Breast Cancer Research Volume 8 Supplement 2, November 2006

Meeting abstracts

Breast cancer research: the past and the future

The Royal Society, London, UK

1 November 2006

Published online: 1 November 2006

Keynote lectures

S1

Breast cancer susceptibility after BRCA1/2: finding the genes and potential practical applications

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Breast Cancer Research 2006, **8(Suppl 2):**S1 (DOI 10.1186/bcr1544)

Background Epidemiological studies have shown that only about 20% of the familial clustering of breast cancer is explained by the known highly penetrant mutations in BRCA1 and BCRA2. We have set out to search for the genes for the remaining 80%. Twin studies indicate a predominant role of shared genes rather than a shared environment; the patterns of occurrence of breast cancer in families are consistent with a major polygenic component.

Methods We have assembled a population based set of 5,000 breast cancer cases and 5,000 controls from the East Anglian population. We have simple clinical and epidemiological information, including family history, and samples of blood and paraffin embedded tumour.

We have used association studies based on single nucleotide polymorphisms, first with candidate genes and then in a genome-wide scan of 266,000 single nucleotide polymorphisms, to search for the putative predisposing genes. We have as yet searched only for common variants (frequency >5%).

Results We have modelled the effects of polygenic predisposition in the East Anglian population, and have shown that the model predicts a wide distribution of individual risk in the population, such that half of all breast cancers may occur in the 12% of women at greatest risk.

Both the candidate gene-based and genome-wide scans have provided provisional identification of a number of novel susceptibility genes, and these are currently being confirmed by a world-wide consortium of independent laboratories totalling 20,000-plus cases and controls. No single gene so far identified contributes more than 2% of the total inherited component, consistent with a model in which susceptibility is the result of a large number of individually small genetic effects.

S2

Translating breast cancer research into clinical practice – new approaches and better outcomes

SRD Johnston

Breast Cancer Research 2006, **8(Suppl 2):**S2 (DOI 10.1186/bcr1545)

Abstract not available at time of printing.

S3

Evolution of aromatase inhibitors as an endocrine treatment for breast cancer

WR Miller

Breast Cancer Research 2006, **8(Suppl 2):**S3 (DOI 10.1186/bcr1546)

Abstract not available at time of printing.

Speaker abstracts

S4

BRCA1 transcriptionally regulates genes associated with the basal breast cancer phenotype

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Breast Cancer Research 2006, **8(Suppl 2):**S4 (DOI 10.1186/bcr1547)

Background Ten to twenty per cent of breast tumours exhibit a basal-like genetic profile and these tumours carry a poor prognosis. Breast tumours which contain germline mutations for BRCA1 commonly exhibit a molecular profile similar to basal breast tumours. BRCA1 is a tumour suppressor gene which is mutated in up to 5–10% of breast cancer cases and is involved in multiple cellular processes including DNA damage control, cell cycle checkpoint control, apoptosis, ubiquitination and transcriptional regulation.

Methods Microarray-based profiling was carried out using the HCC1937EV and HCC1937BR breast cancer cell lines. Basal gene and protein expression levels were analysed by qRT-PCR and western blotting. ChIP analyses were performed and demonstrated that BRCA1 regulates basal gene expression through a transcriptional mechanism involving c-myc.

Results We have previously carried out microarray-based expression profiling to examine differences in gene expression when BRCA1 is reconstituted in BRCA1 mutated HCC1937 breast cancer cells. We observed that *p*-cadherin and the cytokeratin 5 and cytokeratin 17 genes, which are strongly correlated with the basal phenotype, are differentially expressed when BRCA1 is reconstituted. In addition, qRT-PCR and ChIP analysis of BRCA1 reconstituted cells show that BRCA1 represses the expression of these basal genes by a transcriptional mechanism. Furthermore, abrogation of endogenous BRCA1 protein in the T47D cell line using siRNA results in re-expression of these basal genes, suggesting that BRCA1 expression levels may be important in basal gene expression.

We have also demonstrated that BRCA1 is physically associated with the promoter regions of basal genes through an association with c-myc. Consequently, we have confirmed that siRNA inhibition of c-myc in T47D cells results in re-expression of these genes.

Conclusions Our results suggest that BRCA1 is involved in the transcriptional regulation of genes associated with the basal phenotype and that BRCA1 controls basal gene expression through a transcriptional mechanism involving c-myc. Further work is now concentrating on defining the relationship between BRCA1 and basal gene expression and how this may affect clinical responses to breast cancer chemotherapy.

Acknowledgement This work is funded by Breast Cancer Campaign.

S5

Regulation of recombinational repair by the familial breast cancer susceptibility protein BRCA2

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Breast Cancer Research 2006, **8(Suppl 2):**S5 (DOI 10.1186/bcr1548)

Background Inherited mutations in *BRCA2* are associated with a predisposition to early-onset breast cancers. The underlying basis of tumourigenesis is thought to be linked to defects in DNA double-strand break repair by homologous recombination (HR), as indicated by the spontaneous chromosomal instability phenotype of *BRCA2*-defective cell lines. The BRCA2 protein interacts with ssDNA and the RAD51 recombination protein, and is proposed to recruit RAD51 to the damage site for the HR repair.

Methods Recombinant BRCA2 fragments that cover the entire length of BRCA2 were tested for interaction with RAD51 and for their phosphorylation using cell free extracts. An antibody that specifically recognises BRCA2 phosphorylated at serine 3291 was generated and used to analyse the phosphorylation status of endogenous BRCA2 during the cell cycle and after DNA damaging treatment. A cell line that stably expresses a C-terminal BRCA2 fragment was generated, to allow the analysis of RAD51 interactions and ability to promote homologous recombinational repair (HRR).

Results We found that the C-terminal region of BRCA2, which directly interacts with RAD51, contains a site (S3291) that is phosphorylated by cyclin-dependent kinases. Phosphorylation of S3291 increases as cells progress towards mitosis, and was shown to block C-terminal interactions between BRCA2 and RAD51. However, DNA damage overcomes cell cycle regulation by reducing S3291 phosphorylation and stimulating interactions with RAD51. HRR is defective in cells overexpressing the C-terminal fragment of BRCA2, indicating that interactions between RAD51 and the C-terminal region of endogenous BRCA2 are important for repair.

Conclusion We suggest that S3291 phosphorylation provides a molecular switch that can regulate RAD51-mediated HRR. Loss of phosphorylation in response to DNA damage allows interactions between RAD51 and the C-terminal region of BRCA2 and may facilitate the loading of RAD51 on damaged DNA [1]. Importantly, a S3291 nonphosphorylatable mutation (P3292L) has been found in familial breast cancer patients, implicating a role of S3291 phosphorylation in the maintenance of genome integrity.

Acknowledgements This research was supported by Breast Cancer Campaign (SCW), Cancer Research UK (SCW, FE), the Human Frontiers Science Program (FE) and the Japan Society for the Promotion of Science (FE).

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S6

Chromosome translocations may play a significant role in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**S6 (DOI 10.1186/bcr1549)

Chromosome translocations that form fusion transcripts and/or activate expression of genes by promoter insertion are key events in leukaemias and lymphomas, and mesenchymal tumours, but it has been fashionable to think they are irrelevant to the common epithelial cancers such as breast cancer. However, that view is now being challenged [1-4]; in particular, we have shown that *NRG1* is translocated in breast cancers [3]. It seems likely that some translocations in breast cancers target specific genes at their breakpoints, and this is particularly likely for reciprocal translocations.

We are cataloguing translocation breakpoints in breast cancer cell lines and tumours. We use array painting, in which individual chromosomes are purified in a cell sorter and their DNA hybridized to microarrays. We have analysed all the chromosomes of three breast cancer lines to 1 Mb resolution or better.

A striking finding was that reciprocal and more complex balanced translocations are far more frequent than expected. Together the three lines had at least 14 balanced translocations, almost three times more than identified by cytogenetics – the cryptic ones involved small fragments, or were obscured by subsequent rearrangement. Furthermore, several translocation breaks were in genes, including known cancer-critical genes such as *EP300*/p300 and *CTCF*. This supports the emerging idea that chromosome rearrangement plays a major role in the gene changes that cause breast cancer.

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S7

Regulation of human breast stem cells

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Breast Cancer Research 2006, **8(Suppl 2):**S7 (DOI 10.1186/bcr1550)

Breast epithelial stem cells are thought to be the primary targets in the etiology of breast cancer. Since breast cancers mostly express estrogen receptor-alpha (ERα), we examined the biology of these cells and their relationship to stem cells in normal human breast epithelium.

We employed several complementary approaches to identify putative stem cell markers, to characterise an isolated stem cell population and to relate these to cells expressing ERα. ERα-positive cells were found to coexpress the putative stem cell markers $p21^{CIP1}$ and Msi-1. Human breast epithelial cells with Hoechst dye-effluxing 'side population' (SP) properties characteristic of mammary stem cells in mice were demonstrated to be undifferentiated cells by lack of expression of myoepithelial and luminal epithelial membrane markers. These SP cells were sixfold enriched for ERα-positive cells and expressed several-fold higher levels of the ERα, $p21^{CIP1}$ and Msi1 genes than non-SP cells. In contrast to non-SP cells, SP cells formed branching structures in matrigel which included cells of both luminal and myoepithelial lineages. The data suggest a model where scattered ERα-positive cells are stem cells that self-renew through asymmetric cell division and generate patches of transit amplifying and differentiated cells.

In recent studies we have been investigating breast cancers for the presence of a stem cell population. Using a nonadherent culture method analogous to neurosphere culture that enriches for neural stem cells, we have demonstrated that breast cancer cell lines and primary tumours contain a self-renewing population that is highly regulated by the Notch receptor signaling pathway. Inhibitors of this pathway could represent a new therapeutic modality in breast cancer, perhaps through combination with current treatments.

In order to discover novel pathways that regulate stem cell self-renewal, we have applied functional genomics using an RNAi library targeting ~8,000 genes involved in cancer. This has revealed the importance of several pathways not previously associated with stem cell self-renewal. These pathways may represent novel targets for breast cancer therapy aimed at the breast cancer stem cells that survive conventional therapies.

S8

Aberrant activation of Notch signalling in human breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**S8 (DOI 10.1186/bcr1551)

Background Like many developmental signalling pathways, the Notch pathway has been linked to the aetiology of several different human cancers. The development of focal adenocarcinomas in the murine mammary gland [1] and the transformation of both normal murine and human breast epithelial cell lines following Notch activation [1,2] have long suggested that the pathway may play a role in human breast cancer. However, this question has received little attention.

Methods Activation of the Notch pathway in human breast cancer cell lines and breast carcinoma samples was monitored by western blotting with an antibody that recognises the cleaved Notch1 intracellular domain which is produced during signalling. Regulation of apoptosis by Notch was studied in MCF 10A cells transformed by overexpressing the Notch1 intracellular domain. Apoptosis was triggered by treating cells with the kinase inhibitor staurosporine or the DNA damaging agents melphalan and mitoxantrone, and monitored by nuclear fragmentation or cleavage of caspase 3. Changes in the apoptotic machinery were examined by western blotting using a range of antibodies that recognise both total and phosphospecific forms of different components.

Results We will present data showing that Notch signalling is activated in a wide range of breast cancer cell lines and in a panel of 20 human breast carcinomas of different pathological grade and prognosis. In addition, we will demonstrate that sustained signalling is required to maintain the transformed phenotype of breast cancer cell lines, as its inhibition by expressing Numb, a natural inhibitor of the pathway, causes both MCF7 and MDA-MB-231 cells to adopt a normal phenotype. Our data with the normal breast epithelial cell line MCF 10A indicate that Notch signalling contributes to the transformed phenotype by inhibiting apoptosis. Activation of Notch signalling in these cells by overexpressing the Notch1 intracellular domain prevents apoptosis in response to growth factor withdrawal, removal from the extracellular matrix and DNA damage. Finally, we will provide evidence that the apoptosis resistance seen in Notch transformed MCF 10A cells is through the activation of the Akt survival pathway.

Conclusion Altogether this suggests that targeting Notch signalling may be a novel therapeutic strategy for the treatment of breast cancer.

Acknowledgements This work was supported by Breast Cancer Campaign. KB was a Wellcome Trust Research Career Development Fellow.

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S9

Novel roles for integrins in tumour angiogenesis

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Breast Cancer Research 2006, **8(Suppl 2):**S9 (DOI 10.1186/bcr1552)

The laminin receptors $\alpha_3\beta_1$ and $\alpha_6\beta_1$ are expressed by endothelial cells, but their direct roles in tumour angiogenesis and especially breast cancer angiogenesis remains unexplored. We show that $\alpha_6\beta_1$-integrin is expressed in 80–90% of blood vessels associated with normal breast or ductal carcinoma *in situ*. However, the proportion of vessels that express $\alpha_6\beta_1$ drops to less than 30% in invasive ductal carcinoma samples, suggesting that loss of this laminin receptor can enhance invasive carcinoma angiogenic events. Furthermore, the deletion of α_6-integrin or α_3-integrin in *ex vivo* angiogenic assays can promote VEGF-mediated microvessel sprouting. Taken together these results implicate these integrins in the negative control of angiogenesis. Since global deletion of the α_3-integrin or α_6-integrin genes in mice is lethal, we have generated mice where these genes are deleted on endothelial cells only.

Our data indicate that mice deficient in individual laminin receptors on endothelial cells *in vivo* not only support tumour growth but have enhanced tumourigenesis. Moreover, tumour angiogenesis is elevated in these mice, suggesting strongly that laminin receptors are not required for tumour angiogenesis. We also observed that angiogenic responses to hypoxia are enhanced in mice deficient for laminin receptors on endothelial cells and have evidence that, at least in α_3-null endothelial cells, VEGF-receptor 2 (FLK1) levels are elevated when compared with controls. We provide the first evidence that α_3-integrin and α_6-integrin can be differentially expressed in the angiogenic vessels associated with invasive carcinoma of the breast and suggest that these laminin receptors can negatively regulate angiogenesis *in vivo* and *ex vivo*.

