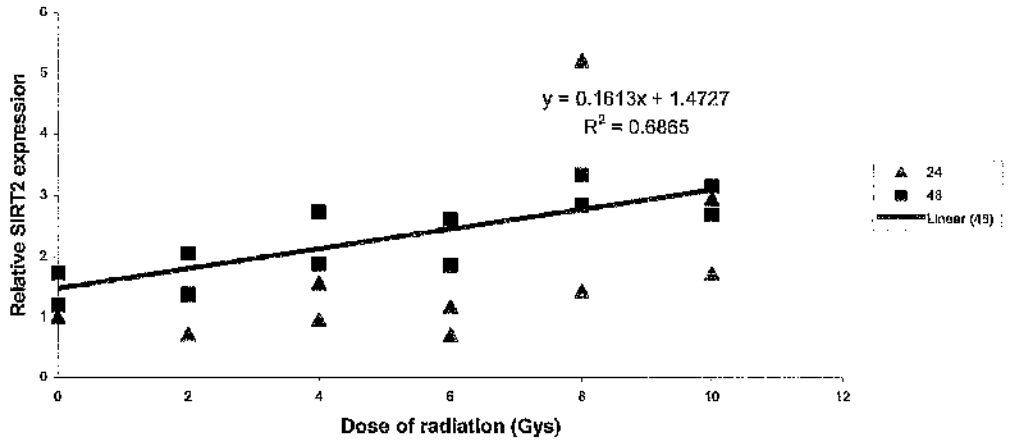


Figure 5.6 SIRT2 expression in irradiated mammary cancer cell lines. The scatterplots show irradiation dose plotted against *SIRT2* transcriptional expression at 24 and 48 hours after treatment in MCF-7 and MDA-MB-231 mammary cancer cell lines. Lines of 'best fit' are displayed where tests of linear regression show a statistically significant relationship. (n=2)

MCF-7



MDA-MB-231

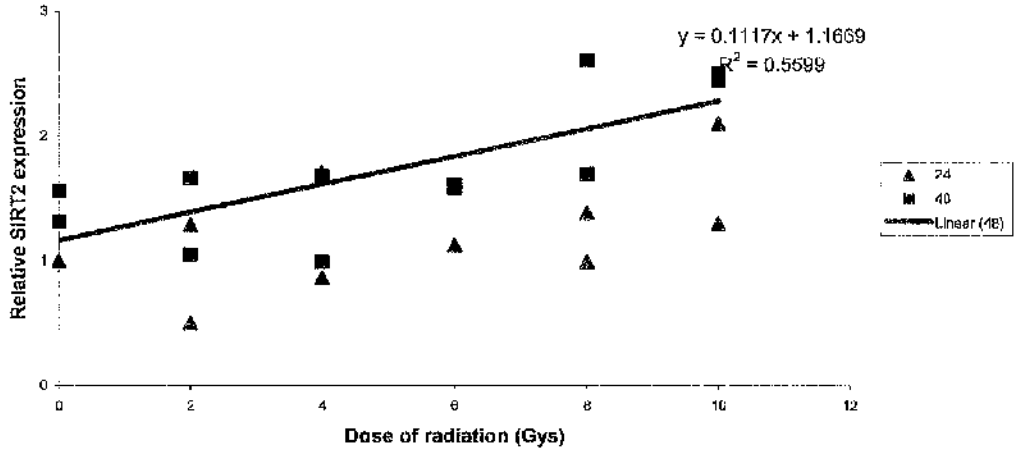
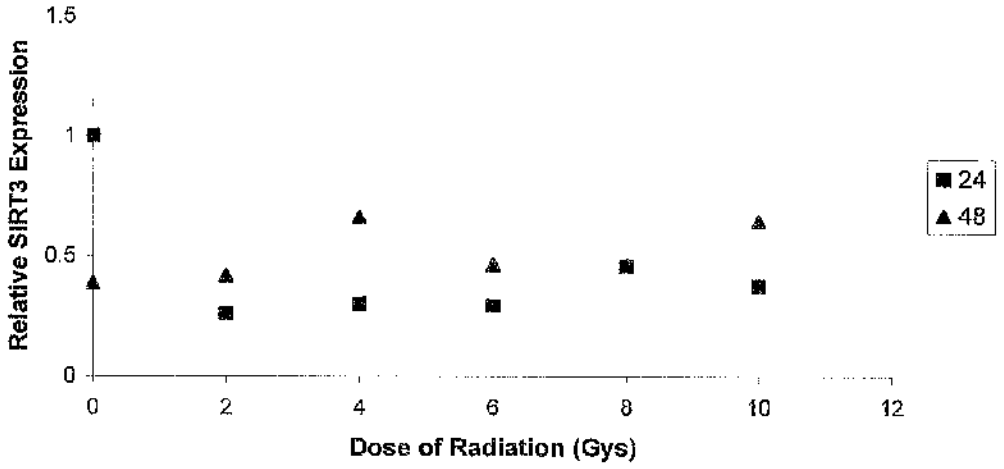


Figure 5.7 SIRT3 expression in irradiated mammary cancer cell lines. The scatterplots show irradiation dose plotted against *SIRT3* transcriptional expression at 24 and 48 hours after treatment in MCF-7 and MDA-MB-231 mammary cancer cell lines. Lines of 'best fit' are displayed where tests of linear regression show a statistically significant relationship. (n=2)

MCF-7



MDA-MB-231

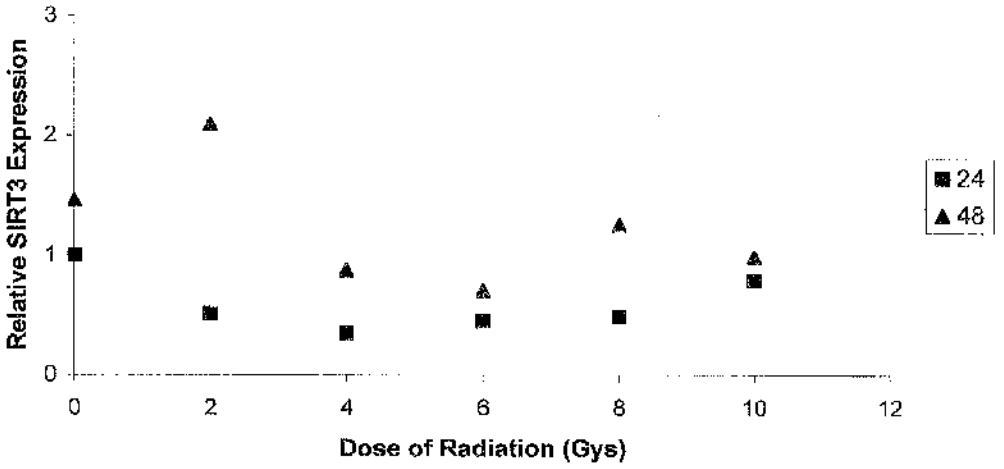
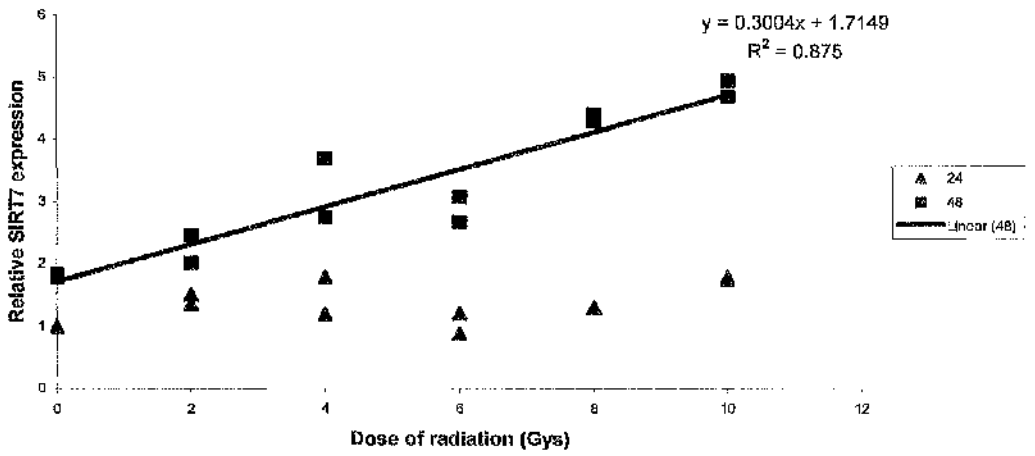