S10

Genome-wide RNAi to identify genes that confer synthetic lethality with BRCA1

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Breast Cancer Research 2006, **8(Suppl 2):**S10 (DOI 10.1186/bcr1553)

We have previously demonstrated that a functional orthologue of the breast cancer tumour suppressor gene BRCA1 exists in *C. elegans* (brc-1). Deletion mutants in *C. elegans* brc-1 or its heterodimeric partner, brd-1, share many of the phenotypic hallmarks of BRCA1-deficient human cells, yet are homozygous viable thus permitting extensive reverse genetic analysis. Using a rapid and inexpensive genome-wide screen in *C. elegans* we set out to identify genes that could be targeted in human patients to selectively kill tumours defective in the BRCA pathway. To this end we have utilized the complete *C. elegans* RNA-mediated interference library to systematically inactivate all 19,500 *C. elegans* genes and have identified those genes whose depletion confers synthetic lethality in combination with brc-1 and brd-1 mutations. In total, this screen identified 20 genes including pme-1 and pme-2, the *C. elegans* counterparts of PARP, a gene whose inhibition selectively kills BRCA defective tumour cells. We are currently using siRNA to knockdown all human homologues to identify those genes whose inactivation specifically kills mammalian cells harbouring mutations in BRCA1. These results and our current progress will be presented.

S11

Microarray studies reveal novel genes associated with endocrine resistance in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**S11 (DOI 10.1186/bcr1554)

Background Endocrine resistance is a major hurdle in breast cancer management, and determining the underlying factors driving its growth and aggressive behaviour should vastly improve treatment.

Methods Microarray technology (BD Atlas Plastic Human 12K Microarrays; GeneSifter software), verified by PCR, western blotting and immunocytochemisty, was used to identify genes increased in acquired resistant models to tamoxifen (TamR) or faslodex (FasR) as potential predictive/prognostic markers and new therapeutic targets.

Results Alongside known breast cancer genes (β-catenin, PEA3, vitronectin, CD44), two novel genes in endocrine resistance were revealed (the latter never previously described in breast cancer): a securin/cell cycle regulator Pituitary Tumour Transforming Gene-1 (PTTG1), and GDNF receptor-alpha 3 (GFRα3) reported to promote cell survival signalling via RET coreceptor. Altered levels of PTTG1, GFRα3, or their associated family members were observed in further endocrine resistant states, including an additional faslodex resistant model that has progressed to a highly-aggressive state (FasR-Lt) and XMCF-7 cells resistant to oestrogen deprivation. PTTG1 and GFRα3 induction were also implicated in limiting response to anti-EGFR agents currently in breast cancer trials, with GFRα3 ligand (artemin) largely overcoming drug response. mRNA studies in clinical disease revealed PTTG1 associated with lymph node spread, high tumour grade and proliferation, while GFRα3 was enriched in ER-negative tumours and those expressing EGFR, profiles implying roles in clinical resistance and aggressive tumour behaviour. Promisingly, PTTG1 or GFRα3 siRNA knockdown promoted cell kill and inhibited proliferation in the resistant models.

Conclusion Cumulatively, these data indicate PTTG1 and GFRα3 may provide useful biomarkers, and perhaps clinically relevant therapeutic targets for multiple resistant states.

Acknowledgement Funding from Breast Cancer Campaign is gratefully acknowledged.

S12

Benefits of combined treatments using antiresorptive agents and cytotoxic drugs

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Breast Cancer Research 2006, **8(Suppl 2):**S12 (DOI 10.1186/bcr1555)

Background Breast cancer patients often receive a combination of different therapies, but our understanding of how best to utilise such combinations to achieve maximal benefit for the patients is incomplete. We have investigated the ability of the antiresorptive agent zoledronic acid (Zol) and the commonly used chemotherapy agents paclitaxel (Pac) and doxorubicin (Dox) to induce apoptotic breast cancer cell death *in vitro*.

Methods Hormone-dependent (MCF7) and hormone-independent (MDA-MB-436) breast cancer cells were treated with increasing doses of Zol, alone and in sequence or combination with a low dose of Pac (2 nM) or Dox (0.05 μM) for 1–72 hours. The following treatment groups were used: (A) untreated controls, (B) each drug given as a single agent, (C) the drugs given simultaneously, (D) chemotherapy agent followed by Zol, and (E) Zol followed by the chemotherapy agent. In some cases Zol was given together with GGOH, a downstream component of the mevalonate pathway targeted by Zol. The effects of the different treatments on both apoptotic and necrotic cell death were determined at 72 hours, by evaluation of nuclear morphology following staining with Hoechst and PI. The effects of the various treatments on the cell cycle distribution were also determined.

Results Our data show that exposing breast cancer cells to the chemotherapy agent prior to Zol results in a synergistic increase in tumour cell death, compared with when the drugs are used as single agents. This was seen both for paclitaxel and doxorubicin, and the effect was found to be associated with changes in the cell cycle distribution following pretreatment with the cytotoxic drug. The synergistic increase in tumour cell death could be reversed by addition of GGOH, a compound that counteracts the effects of Zol on a key metabolic pathway, supporting an essential role of Zol in the toxic effects of the combined treatments. We also show that these effects are significant using clinically achievable doses and exposure times, suggesting that sequential treatments may be relevant also in a clinical setting.

Conclusions We have shown that combining chemotherapy agents and the antiresorptive drug Zol results in a synergistic increase in breast cancer cell death *in vitro*. We are currently investigating whether the same is seen using more complex *in vivo* model systems. Our data suggest that in order to achieve maximum benefit from combined treatments, the order and timing of the combinations are crucial.

Poster abstracts

P1

Loss of C-terminal binding protein transcriptional corepressor leads to aberrant mitosis and cell death in breast cancer cells

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Breast Cancer Research 2006, **8(Suppl 2):**P1 (DOI 10.1186/bcr1556)

C-terminal binding proteins (CtBPs) are transcriptional corepressors that regulate the activity of proteins important for a wide variety of cellular processes, including development, proliferation, differentiation, and transformation. Many targets of CtBP corepression are members of pathways involved in tumorigenesis, and evidence is emerging that CtBPs also play a role in cell survival. Loss of CtBP in different experimental systems leads to upregulated expression of a number of proapoptotic genes and increased sensitivity to apoptosis.

In this study, we have continued investigation into the role of CtBPs in breast cancer cell survival, identifying a previously unknown function for CtBPs in the regulation of the mitotic spindle checkpoint. Loss of CtBP expression by RNAi results in a marked decrease in cell number, and in reduced cell viability and clonogenicity. We find that this apparent cell death does not occur by a traditional caspase-mediated apoptotic pathway.

Detailed microscopic analysis of the morphology of MCF7 breast cancer cells lacking CtBPs reveals an increase in the number of cells containing abnormal micronucleated cells and dividing cells with lagging chromosomes, indicative of aberrant mitotic chromosomal segregation. Live cell imaging reveals defects in cell abcission after mitosis following CtBP knockdown. Furthermore, cells lacking CtBP fail to undergo mitotic arrest induced by spindle toxins, indicating a spindle checkpoint defect. The loss of cell viability in breast cancer cells following CtBP inhibition is most probably a consequence of aberrant mitosis and cell death by mitotic catastrophe. Here we present a detailed characterization of the mechanism by which CtBPs are involved in mitosis and cell survival, which we hope will increase our understanding of how breast cancer cells evade cell death, and ultimately lead to new treatments for patients.

Acknowledgement This research was funded by Breast Cancer Campaign.

P2

Phenotypic characterization of mouse mammary epithelial stem and progenitor cells

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Breast Cancer Research 2006, **8(Suppl 2):**P2 (DOI 10.1186/bcr1557)

Elucidation of the genes controlling the proliferation and differentiation of mouse mammary epithelial stem (MaSC) and progenitor (Ma-CFC) cells is paramount to understanding the processes that regulate mammary gland development and breast cancer progression. We have previously described a strategy in which MaSC and Ma-CFC can be purified to 5% and 15%, respectively, on the basis of lack of expression of the hematopoietic and endothelial markers CD45, Ter119 and CD31 and on the differential expression of CD24 and CD49f [1], with the MaSC having a $CD24^{med}CD49f^{high}$ phenotype and the Ma-CFC having a $CD24^{high}CD49f^{low}$ phenotype. Currently, a definitive analysis of the gene expression profiles of MaSC and Ma-CFC is not possible due to the presence of large numbers of contaminating cells in these enriched subpopulations. However, a preliminary microarray analysis of these subpopulations has identified potential new cell surface markers that can be exploited to further purify MaSC and Ma-CFC. We have initiated a screening program using the markers identified in the microarray analysis as well as markers used to identify other adult tissue stem cells to further purify and characterize MaSC and Ma-CFCs. Results of this screen will be presented.

Acknowledgement This work is supported by Breast Cancer Campaign.

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P3

Characterisation of the tumour suppressor gene *ZAC* in breast tissue

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Breast Cancer Research 2006, **8(Suppl 2):**P3 (DOI 10.1186/bcr1558)

ZAC (also known as *PLAGL1/LOT1*) is a transcription factor gene located on chromosome 6q24, a region that is frequently deleted in solid tumours. *ZAC* is known to promote cell cycle arrest and apoptosis, and loss of expression has been observed in several different cancers including primary breast tumours and breast cancer cell lines. Due to its antiproliferative properties, the downregulation or loss of this gene would be expected to deregulate cell growth. *ZAC* has also been shown to act as a transcriptional coactivator of nuclear receptors, including oestrogen receptors which are important as prognostic indicators and therapeutic targets in breast cancer.

ZAC is maternally imprinted in most tissues. Its promoter is believed to be located within a differentially methylated CpG island, and it directs transcription exclusively from the unmethylated paternal allele. As this imprinted promoter has been shown to be hypermethylated in ovarian cancer and breast cancer cell lines, similar epigenetic changes may occur in primary breast tumours, and may contribute to altered cell cycle regulation and thus tumour growth. In some tissues, however, *ZAC* expression is biallelic. We are currently studying the mechanism underlying this tissue-specific phenomenon. A detailed understanding of the way in which the expression of imprinted and nonimprinted transcripts is regulated in normal breast tissue will be required in order to allow analysis of the epigenetic mechanism for *ZAC* inactivation in breast tumours.

Acknowledgements This study is funded by Breast Cancer Campaign and The West Riding Medical Research Trust.

P4

Role of BRCT motif containing proteins in Chk1 activation

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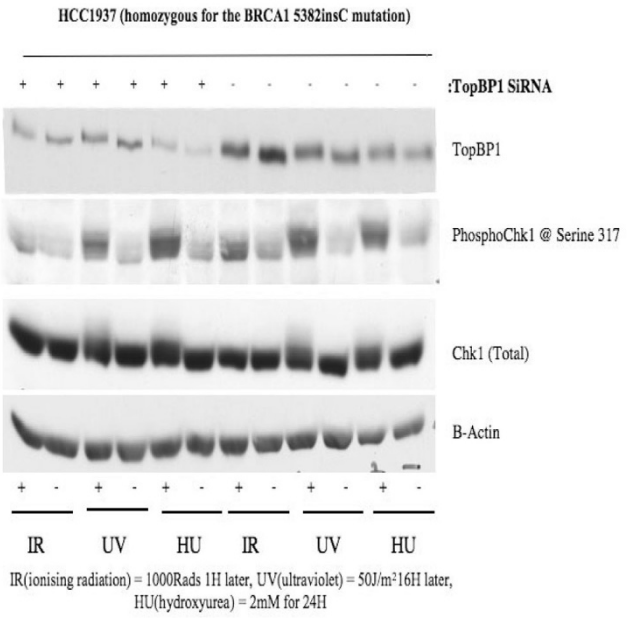
Breast Cancer Research 2006, **8(Suppl 2):**P4 (DOI 10.1186/bcr1559)

Introduction Chk1, along with Chk2, regulates processes such as DNA replication, cell cycle control, chromatin restructuring and apoptosis. DNA damage/replication stress activates Chk1 by phosphorylation from the PI3/PI4 family of kinases. Activation of Chk1 is thought to be mediated by proteins containing the BRCA1 C-terminal domain (BRCT). We previously identified a potential complex of four Chk1-associated proteins by immunoprecipitation, western blotting and mass spectrometry, one of which is BRCA1. Germline mutations in BRCA1 are responsible for many cases of hereditary breast cancer, and cells deficient in BRCA1 sustain spontaneous aberrations in chromosome structure. Such findings indicate that BRCA1 is essential for suppressing genome instability.

Method and results Studies have concentrated on the role of BRCA1, with other BRCT-motif proteins, in the regulation of Chk1. Through immunoprecipitation assays and analysis of the phosphorylation status of Chk1, in both wildtype and mutated *BRCA1* cell lines, we have shown that although BRCA1 forms a complex with Chk1, it is not essential for the activation of Chk1 in response to either stalled replication forks (induced by hydroxyurea) or double-stranded DNA breaks (induced by ionising radiation). In contrast, we have observed that the loss of both BRCA1 and the knockdown of the fission yeast rad4/Cut5 related protein Topisomerase II binding protein 1 (TopBP1) inhibit activation in response to DNA damage but not stalled replication forks (Figure 1). However, the knockdown of TopBP1 alone was insufficient to inhibit activation.

Conclusion Inhibition of Chk1 activation in response to ionising radiation requires the loss of both TopBP1 and BRCA1, suggesting redundancy. In addition, as the response to hydroxyurea, or UV, was unaffected, it seems likely that different proteins are involved in Chk1

Figure 1 (abstract P4)



Loss of Topisomerase II binding protein 1 (TopBP1) and BRCA1 inhibits Chk1 activation after ionising radiation.

activation in response to differing stimuli. Analysis of other Chk1 binding proteins continues determining whether they are involved in Chk1 activation in response to stalled replication forks and/or double-stranded DNA breaks. As Chk1 is involved in maintaining tumor cell viability following activation of the replication checkpoint, the Chk1-regulated checkpoint(s) may protect cells from ionizing radiation-induced killing. The ability to delineate the control mechanisms of Chk1 is of critical importance in order to target Chk1 with the aim of increasing the selectivity and specificity of anticancer drug treatments.

Acknowledgement Breast Cancer Campaign funded the project.

P5

The *NEUREGULIN1* gene and breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P5 (DOI 10.1186/bcr1560)

Background It has long been suspected that there is a tumour suppressor gene on chromosome 8p, and our array CGH data [1] suggest that it may be close to the *WRN* and *NEUREGULIN1* (*NRG1*) genes. *NRG1* encodes growth factors that function as ligands for the tyrosine kinases ErbB3 and ErbB4, and can both stimulate cell proliferation, differentiation and apoptosis. We previously showed that many breast carcinoma (that is, 39% of cancer cell lines and 6% of breast tumours) have chromosome breakpoints in *NRG1*, suggesting that the gene plays an important role in tumourigenesis [2,3], and our initial hypothesis was that the translocations activate expression.

Results Our current work shows that *NRG1* expression is silenced in many breast cancer cell lines (17 out of 23 lines), as compared with normal breast cell lines. Western blotting experiments also indicate that *NRG1* is downregulated at the protein level. To investigate whether *NRG1* maybe repressed by epigenetic mechanisms, we examined DNA methylation at a CpG island present in the promoter and the first exon of the gene using bisulphite sequencing. This region is heavily methylated in 76.5% (13/17) of breast cancer cell lines that have no *NRG1* expression. In contrast, the region is relatively unmethylated in normal breast lines, and in cancer cell lines expressing *NRG1*. Treatment of cancer cell lines with 5-aza-2-deoxycytidine, which abolished DNA methylation, activated the expression of *NRG1* by 7–100 times.

Conclusion These results suggest that DNA methylation is a key mechanism that silences *NRG1* expression in breast cancer cells, and our current view is that *NRG1* could be the long-sought tumour suppressor on 8p, with the translocations either inactivating the gene or producing aberrant transcripts.

Acknowledgement The authors thank Breast Cancer Campaign for funding.

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P6

hCLK2 couples FANCD2 to stalled replication forks and functions in the mammalian S-phase checkpoint

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Breast Cancer Research 2006, **8(Suppl 2):**P6 (DOI 10.1186/bcr1561)

Background Recent work has highlighted interplay between components of the Fanconi anemia (FA) pathway, an inherited genome instability syndrome characterized by hypersensitivity to DNA interstrand cross-links (ICLs), and the breast cancer susceptibility proteins BRCA1 and BRCA2/FANCD1. Is has also been suggested that certain defects within FANCD2, which is the central factor in the FA pathway, may lead to an increased risk of sporadic breast cancer.

Methods Mass spectrometry and candidate western blotting analyses were carried out on FCD-2 immunoprecipitates from untreated and cisplatin-treated whole worm extracts.

Results Using the nematode worm as a model system, we have identified the circadian protein CLK-2 and ATL-1 (*C. elegans* ATR) as factors that coimmunoprecipitate with *C. elegans* FANCD2 (FCD-2) following ICL damage. *C. elegans atl-1* and *clk-2* mutants and siRNA depletion of human hCLK2 (KIAA00693) compromises FCD-2/FANCD2 recruitment to blocked replication forks and confers ICL sensitivity, a hallmark of FA. Cells deficient for hCLK2 are also defective for damage-induced mono-ubiquitylation of FANCD2 and exhibit radio-resistant DNA synthesis indicative of an S-phase checkpoint defect. ATR activation leading to BRCA1-mediated ubiquitylation remains intact in hCLK2 depleted cells, yet ATR-dependent phosphorylation of Chk1 and Claspin is severely attenuated following S-phase insults. Finally, recruitment of the homologous recombination factor RAD51 is also impaired in cells depleted of hCLK2, which leads to a reduced homologous recombination frequency at sites of DNA damage.

Conclusion These data indicate that the novel factor hCLK2 is an essential component of the mammalian S-phase checkpoint required to coordinate both FA and HR-mediated repair responses following replication stress.

P7

Poly(ADPribosyl)ation of CTCF: role in breast tumourigenesis

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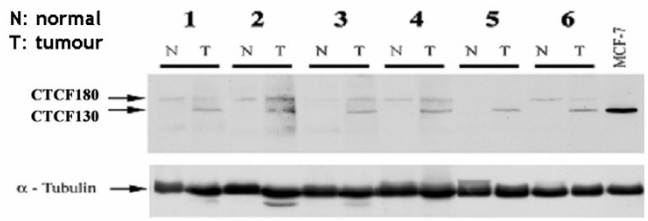
Breast Cancer Research 2006, **8(Suppl 2):**P7 (DOI 10.1186/bcr1562)

Background CTCF is a conserved, ubiquitous and multifunctional 11 Zn finger (ZF) transcription factor with features of a tumour suppressor. CTCF regulates transcription in diverse modes, such as promoter activation and repression, silencing, constitutive and methylation-dependent chromatin insulation. We have previously reported that CTCF can be post-translationally regulated by poly(ADPribosyl)ation and that this modification modulates the insulator function of CTCF [1,2]. The purpose of the present study is to investigate the role of CTCF poly(ADPribosyl)ation in normal and breast cancer cells.

Methods The following techniques have been used in this investigation: western analysis, mass spectrometry, immunoprecipitation, cell cultures, transient transfection, primary cultures from normal and tumour tissues, cellular fractionation and laser capture microdissection.

Results Using a large panel of breast tumours and paired peripheral tissues, we have discovered that only the poly(ADPribosyl)ated isoform of CTCF (called CTCF180) is detected in normal breast tissues, whereas the other isoform of CTCF (called CTCF130) only appears in breast tumour tissues and immortalised cell lines (see Figure 1). The identity of the poly(ADPribosyl)ated isoform of CTCF was further verified by mass spectrometry. We are currently establishing primary cultures from normal and tumour tissues in order to investigate whether the appearance of CTCF130 is linked to immortalisation. The histological type of cells containing CTCF180 and CTCF130 is being determined by cellular fractionation and laser capture microdissection of breast

Figure 1 (abstract P7



Western blot analysis of six paired samples (1–6) of normal and tumour tissues (a representative western blot of total 56 paired samples is shown). Fifty micrograms of the total protein were used in this assay. The bands of the CTCF 180 kDa and 130 kDa isoforms were quantified and their additive values were normalized to the corresponding values of α-Tubulin used as loading control (bottom panel).

tissues. Finally, experiments using cell culture models suggest that the generation of the CTCF180 can be associated with cell cycle arrest.

Conclusion This research addresses the molecular mechanisms of breast tumourigenesis: CTCF180 and CTCF130 may regulate different sets of genes and/or different cell functions specific for normal and cancer cells, respectively. The loss of CTCF poly(ADPribosyl)ation could also lead to epigenetic disturbances. Our data obtained so far indicate that the transition from CTCF180 to CTCF130 could be a hallmark of tumour development. We envisage the potential use of both CTCF isoforms as biological markers for breast tumourigenesis.

Acknowledgements This work was funded by Breast Cancer Campaign, The Medical Research Council, and The University of Essex.

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P8

Investigating the role of Wnt signalling in lobuloalveolar development of the mammary gland

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Breast Cancer Research 2006, **8(Suppl 2):**P8 (DOI 10.1186/bcr1563)

The Wnt signalling pathway regulates postnatal lobuloalveolar development. Expression of Wnt inhibitors blocks lobuloalveolar development, whereas expression of Wnt pathway activators induces precocious lobular development. Wnt ligands have been suggested to operate by regulating the proliferation and differentiation of lobuloalveolar progenitor cells during pregnancy. However, the lobular developmental switch is difficult to study using current experimental systems due to a mammary-specific 'Catch 22' in which promoters such as MMTV and WAP are only expressed after commitment to the lobular lineage. We are therefore developing an inducible transgene expression system which expresses Wnt regulators in all mammary epithelial cell types prior to and during lobuloalveolar development. In addition we are using Wnt-reporters to identify Wnt-responsive cells during these early developmental stages and aim to use stem cell markers to further characterise this subset of cells. Many studies support the idea that breast cancer results from oncogenic changes to mammary stem cells. This work should help establish the role Wnt signalling plays in the expansion of lobular progenitor cells and investigate the effect that switching the Wnt pathway on or off has on lobuloalveolar development.

P9

Inhibitor of apoptosis proteins in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P9 (DOI 10.1186/bcr1564)

Background and methods Resistance to apoptosis is a hallmark of cancer. Decreased sensitivity to apoptosis leads to an elevated therapeutic threshold for classical interventions such as chemotherapy or radiotherapy. The Inhibitor of Apoptosis Proteins (IAPs) are a family of proteins that prevent caspases from inducing apoptosis. Targeting IAPs therefore represents a potential avenue for reducing the threshold to apoptosis and improving therapeutic effectiveness. There are eight human IAP family members, including XIAP, Survivin, cIAP1, cIAP2, Livin, NAIP and Apollon. Although some studies have indicated altered levels of Survivin and XIAP in several tumour models, no study to date has examined the role of all members of the IAP family in cancer progression. We aim (i) to investigate the expression of the whole family of IAPs across a wide range breast cancer cell lines and tumour samples at both the RNA and protein level, and (ii) to determine whether targeting IAPs alters susceptibility to apoptosis.

Results Preliminary results confirm that the levels of Survivin and XIAP vary across a breast cancer cell line panel. Expression of the other IAP family members is currently being determined.

IAP expression will be correlated to the ER, PR, p53, Erb2, and EGFR status of the cell lines and tumours, to determine whether there is a relationship between IAP expression and prognostic indicators. Overexpression and siRNA-induced knockdown approaches will be used to investigate whether altering the expression of IAPs identified in our original screen affects the apoptotic threshold in response to various chemotherapeutic agents. This will be examined using both 2D and 3D cell culture systems.

P10

Characterization of peptide aptamers to the Anterior Gradient 2: a novel inhibitor of the tumour suppressor protein p53

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Breast Cancer Research 2006, **8(Suppl 2):**P10 (DOI 10.1186/bcr1565)

Anterior Gradient-2 was identified using proteomic technologies as a protein overexpressed in human cancers. We show here properties of AG2 suggesting it has proto-oncogenic properties:(i) the AG2 protein and gene are unregulated in a tamoxifen resistance panel of breast cancer cells lines, (ii) cell lines overproducing AG2 have an elevated clonogenic activity *in vitro* and also increase cell growth in a xenograft model, (iii) AG2 protein inhibits the tumour suppressor protein p53, and (iv) AG2 localizes to the endoplasmic reticulum, suggesting a proto-onocogenic signalling pathway exists from endoplasmic reticulum to the nucleus to inhibit p53.

To validate the AG2-mediated endoplasmic reticulum pathway as a possible drug target, we developed peptide aptamers to AG2 protein in order to determine whether the oncogenic properties of the protein can be altered by the peptide ligand.

These studies hold promise for developing new types of drugs that can release and reactivate the tumour suppressor p53 in breast cancers.

P11

Role of CASP8 D302H and other apoptosis gene variants in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P11 (DOI 10.1186/bcr1566)

Background It is well established that perturbations in high penetrance genes such as *BRCA1* and *BRCA2* predispose to breast cancer. However, low penetrance genes are still under investigation. Some

apoptotic genes (for example, *BIRC5*, *BCL2*, *DR4* and *DR5*) have been implicated, and we reported that a coding single nucleotide polymorphism (SNP) in the caspase 8 gene (*CASP8* D302H) is associated with a reduced risk of breast cancer [1]. We hypothesise that *CASP8* and other apoptotic genes may play an important role in breast cancer susceptibility. The objectives were to study the functional effect of *CASP8* D302H on apoptosis, and to perform a case–control analysis of other *CASP8* variants to determine their effect on breast cancer susceptibility.

Methods Apoptotic activity in peripheral blood lymphocytes (PBLs) was measured using Annexin-V FITC with propidium iodide and FACs analysis. Genotyping was conducted by TaqMan™ (ABI, UK).

Results We detected a 68% increase in apoptosis in PBLs after treatment with CD95 ligand (R & D Systems, UK) with anti-CD95 antibody (BioLegend, UK), and are currently optimising this assay as a functional screening tool. We identified 50 SNPs in *CASP8* by database searching, and 15 more putative SNPs were sequenced, one of which is novel (T51087A in *exon 13*). Using data from 33 SNPs with a minor allele frequency >0.05 and various haplotype-tagging SNP (htSNP) selection programs, results suggested that 11 htSNPs (PCA method) need to be genotyped to adequately capture common genetic variation within *CASP8*. A case–control study of these 11 htSNPs is in progress.

Conclusion These methods will be used to address the hypothesis that apoptotic genes are involved in breast cancer susceptibility and treatment outcome. In the future, this research will help us understand the role of the whole pathway and whether it will be amenable to manipulation by targeted treatments.

Acknowledgements This work was funded by Breast Cancer Campaign and Yorkshire Cancer Research.

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P12

Functional analysis of the breast cancer associated transcriptional repressor PLU-1/JARID1B

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Breast Cancer Research 2006, **8(Suppl 2):**P12 (DOI 10.1186/bcr1567)

Background The *PLU-1/JARID1B* gene, which is upregulated in breast cancers, encodes for a 1,544-amino-acid multidomain protein that is exclusively localised to the nucleus. The protein contains several conserved domains, including the ARID DNA binding domain, both N and C jumonji domains, three PHD domains and putative nuclear localisation signals, indicating that it could regulate the transcription of specific genes either through direct binding or through other transcription factors [1,2].

In this study, we aim to identify the target genes regulated by PLU-1/JARID1B and the possible mechanism of PLU-1/JARID1B-mediated transcriptional regulation.