Figure 5.8 SIRT7 expression in irradiated mammary cancer cell lines. The scatterplots show irradiation dose plotted against *SIRT7* transcriptional expression at 24 and 48 hours after treatment in MCF-7 and MDA-MB-231 mammary cancer cell lines. Lines of 'best fit' are displayed where tests of linear regression show a statistically significant relationship. (n=2)

MCF-7



MDA-MB-231

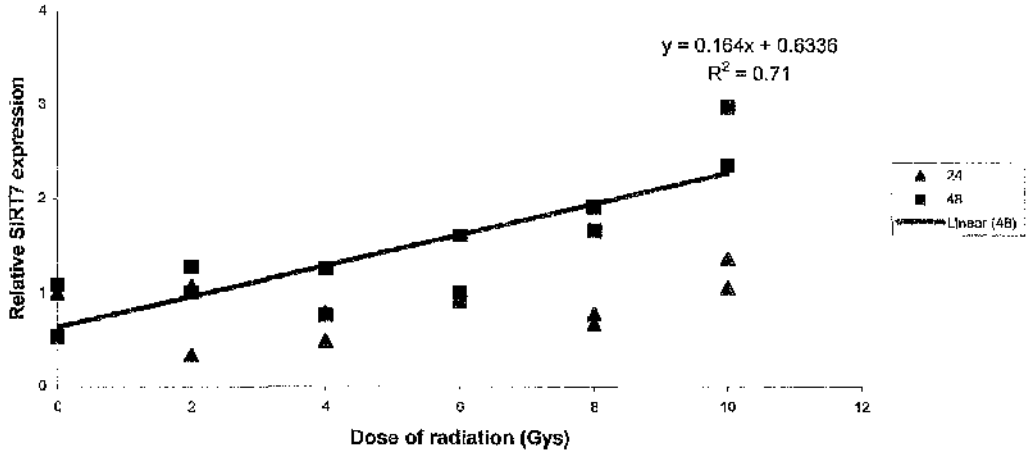


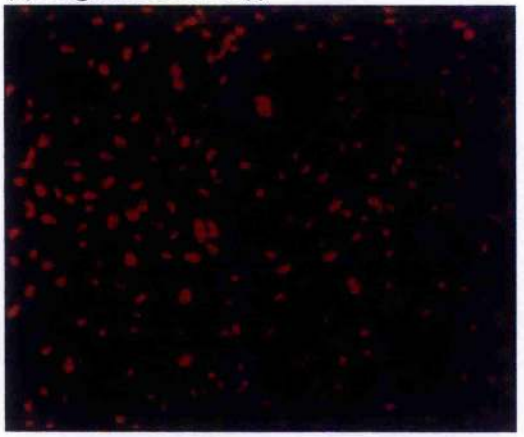
Figure 5.9 TUNEL assay controls.

a) Negative control (i). Apoalert assay image of MDA-MB-231 breast cancer cells at 48 hours after irradiation with 10 Grays in the absence of Tdt enzyme. Red colour represents nuclear Propidium Iodide staining and green colour, staining of terminal DNA strand breaks.

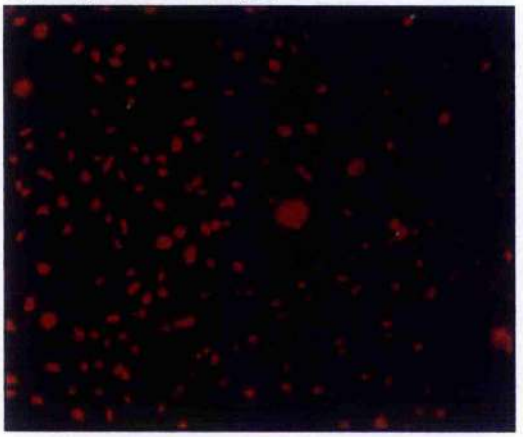
b) Negative control (ii). Apoalert assay image of MDA-MB-231 breast cancer cells at 48 hours not subjected to irradiation. Red colour represents nuclear Propidium Iodide staining and green colour, staining of terminal DNA strand breaks.

c) Positive control. Apoalert assay image of MDA-MB-231 breast cancer cells treated with DNase I. Red colour represents nuclear Propidium Iodide staining and green colour, staining of terminal DNA strand breaks.

(a) Negative control (i)



(b) Negative control (ii)



(c) Positive control

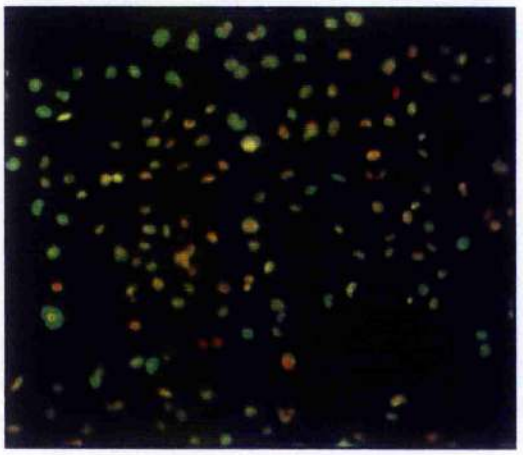
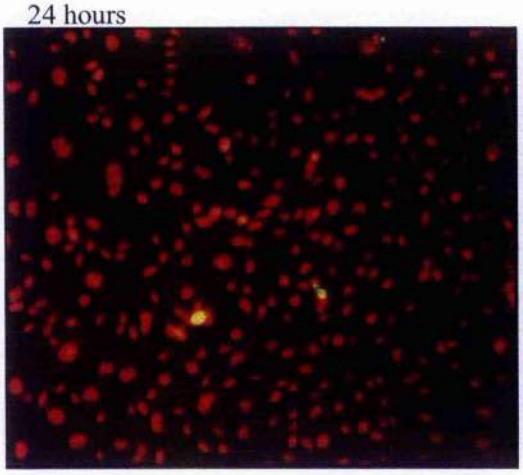


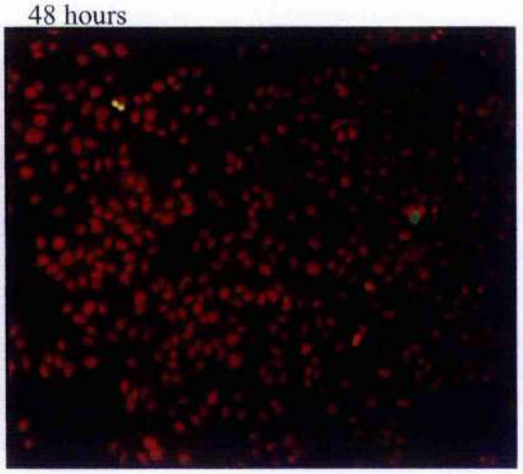
Figure 5.10 TUNEL assays in MCF-7 and MDA-MB-231 cell lines after irradiation. Apoalert assay images of (a) MCF-7 and (b) MDA-MB-231 breast cancer cells at 24 and 48 hours after irradiation with 10 Grays. Red colour represents nuclear Propidium Iodide staining and green colour, staining of terminal DNA strand breaks.

(a) MCF-7

24 hours

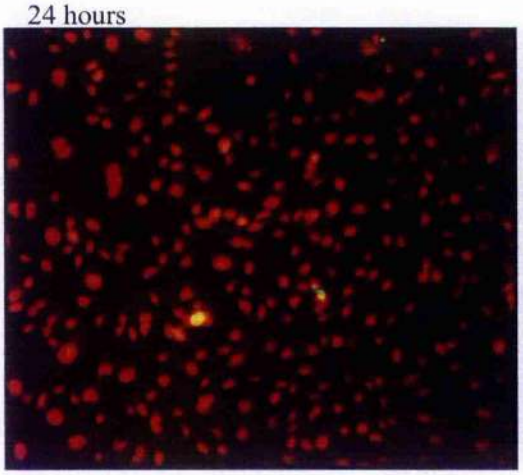


48 hours

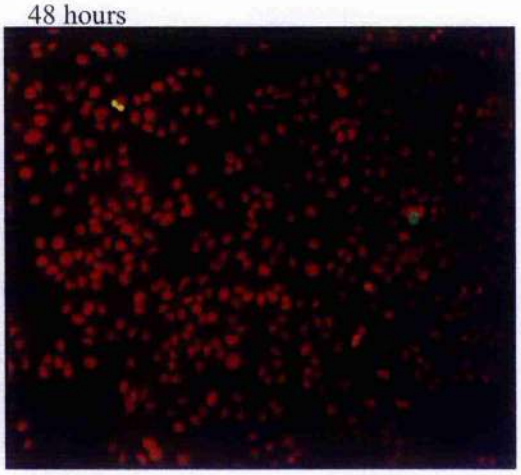


(a) MDA-MB-231

24 hours



48 hours



5.6.2 Paclitaxel treatment

Cells from the MCF-7 breast cancer cell line were exposed to increasing concentrations of the taxane, paclitaxel. The concentrations of paclitaxel used were based on previous studies (Blagosklonny *et al.*, 2002). The cultures were then assessed for the effects of acute stress via real-time Taqman PCR analysis of $p21^{CIP1}$ and *SIRT1,2,3* and 7 transcription. As previously stated, $p21^{CIP1}$ is a good indicator of damage response and *SIRT1,2,3* and 7 are implicated in apoptosis, cell-cycle control, senescence and tumour proliferation respectively (Vaziri et al., 2001, Langley et al., 2002, Dryden et al., 2003, Inoue et al., 2006, Hiratsuka et al., 2003, de Nigris et al., 2002).