Methods Co-immunolocalisation and/or co-immunoprecipitation of PLU-1/JARID1B with HDACs were carried out using anti Myc/HisA antibodies or an antiserum (αPLU-1-C) against PLU-1/Jarid1B after transient transfection of Cos and MCF7 cells with expression vectors coding for Myc or HisA tagged proteins. Direct interactions of PLU-1/JARID1B expressed from a baculovirus with *in vitro* translated HDACs were also demonstrated. *In vitro* mutagenesis and reporter assays were also used. HB2 and MCF7 cells were subjected to microarray using the Affymetrix gene chip HG-U133A after transduction with a recombinant adenovirus or silencing the endogenous gene using a short hairpin RNA (shRNA) expression vector (Imgenex). ChIP assays were carried out using the αPLU-1-C specific antiserum or an antibody against the acetylated form of Histone H3. PCR-assisted DNA binding selection from a random pool of oligonucleotides was carried out using *in vitro* translated full-length PLU-1/JARID1B and GST-PLU-1-ARID.

Results PLU-1/JARID1B binds to chromatin and the nuclear matrix and localises in MAD bodies when co-transfected with class IIa histone deacetylases (HDACs) or *N*-CoR. Direct binding to class I and class IIa HDACs is demonstrated using co-immunoprecipitation assays and binding of PLU-1/JARID1B to *in vitro* translated HDACs. Two PHD domains in PLU-1 were shown to be crucial for binding to a domain in the N-terminal region of HDAC4 and for the transcriptional repression. Approximately 100 target genes were identified by microarray analysis after overexpressing or silencing the human PLU-1/JARID1B gene in human mammary epithelial cells using adenovirus and RNA interference systems, respectively. Most of the candidate genes were downregulated by PLU-1/JARID1B overexpression, including the *mellathionein* (MT) genes, the *BRCA1* gene, and genes involved in the regulation of the spindle and G2/M checkpoints such as *BUBR1*, *BUB3*, *STK6*, *TTK*, *CDC2* and *Cyclin B1*. ChIP assays confirmed that the MT1H, MT1F and MT1X genes are direct transcriptional targets of PLU-1/JARID1B, and that PLU-1/JARID1B affects the level of acetylation of the promoter of the MT1H gene. Some other candidate genes such as BRCA1 may be downregulated indirectly. The PLU-1/JARID1B ARID domain preferentially binds to a GCACA motif, a putative consensus sequence that is abundant in MT promoters.

Conclusion The downregulation of the metallothionein genes, checkpoint genes and BRCA1 by PLU-1/JARID1B overexpression is of great interest and could be highly relevant to any role this protein plays in the development and progression of breast cancer.

Acknowledgements This work was supported by a Programme grant to JT-P and a competitive post doctoral fellowship to AGS, both from Cancer Research UK, and by King's College London.

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P13

Potential role of cyclin D_1 in DNA damage response

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Breast Cancer Research 2006, **8(Suppl 2):**P13 (DOI 10.1186/bcr1568)

Background In mammalian cells, cell cycle progression is governed by distinct cyclin-dependent kinases (cdks) whose activities are regulated by binding of their activating cyclin subunits and through negative regulation by inhibitor proteins such as p21. Cyclin levels oscillate in a phase-dependent manner, ensuring the stage-specific activation of cyclin/cdk complexes. The D-type cyclin levels are thought to act as sensors of the cellular environment: under conditions permissive for proliferation, D-type cyclins accumulate and facilitate the G_1 phase progression; whereas under restrictive conditions, D-type cyclin transcription is attenuated and the protein is destabilised via ubiquitin-mediated proteolysis. In addition to the normal cell cycle regulation, a member of D-type cyclins, cyclin D_1, has been implicated in the DNA damage response. Once activated, DNA damage responses disrupt the function of the cell cycle and can result in a number of outcomes including short-term or long-term cell cycle arrest, apoptosis and necrosis. Cyclin D_1 expression is often found deregulated in cancerous cells, particularly in those of the breast and the head/neck.

Results Preliminary data showed that the expression of cyclin D_1 responds to the DNA damage induced by an environmental carcinogen, 4-nitroquinoline 1-oxide (4NQO), in a biphasic manner. At a low level (2.5 μM), the cyclin D_1 level is unchanged but p21 is induced strongly after 3 hours; at intermediate levels (10–50 μM), there is a dramatic reduction in the level of cyclin D_1 while p21 fails to accumulate; at high levels (100–200 μM), little change in cyclin D_1 or p21 is observed. The cellular responses associated with different 4NQO doses analysed by flow cytometry will be presented.

Conclusion Our findings suggest that the level of cyclin D_1 following the DNA damage induced by 4NQO may play a role in dictating the outcome of the cellular response. Our ongoing research aims to compare and contrast the cellular responses linked to various specific DNA damaging agents in terms of cell cycle regulatory proteins, focusing on cyclin D_1, and ultimately to understand the molecular mechanisms underlying the regulation of such responses.

Acknowledgements Linda Jacobs Breast Cancer Campaign PhD Studentship and Overseas Research Students Award.

P14

Functional analysis of normal and DCIS modified breast myoepithelial cells

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Breast Cancer Research 2006, **8(Suppl 2):**P14 (DOI 10.1186/bcr1569)

Background Normal breast myoepithelial cells have been shown to exhibit tumour-suppressor activity mediated, in part, by downregulation of MMP expression [1]. DCIS myoepithelial cells have an altered phenotype as demonstrated by a different gene expression profile [2]. We have identified upregulation of $\alpha(v)\beta_6$ integrin on myoepithelial cells in a subset of DCIS; however, the role of $\alpha(v)\beta_6$ in this context is not clear. $\alpha(v)\beta_6$ is not expressed by normal epithelial cells, but is expressed in some cancers where it promotes tumour cell invasion and enhances MMP expression.

Methods The purpose of this project is to investigate the hypothesis that DCIS-associated myoepithelial cells lose their tumour suppressor effect and acquire a tumour promoting activity. There are three general aims: (1) to generate a series of myoepithelial cell models to mimic DCIS-associated myoepithelial cells and overexpress $\alpha(v)\beta_6$ to assess the contribution of this integrin; (2) to compare tumour suppressor/promoter properties of normal, $\alpha(v)\beta_6$ overexpressing and DCIS-associated myoepithelial cells; and (3) to examine the effect of *de novo* $\alpha(v)\beta_6$ expression on the biological activity of myoepithelial cells.

Results We have fully characterised an immortalised myoepithelial cell line, engineered it to overexpress $\alpha(v)\beta_6$ and determined that it is functional. We are starting to examine the morphology and phenotype of these cells to determine any differences, and we have been able to show the parental cell line is able to recapitulate the tumour suppressor effect in *in vitro* systems. We are now looking into what effect the expression of $\alpha(v)\beta_6$ has in these systems. We are also in the process of trying to create further myoepithelial cell lines from primary cells isolated from patient tissue.

Conclusion Through this work we hope to identify the role $\alpha(v)\beta_6$ expression has in DCIS myoepithelial cells with the goal of making this integrin a viable therapeutic target in the future.

Acknowledgement This work was funded by Breast Cancer Campaign.

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P15

AGR2, a novel metastasis inducing protein with an effect on breast cancer patient survival

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Breast Cancer Research 2006, **8(Suppl 2):**P15 (DOI 10.1186/bcr1570)

Background In order to provide potential diagnostic markers and to identify potential targets for breast cancer therapy, gene products that are differentially expressed between benign and malignant cells have been isolated and identified by a combination of PCR-selected suppression subtractive libraries [1,2] and inhouse cDNA microarrays, screened using mRNAs from human breast cancer specimens. A number of the cDNAs were differentially expressed by greater than twofold, including the one for AGR2, the secreted human homologue of a Xenopus developmental protein.

Methods and results In an *in vivo* model system of metastasis, AGR2 induced metastases compared with no metastases in the control groups [3]. In immunocytochemistry with an inhouse affinity-purified AGR2 antiserum [3], the presence of AGR2 protein in tumour specimens was statistically significantly associated with malignancy, with oestrogen receptor (ER) alpha-positive carcinomas, with low histological grade and with reduced patient survival over a 10-year period of follow-up of a group of ER-positive cases [4].

Conclusions Our results demonstrate that AGR2 is causatively involved in metastasis and associated with poor outcome in patients with breast cancer, indicating that AGR2 might be a valuable new potential diagnostic marker and possible target for breast cancer therapy. Further studies are essential to understand the mechanism of AGR2-induced metastasis.

Acknowledgements The authors thank Clatterbridge Cancer Research Trust, The Cancer and Polio Research Fund Ltd and the Higher Education Funding Council for financial support.

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P16

Insulin-like growth factor signalling in oestrogen nonresponsive breast cancer cells

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Breast Cancer Research 2006, **8(Suppl 2):**P16 (DOI 10.1186/bcr1571)

Background Insulin-like growth factors (IGFs) regulate normal growth and development. In breast cancer, they stimulate cell proliferation, cell migration and inhibit apoptosis. The IGF signal transduction pathway is, therefore, a potential therapeutic target in the treatment of breast cancer [1,2]. Inhibitors of the IGF pathway may be effective in the treatment of breast cancer with *de novo* or acquired endocrine resistance. We have studied IGF signalling in oestrogen nonresponsive

MDA-MB-231, HBL-100 and BT-20 breast cancer cell lines as models of endocrine resistant breast cancer. Oestrogen responsive MCF-7 cells were also studied.

Results Components of the IGF signalling pathway, type I IGF Receptor (IGF1R), IRS-1, IRS-2, and the three Shc isoforms, were expressed at varying levels, demonstrating a range of phenotypes in the breast cancer cells. IRS-1 is expressed in a truncated form in the BT-20 cells as an antibody to the C-terminus is unable to detect the protein.

IGF-1 activated IGF1R, IRS-1, MAP kinase and Akt in the MCF-7, MDA-MB-231 and HBL-100 cell lines. IGF-1 stimulated phosphorylation of IGF1R in BT-20 cells but did not alter the level of activation of IRS-1, MAP kinase or Akt. The MEK1/2 inhibitor (PD 98059) and the PI-3 kinase inhibitor (LY 294002) decreased the level of phosphorylation of MAP kinase and Akt in BT-20 cells. A phosphospecific antibody to tyrosine 896, the Grb2 SH2 binding site, shows that IRS-1 is constitutively phosphorylated in BT-20 cells.

IGF-1 inhibited staurosporine-induced apoptosis in MCF-7, MDA-MB-231 and HBL-100 cells but not in BT-20 cells. Inhibition of the IGF signalling pathways with PD 98059 and LY 294002 sensitise MDA-MB-231 cells to staurosporine-induced apoptosis. IGF-1 stimulated growth in MCF-7 and MDA-MB-231 cells but not in BT-20 cells.

Conclusion Expression and activation of IGF signalling proteins vary among the oestrogen nonresponsive cells. These differences will affect the response of breast cancer cells to IGF targeted therapy. BT-20 cells provide a useful model for constitutive IRS-1 phosphorylation which is reported to occur in breast tumours [3].

Acknowledgement This project was funded by Breast Cancer Campaign.

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P17

Role of the Brk tyrosine kinase in breast cancer progression

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Breast Cancer Research 2006, **8(Suppl 2):**P17 (DOI 10.1186/bcr1572)

Background The Brk tyrosine kinase is expressed in approximately two-thirds of human breast carcinomas, including lymph node metastases, but neither in normal mammary tissue nor benign lesions. This study tested the hypothesis that Brk is involved in regulating the tumour cell environment during progression and investigated the effects of suppressing Brk in breast carcinoma cells to determine in which contexts Brk may be a valid therapeutic target.

Methods We investigated whether Brk regulates the production of extracellular matrix enzymes and angiogenic cytokines, and whether Brk influences cell migration and chemotaxis. Studies to determine whether modification of Brk expression affects tumour behaviour *in vivo* are currently ongoing.

Results We have shown that suppression of Brk expression by RNA interference significantly decreases the secreted level of the matrix degrading enzyme MMP9 and the cytokine VEGFA, suggesting a role for Brk in regulating some of the processes involved in metastasis (proteolytic activity and neo-angiogenesis). As well as being able to modify the extracellular environment and to regulate angiogenic cytokine production, disseminating tumour cells must be able to survive in the circulation. We have also shown that Brk suppression increases the levels of cell death observed in breast carcinoma cells in suspension culture, implicating Brk in promoting anchorage-independent survival. In addition, suppression of Brk in suspension culture alters the relative levels of Bcl-x proteins in favour of Bcl-x_S. As elevated Bcl-x_L levels have been linked to chemotherapeutic resistance, targeting Brk may have benefits in overcoming chemoresistance in disseminating breast tumour cells.

Conclusions Taken together these data propose key functions for Brk in breast tumour development and progression. Therapeutically targeting Brk may have multiple effects in controlling the spread of breast cancer.

Acknowledgement This work was funded by a project grant from Breast Cancer Campaign.

P18

The emerging role of CD44 in regulating skeletal metastasis

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Breast Cancer Research 2006, **8(Suppl 2):**P18 (DOI 10.1186/bcr1573)

Background Bone metastasis is a frequent and often incurable complication of breast cancer causing severe bone pain, pathological fractures, spinal cord compression and hypercalcaemia. We have focused on establishing the significance of the cell surface hyaluronan receptor CD44 in underpinning the preferential metastasis of breast cancer cells to bone. In prior *in vitro* studies, we demonstrated that depletion of CD44 expression in breast cancer cells attenuates their adhesion to bone marrow endothelial cells (BMECs). Our recent experiments have also determined that the expression of CD44 is elevated in the bone homing breast cancer subline MDA231BO relative to that detected in the parental MDA231 breast cancer cell line. Together these experiments suggest a physiological role for this receptor in promoting the entry of breast cancer cells into the bone microenvironment.

Methods To further understand the potential significance of CD44 signalling to breast cancer metastasis, we established a tetracycline-regulated CD44 expression system in the minimally invasive, CD44-negative MCF7F cell line. Removal of tetracycline from the growth media resulted in time-dependent increases in CD44 expression in MCF7F cells, promoting increased cell invasion and migration responses in addition to potentiating the adhesion of MCF7F cells to BMECs. Subsequent microarray analysis was conducted using this expression system to identify CD44/HA regulated genes in breast cancer cells.

Results The expression and activation of CD44 was associated with increased expression of a subset of genes implicated in metastasis including proteolytic enzymes, growth factors and cytoskeletal proteins (for example, cortactin). Interestingly, the cysteine protease cathepsin K and the matrix metalloprotease MT1MMP were identified as CD44/HA regulated genes. These proteases target collagen I, a major component of the bone matrix whose degradation is a major consequence of osteolytic metastasis of breast cancer. Consistent with their respective metastatic potential, immunoblotting and ELISA based experiments have confirmed that the expression of MT1MMP and cathepsin K are both elevated in the MDA231BO bone homing cells relative to the parental MDA231 cells. In addition, the expression of cathepsin K and MT1MMP in the MDA231BO cells was significantly decreased upon RNAi-mediated suppression of CD44. Quantitative real-time PCR, immunoblotting and ELISA based experiments have also demonstrated that the transcript and protein expression of cathepsin K and MT1MMP increase in response to CD44/HA signalling in a panel of CD44-expressing breast cancer cell lines (MDA231, MDA157 and MCF7F). Currently, we are (i) investigating the mechanistic basis underpinning the transcription of these target genes in breast cancer cells, (ii) determining the functional significance of their overexpression in facilitating breast cancer cells to degrade a collagen I matrix, and (iii)

using the MDA231BO cell line to determine the *in vivo* significance of CD44 expression to osteolytic metastasis.

Conclusions It is consequently our hypothesis that CD44 may not only promote extravasation into the bone marrow but may also confer an osteoclast-like phenotype to the cancer cell, thus orchestrating the ability of cancer cells to initiate and regulate the modification of the bone matrix. The long-term objective of our research will be to determine whether CD44 expression and that of its transcriptional targets may be predictive for those breast cancer patients at higher risk of developing skeletal disease and/or potentially lead to the development of novel and more effective therapeutic strategies to attenuate bone metastasis.

Acknowledgement This work is funded by Breast Cancer Campaign.

P19

Evaluation of migration-stimulating factor expression for breast cancer diagnosis and prognosis

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Breast Cancer Research 2006, **8(Suppl 2):**P19 (DOI 10.1186/bcr1574)

Migration-stimulating factor (MSF) is a novel angiogenic factor present in most breast tumours but not in normal breast [1]. The purpose of this study is to ascertain the presence of MSF in serum and to determine its possible value for breast cancer diagnosis and prognosis. MSF bioactivity has been detected in the serum of 90% (27/30) of breast cancer patients, compared with 13% (4/30) of healthy controls. MSF-specific antibodies have enabled the identification of MSF in serum using immunoprecipitation and ELISA. Unexpectedly, quantification of immunoreactive MSF in serum showed no difference between cancer patients and controls. This discrepancy between bioactive MSF and immunoreactive MSF is due to the presence of two forms of MSF in serum, as well as a potent inhibitor of MSF (MSFI). Two isoforms of MSF have been cloned; these differ by a 15-amino-acid deletion and are referred to as MSF+aa and MSF−aa. MSF isolated from control serum behaves like rhMSF+aa, in that it is inhibited by MSFI and therefore is not bioactive in serum. MSF from cancer patient serum and rhMSF−aa are not inhibited by MSFI, and are bioactive in serum. Our next goal is to ascertain the biochemical difference between patient and control MSF and to assess the diagnostic and prognostic value of MSF-based serum measurements.

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P20

Functional analysis of altered Tenascin isoform expression in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P20 (DOI 10.1186/bcr1575)

Background Cellular interactions with the extracellular matrix (ECM) control many aspects of cell function. The complex ECM protein Tenascin-C (TN), which exists as multiple isoforms, is upregulated in breast cancer. We previously have identified a change in the TN isoform profile in breast cancer, with detection of two additional isoforms – TN16 and TN14/16 – not seen in normal breast [1]. The purpose of this study was to investigate directly the effects of these tumour-associated TNC isoforms on breast cancer cell behaviour.

Methods A PCR-ligation approach was used to generate specific TNC isoform sequences which were Flag tagged and inserted into a pCMV vector. Transient transfection into breast cancer cell lines or primary normal fibroblasts was confirmed by RT-PCR, western blotting and immunohistochemistry. The effect of different TNC isoforms on breast cancer cell invasion, proliferation and gene expression was analysed.

Results Expression of TN16 and TN14/16 in breast cancer cells (MCF-7, T47D, MDAMB231) resulted in significantly enhanced tumour invasion compared with adult-type truncated TN, large TN and vector-only controls. A similar increase in tumour cell proliferation was detected. Coculture of tumour cells with primary breast fibroblasts overexpressing TN16 or TN14/16 or conditioned medium from these fibroblasts also led to enhanced tumour cell invasion. Expression of TN resulted in upregulation of MMP-1; however, this was equivalent for all TN isoforms. The invasion-promoting effect of TN16 and TN14/16 was dependent on direct interaction between tumour cells and was blocked by incorporation of anti-TN blocking antibodies. Furthermore, TN appears to be essential for tumour cell invasion, since with all isoforms invasion was minimal in the presence of anti-TN antibodies.

Conclusion This study has demonstrated that the tumour-associated TN isoforms TN16 and TN14/16 significantly enhance breast cancer cell invasion and that blocking TN inhibits invasion. We aim to further investigate the invasion-promoting activity of these isoforms and to explore their therapeutic potential in more sophisticated tumour models.

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P21

Role of the metastasis suppressor tetraspanin CD82/KAI 1 in regulation of signalling in breast cancer cells

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Breast Cancer Research 2006, **8(Suppl 2):**P21 (DOI 10.1186/bcr1576)

Four transmembrane domain proteins of the tetraspanin superfamily are the organisers of specific microdomains at the membrane (tetraspanin-enriched microdomains (TERM)) that incorporate various transmembrane receptors and modulate their activities. Tetraspanin CD82 is frequently downregulated or absent in the metastatic cancers. In human prostatic cancer, downregulation of CD82 has been correlated with tumour progression, providing evidence for its role as a metastasis suppressor. We have shown recently that the overexpression of metastasis suppressor tetraspanin CD82/KAI1 led to the attenuation of epidermal growth factor receptor (EGFR) signalling, to an increased internalisation rate of the receptor and to the redistribution of EGFR at the plasma membrane [1]. Moreover, our latest data suggested that the effect of CD82 on the EGFR signalling is mediated by gangliosides [2]. Gangliosides (a subclass of glycosphingolipids) are essential structural components of distinct microdomains at the membrane. In addition, these glycosphingolipids are also involved in the regulation of signalling and tumour progression.

We currently demonstrate that inhibition of the glycosphingolipid biosynthetic pathway with specific inhibitors of glucosylceramide synthase (NB-DGJ and PPMP) resulted in specific weakening of the interactions involving tetraspanin CD82, including CD82–EGFR association. Furthermore, ectopic expression of the plasma membrane-bound sialidase Neu3 in mammary epithelial cells destabilised CD82-containing complexes. The destabilisation of these complexes correlated with the redistribution of the proteins within the plasma membrane. Importantly, depletion of gangliosides affected EGF-induced signalling only in the presence of CD82. Taken together our results provide strong evidence that gangliosides play an important role in supporting the integrity of CD82-enriched microdomains [3]. Furthermore, these data

demonstrate that the association between different proteins in TERM in mammary epithelial cells is controlled by distinct mechanisms.

In further experiments we are going to investigate the role of TERM, and specifically CD82-enriched microdomains, in the signalling through the ErbB3 receptor. The ErbB3 receptor is considered a major partner for the ErbB2 receptor and is involved in the progression of breast cancer.

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P22

Expression of adrenomedullin in long-term oestrogen-deprived human breast cancer cells

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Breast Cancer Research 2006, **8(Suppl 2):**P22 (DOI 10.1186/bcr1577)

Oestrogen is a major requirement for the growth of human breast cancer cells. Current treatments are aimed at reducing the action of oestrogen with antioestrogen therapy. However, many patients are able to progress to a state where they no longer respond to antioestrogen therapy. Long-term growth of breast cancer cell lines in the absence of oestrogen leads to the development of acquired resistance where the cells are able to grow without the addition of oestrogen, they can still be inhibited by antioestrogens and there is no loss of oestrogen receptor alpha. The aim of this work was to identify novel molecular markers that could indicate impending failure to endocrine therapy. Adrenomedullin is a 52-amino-acid peptide which may play a role in tumour survival and angiogenesis. Microarray data comparing oestrogen-maintained MCF7 cells with long-term oestrogen-deprived MCF7 cells showed that the expression of adrenomedullin mRNA was 12-fold upregulated after more than 1 year of culture in the absence of oestrogen. Real-time RT-PCR data were able to confirm the increase in the levels of adrenomedullin mRNA in long-term oestrogen-deprived cells. Immunocytochemistry using a monoclonal antibody specific for adrenomedullin was also able to show an increase in the amount of adrenomedullin protein in long-term oestrogen-deprived cells. Furthermore, long-term treatment of oestrogen-maintained cells with tamoxifen and fulvestrant led to an increase in the level of adrenomedullin mRNA which was not observed in long-term oestrogen deprived cells. Further validation with tumour samples is required to examine the importance of adrenomedullin as a possible marker of endocrine resistance in human breast cancer.

P23

Role of protein kinase B in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P23 (DOI 10.1186/bcr1578)

Breast cancer is the most common cancer in women and is increasing in both the developed and developing countries. There is an urgent need to understand the precise mechanisms of tumour development in breast cancer, to develop new treatment strategies and to identify predictive markers for tumour aggressiveness and therapy resistance. A protein called protein kinase B (PKB, also called Akt) is frequently elevated in breast cancers and has been implicated as a key player in breast cancer development and progression. The activation level of PKB is also thought to correlate with patient outcome. However, the function of the three isoforms of PKB in mediating the crucial responses is unknown. We have developed a set of antisense phosphorothioate oligonucleotide probes that target antisense-active regions in PKB and that enable >90% knockdown of all three known PKB isoforms (alpha, beta and gamma), either individually or in various combinations, including removal of all three isoforms together. We have demonstrated that these agents specifically and potently prevent the growth of breast cancer cells. Application of these antisense agents offers a unique opportunity to understand how PKB works and contributes to breast cancer, and to provide insight into the role of signalling by individual PKB isoforms in breast cancer cells. Such work may also identify clinically relevant markers of disease, thereby enabling better predictors of patient outcome, and provide the necessary intellectual framework for the development of PKB-isoform selective inhibitors (for example, antisense oligonucleotides, small chemical inhibitors) as novel therapeutic agents.

P24

Progress towards unlocking the secrets of oestrogen receptor beta in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P24 (DOI 10.1186/bcr1579)

Oestrogen receptor (ER) alpha remains the only reliable biological prognostic marker in breast cancer. A sister molecule, ERβ, has been described, but while ERα predicts a favourable disease outcome, the utility of ERβ as a clinical prognosticator is unclear. ERβ exists as five isoforms (ERβ1–ERβ5), each with a unique exon 8. The aim of our research is to understand the function of ERβ and its isoforms in the normal mammary gland and in breast cancer. We have previously shown high expression of total ERβ in normal gland with declining expression in the transition to breast tumours. LOH analysis in 27 paired samples of tumours and normal breast showed no correlation between LOH and loss of total ERβ expression by immunohistochemistry, indicating the latter was not a mutational event. Instead this was due to methylation as treatment of ERβ-negative cell lines resulted in re-expression of total ERβ at the protein and mRNA level. Furthermore, using methylation-specific PCR, ERβ was methylated in up to 50% of all tumours but not in matched normal gland. Recent immunohistochemical analysis of isoforms ERβ1–ERβ5 using specific well-validated antibodies in 777 invasive breast cancers with long-term clinical follow-up showed nuclear expression of ERβ2 was significantly correlated with tumour size, grade, NPI, overall survival, distant metastasis, death from breast cancer and ERα, PR, AR and BRCA1 expression. ERβ5 was predominantly expressed in high-grade cancers and showed a significant positive correlation with ERβ1. ERβ1, however, was not associated with any other pathological parameters. Using an antibody to detect total ERβ, positive tumours were more likely to develop distant metastasis. Notably, this study also highlighted the importance of cytoplasmic expression of ERβ in dictating outcome, a feature that had previously been reported but the significance of which had not been elucidated. In our study cytoplasmic staining, whether alone or in combination with nuclear staining, was associated with decreased overall survival. In summary, ERβ and its variants do seem to influence the breast cancer outcome. The data accumulated thus far and the importance of its sib ERα in directing breast cancer therapy create an imperative for us to continue to unlock its secrets.

P25

New molecular tools and optical technologies to dissect CXCR4 function in breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P25 (DOI 10.1186/bcr1580)

Background The objective was to study the relationship between CXCR4 expression (total and conformational subsets) and disease outcome in malignant breast disease. Initially, a retrospective study evaluating the clinical significance of CXCR4 expression (as determined by immunohistochemistry and immunofluroscence) with histopathological grade and clinical outcome of breast cancer patients were evaluated.

Methods Tumour specimens from breast cancer patients treated at the Breast Unit at Guy's Hospital London, with prospectively acquired long-term follow-up (25 years) were used in this study.