A statistically significant dose related increase in $p21^{CIP1}$ ($R^2 = 0.817$, p=0.002), *SIRT2* ($R^2 = 0.861$, p=0.001) and *SIRT7* ($R^2 = 0.821$, p=0.002) expression was seen in the MCF-7 cells after paclitaxel treatment (Table 5.2 and Figures 5.11 and 5.12). No association was seen between paclitaxel treatment and *SIRT1* or *SIRT3* expression (Figure 5.12). These results mirrored those from the irradiation experiments.

Table 5.2 Relationship between paclitaxel exposure and sirtuin and p21 expression. The table shows linear regression analyses for the relationship between paclitaxel dose and sirtuin and $p21^{CIP1}$ transcriptional expression in MCF-7 mammary cancer cell lines. R^2 values and p-values are displayed. * $p<0.01$

MCF-7	R^2	p-value
P21	0.817	0.002*
SIRT1	0.038	0.645
SIRT2	0.861	0.001*
SIRT3	0.034	0.660
SIRT7	0.821	0.002*

Figure 5.11 p21 expression in MCF-7 cells treated with paclitaxel. The scatterplots show paclitaxel dose plotted against $p21^{CIP1}$ transcriptional expression in the MCF-7 cell line. Lines of 'best fit' are displayed where tests of linear regression show a statistically significant relationship. (n=2)

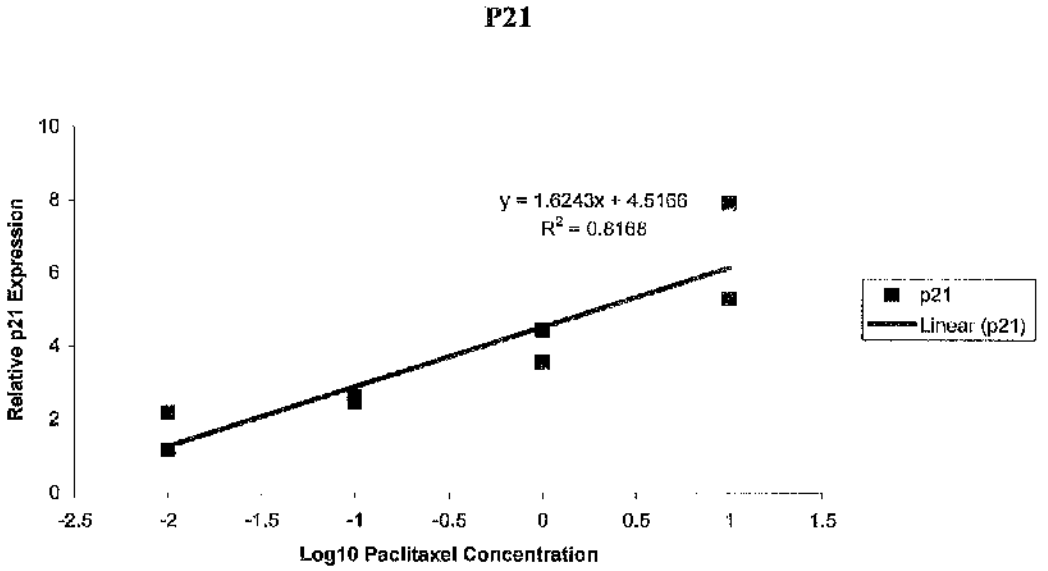
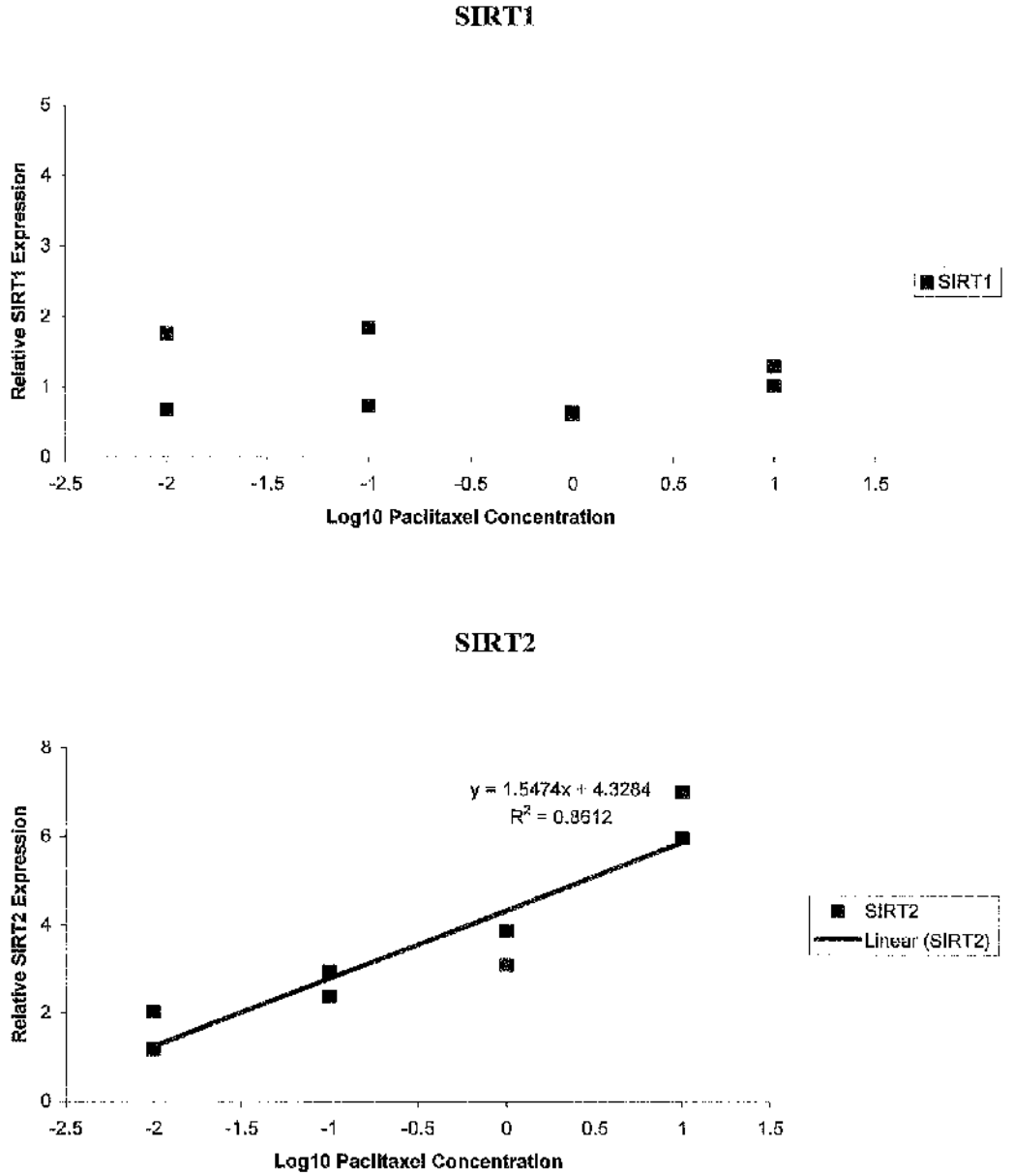
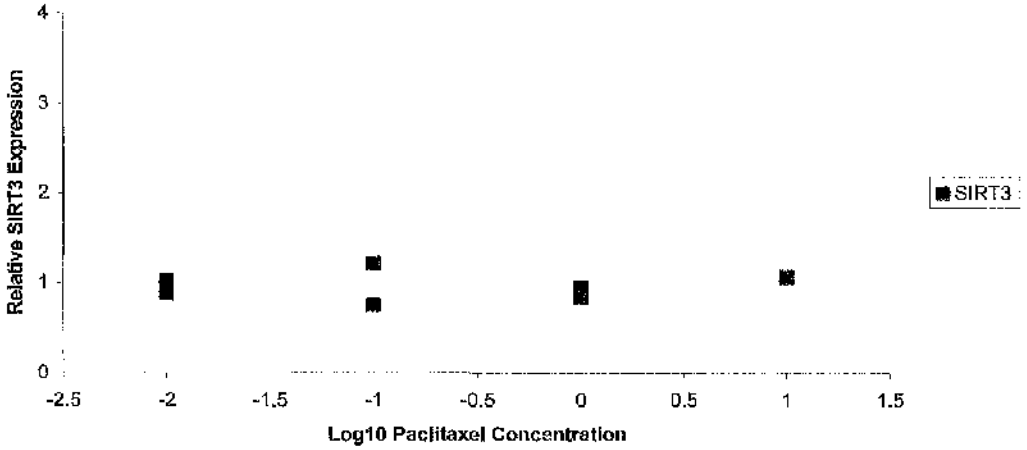