Using tissue microarrays (TMAs), of primary breast tumour specimens from a series of 252 invasive ductal and lobular carcinomas were immunolabelled for CXCR4. Polyclonal antibodies to human CXCR4 (Anti-Human CXCR4 ARP4016){CXCR4 peptide ARP 7039 N-terminal extracellular domain (1-38)} and two further anti-human CXCR4 cytoplasmic antibodies against two distinct peptides based on the membrane proximal sequence (GAKFKTSAQHALTSVSRG) and distal cytoplasmic sequence (VSTESESSSFHSS) of huCXCR4 cytoplasmic domain, were used to detect CXCR4.

The immunohistochemical detection of CXCR4 expression, was assessed by 2 independent pathologists (with consensus agreement). Both the proportion and intensity of expression was recorded for the total and subpopulations of CXCR4 recognised by ARP4016 and the two cytoplasmic antibodies, respectively.

For immunofluroscence the average fluorescence intensity/unit area of cells stained with the respective antibodies were plotted and quantified.

Results The proportion and intensity of invasive cells expressing CXCR4 was significantly less in Grade III infiltrating ductal carcinoma compared with Grades I, II and lobular types ($P < 0.0001$ by Kruskal-Wallis). There is a complex relationship between survival and total CXCR4 expression, with a subset of high CXCR4 expressing, Grade III tumours showing a trend towards poor prognosis. This association will be further elucidated by results of the CXCR4 cytoplasmic antibody staining.

Conclusions CXCR4 was expressed uniformly across a spectrum of normal, and a panel of invasive breast tumour cells but only a subset of Grade III tumours expressing high CXCR4 correlated with poor prognosis. It may be that only highly invasive cells that are metastatic and very poorly differentiated express functional CXCR4 receptors. CXCR4 function is subject to complex and potentially tightly controlled regulation in breast cancer cells via differential G protein receptor complex formation and this regulation may play a role in the transition from non metastatic to malignant transformation [1]. The application of new antibody tools and optical technologies to these pathological samples will assist the discovery of new biomarkers that will report on the function of CXCR4 *in situ*.

Acknowledgement This study was funded by Breast Cancer Campaign.

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P26

Regulation of cytochrome P450s in breast cancer and their role for tumour growth and anticancer chemotherapy

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Breast Cancer Research 2006, **8(Suppl 2):**P26 (DOI 10.1186/bcr1581)

Mammary cancer can develop for many reasons; one is the exposure to environmental carcinogens and/or steroid hormones. The cytochrome P450 enzyme family catalyses not only the metabolism of a wide range of carcinogens but is also involved in the metabolism of steroids. This process alters their steroidogenic properties, a mechanism important for mammary carcinogenesis.

At the centre of this research are cytochrome P450 1B1 (CYP1B1) and cytochrome P450 1A1 (CYP1A1). Unlike many other P450s, these isoforms are expressed extrahepatically. CYP1B1 protein is found to be overexpressed in tumours compared with the corresponding healthy tissues. Special regulatory mechanisms are likely to cause this difference.

In this study we employed TaqMan analysis, immunoblotting and reporter assays to investigate the expression patterns of CYP1B1 and CYP1A1 in a panel of breast cancer cell lines derived from different stages of mammary carcinomas. Furthermore, we investigated the expression of these P450s in cell lines derived from primary human mammary epithelial cells (HMECs) that have been transfected with various combinations of oncogenes and telomerase. In the transformed HMECs we found that the expression of CYP1B1, CYP1A1 and their inducibility by TCDD was differentially affected by the different oncogenes. We are presently investigating the regulatory mechanisms that cause this response.

In a second investigation, we analysed the relevance of P450 expression for mammary-tumour development and tumour therapy. For this purpose we have developed MCF-7-derived cell lines in which the expression of CYP1A1 and CYP1B1 can be switched on by treatment with low doses of doxycycline. We demonstrated that expression of these P450s altered the effects of estrogens and antiestrogens on cell cycle and apoptotic markers. Currently, the MCF-7-derived cell lines are being grown in xenografts. P450 expression will be induced by doxicycline in the drinking water, and animals will be treated with or without tamoxifen. Subsequently, the effects of P450 expression on tumour growth, angiogenesis and apoptosis will be measured.

It is anticipated that the results of these investigations will greatly enhance our understanding about the aetiology of breast cancer and may provide strategies to improve treatment.

P27

High-grade ER-negative tumour breast cancers are characteristic of both very young onset cases and patients with hereditary breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P27 (DOI 10.1186/bcr1582)

Background The Prospective Study of Treatment Outcomes in Sporadic versus Hereditary Breast Cancer (POSH) will have recruited 2,000 women over a 5-year interval from over 100 participating UK centres who have newly diagnosed breast cancer before age 41 years.

Methods The first 1,200 cases from the study in whom diagnostic pathology reports were submitted were analysed. We looked at the distribution of the reported tumour phenotype (major prognostic histopathological features) in women aged ≤ 35 years (43% of the total cohort) compared with women diagnosed age 35–40 years in order to further explore biological explanations for the known worse clinical prognosis for women aged under 36 years compared with older women. The χ^2 statistic was used to compare groups; genetic risk for

each recruit was derived using software that incorporates a general genetic model rather than a gene specific model. The highest genetic risk groups are likely to harbour most of the BRCA1 and BRCA2 gene carriers.

Results The majority of women at all ages were treated with anthracycline-based adjuvant chemotherapy and there was no difference in the choice of immediate surgical management between either age groups or between genetic risk groups.

Significantly more women in the ≤35 years age group had grade 3 ($P < 0.01$) and ER-negative ($P < 0.02$) tumours compared with women diagnosed in the older age group (>35 but ≤40 years). There was no significant difference in tumour size or lymph node status based on age categories. Compared with women with no family history, women falling into the 10% of the cohort estimated from family history to be most likely to carry *BRCA1* or *BRCA2* gene mutations, high genetic risk women had significantly more grade 3 tumours ($P < 0.001$) and a nonsignificant trend towards more ER-negative tumours.

Conclusion These data are from a preliminary pending systematic pathology review but bear out the observations by others that very young age of onset and host genotype affect the tumour phenotype and are therefore likely to have an impact on prognosis. Longer follow-up of this cohort is planned and outcome data based on age and based on genetic risk category and genetic mutation status will be available in a further 12 months time.

Acknowledgements Funding for the work presented in this abstract was from Wessex Cancer Trust, NCRN. Additional funding for further work on the POSH study was from CRUK, Breast Cancer Campaign, Breast Cancer Research Trust.

P28

Association of gene variants in the transforming growth factor beta signalling pathways with invasive breast cancer risk

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Breast Cancer Research 2006, **8(Suppl 2):**P28 (DOI 10.1186/bcr1583)

We are performing comprehensive association studies of single nucleotide polymorphisms (SNPs) in genes in the transforming growth factor beta (TGFβ) signalling pathways in a female breast cancer case–control study.

TGFβ acts as a suppressor of primary tumour initiation but is implicated as a promoter of the later malignant stages. A hypothesis explaining this dual action proposes that TGFβ acts through the ubiquitous ALK5/SMADs2&3 signalling pathway to inhibit proliferation of primary tumour cells, but acts through the endothelial-specific ALK1/SMADs1&5 pathway to promote angiogenesis, which is required for progression to malignancy.

In a previous study we showed that a SNP generating a leucine to proline substitution in the signal peptide of the TGFB1 protein was associated with a 1.24-fold increase in the risk of invasive breast cancer, with increased levels of the protein in human serum and with a 2.8-fold increase in amount of the protein secreted *in vitro*.

It is also plausible that other genes in the TGFβ signalling pathways might be associated with altered risk of breast cancer. We have initiated systematic breast cancer association studies with SNPs in the genes encoding proteins directly implicated in TGFβ signalling pathways, including the LTBP and TGFβ isoforms, ALK1, ALK5 and TGFBR2 receptors, and SMADs 1–7. A comprehensive SNP tagging approach was used to select variants for genotyping in a staged study design using up to 4,600 cases and 4,600 controls, all from the East Anglian region of the United Kingdom (>98% of northwestern European ancestry).

To date, nine genes (*TGFB1*, *TGFB2*, *TGFB3*, *ALK1*, *ALK5*, *TGFBR2*, *Endoglin*, *SMAD2* and *SMAD4*) have been analysed. From 285 common SNPs (minor allele frequency >0.05), identified from the International HapMap project data, 83 tagging SNPs have been defined and genotyped. Statistically significant associations with cancer susceptibility have been identified with at least one variant in five of the genes. The *TGFB2*, *TGFB3*, *Endoglin* and *SMAD4* genes are not associated. A further eight genes will be studied.

Acknowledgements Breast Cancer Campaign funded this work. The authors are grateful to the SEARCH Breast Cancer Study Team for sample collection.

P29

Effect of intermittent versus chronic energy restriction on breast cancer risk biomarkers in premenopausal women: a randomised pilot trial

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Breast Cancer Research 2006, **8(Suppl 2):**P29 (DOI 10.1186/bcr1584)

Background Postmenopausal breast cancer risk increases twofold in women who gain significant amounts of weight [1] and there is evidence that energy restriction may reduce risk [2]. Animal studies indicate that intermittent energy restriction (IER) reduces risk and may be superior to continuous energy restriction (CER) [3]. We have shown chronic energy restriction reduces biomarkers of breast cancer in women at risk but is hard to maintain. We hypothesise that IER may be superior to CER in reducing biomarkers of breast cancer risk and may also be more acceptable to women.

Methods We are undertaking a 6-month randomised trial to compare the two approaches in 104 premenopausal women aged 30–45 years at high risk of breast cancer because of adult weight gain >7 kg. Women will be randomly assigned to either CER (75% estimated energy requirements: ~1,500 kcal 7 days/week) or IER (75% estimated energy requirements: 650 kcal for 2 days and ~1,800 kcal 5 days/week) over 6 months. Study end points will be measures of insulin sensitivity (HOMA, SHBG and testosterone), potential breast cancer growth factors (IGF axis, leptin and adiponectin), inflammatory markers (C-reactive protein and sialic acid), oxidative stress marker (urinary F2 isoprostane), weight and body composition (waist/hip circumference, fat free and total fat mass). The relative acceptability of IER and CER will be assessed using a quality of life questionnaire (RAND SF-36) and scales of behaviour change and adherence. The relative efficacy and acceptance of intermittent and chronic calorie restriction will inform future weight loss programmes to prevent breast cancer. Thirty-seven women have currently been recruited to the study (18 CER and 19 IER) and recruitment is planned to be completed by December 2006.

Acknowledgements This study is funded by the Breast Cancer Campaign, the World Cancer Research Fund and Genesis.

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P30

Breast cancer in relation to childhood parental divorce and early adult psychiatric disorder in a British birth cohort

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Breast Cancer Research 2006, **8(Suppl 2):**P30 (DOI 10.1186/bcr1585)

Background Jacobs and Bovasso reported [1] that maternal death in childhood and chronic, severe depression in adulthood was associated with subsequent breast cancer. We examined the effects of parental loss in childhood and psychiatric disorder in adult life on breast cancer risk using a national birth cohort study.

Methods Eighty-three cases of breast cancer were diagnosed in a study of 2,253 women followed from birth to age 59 years. Cox's proportional hazards models were used to test whether breast cancer rates were higher in women who experienced parental death and divorce before age 16, psychiatric illness between 15 and 32 years, symptoms of anxiety and depression at 36 years, or use of anti-depressant medication at 31 or 36 years than in women who did not have these experiences.

Results There was no overall association between parental death, parental divorce, or psychiatric disorder on the incidence of breast cancer. There was some evidence that women with severe psychiatric illness were more likely to develop breast cancer early. The interaction between parental divorce and severe psychiatric illness was non-significant ($P = 0.1$); however, the group who experienced both these events had an increased breast cancer risk compared with those who experienced neither (HR = 2.64, 95% CI = 1.13–6.19) [2].

Conclusions Our study does not provide strong support of the hypothesis that early loss or adult psychiatric disorders are associated with breast cancer. A meta-analysis is needed that uses data from all available cohort studies and investigates possible interactive effects on breast cancer risk.

Acknowledgements RH, SB, MEJW, and DK are funded by the UK Medical Research Council. GM is funded by Breast Cancer Campaign and the World Cancer Research Fund, UK.

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P31

Increased regulatory T-cell numbers distinguish high-risk breast cancer patients and those at risk of late relapse

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Breast Cancer Research 2006, **8(Suppl 2):**P31 (DOI 10.1186/bcr1586)

Background We aimed to assess the clinical significance of tumour-infiltrating FOXP3+ regulatory T cells (T_R) in breast cancer patients with long-term follow-up.

Methods FOXP3+ T_R were detected by immunohistochemistry with our new FOXP3 monoclonal antibody, 236A/E7. Numbers of FOXP3+ lymphocytes in tissue microarray cores from pure ductal carcinoma *in situ* (DCIS) ($n = 62$), from invasive breast cancer ($n = 237$) or from comparable areas of normal terminal duct lobular breast tissue from patients without cancer ($n = 10$) were determined. A median cutoff value of 15 defined patients with high numbers of T_R.

Results T_R numbers were significantly higher in DCIS and invasive breast carcinomas when compared with normal breast, with invasive tumours having significantly higher numbers than DCIS ($P = 0.001$). High numbers of FOXP3+ T_R identified patients with DCIS at increased risk of relapse ($P = 0.04$) and patients with invasive tumours having both shorter relapse-free ($P = 0.004$) and overall survival ($P = 0.007$). High T_R numbers were present in high-grade tumours ($P < 0.001$), in patients with lymph node involvement ($P = 0.01$) and in estrogen receptor alpha (ER)-negative tumours ($P = 0.001$). Importantly, quantification of FOXP3+ T_R identified a group at high risk of relapse, within the so-called good prognostic group of ER-positive patients ($P = 0.005$) and these patients have a prognosis as poor as those that lack ER expression. Multivariate analyses, in ER-positive patients, demonstrated that greater T_R numbers independently conferred a significantly higher hazard ratio than that of tumour grade and nodal status for relapse-free and overall survival, respectively. Unlike conventional clinicopathological factors, high numbers of FOXP3+ T_R identified patients at risk of late relapse after 5 years disease-free survival.