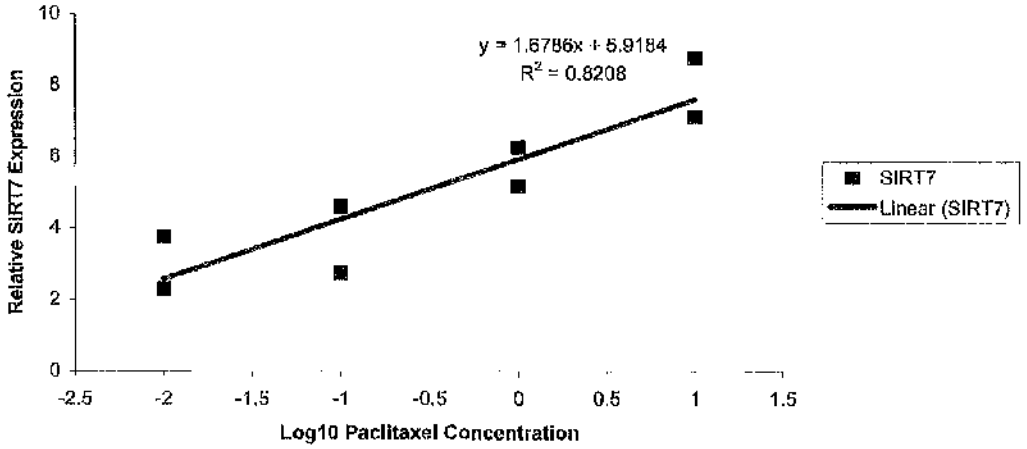
Figure 5.12 Sirtuin expression in MCF-7 cells treated with paclitaxel. The scatterplots show paclitaxel dose plotted against sirtuin transcriptional expression in the MCF-7 cell line. Lines of 'best fit' are displayed where tests of linear regression show a statistically significant relationship. (n=2)



SIRT3


Relative SIRT3 Expression
4
3
2
1
0
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
Log10 Paclitaxel Concentration
SIRT3

SIRT7


y = 1.6786x + 5.9184
R2 = 0.8208
Relative SIRT7 Expression
10
8
6
4
2
0
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
Log10 Paclitaxel Concentration
SIRT7
Linear (SIRT7)

5.7 Discussion

5.7.1 Radiotherapy

Ionising radiation results in cellular damage including damage to DNA. At the centre of the radiation response, is the p53 protein, which induces cell-cycle arrest and apoptosis (Pawlik and Keyomarsi, 2004). The activity of p53 is inhibited by the Sirt1 deacetylase which may function, like its yeast Sir2 orthologue, in facilitating DNA damage repair (Tsukamoto et al., 1997, Lee et al., 1999b). In addition, p53 has itself been shown to control *SIRT1* expression (Nemoto *et al.*, 2004). It has, therefore, been hypothesised that p53 activity may also impact on the expression of the other sirtuins. In order, to determine the role of p53 in sirtuin expression in mammary cancer cells in response to ionising radiation, the expression of sirtuins was studied in MCF-7 cells expressing active, wild-type *p53*, as well as in MDA-MB-231 cells, expressing inactive, mutant *p53* (O'Connor *et al.*, 1997). Although sirtuin responses to ionising radiation occur early they may also be influenced by cell-cycle progression and cell-cycle checkpoints, that result in replicative cell death (Dryden et al., 2003, Inoue et al., 2006, Michishita et al., 2005, Mostoslavsky et al., 2006). Sirtuin expression was therefore studied at both 24 and 48 hour time points post irradiation in order to allow for the accumulation of cell-cycle arrested, damaged cells.

The dose-dependent increase in $p21^{CIP1}$ expression in the MCF-7 cells, but not in the MDA-MB-231 cells at 24 hours after irradiation reflects the difference in functioning p53

(Bartek et al., 1990, Watson et al., 1997). There was a smaller increase in $p21^{CIP1}$ expression in the MDA-MB-231 cells at 48 hours after irradiation, independent of p53, in keeping with previous observations (Macleod *et al.*, 1995). Despite these changes, both cell lines were resistant to radiation-induced apoptosis (Figure 5.8). This observation confirms those from previous studies, that have shown both cell lines to be relatively resistant to radiation-induced apoptosis independent of p53 activity (Watson *et al.*, 1997).

The dose-dependent increase in *SIRT2* expression observed in both breast cancer cell lines, is consistent with the role of Sirt2 in arresting the cell-cycle in response to chromosomal damage. Cellular irradiation is known to result in chromosomal instability and an increase in chromosomal abnormalities (Revell, 1983). During the M-phase of mitosis, these abnormalities would result in mitotic arrest (Dryden *et al.*, 2003). Sirt2 has recently been shown to prevent drug-induced hyperploid cell formation via a block of entry to chromosome condensation (Inoue *et al.*, 2006). The increased *SIRT2* expression in breast cancer cell lines, is in keeping with such a view point. Increased *SIRT2* expression, secondary to radiation-induced chromosomal damage, may confer a survival advantage to these cells. This increased transcriptional expression, appears to be independent of p53 activity, as increased *SIRT2* expression was observed in both types of mammary cancer cell line. This confirms previous observations in which Sirt2 was shown to regulate radiation-induced cell death in a p53-independent manner in a chicken cell line (Matsushita *et al.*, 2005). The delay in observing increased *SIRT2* expression, may reflect tumour cell replication leading to an accumulation of cells with chromosomal abnormalities. Furthermore, nucleo-cytoplasmic shuttling of Sirt2 also occurs within 24

hours of exposure to ionising radiation suggesting a possible role for it in the DNA damage response (Inoue *et al.*, 2006). Our data, indicating a delay in the elevation of *SIRT2* transcription is consistent with such a role.

Similarly, *SIRT7* expression also displayed a delayed dose-dependent increase with irradiation in both cell lines. As with Sirt2, both Sirt6 and Sirt7 closely associate with chromosomes during mitosis and, may similarly, participate in a cell-cycle checkpoint. Furthermore, Sirt7 may also have a role in the repair of damaged DNA. Indeed, decreased levels of Sirt7 are not only associated with decreased cell proliferation but are also associated with an increase in apoptotic cell death (Ford *et al.*, 2006). Conversely, increased Sirt7 levels are associated with both cell proliferation and survival. The increase in *SIRT7* expression observed in the experiments in this chapter, may reflect an indirect consequence of cell stress responses, induced to help in cell survival or, alternatively, may be a direct response to radiation-induced DNA damage and growth arrest. This remains to be proven.

The anti-apoptotic sirtuin, *SIRT1* showed no change in transcriptional expression with ionising radiation and, therefore, does not appear to participate in survival responses in response to radiation in either, the MCF-7 or MDA-MB-231 breast cancer cells. The resistance to apoptosis in response to ionising radiation in these cell lines is independent of p53 activity and linked to abnormalities in mitochondrial caspase (Essmann *et al.*, 2004). Sirt1 inhibits apoptosis by interacting with factors upstream from caspase, including p53, Ku and E2F1 (Luo et al., 2001, Cohen et al., 2004b, Wang et al., 2006).