Conclusion These findings indicate that quantification of FOXP3+ T_R in breast tumours is valuable for assessing disease prognosis and progression, and represents a novel marker for identifying late-relapse patients who may benefit from aromatase therapy after 5 years of tamoxifen treatment. Furthermore, tumour vaccination approaches in combination with targeting T_R cells are just entering clinical trials and our data strongly suggest that such therapy would be beneficial for a significant proportion of breast cancer patients.

Acknowledgement The authors would like to thank Breast Cancer Campaign for their research support.

P32

Development of anti-MUC1 DNA aptamers for the imaging and radiotherapy of breast cancer

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Breast Cancer Research 2006, **8(Suppl 2):**P32 (DOI 10.1186/bcr1587)

Background Aptamers are novel oligonucleotide-based recognition molecules which can bind to almost any target, including extracellular proteins, antibodies, peptides and small molecules. Aptamers can be rapidly generated, and offer reduced immunogenicity, good tumour penetration, rapid uptake and clearance, and can thus be used as alternatives to monoclonal antibodies in molecular targeted radiotherapy and diagnostic imaging.

Methods We have previously reported the isolation of high affinity and specificity DNA aptamers against the protein core of the MUC1 glycoprotein as a tumour marker on breast cancer cells. Once conjugated with a chelating agent and labelled with a radionuclide (^{99m}Tc or ^{188}Re), such aptamers can be particularly useful in the diagnosis and targeted radiotherapy of breast cancer. The conjugation is achieved using standard peptide coupling reactions between an amino modification on the aptamer and the carboxylic group on the ligands.

Results We have coupled the aptamer with the highest affinity for the MUC1 glycoprotein to different ligands (MAG2 or *meso*-2,3-dimercaptosuccinic acid) and labelled it with ^{99m}Tc and ^{188}Re to obtain stable complexes. An efficient and convenient labelling of the aptamer with short half-life radioisotopes was achieved as the last step of the synthesis (postconjugation labelling).

Conclusions The selected ligands have strong ^{99m}Tc and ^{188}Re binding properties and the resulting complexes are highly stable *in vivo* both in terms of nuclease degradation and leaching of the metal. The presence of more than one molecule of aptamer per complex or the conjugation of the aptamer to high molecular weight polyethylene glycol modifies the pharmacokinetic properties of the radiolabelled products, allowing the complex to remain longer in circulation and thus offering improved tumour imaging properties and further possibilities for development into a targeted radiopharmaceutical for breast cancer therapy.

Acknowledgement The authors thank Breast Cancer Campaign for financial support.

P33

Global histone modifications in breast cancer and their prognostic significance

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Breast Cancer Research 2006, **8(Suppl 2):**P33 (DOI 10.1186/bcr1588)

Background Post-translational modification of histones is a common mode of regulating chromatin structure and gene activity in normal tissues. In malignant cells, aberrant modifications through acetylation and methylation at the promoter regions of individual histones have been reported. Global changes in histone modification have recently been shown to be predictive of clinical outcome in prostate cancer. However, the expression and prognostic significance of modified histones in breast cancer has not been previously explored.

Methods Global histone modification in a large well-characterised series of breast carcinomas (n = 880) with long-term follow-up was therefore assessed using immunohistochemistry and tissue microarray. Specific antibodies were used to detect acetylation of H3 (Lys9 and Lys18) and H4 (Lys12), and dimethylation of histone H4 (Arg3) and H3 (Lys4). The presence of these chromatin 'marks' was correlated with clinicopathological variables and patients' outcome.

Results Reduced levels of histone acetylation/dimethylation were observed in medullary-like carcinomas, whereas they were readily detected in lobular and tubular carcinomas. Reduced global histone acetylation/dimethylation was significantly associated with established poor prognostic variables; larger tumour size, higher stage, recurrence, distant metastases and higher mortality rate. Survival analyses showed low detection of the histone modifications, with the exception of acetylated H3K9, was associated with shorter overall survival and shorter disease-free interval.

Conclusion Our results show, for the first time, that global changes in specific histone modification patterns may play an important role in breast cancer development and progression and their reduced expression is associated with poor prognosis and shorter survival.

P34

The Cambridge breast intensity modulated radiotherapy trial

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Breast Cancer Research 2006, **8(Suppl 2):**P34 (DOI 10.1186/bcr1589)

Background Radiotherapy (RT) following conservation surgery for breast cancer has been proven to improve both local control and survival. Currently, the challenge is to minimise RT-induced side effects without losing efficacy. Conventional 2D RT breast plans can lead to substantial dose inhomogeneities, which may cause a worse cosmetic result. This is important to patients, as a poor cosmetic result can cause significant psychological morbidity. Planning studies have shown that breast dose homogeneity can be improved with 3D planning and intensity modulated radiotherapy (IMRT). However, there is very little evidence regarding the *clinical* benefit of IMRT for breast cancer. This unique NCRN-adopted randomised controlled trial will test the clinical benefit of IMRT for women with early breast cancer.

Methods The primary question is: does correction of dose homogeneity using forward-planned IMRT improve the cosmetic outcome in patients with early breast cancer? Patients with significant dose inhomogeneities with 2DRT are randomised to IMRT or standard 2D RT. High-quality normal tissue toxicity and cosmesis data are being collected, including a novel analytical method of breast volume measurement using a 3D laser camera.

Results Eight hundred and eighty-five patients have been recruited to date, and accrual of 1,000 patients is on target for January 2007. A high-quality radiographer-led 3D breast radiotherapy service has developed as a direct result of the trial. Blood DNA samples from trial patients will enable investigation of individual genetic variation in normal tissue radiosensitivity within a multicentre translational radiogenomics study.

Conclusion The results from this trial could provide impetus to improve the quality of breast radiotherapy for many women worldwide. The DNA database will greatly contribute to the ultimate aim of individualised radiotherapy based on genetics.

Acknowledgement Jenny Wilkinson, Trial Radiographer, is funded by Breast Cancer Campaign.

P35

Why do most c-erbB-2/HER-2-positive breast cancer patients fail to respond to Herceptin?

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Breast Cancer Research 2006, **8(Suppl 2):**P35 (DOI 10.1186/bcr1590)

Objective The purpose of this study is to explore possible molecular and cellular mechanisms involved in the development of resistance to Herceptin in breast cancer patients.

Background Herceptin is a humanized monoclonal antibody targeted against the human epidermal growth factor receptor c-erbB-2 (HER-2) which is overexpressed in approximately 25–30% of invasive breast cancer. Herceptin recognizes an epitope on the extracellular domain of c-erbB-2 and blocks downstream signaling. Approximately 50% of patients respond to Herceptin therapy; however, the majority of these will demonstrate disease progression within 1 year of treatment initiation. Several molecular mechanisms contributing to Herceptin resistance have been proposed. This research aims to define the effects of Herceptin on subcellular c-erbB-2 receptor trafficking.

We have created a *c-erbB-2* plasmid fused to Yellow Fluorescent Protein (*c-erbB-2*-YFP) and an epidermal growth factor receptor fused to Green Fluorescent Protein (*EGFR*-GFP). Both constructs were sequenced and the correct sequence obtained. Both constructs were shown to react with specific antibodies and to have the predicted molecular weight using western blotting.

Methods Both *EGFR*-GFP and *c-erbB-2*-YFP plasmids were used to transiently transfect COS-7 cells. Time-course studies using low-light fluorescent microscopy revealed maximal membrane receptor expression between 18 and 24 hours after transfection. Herceptin immunoglobulin (Genentech Inc, South San Francisco, USA) was conjugated to Alexa Fluor 568 (Invitrogen Molecular Probes, Inc, USA) to allow visualization of the antibody. After 20 hours, *c-erbB2*-YFP transfected COS-7 cells were incubated with Alexa Fluor-labeled Herceptin for 2 hours. Serial fluorescent images were recorded over 12 hours allowing real-time visual localization of both the receptor and Herceptin.

Results These preliminary studies indicate that Herceptin induces receptor internalization. Further studies are planned whereby cells will be co-transfected with both *c-erbB2*-YFP and *EGFR*-GFP and exposed to an anti-EGFR antibody as well as Herceptin. Confocal microscopy will be utilized in mapping the fate of receptors and their antibodies. It may be that this dual targeting will exaggerate receptor internalization and degradation.

Conclusion We demonstrate that both constructs can be expressed in mammalian cells and receptor trafficking can be observed using digital fluorescent microscopy. In addition, we have fluorescently labeled Herceptin and its ability to bind c-erbB-2 is retained. This study of receptor and antibody trafficking may lead to further knowledge of Herceptin's mechanism of action as well as that for drug resistance and the possible effects of the use of combined therapies.

P36

Synergistic effects of cytotoxic drugs and antiresorptive agents *in vitro* and *in vivo*

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Breast Cancer Research 2006, **8(Suppl 2):**P36 (DOI 10.1186/bcr1591)

Background Breast cancer patients commonly receive a combination of different therapies; however, our understanding of how such combined treatments work is incomplete. In an attempt to optimize treatment strategies we have focused on determining how anticancer agents can be combined in order to induce maximum levels of tumour cell death. The antiresorptive agent zoledronic acid (zol) (Novartis Pharma, Basel, Switzerland) and the chemotherapeutic agent doxorubicin (dox) (Parmachemie BV, Haarlem, The Netherlands) have been shown to synergistically increase apoptosis in breast cancer cells *in vitro*. In order to determine whether sequential treatment with dox and zol could have potential clinical relevance and to determine the cellular mechanisms responsible for this synergy, we have further investigated combination treatments *in vitro* and *in vivo*.

Methods To enable visualization of intratibial tumours, MDA MB 436 breast cancer cells were stably transfected with GFP (MDA GFP 2 cells). Following sequential treatment with dox and zol, levels of MDA GFP 2 apoptosis were assessed by microscopic analysis following Hoechst and propidium iodide (PI) staining and by flow cytometry after annexin and PI staining. For *in vivo* dose–response studies, MDA GFP 2 cells were inoculated subcutaneously into the right flanks of female MF1 nude mice ($n = 8$). Mice were administered 2.5, 3, 30 or 150 μM zol intraperitoneally, or 2, 4 or 8 mg/kg dox intravenously. Combination studies were carried out against subcutaneous ($n = 16$) and intratibial ($n = 8$) MDA GFP 2 xenografts using a dosing regime of 2 mg/kg dox and/or 2.5 M zol once per week for 6 weeks, with zol being administered 24 hours after dox. The tumour volume was measured once per week for 6 weeks and mice were sacrificed 24 hours following final treatment.

Results and conclusions *In vitro* sequential treatment with dox then zol synergistically increased apoptosis in MDA GFP 2 cells. *In vivo* combination treatment with dox then zol resulted in a significant reduction of tumour growth compared with control mice or mice treated with dox or zol alone.

P37

Development of small-molecule transforming growth factor beta antagonists

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Breast Cancer Research 2006, **8(Suppl 2):**P37 (DOI 10.1186/bcr1592)

Background Transforming growth factor beta (TGFβ) is a multifunctional cytokine that regulates a wide variety of cellular processes, such as proliferation, differentiation and apoptosis. The role of TGFβ in breast cancer is complex. In the early stages of the disease TGFβ functions as a tumour suppressor, but later the protein switches to a prometastatic factor, suggesting that the inhibition of TGFβ activity may be of benefit in the treatment of stage IV metastatic disease. There is much interest at the present time in the development of strategies to inhibit the TGFβ signalling pathway for the treatment of metastatic cancer and other diseases.

We are using an *in silico* approach to identify small molecules capable of disrupting the TGFβ signalling pathway. In particular, we are searching for compounds with the ability to bind to the same site on the type II receptor (TβR-II) as TGFβ itself, thus preventing recruitment of the type I receptor, effectively blocking the ensuing signalling cascade.

Methods Molecular docking was performed using the commercially available docking program FlexX [1]. We attempted to dock 250,251 molecules from the NCI compound library against the extracellular domain of TβR-II, coordinates for which were taken from a crystal structure of the TGFβ3:TβR-II complex (Protein Data Bank accession number 1KTZ [2]). The consensus scoring function embedded within the software was used to assign each compound with a score, allowing them to be ranked, such that the highest ranking compounds could be prioritised for *in vitro* assessment.

The ability of the compounds to inhibit TGFβ signalling was tested in a cell-based reporter assay [3]. Any compounds shown to bring about a reduction in TGFβ signalling were taken forward for IC_{50} determination, performed in tandem with an MTT cell viability assay.

Results From the NCI compound database, a total of 219,567 molecules were successfully docked and scored by FlexX. Eighteen of the highest-ranking 40 compounds were obtained from the NCI Developmental Therapeutics Program and assessed for their ability to inhibit TGFβ signalling. One of these compounds was shown to inhibit TGFβ signalling without displaying any significant cytotoxicity.

Conclusion We have discovered a novel, small molecule capable of inhibiting TGFβ signal transduction. Our current work is focused on identifying the mode of action of this molecule and on the exploration of the surrounding chemical space, with a view to discovering more potent compounds and developing structure–activity relationships.

Acknowledgements The authors would like to thank Breast Cancer Campaign for funding and the NCI Developmental Therapeutics Program for supplying the small molecules.

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P38

Development of breast cancer immunotherapy using MUC1 retargeted T lymphocytes

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Breast Cancer Research 2006, **8(Suppl 2):**P38 (DOI 10.1186/bcr1593)

Background The MUC1 mucin represents an excellent target for breast cancer immunotherapy since it is overexpressed and underglycosylated in 90% of cases. To exploit this, we are developing a genetic approach to retarget T-cell specificity to MUC1, using chimeric antigen receptor (CAR) technology.