Changes in the transcriptional expression of *SIRT1* would, therefore, still be expected if Sirt1 functions as a sensor of DNA damage in breast cancer cells. The transcriptional expression of *SIRT1* remained relatively stable with increasing radiation dose suggesting that Sirt1 does not perform this function in the cell lines studied. This observation is supported by studies that have shown deficiencies in *SIRT1* expression to only impact on sensitivity to apoptosis in thymocytes and spermatogonia but not in other tissue (Cheng et al., 2003, McBurney et al., 2003).

In both cancer cell lines, *SIRT3* expression showed no change in pattern of expression with increasing radiation exposure. This suggests that Sirt3 does not play a role in the prevention of radiation-induced damage in either of these cell lines. Although ionising radiation results in global cell damage, including that at cell membranes and within mitochondria, it primarily causes cell death and growth arrest by the induction of DNA damage (Ward, 1988). This may explain the absence of changes in *SIRT3* expression in the breast cancer cell lines studied. The location of Sirt3 within the mitochondrion and its NAD-dependence make it a potential candidate for mediating the apoptotic response (Onyango et al., 2002, Schwer et al., 2002). This relationship cannot be confirmed as neither cell line displayed radiation-induced apoptosis. It is, therefore, not possible to exclude a role for Sirt3 in radiation-induced apoptosis.

5.7.2 Taxane therapy

Taxols induce apoptosis (Schimming *et al.*, 1999) as well as mitotic catastrophe, the primary mechanism of cell death (Morse *et al.*, 2005). Sirtuin expression was studied in response to paclitaxel exposure, as sirtuins are implicated in both of these processes. Increasing $p21^{CIP1}$ expression in the MCF-7 cell line with paclitaxel exposure suggests that these cells sustained increased damage with increasing paclitaxel dosage. In response to cell damage, the $p21^{CIP1}$ cdk inhibitor prevents phosphorylation of pRb, thereby repressing expression of cell-cycle genes and resulting in cell-cycle arrest (Samuel *et al.*, 2002).

SIRT2 expression displayed a dose-dependent increase with paclitaxel exposure. Taxane therapy induce cell damage by microtubule disruption and is associated with increased microtubule acetylation (Marcus *et al.*, 2005). Sirt2 deacetylates microtubules at the M phase of the cell-cycle resulting in mitotic arrest. It is hypothesised that, in this way, Sirt2 prevents cell division in cells harbouring chromosomal abnormalities (Dryden *et al.*, 2003). Sirt2 has been shown to prevent microtubule poison-induced hyperploid cell formation via a block of entry to chromosome condensation (Inoue *et al.*, 2006). Increased transcriptional expression of *SIRT2* may, therefore, be expected after taxane treatment. Our data are consistent with this and, in fact show that increased *SIRT2* expression occurs 24 hours after treatment. This may reflect the accumulation of breast cancer cells as they undergo replicative cell death.

Similarly, *SIRT7* expression displayed a dose-dependent increase with paclitaxel exposure. Sirt7 may also participate in a mitotic check point and cell-cycle control (Michishita *et al.*, 2005), and its increased transcriptional expression may be expected after paclitaxel treatment. Sirt7 may also have a role in the repair of DNA damage as decreased levels of Sirt7 are associated with apoptotic cell death (Ford *et al.*, 2006). Paclitaxel is known to induce apoptotic as well as non-apoptotic cell death in breast cancer cells (Blagosklonny *et al.*, 2002). This again suggests that increased *SIRT7* expression may be expected in response to paclitaxel exposure. Our data are, again, consistent with this and show increased *SIRT7* expression to occur after 24 hours. These results mirror that for Sirt2 and suggest that Sirt7 may participate in a similar mitotic checkpoint (Michishita *et al.*, 2005).

SIRT1 has been shown to control drug resistance by regulating the multidrug resistance gene, *mdr1*, in response to anthracycline therapy (Chu *et al.*, 2005). A correlation with *SIRT1* expression was only observed at low drug levels and was associated with $p21^{CIP1}$ but not caspase-3 expression, suggesting that Sirt1-mediated resistance may only occur at sublethal stress levels. Furthermore, increased levels of *SIRT1* expression were also observed in various types of cancer cell line and tumour biopsy subjected to anthracycline, etoposide and cisplatin therapies (Chu *et al.*, 2005). Treatment with paclitaxel may be expected to promote *SIRT1* transcription, as the *mdr1* gene is also implicated in resistance to paclitaxel in breast cancer (Mechetner *et al.*, 1998). This was not observed and may reflect the very high doses of paclitaxel used. It may be that lower

levels of paclitaxel treatment, resulting in sub-cytotoxic stress, may be associated with *SIRT1* and *mdr1* expression.

In MCF-7 cells, paclitaxel exposure can also result in apoptosis via a Foxo3a-dependent mechanism (Sunters *et al.*, 2003). Sirt1 increases the ability of Foxo3a to induce cell-cycle arrest thereby helping resist oxidative stress and inhibiting apoptosis (Brunet et al., 2004, Motta et al., 2004, Nemoto et al., 2005). Our data show no change in the transcriptional expression of *SIRT1* and suggest that Sirt1 does not play a part in Foxo3a mediated resistance to paclitaxel-induced cell death. This is in keeping with other studies that suggest that Sirt1 represses Foxo4 rather than Foxo3a-mediated apoptosis (Ford *et al.*, 2005)

SIRT3 did not show any specific pattern of expression with paclitaxel treatment. Although paclitaxel can affect the cellular redox state to induce apoptosis (Varbiro *et al.*, 2001) its primary mechanism of action is by the microtubular damage and disruption of mitotic cell division (Horwitz, 1992). Our data, are in keeping with a scenario, whereby Sirt3 is not involved in the paclitaxel damage response and its expression would, therefore, not be expected to increase with paclitaxel therapy. The study of Sirt3 as well as of the other mitochondrial sirtuins, Sirt4 and Sirt5, may be of interest in breast cancer cells exposed to direct oxidative insult.

These experiments have shown increased transcriptional expression of *SIRT2* and *SIRT7* occurs in mammary cancer cell lines in response to both irradiation and paclitaxel

therapy. These results parallel the observations on anti-oestrogens that show increased *SIRT2*, *4* and *7* transcription in breast cancer cells after exposure to these drugs (Reid *et al.*, 2005). This suggests that these damage responses affect sirtuin responses within different components of the MTR. These data further suggest that *SIRT2* and *SIRT7* expression may be a useful evaluator of breast cancer therapy.

Chapter 6

General Discussion

The aim of the research presented within this thesis was to study breast cancer as an aberration of normal biological ageing, with respect to sirtuin expression. It specifically sought to determine whether there was an association between the transcriptional expression of sirtuins and breast cancer pathogenesis and treatment.

The transcriptional expression of sirtuins was initially investigated in primary and immortalised mammary epithelial cells, as a function of *in vitro* growth (Chapter 3). In order to determine whether changes in biological ageing, observed in the primary mammary epithelial cells, were pertinent to breast cancer *in vivo*, transcriptional expression of sirtuins was studied in non-malignant and malignant breast tissue biopsies (Chapter 4).

Transcriptional studies were also performed to determine the relationship between common therapies used for the treatment of breast cancer, namely radiotherapy and chemotherapy, and sirtuin expression. Specifically, sirtuin expression was investigated in breast cancer cells in response to cellular irradiation and paclitaxel treatment (Chapter 5).

6.1 Introduction

In this thesis, breast cancer has been studied as an aberration of normal biological ageing. Specifically, using real-time PCR, it has sought to determine whether there was an association between the transcriptional expression of sirtuins and breast cancer pathogenesis and treatment.

Real-time PCR is a well established, powerful technique that allows the rapid transcriptional analysis of a large number of genes (Heid et al., 1996). To ensure the reproducibility of this technique a number of measures were taken. An internal housekeeping reference gene, against which each sample was normalised, was analysed for each sample. Negative controls lacking cDNA template as well as primer/probes were routinely analysed for each experiment. Validation experiment were initially performed to confirm that the efficiencies of the primer/probe sets for both housekeeping gene and gene of interest was similar at the range of dilutions used. Furthermore, all samples were analysed in duplicate and gave results that showed coefficients of variation of less than 0.01. Finally, all experiments were performed in duplicate.

These measures ensured that the results from the transcriptional studies were truly representative for our breast cancer experiments.

6.2 Sirtuins, senescence and breast cancer

The study of breast cancer biology has traditionally focused on transformation as a result of abnormalities in normal growth and proliferation. The study of this disease as an aberration of biological ageing provides an alternative platform for understanding breast cancer. Components of the MTR, are inextricably linked and allow the cell to tie damage responses to energy production, fuel utilisation and protein synthesis (Shiels and Davies, 2003). Furthermore, aberrations in any one of these may contribute to ageing-related diseases, including cancer. The study of sirtuins, genes that link components of the MTR, is informative in this context (Sklavounou et al., 2006).

This thesis has demonstrated that sirtuins, associated with individual components of the MTR, show changes in transcription with mammary epithelial cell senescence. This has been demonstrated for *SIRT3* and *SIRT7* in primary mammary epithelial cell senescence. As with yeast Sir2, nucleolar levels of Sirt7 may determine when cells become senescent. Although Sirt7 activates Pol I-mediated rRNA transcription, thereby encouraging growth and proliferation (Ford *et al.*, 2005), senescent cells have been shown to exhibit a higher rate of rRNA synthesis compared to younger cells (Halle *et al.*, 1997). Increasing *SIRT7* expression may reflect increased rRNA synthesis that occurs in senescent cells. In addition, rRNA transcription may also act as a direct sensor for accumulation of DNA damage over a cell's lifespan (Shiels and Davies, 2003). These data are consistent with those of Ford *et al.* (2006), that showed increased levels of Sirt7 to be associated with

growth and proliferation and decreased levels of Sirt7 with growth arrest and apoptosis (Ford *et al.*, 2006).

Sirt3 is implicated in mitochondrial energy production and fuel utilisation in times of stress. In particular, high levels of Sirt3 are observed in starvation and diabetic ketogenesis (Hallows *et al.*, 2006). Cells undergoing stresses inherent in replicative ageing are unable to utilise fuel efficiently and increased *SIRT3* expression may occur as a compensatory mechanism to counter this (Onyango et al., 2002, Schwer et al., 2002). Our data support this view, with increased *SIRT3* transcriptional expression being observed in replicative ageing mammary epithelial cells. These changes may result in increased efficiency of fuel utilisation and energy production in an attempt to prolong lifespan as cells age. This is further supported by *in vivo* studies that have shown increased *SIRT3* expression to be associated with longevity (Bellizzi et al., 2005, Rose et al., 2003).

The changes in sirtuin expression in replicatively ageing mammary epithelial cells parallel those observed in the breast cancer biopsies. The data in this thesis showed *SIRT7* expression to be higher in breast cancer biopsies compared to non-malignant breast biopsies. This result mirrors the observation that increased *SIRT7* expression is a feature of thyroid cancer (de Nigris et al., 2002, Frye, 2002). Furthermore, subdividing breast cancer biopsies by lymph node status revealed that increased expression of both *SIRT3* and *SIRT7* is associated with prognostically poorer lymph node positive disease. The research in this thesis has, for the first time, indicated that increased expression of these

sirtuins may provide mammary epithelial cells with a survival advantage and thereby contribute to breast tumorogenesis. These data also suggest that increased sirtuin expression may reflect the greater degree of biological ageing that occurs in breast cancers, despite the ability of tumour cells to continue proliferating. The increased expression of *SIRT3* and *SIRT7* observed in lymph node positive breast cancers indicates that tumours that are more aggressive and have the worse prognosis display the most biological ageing.

The expression of *SIRT1* and *SIRT2*, sirtuins associated with senescence and certain tumour types, was not found to be different in ageing mammary epithelial cells or between non-malignant and malignant breast tissue, at the transcriptional level. These data contrast with the observations that altered expression of these sirtuins is a feature of certain other malignancies (Luo et al., 2001, Lim, 2006b, Yeung et al., 2004, Kuzmichev et al., 2005, Chen et al., 2005, Hiratsuka et al., 2003, Inoue et al., 2006). Despite this, the role for these sirtuins in cancer pathogenesis is by no means clear, as their expression is not altered in a number of other malignancies (Lim, 2006b). Likewise, our data suggest that these sirtuins are not implicated in biological ageing in mammary epithelial cells or in breast cancer pathogenesis. These results must, however, be interpreted within the context of a transcriptional study of limited size. Increasing the number of biopsies studied and performing post-transcriptional studies may help to confirm these results.

6.3 Sirtuins and breast cancer therapy

Therapies commonly used for the treatment of breast cancer often induce senescence and apoptosis, processes that also contribute to the pathogenesis of ageing-related diseases (Shay and Roninson, 2004, Chang et al., 1999, Ota et al., 2006, Chu et al., 2005). The ability of breast cancer cells to withstand the insult of cytotoxic therapies may, therefore, be influenced by the expression of sirtuins; genes involved in DNA repair, cell-cycle regulation and ageing (Mostoslavsky et al., 2006, Dryden et al., 2003, Howitz et al., 2003). Cancer therapies can affect the different components of the MTR, by inducing DNA and telomeric damage, cell-cycle arrest and interfering with mitochondrial energy production and protein synthesis (Russo and Russo, 2003). The sirtuins that link individual components of the MTR may, therefore, be useful in evaluating breast cancer therapies (Sklavounou et al., 2006).

A role for sirtuins in promoting cell survival in response to stress is suggested by our data, that showed changes in sirtuin expression in breast cancer cells after exposure to cytotoxic agents. Specifically, evaluation of individual sirtuins indicated that *SIRT2* and *SIRT7* expression was associated with cell damage from both ionising radiation and paclitaxel treatment.

The increased *SIRT2* expression observed is consistent with a scenario whereby increased chromosomal abnormalities occur as a result of irradiation and paclitaxel therapy (Suzuki et al., 2003, Pawlik and Keyomarsi, 2004, Piperno et al., 1987). Sirt2 is involved in the M

phase cell-cycle checkpoint and may function to arrest the cell-cycle in response to chromosomal abnormalities (Dryden et al., 2003, Inoue et al., 2006). The nucleo-cytoplasmic shuttling of Sirt2 in response to ionising radiation also suggest a possible role for it in the DNA damage response. Treatment of gliomas and gastric cancers with histone deacetylase inhibitors, results in tumour cell death and is associated with increased *SIRT2* expression (Inoue *et al.*, 2006). Furthermore, increased *SIRT2* expression is associated with puromycin-induced cell death (Grubisha *et al.*, 2006). Our data also demonstrated an increase in *SIRT2* expression in breast cancer cells, after irradiation and paclitaxel treatment and is supportive of a role for Sirt2 in the damage response. These observations suggest that targeting Sirt2, a protein that participates in mitotic control and possibly DNA damage responses, may provide therapeutic benefit.

The increased levels of *SIRT7* expression observed may reflect stresses experienced by damaged cancer cells suffering replicative death and unable to proliferate as a result of these therapies. In particular, the increase in *SIRT7* expression after these cytotoxic agents may reflect accelerated ageing as a result of these treatments (Shiels and Davies, 2003). This is analogous to the increased *SIRT7* expression observed in mammary epithelial cells as they undergo replicative ageing. In addition, the repetitive nature of the rRNA genes provides a high probability of a polymerase encountering, and stalling at sites of DNA damage (Russell and Zomerdijk, 2005). The accumulation of signals associated with such stalled DNA polymerases may activate DNA-damage response pathways that contribute to apoptosis and growth arrest. The data in this thesis, showing

increased *SIRT7* expression, and as a consequence, increased rRNA transcription, is supportive of this.

Altered *SIRT2* and *SIRT7* expression have previously been shown to be associated with microtubule disrupting drugs, cisplatin therapy and anti-oestrogen therapy (Matsushita et al., 2005, Kyrylenko et al., 2003, Inoue et al., 2006). Our findings have shown that these same sirtuins are also good evaluators of irradiation and paclitaxel therapy in breast cancer.

The lack of changes in *SIRT1* and *SIRT3* expression in breast cancer cells subjected to irradiation and paclitaxel treatment, suggest that these sirtuins are not implicated in damage responses as a result of these therapies. Although the cell's response to these treatments include apoptosis, their primary mode of action is by inducing replicative cell death (Steel, 2001, Pawlik and Keyomarsi, 2004, Blagosklonny et al., 2002). Indeed, our data have confirmed that apoptosis is not the principal method of death in the irradiated breast cancer cells used in this study, and have, for the first time, shown that increased *SIRT2* and *SIRT7* expression occurs in irradiated mammary epithelial cells in a p53-independent manner. These observations do not, however, exclude a role for Sirt1 and Sirt3 in the response of breast cancer cells to these treatments. This was a transcriptional study and it may be that performing post-translational studies show that changes in sirtuins are reportive of these therapies. Studying breast cancer treatments that primarily induce apoptosis may also shed light on the role of sirtuins, in particular of the anti-apoptotic Sirt1, in protecting against these therapies.