Methods A panel of MUC1-specific CAR have been generated using scFv derived from the SM3 and HMFG2 hybridomas. All CAR were generated by overlap extension PCR and incorporate a fused signalling domain comprising CD28 and CD3ζ. Stable CAR expression was

achieved in up to 75% of human T cells using the SFG oncoretroviral expression vector, following activation using PHA or CD3+28 beads.

Results Our first-generation MUC1-specific CAR, termed S28z, contained an SM3 scFv fused to a CD28 hinge. Surprisingly, however, S28z grafted T cells were poorly activated by a MUC1 + IgG fusion protein or MUC1 expressing T47D breast cancer cells. By contrast, S28z enabled T-cell activation when the MUC1 epitope was presented as a crosslinked peptide. Together, these findings suggested that steric hindrance and/or poor access to the epitope are limiting factors in CAR-based targeting of MUC1. To overcome this, a flexible monomeric hinge derived from IgD was introduced, thereby creating SD28z. Despite reduced stability, the SD28z CAR enabled T cells to proliferate in response to MUC1 glycoforms found in breast cancer. Stability of SD28z was further improved by inclusion of IgG_1 Fc sequences in the extracellular domain (giving SDF28z). SDF28z exhibited greater functional activity, enabling T cells to kill T47D tumour cells. In a second approach to optimize function, a scFv was cloned from the MUC1-specific HMFG2 hybridoma. HMFG2 binds breast tumour cells with greater intensity than SM3. In keeping with this, all HMFG2-derived CAR exhibited greater functional activity than their SM3 counterparts. In the MUC1-specific CAR that exhibits greatest activity (HDF28z), an HMFG2 scFv has been fused to the IgD hinge and IgG_1 Fc (HDF28z). HDF28z grafted human T cells exhibit potent cytolytic activity against MUC1 expressing breast cancer cells, associated with cytokine production and subsequent T-cell clonal expansion.

Conclusion Following extensive protein engineering, we have developed a stable and highly potent CAR to retarget human T cells to the ubiquitous tumour antigen MUC1.

Acknowledgements This work is supported by a Health Foundation/ Royal College of Pathologists Senior Clinician Scientist Research Fellowship and a Project Grant awarded by Breast Cancer Campaign.

P39

Effects of combined treatment with Zometa and Taxol on endothelial cells *in vitro*

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Breast Cancer Research 2006, **8(Suppl 2):**P39 (DOI 10.1186/bcr1594)

Background Zoledronic acid (ZOL) (Novartis Pharma, Basel, Switzerland) is a N-containing bisphosphonate currently used in the treatment of osteoporosis and tumour-induced bone disease in a wide range of solid and haematologic malignancies. Previous studies have shown that ZOL interferes with endothelial cell (EC) function; however, little is known about the effect of ZOL on ECs of the microvascular network that resemble the tumour vasculature. Taxol (Bristol-Myers Squibb Company, New York, USA), also known as paclitaxel (PAC) (Sigma, UK), is a chemotherapeutic agent currently used in anticancer therapy. Previous studies on tumour cells have shown that it specifically interferes with microtubule assembly. Its antiangiogenic properties *in vitro* or *in vivo* remain unestablished. Combination treatments with ZOL and PAC have also been shown to have a synergistic effect on apoptosis in tumour cell lines.

Methods We have investigated the effects of ZOL and PAC on ECs *in vitro*, both alone and in combination, therefore determining the effects on EC death and the ability of ECs to adhere onto membranes of various components of the extracellular matrix as well as on EC proliferation, migration and Rap1a prenylation.

Results Human dermal microvascular ECs (HuDMECs) were treated with increasing doses of ZOL (0–50 μM) and PAC (0–10 nM) alone and in combination. ZOL affected EC proliferation (50 μM for 48 and 72 hours, $P < 0.05$), tube formation (50 μM for 24 hours, $P < 0.05$) and Rap1a prenylation. EC adhesion or apoptosis was not affected. PAC interfered with EC tube formation but not proliferation (24 and 48 hours). PAC also affected apoptosis; however, the exact level was dependent on the cell batch. Apoptosis was induced (50 μM ZOL and 2 nM PAC for 24 hours, $P < 0.05$) in cultures treated with ZOL and PAC together. Migration was inhibited at very low doses of PAC (500 pM) and 25 μM ZOL with combination treatment.

Conclusions These data suggest that combinations of ZOL and PAC may have increased antiangiogenic effects compared with that caused by the single agents.

P40

Breast cancer follow-up: a focus group and interview study

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Breast Cancer Research 2006, **8(Suppl 2):**P40 (DOI 10.1186/bcr1595)

Background The aim was to explore the experiences of women with breast cancer in relation to routine follow-up appointments in different settings, including the issues surrounding discharge from hospital care.

Methods A qualitative focus group and interview study in the area of Norfolk serviced by the Norfolk and Norwich University Hospital Healthcare Trust. The participants were 46 women, 2 years or more post diagnosis of breast cancer (range 2–20 years), aged 30–85 years, with no active recurrent disease. The women were undergoing follow-up in hospital or in general practice, or no follow-up.

Six focus group meetings were held initially, transcribed and themes derived using N*Vivo software and constant comparison. Individual interviews were then carried out to explore the themes, and to widen the range of participants.

Results Themes identified fell into two categories: discharge from hospital care, and information. Themes related to the former included initial disease experience, whether the cancer was detected mammographically or self-detected, uncertainty about recurrence, 'expert' care', and continuity of care. Themes related to information included follow-up protocols, breast care nurses, tamoxifen, mammography, lymphoedema, and the role of support groups.

Conclusions Women wished to participate in decisions on follow-up. A small group of women preferred hospital follow-up long term. Most others would value a final hospital appointment generating a plan for further follow-up. They would then be content to be discharged to GP care, preferably with telephone access to a breast care nurse. A few women were confident to be discharged fully to self-examination and mammography with no formal follow-up. The breast care nurses were a popular choice to provide 'expert' information at every stage of the process, and were perceived as easily accessible. This study supports increasing the role of breast care nurses in the community after discharge from hospital care.

Acknowledgements This study was funded by a grant from Breast Cancer Campaign. The research idea was developed in conjunction with Mr D Ralphs, Consultant Breast Surgeon, and the Breast Care Nurses at the Norfolk and Norwich University Hospital NHS Trust. The design and recruitment were aided by local volunteers from Breast Cancer Care.

P41

'I haven't had breast cancer but I've had a mastectomy anyway': do women with ductal carcinoma *in situ* have appearance concerns?

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Breast Cancer Research 2006, **8(Suppl 2):**P41 (DOI 10.1186/bcr1596)

Background This study explored the psychosocial impact of being diagnosed and treated for ductal carcinoma *in situ* (DCIS), with the aim to improve the current knowledge and understanding of DCIS from the patient's perspective. DCIS is a preinvasive breast condition increasingly detected by mammogram screening and has an uncertain natural history (some DCIS cells may develop into invasive cancer, but there is no marker to determine which DCIS cells will). Although DCIS is not an invasive condition, many women undergo extensive surgery (including mastectomy); therefore, this is a paradoxical situation – these women are reassured that it is noninvasive, caught early and not life-threatening, but they are offered similar treatment as women with

invasive breast cancer. The presentation aims to disseminate the initial findings of an exploratory qualitative study.

Methods In-depth semistructured interviews with 16 women previously diagnosed with DCIS explored their experience. Thematic analysis highlighted the important issues from the women's own perspective.

Results This study identified seven themes, which included two subthemes relating to appearance that are presented here. The paradox of DCIS and concerns about appearance were clearly evident in several participants.

Conclusion The results emphasise that women may have post-treatment concerns and appearance issues following surgery for DCIS; these women may require specific support and advice in order to adjust for and accept the impact that the treatment may have on their appearance and feelings following surgery. Further research is needed to explore this area. The research team plans to address this by following a group of DCIS patients prospectively in order to identify how women's feelings and concerns (including appearance) change during the diagnosis and treatment for DCIS.

Acknowledgement Funded by Breast Cancer Campaign.

P42

Setting a lower risk threshold for surveillance within breast cancer family services

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Breast Cancer Research 2006, **8(Suppl 2):**P42 (DOI 10.1186/bcr1597)

Background Counselling, risk assessment and surveillance are provided for women with a significant family history of breast cancer through a network of clinical centres across the United Kingdom. Before 2004, the recommended minimum 'threshold' for significant familial risk was set by a number of guidelines issued, which broadly required one first-degree relative diagnosed with breast cancer before age 40 or two close relatives both diagnosed before age 60. In 2004, NICE issued detailed guidelines in which the age requirement for two affected relatives was removed. However, it is widely recognised that the evidence base for any specific minimum threshold is limited and that there is a need for empirical studies to validate current and future recommendations. That is the object of the present study.

Methods Records of the four Scottish Breast Cancer Family clinics were scrutinised for the period January 1994–December 2003 to identify any women referred but discharged because the level of familial risk was judged to fall below the (pre-NICE) threshold. From dates of birth and dates of discharge, the number of women-years of observation (to December 2003) within each 5-year age group (35–39 years, 40–44 years, and so on) was calculated. With permission from the Privacy Committee, the list was then checked against Scottish Cancer Registry records and any breast cancers recorded were rechecked from hospital notes. Expected cancer rates for an age-matched Scottish population were derived from Cancer Registry Statistics.

Results A total of 2,074 'low risk' women were identified, giving over 8,000 woman-years of observation. Twenty-eight invasive breast cancers were recorded while 14.4 would have been expected (relative risk = 1.9 assuming complete ascertainment). A further eight invasive breast cancers have been recorded since 2003 (records incomplete). One-third of the cancers were in women who would have met the new NICE criteria for surveillance, whereas only some 10% of the total cohort had 'NICE moderate' family histories. The great majority of the cancers occurred in women between age 45 and 56. For them the relative risk approached 2 even when 'NICE moderate' women were excluded.

Conclusion The new NICE family history guidelines are more accurate than previous ones in identifying women who should be included in breast surveillance programmes, but consideration should be given to making some provision particularly for women between age 45 and 56 with 'limited' family histories of breast cancer. The cohort we have identified should continue to be followed up since cancers are continuing to accrue and each year provides a further 2,000+ woman-years of observation.

P43

Role of macrophages in breast cancer angiogenesis *in vivo*

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Breast Cancer Research 2006, **8(Suppl 2):**P43 (DOI 10.1186/bcr1598)

Background The objective was to establish a murine model to study the role of macrophages in the initiation of angiogenesis by human breast tumour spheroids *in vivo*. Despite the increasing body of evidence, both experimental and clinical, implicating macrophages in breast tumour angiogenesis, there have been no previous *in vivo* studies demonstrating proangiogenic tumour activity.

Methods Human breast tumour spheroids (600 μm) were infiltrated with human monocytes *in vitro*, allowed to differentiate into macrophages, coated with alginate to isolate from the host (murine) cells and implanted into dorsal skin-fold chambers on nude mice. The resultant angiogenesis surrounding the spheroids infiltrated with human macrophages prior to implantation was quantified using image analysis (Angiosys), and compared with that induced by spheroids consisting of tumour cells alone.

Results The presence of macrophages resulted in at least a threefold upregulation in the release of vascular endothelial growth factor (VEGF) *in vitro* when compared with spheroids composed only of tumour cells. A homogeneous distribution of macrophages surrounding the hypoxic centre was observed in the majority of spheroid sections assessed. The angiogenic response measured around the spheroids 3 days after *in vivo* implantation was significantly greater in the spheroids infiltrated with macrophages; the number of vessels increased (macrophages vs no macrophages, 34 ± 1.9 vs 26 ± 2.5, $P < 0.01$), and were shorter in length (macrophages vs no macrophages, 116 ± 4.92 vs 136 ± 6.52, $P < 0.008$) with an increased number of junctions (macrophages vs no macrophages, 14 ± 0.93 vs 11 ± 1.25, $P < 0.025$), all parameters indicative of new vessel formation. By day 7 no significant differences were seen. Viable human but no murine macrophages were identified in the tumour spheroids at the end of the study, using immunohistochemistry.

Conclusions This is the first *in vivo* study to demonstrate that macrophages modulate breast tumour angiogenesis, in the early stages of development, with an increased number of vessels and branches.

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Is transforming growth factor beta signalling required for breast cancer metastatic cell motility?

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Breast Cancer Research 2006, **8(Suppl 2):**P44 (DOI 10.1186/bcr1599)

Background In many cell types, transforming growth factor beta (TGFβ) results in a growth inhibitory signal, which is mediated by transducers of the Smad family. In tumour cells, however, TGFβ-dependent antiproliferative control is lost and cells acquire the ability to replicate in TGFβ-rich environments. Furthermore, molecular and clinical evidence points to a role for TGFβ signalling in cancer progression and metastasis; however, it is unclear at which points of the metastatic process TGFβ signalling occurs and whether it is necessary and/or sufficient to elicit cancer cell motility.

Methods To address these questions, MTln3E rat breast cancer cells were used as a relevant model system. When injected into the

mammary fat pad of nude mice, these cells form a primary tumour from which motile cells will depart to form metastasis in the lymph nodes and the lungs. To gain insight into TGFβ signalling *in vivo*, MTln3E cells were engineered to express GFPSmad2. This allowed monitoring Smad-dependent TGFβ signalling *in vivo* by imaging the primary tumour and in lymph-node metastasis using multiphoton confocal microscopy.

Results The results indicate that TGFβ signalling, measured by cytoplasmic to nuclear translocation of GFPSmad2, does not occur ubiquitously within the primary tumour. On the contrary, TGFβ signalling appears most prominent in movement-rich areas. Within these areas, all the cells that have acquired a motile phenotype display active TGFβ signalling. Furthermore, none of the motile cells display nuclear exclusion of GFPSmad2.

Conclusions Together these data suggest that TGFβ signalling may be required in metastatic cells, possibly to enable acquisition of the motility phenotype. However, as nuclear localisation of GFPSmad2 is observed also in nonmotile cells, TGFβ signalling alone may not be sufficient to elicit cell motility in primary tumour cells.