Furthermore, inhibition of sirtuin activity with the Sirt1 inhibitor, Sirtinol, has been shown to induce senescent growth arrest in human breast and lung cancer cells (Ota *et al.*, 2005). This may act, not only by targeting tumour cells that may have upregulated *SIRT1* in order to achieve transformation, but also by sensitising tumour cells to p53-mediated senescence and apoptosis (Luo et al., 2001, Heltweg et al., 2006). A recent study has also shown that inhibition of Sirt1 causes the reactivation of silenced genes in cancer cells resulting in growth arrest (Pruitt *et al.*, 2006). Paradoxically, the sirtuin activator, resveratrol, has also been shown to beneficial in the treatment of cancer and has been shown to induce tumour cell apoptosis (Signorelli and Ghidoni, 2005). This has been shown to occur via Sirt1-mediated augmentation of TNFα-mediated tumour cell apoptosis (Yeung *et al.*, 2004).

The association between sirtuin expression in breast cancer biopsies as well as its relationship to breast cancer therapy raises the question as to whether targeting of sirtuins may be beneficial in the treatment of the disease. Previously, Sirt1 has been shown to be essential for cancer cell viability but not for non-cancer cell viability (Ford *et al.*, 2005). Furthermore, overexpression of *SIRT1* has been shown to be a feature of drug-resistant cancer cell lines and tumour biopsies from patients subjected to chemotherapy (Chu *et al.*, 2005). Sirt1 may therefore be a selective target in tumour cells. Similarly, other sirtuins, in particular Sirt2, 3 and 7, may also be selective targets in breast cancer.

Already, histone deacetylase inhibitors (HDACi) have been shown to exert anti-tumour effects and are undergoing phase II clinical trials (Lin et al., 2006, Carey and La

Thangue, 2006, Marks et al., 2001). HDACi that target sirtuins have also been shown to induce senescence in a number of cancers including breast and lung cancer (Ota et al., 2005, Porcu and Chiarugi, 2005). These drugs are not selective for Sirt1 and may mediate their effects by inhibiting the activity of other sirtuins. The data in this thesis is supportive of such as notion.

6.4 Future work

In the study of sirtuins in replicative ageing and breast biopsies, the transcriptional expression of Sirtuins 4, 5 and 6 have yet to be examined. Sirt6 is particularly interesting as it has been shown to participate in DNA damage repair and its deletion in transgenic mice results in a progeroid syndrome (Mostoslavsky *et al.*, 2006). Sirt4 is a mitochondrial sirtuin that is implicated in fuel utilisation and insulin metabolism in response to calorie restriction (Haigis *et al.*, 2006). Sirt5, also a mitochondrial sirtuin, may have a similar role, or like Sirt1, may participate in resistance to oxidative stress. The analysis of these genes may provide insight into the role of sirtuins in mammary epithelial cell senescence and breast cancer.

These sirtuins may also play a role in the response of mammary cancer cells to the breast treatments studied as well as other treatments commonly used in breast cancer. The discovery that the transcriptional expression of sirtuins is altered in response to anti-oestrogenic treatments in breast cancer cells, suggests that these sirtuins may also participate in tumour cell damage responses (Kyrylenko *et al.*, 2003). The role of Sirt6 in assisting DNA repair is supportive of this (Mostoslavsky *et al.*, 2006).

The role of Sirt1 in mediating apoptosis in breast cancer cells in response to ionising radiation has not been fully investigated, as cell lines studied displayed resistance to radiation-induced apoptosis. Repeating the experiments in a mammary cancer cell line that displays radiation-induced apoptosis may clarify the roles of sirtuins in this process.

The MCF-7 cell line was used to study the effect of paclitaxel on the transcriptional expression of sirtuins in breast cancer. This cell line is a good *in vitro* representative of ER positive breast cancer. This type of tumour is normally treated with hormone therapy although chemotherapy may also be used if the patient has node positive disease. Chemotherapy, however, is usually administered to patients with ER negative or ERBB2 positive disease. It would, therefore, also be of value to study the effect of paclitaxel on the transcriptional expression of sirtuins in both ER negative and ERBB2 positive cell lines.

It would be also be interesting to determine the role of sirtuin activators, such as resveratrol, as well as sirtuin inhibitors, such as sirtinol and nicotinamide, on sirtuin expression, survival and apoptosis in breast cancer cells. Although the activity of these agents is not specific to sirtuins it may provide an interesting study on the role of human sirtuins in breast cancer. Specific targeting of sirtuins using siRNA may provide a more accurate way of determining the role of these sirtuins in breast cancer therapy.

Finally, whilst these studies of sirtuins in breast cancer have been transcriptional, it would be interesting to investigate the changes in sirtuin protein expression, as changes in mRNA do not necessarily reflect changes in protein expression.

6.5 Concluding remarks

Prior to the start of this study, sirtuins were not associated with malignancy or cancer treatment. In this study, I have presented data that show changes in sirtuin expression to be a feature of replicative ageing in mammary epithelial cells and breast cancer. Furthermore, changes in sirtuin expression have been shown to be associated with common therapies used to treat this condition. This transcriptional analysis of sirtuins in breast cancer has added to our understanding of breast cancer biology and may help in identifying new ways for managing the disease.

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Appendix I

(i) Tissue culture and all molecular work were performed in the Department of Surgery, Western Infirmary, University of Glasgow. Taqman PCR was performed on an ABI Prism 7700 sequence detector in the Department of Immunology, Western Infirmary, Glasgow. Cellular irradiation and Apoalert TUNEL assays were performed at the Department of Radiation Oncology at the Cancer Research UK, Beatson Laboratories, Garscube, Glasgow.

(ii) All work was carried out by myself, however cellular irradiation was performed with the assistance of Lesley Wilson and Anne Green from the Department of Radiation Oncology at the Cancer Research UK, Beatson Laboratories, Garscube, Glasgow.

(iii) Research funding was obtained from the Marjorie Wilkinson Bequest and supervision from Dr Paul Shiels, Department of Surgery, Glasgow Royal Infirmary, Glasgow. Adviser of studies was Professor WD George, Western Infirmary, Glasgow.

(iv) Ethical approval for work on archival human breast biopsies was obtained from the West of Scotland Ethics Committee.

Appendix II

Western Analyses

In addition to the transcriptional studies described, studies on sirtuin protein were also attempted. Unfortunately, problems were encountered with the human anti-Sirt1 antibody obtained from Abcam. A number of experiments were performed but, unfortunately, gave no interpretable results.

The anti-Sirt1 antibody was found to be non-specific, giving a great deal of background staining on western analyses. Specifically, two distinct bands of 100kDa and 160kDa could be discerned. These did not match the expected weight of Sirt1 (120kDa). Furthermore, the detection of these particular bands was not always reproducible even within the same protein sample.

On approaching Abcam and reviewing technical enquiries relating to this antibody, it was found that others had experienced similar problems. Abcam acknowledged that their original antibody was non-specific and in some cases did not work at all. Since completing my research, Abcam have produced a new anti-Sirt1 antibody and numerous other anti–Sirt1 antibodies have come onto the market including those from Santa Cruz, Upstate and Everest Biotech.

Appendix III

Senescence data (Chapter 3)

Real-time PCR

Gene	P16			P21			SIRT1		
Passage/ Cell Line	1	2	3	1	2	3	1	2	3
HMEC	0.8467	2.2736	2.7511	0.9461	1.7715	2.3295	1.0570	1.7471	1.4743
	1.1810	2.0350	3.3870	1.0570	1.5210	2.0420	0.9461	2.3376	1.5911
MCF-12A	0.0001	0.0003	0.0000	1.0461	0.7397	0.8123	1.1057	1.6644	1.3195
	0.0000	0.0000	0.0000	0.6902	0.3673	0.5249	1.4093	1.4489	1.6702
MCF-7	0.0001	0.0001	0.0000	0.4132	0.3724	0.6874	1.8340	1.8987	1.8150
	0.0000	0.0000	0.0000	0.5017	0.4368	0.6029	1.1851	1.2269	1.4540

Gene	SIRT2			SIRT3			SIRT7		
Passage/ Cell Line	1	2	3	1	2	3	1	2	3
HMEC	1.0607	2.4623	1.6077	0.6462	2.2579	3.2490	0.5529	2.0420	4.6107
	0.9428	2.0849	1.5966	1.5476	2.1067	2.5669	1.8088	3.8637	7.3871
MCF-12A	1.8404	0.9138	3.0420	0.8796	0.8796	1.2658	1.3379	1.3013	1.9386
	2.3784	0.7320	2.4368	1.0755	1.4290	1.8404	1.0792	2.5669	1.9119
MCF-7	2.0420	1.4241	2.4368	1.3613	0.6395	1.5263	0.9266	0.5743	0.3046
	2.6390	1.1408	3.1492	0.9897	0.8039	0.7579	1.3566	0.6926	0.3909

SA β-Gal Assay

Passage/ Cell Line	1	2	3
HMEC	15	38	79
	20	43	82
MCF-12A	24	22	25
	30	28	27

Biopsy data (Chapter 4)

Gene/ Cell Line	SIRT1	SIRT2	SIRT3	SIRT7
Cancer 1	0.572362	0.959264	0.410371	1.090508
Cancer 2	0.993092	1.197479	1.128964	0.949342
Cancer 3	0.655197	0.366021	0.526681	2.034959
Cancer 4	0.301452	0.712025	0.590496	2.566852
Cancer 5	1.22264	0.861546	0.675955	1.993081
Cancer 6	0.063153	0.162668	0.281265	2.522755
Cancer 7	0.345079	2.281527	0.271684	1.180993
Cancer 8	0.986233	0.64842	0.617709	1.681793
Cancer 9	0.867539	0.712025	0.809442	2.313376
Cancer 10	0.537747	0.76313	0.449066	0.920188
Cancer 11	1.090508	0.750019	0.435275	0.879649
Cancer 12	0.732043	1.185093	0.369847	1.438934
Cancer 13	0.150205	0.582367	0.133508	0.768438
Cancer 14	0.259715	0.650671	0.408951	1.060688
Cancer 15	0.939523	1.324089	0.464902	1.652901
Cancer 16	0.098413	0.17254	0.135842	0.867539
Cancer 17	0.143091	0.417544	0.153361	0.755236
Cancer 18	1.000000	1.000000	1.000000	1.000000
Cancer 19	0.367292	0.269807	0.524858	0.923382
Cancer 20	0.316439	3.149232	0.396392	1.840375
Cancer 21	0.075102	0.476319	0.079937	0.760489
Cancer 22	0.747425	0.732043	0.564482	1.443929
Cancer 23	0.336808	0.578344	0.812252	1.484524
Cancer 24	0.421908	1.265757	0.5	2.61172
Non-malignant 1	0.464902	0.643941	0.316439	0.673617
Non-malignant 2	0.855595	1.510473	0.447513	0.61132
Non-malignant 3	0.685391	0.473029	0.240649	0.369847
Non-malignant 4	0.795536	0.716978	0.406126	0.486327
Non-malignant 5	0.403321	0.650671	0.240649	0.574349
Non-malignant 6	0.496546	0.643941	0.067921	0.898132
Non-malignant 7	0.517632	1.558329	0.37501	1.028114
Non-malignant 8	1.292353	1.226885	0.283221	0.726986
Non-malignant 9	0.91067	1.22264	0.313166	0.809442
Non-malignant 10	0.528509	1.536875	0.479632	1.105731
Non-malignant 11	0.835088	0.933033	0.600818	0.882703
Non-malignant 12	1.257013	0.972655	0.427798	1.252664
Non-malignant 13	0.855595	0.823591	0.469761	0.846745
Non-malignant 14	0.707107	1.270151	0.707107	1.433955
Non-malignant 15	0.668964	0.607097	0.639493	1.399586
Non-malignant 16	0.271684	0.580352	0.369847	1.090508
Non-malignant 17	1.464086	0.852635	0.989657	1.226885
Non-malignant 18	0.095722	0.174343	0.108442	0.332171
Non-malignant 19	0.353553	0.750019	0.185565	0.261521
Non-malignant 20	1.265757	0.768438	0.494828	1.003472
Non-malignant 21	0.346277	0.503478	0.146097	0.493116

Irradiation data (Chapter 5)

MCF-7 cell line

Gene	Radiation Dose (Gys)/ Time	0	2	4	6	8	10
P21	24hrs	1.0000	0.8615	1.3660	1.7901	2.6574	3.1383
		1.0000	1.0317	2.1585	2.4880	3.5801	3.8371
	48hrs	0.5529	0.9107	1.9386	2.9180	3.8504	4.6428
		0.4005	0.7022	1.0644	1.8921	3.1275	4.1554
SIRT1	24hrs	1.0000	0.7711	0.4730	0.8497	0.9526	1.0792
		1.0000	1.0105	1.3195	1.0867	1.1933	1.4743
	48hrs	0.2726	0.8888	1.6935	0.7448	1.3472	0.9138
		1.1851	1.0905	0.5141	1.3660	0.8322	1.1607
SIRT2	24hrs	1.0000	0.7071	0.9461	1.1607	1.4191	1.7112
		1.0000	1.3519	1.5529	0.6998	5.2054	2.9383
	48hrs	1.7291	2.0350	2.7132	2.6027	2.8284	3.1492
		1.1975	1.3803	1.8661	1.8468	3.3288	2.6759
SIRT3	24hrs	1.0000	0.2588	0.2994	0.2932	0.4538	0.3737
		1.0000	1.1607	1.2184	0.6807	0.8950	0.6760
	48hrs	0.3869	0.4175	0.6598	0.4649	0.4601	0.6439
		0.6071	0.6199	1.0140	0.6926	1.1975	0.8151
SIRT7	24hrs	1.0000	1.3566	1.1975	1.2100	1.3059	1.7471
		1.0000	1.5105	1.7839	0.8796	1.3059	1.7901
	48hrs	1.8340	2.4623	3.6808	3.0738	4.4076	4.6913
		1.7777	2.0209	2.7511	2.6759	4.2871	4.9417

MDA-MB-231 cell line

Gene	Radiation Dose (Gys)/ Time	0	2	4	6	8	10
P21	24hrs	1.0000	1.0534	0.9428	1.1487	0.8645	0.8236
		1.0000	1.0000	1.2058	1.4191	1.4743	1.4389
	48hrs	1.0353	0.8950	0.9693	1.1527	1.7777	1.4340
		1.2058	1.2702	1.5369	1.2397	1.5801	1.7112
SIRT1	24hrs	1.0000	0.5141	0.3572	0.4763	0.5141	0.8236
		1.0000	1.1728	1.6818	1.8213	0.8011	1.1810
	48hrs	1.5422	2.2736	0.9428	0.7955	1.2614	1.0425
		2.0777	2.4794	2.6117	2.0279	2.5403	2.0139
SIRT2	24hrs	1.0000	1.2879	1.7053	1.5856	1.3851	2.0994
		1.0000	0.5000	0.8645	1.1251	0.9931	1.3013
	48hrs	1.5583	1.6644	1.6818	1.6133	2.6027	2.4453
		1.3149	1.0534	0.9931	1.5801	1.6935	2.5053
SIRT3	24hrs	1.0000	0.5070	0.3451	0.4506	0.4830	0.7928
		1.0000	0.7397	1.1290	1.2879	0.8615	0.8950
	48hrs	1.4540	2.0922	0.8766	0.7071	1.2527	0.9897
		1.5476	0.7846	1.8340	0.8526	0.9897	1.0281
SIRT7	24hrs	1.0000	1.0718	0.7900	0.9559	0.7765	1.3613
		1.0000	0.3403	0.4897	0.9170	0.6666	1.0534
	48hrs	1.0867	1.2790	1.2570	1.6077	1.9119	2.3538
		0.5340	1.0070	0.7684	1.0070	1.6586	2.9690

Paclitaxel data (Chapter 5)

Paclitaxel Concentration (ng/ml)/ Gene	0	0.01	0.1	1	10
P21	1.0000	1.1607	2.4538	4.4229	7.9173
	1.0000	2.1962	2.6208	3.5677	5.2964
SIRT1	1.0000	0.6736	1.8404	0.6113	1.0210
	1.0000	1.7593	0.7270	0.6462	1.3013
SIRT2	1.0000	2.0279	2.9282	3.8637	7.0128
	1.0000	1.1810	2.3702	3.0951	5.9587
SIRT3	1.0000	1.0175	1.2142	0.9461	1.0425
	1.0000	0.8766	0.7500	0.8293	1.0681
SIRT7	1.0000	3.7451	4.5948	6.2550	8.7543
	1.0000	2.2894	2.7321	5.1515	7.1107